Journal Pre-proof

Drum drying-and extrusion-black rice anthocyanins exert anti-inflammatory effects via suppression of the NF-κB /MAPKs signaling pathways in LPS-induced RAW 264.7 cells

Tingting Qiu, Yong Sun, Xiaoya Wang, Liufeng Zheng, Hua Zhang, Li Jiang, Xuemei Zhu, Hua Xiong

PII: S2212-4292(20)31179-2

DOI: https://doi.org/10.1016/j.fbio.2020.100841

Reference: FBIO 100841

To appear in: *Food Bioscience*

Received Date: 18 May 2020

Revised Date: 2 November 2020

Accepted Date: 10 December 2020

Please cite this article as: Qiu T., Sun Y., Wang X., Zheng L., Zhang H., Jiang L., Zhu X. & Xiong H., Drum drying-and extrusion-black rice anthocyanins exert anti-inflammatory effects via suppression of the NF-κB /MAPKs signaling pathways in LPS-induced RAW 264.7 cells, *Food Bioscience*, https://doi.org/10.1016/j.fbio.2020.100841.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2020 Published by Elsevier Ltd.

Journal Pre-proof

Drum drying-and extrusion-black rice anthocyanins exert anti-inflammatory effects via suppression of the NF-κB /MAPKs signaling pathways in LPS-induced RAW 264.7 cells

Tingting Qiu[a,b,1], Yong Sun[a,1], Xiaoya Wang[a,c], Liufeng Zheng[a], Hua Zhang[c], Li Jiang[c], Xuemei Zhu[d,*], Hua Xiong[a,*]

[a] State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang 330047, Jiangxi, China

[b] Jiangxi Provincial Institute of Parasitic Diseases, Nanchang 330096, Jiangxi, China

[c] Jiangxi University of Traditional Chinese Medicine, Nanchang 330004, Jiangxi, China

[d] School of Food Science and Technology, Dalian Polytechnic University, Dalian 116034, China

Journal Pre-proof

[1] These authors contribute equally to this manuscript.

[*] Corresponding authors.

E–mail addresses: zhuxuemei2005@hotmail.com (Xuemei Zhu), huaxiong100@126.com (Hua Xiong).

Journal Pre-proof

Abstract

Different processing methods significantly affect the content of bioactivities and the anti-oxidant activities in food sources, including black rice, one of the world's major food sources of black rice anthocyanin extract (BRAE). In this study, the effect of drum-drying and extrusion processes on lipopolysaccharide (LPS)-induced inflammatory responses by bioactive compounds and antioxidants from black rice extract was determined. This study identified the total phenolic, flavonoid, and anthocyanin contents and antioxidant activities in vitro. The phytochemical constituent analysis of three anthocyanin-enriched extracts from raw (BRAE), drum-dried (D-BRAE), and extruded black rice (E-BRAE) using UPLC-LTQ-Orbitrap-MS/MS tentatively identified nine compounds. Cyanidin-3-glucoside was the major anthocyanin in black rice extracts. In contrast, significant reduced levels of cyanidin-3-glucoside and peonidin-3-glucoside were found in D-BRAE and E-BRAE, and the content of protocatechuic acid was increased obviously in E-BRAE. The anti-inflammatory effects of differently processed rice extracts in LPS-stimulated RAW264.7 cells demonstrated that BRAE, D-BRAE, and E-BRAE (400 μg/mL) significantly inhibited NO and PGE2 secretion ($p<0.001$) by down-regulating iNOS and COX-2 mRNA and protein expression levels. mRNA expression of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) were also decreased by BRAE, D-BRAE, and E-BRAE. Therefore, the anti-inflammatory activities of BRAE were not affected by drum-dried or extrusion process. Activation of MAPK and NF-κB pathways were inhibited by BRAE that influenced the

regulation of the phosphorylation of JNK, ERK, p65, and IκBα. These pathways were not affected by the drum-dried process but were significantly enhanced by the extrusion process. This study will provide scientific and meaningful basics for the application of BRAE using different processing methods in anti-oxidant and anti-inflammation.

Keywords: black rice, drum-drying, extrusion, UPLC-LTQ-Orbitrap-MS/MS, anti-inflammatory activity

Journal Pre-proof

Journal Pre-proof

1. Introduction

Inflammation is a series of defense-based immune responses that are produced by the body in response to various stimuli. Excessive inflammatory reactions may lead to diabetes, insulin resistance, and cardiovascular and metabolic diseases (Liu, et al., 2019). Several drugs have been approved for the treatment of inflammatory patients, such as aminosalicylates, corticosteroids, antibiotics, and non-steroidal anti-inflammatory drugs (NSAIDs). However, the long-term and high-dose usage of these drugs may cause side effects such as gastrointestinal or renal damage (Montoya, et al., 2019; Rainsford, 2003). Therefore, natural substances have been widely investigated for anti-inflammatory treatment. Studies have shown that daily dietary intake of fruits, vegetables and grains could prevent inflammation and other chronic diseases caused by inflammation (Lee, et al., 2017; Limtrakul, et al., 2015; Peng, et al., 2019; Teng, et al., 2017; Zhang, et al., 2019a).

Grains play important roles in daily dietary supplementation and possess various biological functions including anti-inflammatory and antioxidant activities, et al. Zhang et al., (2019b) found that colored maize anthocyanin-rich extracts restored inflammation-mediated oxidative stress and insulin resistance in macrophage-conditioned media-treated adipocytes. Roager et al., (2017) reported that whole grain diet reduced body weight and systemic low-grade inflammation when compared with refined grain diet. Wu et al., (2017) found that the anthocyanin in black rice, black soybean, and purple corn could ameliorate diet-induced obesity by alleviating both oxidative stress and inflammation in C57BL/6 mice fed a high-fat diet.

Journal Pre-proof

Black rice is a special rice cultivar mainly cultivated in Southeast Asia, having a higher phenolic and anthocyanin contents than in white rice. Previous investigations have shown that cyanidin-3-O-glucoside and peonidin-3-O-glucoside are the major anthocyanins in black rice (Hao, et al., 2015; Pang, et al., 2018; Zhu, et al., 2018a). Several studies have reported that black rice anthocyanin extracts possess antioxidants, and anti-inflammatory activities, both *in vitro* and *in vivo* (Pang, et al., 2018; Sangkitikomol, et al., 2010; Sirilun, et al., 2016; Wu, et al., 2017). Zhao et al., (2018) reported that dietary black rice anthocyanin-rich extract and rosmarinic acid, alone and in combination, alleviated the symptoms of inflammation in mice with dextran sulphate sodium salt (DSS)-induced colitis.. Study reported by Limtrakul et al., (2015) demonstrated that black rice anthocyanin extracts suppressed LPS-induced inflammation by inhibiting the activation of the mitogen-activated protein kinases (MAPK) signaling pathway and nuclear factor (NF)-κB translocation, thereby indicating that black rice anthocyanin extracts exhibit therapeutic potential in inflammation-related diseases. With the accelerating rhythm of life, the consumption of whole grain fast food for health benefits is gaining considerable attention by the people. However, these processes may affect the bioactive compounds content and the functional activities of grains. Surh et al., (2014) reported on a significant loss of anthocyanin in black rice during roasting (94%), steaming (88%), pan-frying (86%) and boiling (77%), while the phenolic compound content and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity decreased drastically after cooking. Bhawamai et al., (2016) found that thermal cooking

decreased the total anthocyanin and cyanidin-3-glucoside (C3G) contents and the Ferric ion reducing antioxidant power (FRAP) antioxidative capacity, but did not affect the anti-inflammatory activity of black rice. Although several studies have reported the effect of cooking on the polyphenolics, anthocyanin content, and antioxidative activity of black rice, limited data is available regarding the changes in bioactive compounds and biological activities of black rice by other food-related processing methods, for example, drum-drying and extrusion.

Drum drying and extrusion are two important processing methods in the food industry (Henríquez, et al., 2013). Extrusion is an important food processing technology that is widely used for ready-to-eat breakfast cereals, puffed food, and other snack foods. It was demonstrated that the extruded process would lead to the gelatinization of starch, the protein denaturation, and heat-sensitive components such as vitamins and antioxidant degradation (Ruiz-Gutiérrez, et al., 2015). A previous study reported that the extrusion cooking treatment increased the total phenolic content (TPC) and antioxidant activities of green banana flour (Sarawong, et al., 2014). The total phenolics, anthocyanins, and antioxidant activity in black rice bran were increased by extrusion but decreased in polished and brown rice (Ti, et al., 2015). Additionally, drum drying is an economical technology with high drying efficiency commonly used in grain-based baby foods, potato chips, and fruit slices (Henríquez, et al., 2013). Soison et al., (2014) reported that drum-dried purple-flesh sweet potato flours achieved the maximum phenolic content and antioxidant activities with drum-dried temperature at 140 °C. The thermal degradation of anthocyanin and

phenolics in grains has been studied, and it was shown that their functional activities could consequently be affected. Leem et al., (2014b) found that *Acanthopanax senticosus* leaves (ASL) decreased mRNA expression of anti-inflammatory cytokines and protein levels in HMC-1 cells, reduced nitric oxide (NO); malondialdehyde (MDA); and tumor necrosis factor-α (TNF-α) levels in acute inflammatory rats, and extrusion treatment increased the anti-inflammatory effects of ASL. Montoya-Rodríguez et al., (2014) found that extrusion process treatment improved the anti-inflammatory effect of amaranth pepsin/pancreatin hydrolysates in LPS-stimulated human THP-1 macrophage-like and mouse RAW 264.7 macrophages by decreasing TNF-α, NO, and Prostaglandin E2 (PGE2) secretion and inhibiting the phosphorylation of NF-κB signaling pathway. However, research on the effect of extrusion and drum-drying on black rice extracts phytochemical contents, anti-inflammatory activity, and underlying mechanism is limited.

The aim of the present study was to investigate the effects of drum-drying and extrusion on the chemical constituents, and antioxidant and anti-inflammatory activities of black rice extracts. The chemical constituents of black rice extracts were identified by UPLC-LTQ-Orbitrap-MS/MS techniques, and the antioxidant activities were determined by DPPH, ABTS, and FRAP assays. The inhibitory activities of inflammatory mediators (NO, PGE2) and pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) were investigated using ELISA kits, while the mRNA expression of pro-inflammatory cytokines and inflammation-related enzymes (iNOS, COX-2) were determined by RT-qPCR. The effect of black rice extracts on NF-κB and MAPK

118 signaling pathways were investigated by western blotting. The results obtained from
119 this study can provide scientific evidence for the production of cereal convenience
120 foods and human daily dietary intake.

121 **2. Materials and methods**

122 *2.1. Chemicals*

123 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and LPS
124 (Escherichia coli 055: B5) were purchased from Sigma-Aldrich (St. Louis, USA). NO
125 assay kits were obtained from Beyotime Biotechnology (Shanghai, China).
126 prostaglandin E2 (PGE2), TNF-α, IL-6, IL-1β ELISA kits was purchased from
127 Meimian Biotech (Yancheng, China). The JNK, phosphor-JNK (p-JNK), p38,
128 phosphor-p38 (p-p38), ERK, phosphor-ERK (p-ERK), p65, phosphor-p65 (p-p65),
129 IκBα, phosphor-IκBα (p-IκBα) primary antibodies for western blot were obtained
130 from Cell Signaling Technology, Inc. (Beverly, MA, USA).

131 *2.2. Sample preparation*

132 The raw, drum-dried, and extruded black rice powders were obtained from
133 JiangXi Guwuyuan Food Co., Ltd (JiangXi, China). The anthocyanin-rich fraction
134 extraction of black rice was performed as follows: black rice powder (2 g) was mixed
135 with 40 mL 0.1% HCl (v/v) in 80% methanol and incubated at 4℃ for 12 h in the
136 dark. The mixture was centrifuged at 4500×g for 5 min. The residue was re-extracted,
137 and the supernatants were collected, evaporated at 25℃, , and dissolved in an aliquot
138 of methanol. The sample was then purified using Oasis® HLB 6cc (200mg) Extraction

Cartridges (WATERS, USA) and freeze-dried, stored at −80℃ until use. All procedures were performed in the dark to avoid anthocyanin degradation. The obtained freeze-dried black rice extract powder was named black rice anthocyanin-rich extract (BRAE)., Prefix letters were used to distinguish the two processing BRAEs as the drum-dried BRAE (D-BRAE) and the extruded BRAE (E-BRAE).

2.3. Total phenolic, flavonoid and anthocyanin contents

The total phenolic content (TPC) of BRAEs was measured using the Folin-Ciocalteu method (Ti, et al., 2015). TPC was expressed as milligram of gallic acid equivalent per gram dry weight extract (mg GAE/g DW) using the gallic acid calibration curve. The total flavonoid content (TFC) of BRAEs were carried out by $NaNO_2$-$AlCl_3$ method using catechin as a standard. The TFC was expressed as milligram of catechin equivalent in gram of dry weight (mg CAE/g DW). The total anthocyanin content (TAC) of BRAEs was determined by the pH differential method (Ti, et al., 2015). The TAC was expressed as milligrams of cyanidin-3-glucoside equivalent per gram of dry weight (mg C3G/100 g DW). All samples analyzed in triplicate.

2.4. Qualitative and quantitative analysis

2.4.1 Liquid chromatographic and mass spectrometric conditions

The UPLC-LTQ-Orbitrap-MS^2 consisted of a heated-electrospray ionization probe (HESI-II; Thermo Fisher Scientific, USA) equipped with an ACQUITY UPLC

C18 column (2.1×100 mm, 1.7 μm). The mobile phase consisted of 0.1% formic acid in deionized water (A) and 0.1% formic acid in acetonitrile (B). The gradient elution program was as follows: 0–2 min, 5% B; 2–11 min, 5%–43% B; 11–13 min, 43%–70% B; 13–17 min, 70% B; 17–26 min, 70%–100% B; 26–26.1 min, 100%–5% B; 26.1–30 min, 5% B. The flow rate was 0.3 mL/min, the injection volume was 1 μL, and the column temperature was 25℃. ESI-MS^n experiments were performed using the following conditions: negative ion mode, detection range of m/z was 100–1700. source voltage 5 kV; tube lens voltage, −80 V; capillary voltage, −40 V; capillary temperature, 275℃; sheath and auxiliary gas flow (N2), 42 and 11 (arbitrary units).

2.4.2 Quantification

Cyanidin-3-glucoside, Syringic acid, Protocatechuic acid and Vanillic acid (1.0 mg; Sigma-Aldrich, St. Louis, USA) were accurately weighed and dissolved in 1 mL methanol. Calibration curves were obtained by injecting standards (31.25, 62.5, 125, 500, and 1000 μg/mL) thrice. The quantification of anthocyanin was expressed as cyanidin-3-glucoside equivalents.

2.5 Antioxidant assays

The DPPH radical-scavenging capacity was determined according to Pan et al. (2018). Briefly, 20 μL of BRAEs and control solution were added to 280 μL of 65 μM DPPH (Sigma-Aldrich, St. Louis, USA) solution in methanol. The mixture was shaken in a 96-well plate and incubated in the dark for 30 min at 25℃. The absorbance was determined at 540 nm using a microplate reader (Thermo Scientific

Varioskan Flash, Finland). ABTS and FRAP assays were measured using kits (Beyotime Biotechnology, Shanghai) according to the manufacturer's instructions.

2.6 Anti-inflammatory activities

2.6.1 Cell culture and viability assay

RAW264.7 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Solarbio Life Science, Beijing) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 10% FBS (ExCell Biology, Shanghai) , and maintained in a humidified cell incubator at 37℃ with 5% CO_2. Cell viability on RAW264.7 cells was measured by MTT assay as described previously (Sun, et al., 2015). Briefly, The RAW264.7 cells were seeded in 96-well plates (100 μL, 2×10^4 cells/well) for 12 h, and different concentrations of BRAE samples (100 μL) were added and cultured at 37℃ for 24 h, and MTT solution (10 μL, 5.0 mg/mL) were added and incubated at 37℃ for 4 h. Then, DMSO (150 μL) was added to dissolve the formazan crystals, and the absorbance was determined at 490 nm using a microplate reader (Thermo Scientific Varioskan Flash, Finland).

2.6.2 Determination of nitric oxide (NO) production

The RAW264.7 cells were seeded in 24-well plates (5×10^5 cells/well) for 12 h, after pre-treatment with BRAE, D-BRAE, or E-BRAE medium (50, 200, 400 μg/mL) for 1 h, LPS (1 μg/mL) was added and cultivated for 24 h. NO production was determined using the NO assay kit (Beyotime Biotechnology, China) at 540 nm in a

microplate reader. All assays were performed thrice.

2.6.3 Enzyme-linked immunosorbent assay (ELISA)

The RAW264.7 cells were treated the same as the NO production program. Cell supernatants were obtained for determination of PGE2, TNF-α, IL-6, and IL-1β levels using ELISA kits (Meimian Biotech, Yancheng, China) at 450 nm according to the manufacturer's instructions.

2.6.4 Reverse transcription and quantitative real-time PCR

The RAW264.7 cells were seeded in 24-well plates (5×10^5 cells/well) overnight, treated with BRAE, D-BRAE, E-BRAE medium (50, 200, 400 μg/mL) 1 h before LPS stimulation (1 μg/mL) for 24 h. The cells were collected, and an RNA extraction kit (Beyotime Biotechnology) was used for total RNA extraction according to the manufacturer's protocol. The cDNA was synthesized using the Prime Script™ RT reagent Kit (Takara, Japan) according to the manufacture's protocol. Real-time PCR of inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, Tumor Necrosis Factor-α (TNF-α), Interleukin-6 (IL-6), Interleukin-1β (IL-1β) and β-actin was performed on a CFX96 real-time PCR detection system (Bio-Rad, Singapore). The reaction conditions were as follows: heating to 95℃ retain for 30 s, followed by 39 cycles for 30 s at 95℃ and 60℃, and extension at 65℃ for 5 s and 95℃ for 5 s. The PCR primers were obtained from Sangon Biotech (Shanghai, China), and the sequences are shown in Table 1. β-actin used as a standard to indicate the relative expression levels of target mRNAs.

Journal Pre-proof

223 *2.6.5 Western blotting*

224 The RAW264.7 cells were plated with density of 2×10^6 cell/well in six-well
225 plates for 12 h. Then, the cells were treated with BRAE, D-BRAE, E-BRAE medium
226 (400 μg/mL) for 1 h, and incubated with 1 μg/mL LPS for 24 h. Cell lysis buffer (1
227 mL RIPA + 10 μL PMSF, Beyotime Biotechnology) was used for total protein
228 extraction, and total protein concentration was detected using a BCA protein assay kit
229 (Solarbio Life Science). The protein samples were diluted and boiled for 10 min,
230 isolated on 10%-12% SDS-PAGE, and then transferred to a PVDF membrane
231 (Beyotime Biotechnology, China). The cells were incubated with 5% skim milk
232 powder for 2 h and then incubated with primary antibodies (iNOS antibody, COX-2
233 antibody, c-Jun NH2-terminal kinase (JNK)/p-JNK antibody, p38/p-p38 antibody,
234 extracellular signal-regulated kinase (ERK)/p-ERK antibody, p65/p-p65 antibody,
235 Inhibitor of NF-κB (IκB)/p-IκB antibody, β-actin antibody, and
236 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody, all of which were
237 purchased from Cell Signaling Technology, MA, USA) at 4 °C overnight. The cells
238 were washed thrice with TBST and incubated with the secondary antibodies for 2
239 hours, and then washed with secondary antibody + TBST thrice. Protein bands were
240 scanned using the ChemiDoc™ Touch Imaging System (BIO-RAD, USA).

241 *2.6.6 Immunofluorescence staining*

242 The RAW264.7 cells were plated with density of 2×10^6 cell/well in six-well
243 plates for 12 h cells. The cells were then treated with BRAE, D-BRAE, E-BRAE

medium (400 μg/mL) for 1 h, and LPS (1 μg/mL) incubated for 24 h. The tablets were washed twice with PBS, and incubated with 4% paraformaldehyde solution for 10 min. Then, the cells were incubated with the primary antibody (p65 antibody) at 4 °C overnight. The cells were washed three times with cold PBS and incubated with secondary antibodies at 37 °C for 1 h in the dark. Finally stained and image acquisition.

2.7 Statistical analysis

The data were analyzed using SPSS 25.0 and are expressed as mean±S.D. One-way ANOVA followed by Tukey's test was used to assess the statistical differences among groups. $p<0.05$ means significant statistically. All experiments were performed in triplicate.

3. Results

3.1 Characterization of the phytochemicals in BRAEs

The composition of the 80% methanol extract of the three black rice samples was identified by UPLC-LTQ-Orbitrap-MS/MS techniques. A total of nine compounds were tentatively identified on the basis of retention times (t_R), m/z, and MS^2 data and were compared to existing literature. The data regarding the identified compounds is summarized in Table 2. The DAD chromatogram at 280 nm of BRAEs is shown in Figure 1, and the MS^2 spectra and proposed fragmentation patterns of some identified peaks are presented in Figure 2. The characterization of nine peaks is described as follows.

Journal Pre-proof

Peak 1 (t_R 2.53 min) with m/z =197.81 and showed fragment ion at m/z=153.92 ([M-H-COO]$^-$) and 135.88 ([M-H-H_2O-COO]$^-$) (Donato, et al., 2016) was identified as syringic acid. Peak 2 (t_R 3.48 min) showed the precursor ion at m/z=153.02 and fragment ion at m/z=109.01[M-H-COO]$^-$, identified as protocatechuic acid (Sun, et al., 2015). Peak 3 (t_R 7.95 min) with m/z=609.15 and showed a fragment ion at m/z=447.08 and 284.99, which were related to the loss of hexose moiety and two hexose moieties, this compound was tentatively identified as cyanidin-3,5-O-diglucoside (Hao, et al., 2015; Hou, et al., 2013; Pereira-Caro, et al., 2013). Peak 4 (t_R 8.33 min) possessed the precursor ion at m/z 447.09, which fragmented with a loss of hexose group to produce a daughter ion at m/z 285, peak 4 was tentatively identified as cyanidin-3-glucoside (Hao, et al., 2015; Hirawan, et al., 2011; Hou, et al., 2013; Pereira-Caro, et al., 2013). Peak 5 (t_R 9.07 min) with m/z=461.11 was tentatively identified as peonidin-3-glucoside (Hao, et al., 2015; Hirawan, et al., 2011; Hou, et al., 2013; Pereira-Caro, et al., 2013) and produced fragment ion at m/z 299.05, which correspond to the loss of a hexose moiety. Peak 6 (t_R 9.83 min) was tentatively identified as cyanidin (Hao, et al., 2015) with the negatively charged molecular ion ([M-H]$^-$) at m/z 285.04 and fragment ion at m/z=257.03 and 241.04. Peak 7 (t_R 10.12 min) with the precursor ion at m/z = 167.04 and fragment ions at m/z = 108.01, 123.00 and 151.96 was tentatively suggested as vanillic acid (Wang, et al., 2014). Peak 8 (t_R 10.57 min) showed the precursor ion at m/z = 463.09 and fragment ions at m/z = 301.01 (a loss of a hexose moiety), 281.23, and 395.24, and tentatively identified as delphinidin-3-glucooside (Li, et al., 2012; Oh,

et al., 2008). Peak 9 (t_R 10.83min) was tentatively identified as gingerglycolipid B (Sun, et al., 2017) based on the precursor ion at m/z = 723.50 and fragment ions at m/z = 677.46, 397.17.

A UPLC-LTQ-Orbitrap-MS/MS method was established to quantify the individual compounds in BRAEs. The calibration curves of cyanidin-3-glucoside, syringic acid, protocatechuic acid and vanillic acid were y=6.1959 x+4.2681 (R^2=0.9999), y=8.3515 x-46.26 (R^2=0.9999), y=4.2743 x-25.268 (R^2=0.9999), and y=4.1554 x-40.082 (R^2=0.9997), respectively. As shown in Table 2, the major anthocyanin in BRAE, D-BRAE and E-BRAE was cyanidin-3-glucoside (Fig.1, peak 4; 27.45±0.38, 17.70±0.41 and 7.45±0.12 mg C3G/g DW, respectively.), along with the following four minor components: peonidin-3-glucoside (3.22±0.26, 2.12±0.03 and 1.32±0.01 mg C3G/g DW, respectively.), cyanidin-3,5-diglucoside (not detected, 1.21±0.11 and 1.39±0.14 mg C3G/g DW, respectively.), cyanidin (not detected, 1.13±0.03 and 0.36±0.01 mg C3G/g DW, respectively.) and delphinidin-3-glucooside (1.37±0.04, 0.62±0.01 and 0.70±0.01 mg C3G/g DW, respectively.), These results are consistent with previous studies (Hao, et al., 2015; Zhu, et al., 2018b). Compared with BRAE, the contents of cyanidin-3-glucoside and peonidin-3-glucoside decreased significantly in D-BRAE by 35.52% and 34.16%, respectively ($p<0.05$), and in E-BRAE by 72.86% and 59.01%, respectively. The content of protocatechuic acid was increased obviously in E-BRAE by 3.1-fold. Cyanidin was found in D-BRAE and E-BRAE (1.13±0.03 and 0.36±0.01 mg C3G/g DW, respectively), whereas BRAE did not contain these anthocyanins. Anthocyanins were thermolabile, while the

drum-dried and extrusion were thermally processed. Cyanidin and protocatechuic acid are the degradation products of C3G.

3.2 The TPC/TFC/TAC of BRAEs

The TPC, TFC, and TAC of the BRAEs are shown in Table 3. The BRAE showed higher contents in total phenolic and anthocyanins (138.82±4.21 mg GAE/g DW and 121.79±8.28 mg C3G/100 g DW, respectively) than in D-BRAE and E-BRAE, while the TFC of E-BRAE (68.27±2.78 mg CAE/g DW) was higher than of BRAE and D-BRAE. D-BRAE exhibited the lowest content of phenolics (59.74±2.05 mg GAE/g DW), flavonoids (10.30±0.18 mg CAE/g DW), and anthocyanins (19.62±0.89 mg C3G/g DW) among the different samples. Compared with BRAE, the TPC and TAC of D-BRAE and E-BRAE were significantly decreased.

3.3 Antioxidant activities

As shown in Table 3, D-BRAE and E-BRAE exhibited significant decrease in the DPPH radical scavenging activity (0.05±0.01 mM trolox/g DW and 0.19±0.07 mM trolox/g DW, respectively) compared to that in BRAE (0.29±0.10 mM trolox/g DW). The ABTS radical scavenging activity of BRAE was 3.27±0.36 mM trolox/g DW, higher than that in D-BRAE and E-BRAE (0.30±0.03 mM trolox/g DW and 1.44±0.02 mM trolox/g DW, respectively). The ferric reducing activity of BRAE (1.02±0.16 mM FeSO4/g DW) was significantly higher than that of D-BRAE and E-BRAE (0.24±0.02 mM FeSO4/g DW and 0.40±0.03 mM FeSO4/g DW, respectively, $p<0.05$). It is obvious that drum-dried and extruded treatment decreased

Journal Pre-proof

the anti-oxidant activities of BRAEs *in vitro*, and the the antioxidant activities of E-BRAE significantly higher than those of D-BRAE.

3.4 Anti-inflammatory activities

3.4.1 Cell Cytotoxicity

The cytotoxic effects of BRAE, D-BRAE and E-BRAE were determined using the MTT assay. As shown in Figure 3, the cell viability of the RAW 264.7 cells was above 100% at a concentration of 50–400 μg/mL among the three BRAEs, which demonstrated that the survival rate of RAW 264.7 cells might be influenced by the BRAEs. To investigate the relationship between concentration and anti-inflammatory effect of BRAEs, three concentrations of BRAEs (50, 200, and 400 μg/mL) were selected for subsequent experiments.

3.4.2 Effects of BRAEs on NO/PGE2 production, iNOS and COX-2 mRNA and proteins expression in RAW264.7 cells

NO and PGE2 are the important inflammatory signal transduction molecules, which are generated by iNOS and COX-2, respectively (Meram & Wu, 2017; Oh, et al., 2017). As shown in Fig 4A and B, compared to the untreated control group (NC), LPS stimulation significantly increased the secretion of NO and PGE2 ($p<0.001$). BRAE, D-BRAE and E-BRAE dose-dependently suppressed NO secretion in LPS-stimulated macrophages. When treated with the highest concentration (400 μg/mL), BRAE, D-BRAE and E-BRAE reduced the levels of NO in activated macrophages by 66.5%, 41.8%, and 78.0%, respectively. BRAE (50, 200, 400 μg/mL),

D-BRAE (200, 400 μg/mL), and E-BRAE (50, 200, 400 μg/mL) significantly decreased the PGE2 production in LPS-induced RAW264.7 cells. Pretreatment with 400 μg/mL BRAE and D-BRAE and 50 μg/mL E-BRAE showed the highest inhibitory activities of PGE2 secretion (29.5%, 31.8%, and 27.6%, respectively). These results indicated that drum-dried and extruded BRAE did not affect the anti-inflammatory effects of BRAE on NO and PGE2 secretion.

Studies have shown that NO and PGE2 are synthesized by iNOS and COX-2, respectively, in a pro-oxidant, pro-inflammatory environment (Vendrame & Klimis-Zacas, 2015). Therefore, the changes in iNOS and COX-2 were detected by RT-qPCR and Western blot in this study. Results are shown in Fig 4C, D, E, and F. Compared with the NC group, the mRNA expression of iNOS and COX-2 was dramatically upregulated by LPS ($p < 0.001$). However, BRAE (200 and 400 μg/mL), D-BRAE (400 μg/mL), and E-BRAE (50, 200, 400 μg/mL) significantly suppressed the mRNA expression of iNOS. Pretreatment with 400 μg/mL BRAE and 200 μg/mL showed the highest inhibitory activities of iNOS mRNA expression (69.4%, 65.4%, and 71.6%, respectively). BRAE (200 and 400 μg/mL), D-BRAE (400 μg/mL), and E-BRAE (200, 400 μg/mL) significantly decreased the mRNA expression in COX-2 dose-dependent manner. When treated with the highest concentration (400 μg/mL), D-BRAE and E-BRAE were more effective on COX-2 mRNA levels (0.20±0.01 and 0.11±0.01, respectively) relative to BRAE (0.03±0.01). Similarly, as shown in Fig. 4E and F, incubation of macrophages with LPS alone dramatically increased the expression of iNOS and COX-2 at the protein level than in the untreated cells (NC, p

373 <0.05). BRAE, D-BRAE, and E-BRAE inhibited the protein expression of iNOS and
374 COX-2 at 400 μg/mL; BRAE and E-BRAE showed the optimal inhibitory ability of
375 iNOS expression (0.24±0.03) and COX-2 (0.33±0.12), respectively. In conclusion, it
376 was observed that BRAEs reduced NO and PGE2 secretion (Figure 4A and B) to
377 inhibit the inflammatory response by downregulating iNOS and COX-2 mRNA and
378 protein expression (Fig 4 C, D, E, and F). Furthermore, the inhibitory activities of
379 BRAE were not affected by drum-drying and extrusion.

380 *3.4.3 Inhibitory effects of BRAEs on inflammatory cytokines production in RAW 264.7*
381 *cells*

382 Studies have reported that several pro-inflammatory cytokines, such as TNF-α,
383 IL-6, and IL-1β, play important roles in the inflammatory response, and they can
384 activate macrophages when stimulated by LPS (Xie, et al., 2019). Furthermore, the
385 expression of COX-2 could be promoted by IL-1β decisively, and the secretion of NO
386 could promote the release of pro-inflammatory cytokines, while IL-6 could directly
387 induce the expression of iNOS (Ren, et al., 2019). To explore the anti-inflammatory
388 ability of BRAEs, the release and mRNA expression of pro-inflammatory cytokines
389 including TNF-α, IL-6, and IL-1β were determined by ELISA and RT-qPCR assays.
390 As shown in Figure 5 A-F, the production and mRNA expression levels of TNF-α,
391 IL-6, and IL-1β were significantly increased by LPS stimulation alone compared with
392 those in the NC group ($p<0.01$). Pretreatment with BRAE, D-BRAE (200 μg/mL, 400
393 μg/mL) and E-BRAE (400 μg/mL) significantly decreased TNF-α production in
394 LPS-induced RAW264.7 cells. The TNF-α inhibitory activity of 200 μg/mL BRAE

(33.4%) and 400 μg/mL D-BRAE (41.2%) were obviously superior to 400 μg/mL E-BRAE (13.4%). BRAE, D-BRAE (200, 400 μg/mL) and E-BRAE significantly suppressed the mRNA expression of TNF-α (Fig 5 B). Furthermore, pretreatment with 400 μg/mL BRAE and 50 μg/mL showed the highest inhibitory activity of TNF-α mRNA expression.

IL-6 and IL-1β are macrophage activators and play important roles in inflammatory diseases (Xie, et al., 2019). As shown in Fig 5 C, pretreatment with BRAE (400 μg/mL), D-BRAE, and E-BRAE (200 and 400 μg/mL) dramatically suppressed the secretion of IL-6. BRAE, D-BRAE, and E-BRAE dose-dependently suppressed the mRNA expression of IL-6 (Fig 5 D). When treated at the highest concentration (400 μg/mL), BRAE and E-BRAE showed stronger inhibitory abilities than D-BRAE at IL-6 mRNA level. As shown in Fig 5 E, pretreatment of D-BRAE and E-BRAE at 200 μg/mL significantly decreased IL-1β production. Similarly, the mRNA expression of IL-1β was significantly downregulated by BRAE, D-BRAE, and E-BRAE. BRAE at 400 μg/mL, D-BRAE at 50 μg/mL, and E-BRAE at 200 μg/mL showed the highest inhibitory activities of IL-1β mRNA expression. Taken together, the above results demonstrated that BRAEs could inhibit LPS-induced inflammatory responses by suppressing the secretion and mRNA expression of TNF-α, IL-6 and IL-1β. Therefore, the anti-inflammatory activities of BRAE were not affected by drum-dried and extrusion processes.

3.4.4 Effects of BRAEs on NF-κB and MAPK activation

Journal Pre-proof

The MAPK and NF-κB are two important signaling pathways in inflammation (Montoya, et al., 2019). The MAPK pathway includes three major subfamilies: extracellular signal-regulated kinase (ERK) 1/2, c-Jun NH2-terminal kinase (JNK), and p38, which play important roles in regulating the production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8) and inflammatory mediators (iNOS, COX-2) (Wang, et al., 2018; Zhang, et al., 2019b).Therefore, MAPK and NF-κB signal pathways were investigated to further clarify the anti-inflammatory mechanism of BRAEs. The expression levels of phospho-JNK, JNK, phospho-ERK, ERK, phosphor- IκBα, IκBα, phosphor-p65, and p65 were analyzed by western blotting. Fig 6A shows that LPS induced the phosphorylation of JNK and ERK ($p<0.001$). However, BRAE, D-BRAE and E-BRAE at 400 μg/mL dramatically inhibited the phosphorylation of JNK by 62.43%, 26.28% and 82.24%, respectively ($p<0.001$). The phosphorylation of ERK was significantly inhibited by 400 μg/mL E-BRAE (48.26%, $p<0.01$). However, the expression level of p-p38 was not affected in this study (data not shown). E-BRAE (400 μg/mL) treatment showed the highest inhibitory activities in the phosphorylation of JNK and ERK among BRAEs.

The regulation of the NF-κB pathway of BRAEs was determined by western blotting (Fig. 6B) immunofluorescence analysis (Fig. 6C). The phosphorylation of IκBα and p65 was dramatically upregulated by LPS ($p<0.001$), while the pretreatment with BRAE, D-BRAE and E-BRAE (400 μg/mL) significantly suppressed the phosphorylation of IκBα and p65 (52.19% and 20.46%, 24.43% and 40.14%, 75.92% and 68.91%, respectively). Macrophages pretreated with 400 μg/mL E-BRAE showed

438 the highest inhibitory activities in the phosphorylation of IκBα and p65 among
439 BRAEs. As shown in Fig 6 C, compared with NC group, LPS stimulated significantly
440 increased the p65 nuclear translocation ($p<0.001$), while the pretreatment of BRAE
441 (-47.9%), D-BRAE (-31.3%) and E-BRAE (-25.9%) at 400 μg/mL dramatically
442 inhibited the improvement ($p<0.01$). The results indicated that BRAEs could block
443 the phosphorylation of IκBα via inhibition of NF-κB p65 translocation into the
444 nucleus.

445 **4. Discussion**

446 Black rice is a special cultivar of rice mainly cultivated in Southeast Asia. It has
447 a higher content of phenolic and anthocyanin compounds than white rice. Many
448 studies have shown that cyanidin-3-O-glucoside and peonidin-3-O-glucoside are the
449 major anthocyanins in black rice (Pang, et al., 2018; Pedro, et al., 2016;
450 Sangkitikomol, et al., 2010; Shao, et al., 2014; Sompong, et al., 2011; Sumczynski,
451 Kotásková, et al., 2016; Zhang, et al., 2015). Studies have shown that anthocyanins
452 exert strong biological activities, including antioxidant (Sompong, et al., 2011; Zhang,
453 et al., 2015), anti-inflammatory (Limtrakul, et al., 2015; Zhao, et al., 2018), anticancer
454 (Chen, et al., 2015; Hui, et al., 2010), anti-diabetes (Kang, et al., 2013; Sirilun, et al.,
455 2016), and anti-obesity activities (Kwon, et al., 2007). Drum drying and extrusion are
456 two important processing methods in the food industry (Riaz, et al., 2009) . Studies
457 have shown that the bioactive composition and functional properties of dietary
458 compounds could be affacted by processing (Bhawamai, et al., 2016; Fischer, et al.,
459 2013; Hiemori, et al., 2009). Nonetheless, the chemical constituents, antioxidant and

460 anti-inflammatory abilities of BRAE with different processing methods remain poorly
461 investigated. In this study, the chemical constituents of BRAE, D-BRAE and
462 E-BRAE were analyzed using UPLC-LTQ-Orbitrap-MS^2 techniques. The results
463 showed that the major anthocyanin in BRAE, D-BRAE and E-BRAE was
464 cyanidin-3-glucoside (Fig.1, Peak 4) along with four minor components:
465 peonidin-3-glucoside, cyanidin-3,5-diglucoside, cyanidin and
466 delphinidin-3-glucooside. This is consistent with previous research in black rice (Hao,
467 et al., 2015; Zhu, et al., 2018a). Compared with BRAE, the contents of
468 cyanidin-3-glucoside and peonidin-3-glucoside were evidently decreased in D-BRAE
469 and E-BRAE, and the protocatechuic acid was increased obviously in E-BRAE.
470 Cyanidin was found in D-BRAE and E-BRAE, but not in BRAE. Drum drying and
471 extrusion were thermal processes, whereas anthocyanins were thermolabile, which
472 may be the possible reason for the reduced anthocyanin content in D-BRAE and
473 E-BRAE. Cyanidin and protocatechuic acid are the degradation products of C3G.
474 Interestingly, these results were the same as those of a previous study, which showed
475 that thermal cooking decreased total anthocyanin and C3G contents, but increased the
476 content of protocatechuic acid in black rice (Bhawamai, et al., 2016). Hiemori et al.,
477 (2009) showed that thermal cooking significantly decreased the content of
478 cyanidin-3-glucoside, while the content of protocatechuic acid was higher than that of
479 raw black rice. In the present study, compared with BRAE, the TPC and TAC of
480 D-BRAE and E-BRAE were significantly decreased. Studies have shown that
481 polyphenol and anthocyanins are labile to heat cooking, and the anthocyanin content

Journal Pre-proof

is reduced after thermal cooking in phenol and anthocyanin-rich foods such as black rice and blueberry juice (Bhawamai, et al., 2016; Buckow, et al., 2010; Fischer, et al., 2013). The results obtained by Ti et al. (2015) determined that the TPC and TAC were significantly decreased after extrusion in black rice, which was similar to our results. Results obtained by Surh et al. (2014) indicated that the anthocyanin content was dramatically decreased by roasted, steamed, pan-fried and boiled treatments. Therefore, drum-dried and extrusion significantly decreased the contents of phenolic and anthocyanins in black rice, which was expressed as the loss of C3G and the increase in degradation products including cyanidin and protocatechuic acid.

Research has indicated a high correlation between phenolic content and antioxidant activity (Shao, et al., 2018), owing to the antioxidant activities of anthocyanins. Results indicated that BRAE showed the strongest antioxidant activities, as measured by DPPH, ABTS, and FRAP assays among the three groups. The antioxidant activities of E-BRAE were significantly higher than those of D-BRAE. The results of antioxidant activities were similar to those of TPC and TAC (Table 3). The bioactive compounds in black rice were heat-labile and degraded during drum-drying and extrusion, which may be related to the reduction of antioxidant activities. A study by Mora-Rochin et al. (2010) confirmed that 55% of anthocyanins were lost by extrusion in blue maize, and ORAC decreased by 6.8-24.8%.

Additionally to assess the antioxidant capacity, studies have shown that black rice anthocyanin extracts possess extremely anti-inflammatory effects (Hao, et al., 2015; Limtrakul, et al., 2015; Zhu, et al., 2018a). During inflammation, the secretion

504 of pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β, and inflammatory
505 mediators, including NO and PGE2, were increased (Du, et al., 2018). iNOS and
506 COX-2 are essential enzymes that generate NO and PGE_2. In this study, it was
507 observed that BRAE decreased the production of NO and PGE2 (Fig 4A and B) by
508 downregulating the iNOS and COX-2 expression at the protein and mRNA levels (Fig
509 4 C-F). The production and mRNA expression of proinflammatory cytokines,
510 including TNF-α, IL-6, and IL-1β, were dramatically suppressed by BRAE (Fig
511 5A-F). Additionally, the processes of drum-dried and extrusion did not influence the
512 anti-inflammatory activities of BRAEs on pro-inflammatory cytokines and
513 inflammatory mediator production. The results were similar to those of a previous
514 study by Bhawamai et al. (2016), who reported that both raw and thermal cook black
515 rice extracts possessed similar anti-inflammatory activities on NO, IL-6, and TNF-α
516 secretion in LPS-stimulated RAW264.7 cells. A study by Min et al. (2010) confirmed
517 that black rice extracts, C3G and its metabolites cyanidin and protocatechuic acid
518 dramatically inhibited the secretion of NO, PGE2, TNF-α and IL-1β, as well as the
519 mRNA expression of iNOS and COX-2 in RAW264.7 cells.

520 Studies have shown that the activated MAPK signaling pathway plays an
521 important role in regulating the production of pro-inflammatory cytokines (TNF-α,
522 IL-1β, IL-6, IL-8) and inflammatory mediators (iNOS, COX-2) (Wang, et al., 2018;
523 Zhang, et al., 2019b). Moreover, it has been determined that MAPK signaling
524 pathway could regulate the activation of downstream NF-κB pathway (Cai, et al.,
525 2018). In this study, BRAE, D-BRAE, and E-BRAE (400 μg/mL) exerted

anti-inflammatory effects by inhibiting the phosphorylation of JNK, ERK, IκBα and p65, and inhibiting NF-κB p65 translocation into the nucleus in LPS-induced RAW264.7 cells (Fig 6). The drum-dried and extrusion processes did not reduce the inhibitory consequences. Moreover, the inhibitory activities of BRAE on the phosphorylation of JNK, ERK, IκBα, and p65 was significantly enhanced by the extrusion process. The results were similar to those of a previous study by Leem et al., (2014a), who found that *Acanthopanax senticosus* leaves possessed strong anti-inflammatory activities in HMC-1 cells, thereby decreasing the serum NO, MDA, and TNF-α levels in acute inflammatory rats, and the extruded process enhanced the anti-inflammatory activities in a dose-dependent manner. Bhawamai et al., (2016) reported that thermal cooking decreased the anthocyanin content and antioxidant abilities, but did not affect the anti-inflammatory activities of black rice in LPS-induced macrophages. In brief, it could be considered that although drum-dried and extrusion decreased the contents of anthocyanins in black rice, the enhance of its metabolites cyanidin and protocatechuic acid could also exert anti-inflammatory activities directly.

In conclusion, this study analyzed the effects of drum-drying and extrusion on the contents of bioactive substances, which were related to the antioxidant and anti-inflammatory activities in black rice. First, the extracts of the three black rice samples were identified by UPLC-LTQ-Orbitrap-MS/MS techniques. Compared with BRAE, the species of major bioactive substances were not altered by drum-dried and extrusion; the contents of cyanidin-3-glucoside and peonidin-3-glucoside were

548 significantly decreased in D-BRAE and E-BRAE, while protocatechuic acid was
549 increased obviously in E-BRAE. Cyanidin was found in D-BRAE and E-BRAE,
550 whereas BRAE was not. Meanwhile, the TPC and TAC were significantly decreased
551 by drum-drying and extrusion, while the antioxidant activities exerted the similar
552 trends. The D-BRAE was inferior to E-BRAE in terms of TPC, TAC and antioxidant
553 activities. Moreover, cell assays suggested that the pro-inflammatory cytokines,
554 inflammatory mediators, and enzymes were dramatically suppressed by BRAE while
555 drum-dried and extrusion did not inhibit the anti-inflammatory abilities. BRAE
556 inhibited the inflammatory response via regulating the activation of MAPK and
557 NF-κB inflammatory signaling pathways, which was not affected by drum-dried and
558 extrusion processes. The results obtained may provide scientific guidance for
559 whole-grain resource utilization and daily healthy food intake to regulate oxidant or
560 inflammation-related diseases.

561 **Acknowledgements**

562 This study was financially supported by the National Natural Science Foundation
563 of China (82060781), China Postdoctoral Science Foundation (2020M671975),
564 Department horizontal project of Nanchang University (Grant No. 11002824), the
565 Research Program of State Key Laboratory of Food Science and Technology,
566 Nanchang University (Grant No. SKLF-ZZA-201909) and the Science and
567 Technology Program of Jiangxi Province (Grant No. 5511, 20182ABC28010)

568 **ABBREVIATIONS**

Journal Pre-proof

BRAE, Black rice anthocyanin extracts; D-BRAE, Drum dried Black rice anthocyanin extracts; E-BRAE, Extruded Black rice anthocyanin extracts; LPS, Lipopolysaccharide; NO, nitric oxide; PGE2, prostaglandin E2; ELISA, enzyme-linked immunosorbent; iNOS, nitric oxide synthase; COX-2, cyclooxygenase-2; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; IL-1β, interleukin-1β; MAPK, mitogen-activated protein kinases; NF-κB, nuclear factor-kappa B; JNK, jun-amino-terminal kinase; ERK, extracellular signal-regulated kinase; IκBα, inhibitory factor kappa B alpha; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel; TPC, total phenolic content; GAE, gallic acid equivalent; TFC, total flavonoid contents; CAE, catechin equivalent; TAC, total anthocyanin contents; C3G, cyanidin-3-glucoside equivalent; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FRAP, Ferric reducing antioxidant power; FBS, Fetal Bovine Serum; DMSO, Dimethylsulfoxide.

References

Bhawamai, S., Lin, S.-H., Hou, Y.-Y., & Chen, Y.-H. (2016). Thermal cooking changes the profile of phenolic compounds, but does not attenuate the anti-inflammatory activities of black rice. *Food & Nutrition Research, 60*.

Buckow, R., Kastell, A., Terefe, N., & Versteeg, C. (2010). Pressure and Temperature Effects on Degradation Kinetics and Storage Stability of Total Anthocyanins in Blueberry Juice. *Journal of Agricultural and Food Chemistry, 58*, 10076-10084.

Cai, Z., Xu, P., Wu, Z., & Pan, D. (2018). Anti-inflammatory activity of surface layer

protein SlpA of Lactobacillus acidophilus CICC 6074 in LPS-induced RAW 264.7 cells and DSS-induced mice colitis. *Journal Of Functional Foods, 51*, 16-27.

Chen, X.-Y., Zhou, J., Luo, L.-P., Han, B., Li, F., Chen, J.-Y., Zhu, Y.-F., Chen, W., & Yu, X.-P. (2015). Black Rice Anthocyanins Suppress Metastasis of Breast Cancer Cells by Targeting RAS/RAF/MAPK Pathway. *Biomed Research International, 2015*, 1-11.

Donato, P., Rigano, F., Cacciola, F., Schure, M., Farnetti, S., Russo, M., Dugo, P., & Mondello, L. (2016). Comprehensive two-dimensional liquid chromatography–tandem mass spectrometry for the simultaneous determination of wine polyphenols and target contaminants. *Journal Of Chromatography A, 1458*, 54-62.

Du, L., Li, J., Zhang, X., Wang, L., & Zhang, W. (2018). Pomegranate peel polyphenols inhibits inflammation in LPS-induced RAW264.7 macrophages via the suppression of MAPKs activation. *Journal Of Functional Foods, 43*, 62-69.

Fischer, U. A., Carle, R., & Kammerer, D. R. (2013). Thermal stability of anthocyanins and colourless phenolics in pomegranate (Punica granatum L.) juices and model solutions. *Food Chemistry, 138*(2), 1800-1809.

Hao, J., Zhu, H., Zhang, Z., Yang, S., & Li, H. (2015). Identification of anthocyanins in black rice (Oryza sativa L.) by UPLC/Q-TOF-MS and their in vitro and in vivo antioxidant activities. *Journal Of Cereal Science, 64*, 92-99.

Journal Pre-proof

Journal Pre-proof

611 Henríquez, M., Almonacid, S., Lutz, M., Simpson, R., & Valdenegro, M. (2013).
612 Comparison of three drying processes to obtain an apple peel food ingredient.
613 *CyTA - Journal of Food, 11*(2), 127-135.

614 Hiemori, M., Koh, E., & Mitchell, A. (2009). Influence of Cooking on Anthocyanins
615 in Black Rice (Oryza sativa L. japonica var. SBR). *Journal of Agricultural and*
616 *Food Chemistry, 57*, 1908-1914.

617 Hirawan, R., Diehl-Jones, W., & Beta, T. (2011). Comparative Evaluation of the
618 Antioxidant Potential of Infant Cereals Produced from Purple Wheat and Red
619 Rice Grains and LC-MS Analysis of Their Anthocyanins. *Journal of Agricultural*
620 *and Food Chemistry, 59*(23), 12330-12341.

621 Hou, Z., Qin, P., Zhang, Y., Cui, S., & Ren, G. (2013). Identification of anthocyanins
622 isolated from black rice (Oryza sativa L.) and their degradation kinetics. *Food*
623 *Research International, 50*(2), 691-697.

624 Hui, C., Bin, Y., Xiaoping, Y., Long, Y., Chunye, C., Mantian, M., & Wenhua, L.
625 (2010). Anticancer Activities of an Anthocyanin-Rich Extract From Black Rice
626 Against Breast Cancer Cells In Vitro and In Vivo. *Nutrition and cancer, 62*,
627 1128-1136.

628 Kang, M.-K., Lim, S., Lee, J.-Y., Yeo, K., & Kang, Y.-H. (2013). Anthocyanin-Rich
629 Purple Corn Extract Inhibit Diabetes-Associated Glomerular Angiogenesis. *Plos*
630 *One, 8*, e79823.

631 Kwon, S.-H., Ahn, I. S., Kim, S.-O., Kong, C.-S., Chung, H.-Y., Do, M.-S., & Park,
632 K.-Y. (2007). Anti-Obesity and Hypolipidemic Effects of Black Soybean
633 Anthocyanins. *Journal Of Medicinal Food, 10*, 552-556.

634 Lee, Y.-M., Yoon, Y., Yoon, H., Park, H.-M., Song, S., & Yeum, K.-J. (2017). Dietary
635 Anthocyanins against Obesity and Inflammation. *Nutrients, 9*, 1089.

636 Leem, K.-H., Lee, S., & Kim, H. K. (2014a). Extrusion process enhances the
637 anti-inflammatory effect of Acanthopanax senticosus leaves. *Food Science And*
638 *Biotechnology, 23*(3), 911-916.

639 Leem, K.-H., Lee, S., & Kim, H. K. (2014b). Extrusion Process Enhances the
640 Anti-inflammatory Effect of Acanthopanax senticosus Leaves. *Food Science And*
641 *Biotechnology, 23*, 911-916.

642 Li, H., Deng, Z., Zhu, H., Hu, C., Liu, R., Young, J. C., & Tsao, R. (2012). Highly
643 pigmented vegetables: Anthocyanin compositions and their role in antioxidant
644 activities. *Food Research International, 46*(1), 250-259.

645 Limtrakul, P., Yodkeeree, S., Pitchakarn, P., & Punfa, W. (2015). Suppression of
646 Inflammatory Responses by Black Rice Extract in RAW 264.7 Macrophage Cells
647 via Downregulation of NF-kB and AP-1 Signaling Pathways. *Asian Pacific*
648 *journal of cancer prevention : APJCP, 16*, 4277-4283.

649 Liu, J., Huang, H., Huang, Z., Ma, Y., Zhang, L., He, Y., Li, D., Liu, W., Goodin, S.,
650 Zhang, K., & Zheng, X. (2019). Eriocitrin in combination with resveratrol

ameliorates LPS-induced inflammation in RAW264.7 cells and relieves TPA-induced mouse ear edema. *Journal Of Functional Foods, 56*, 321-332.

Meram, C., & Wu, J. (2017). Anti-inflammatory effects of egg yolk livetins (α, β, and γ-livetin) fraction and its enzymatic hydrolysates in lipopolysaccharide-induced RAW 264.7 macrophages. *Food Research International, 100*, 449-459.

Min, S.-W., Ryu, S.-N., & Kim, D.-H. (2010). Anti-inflammatory effects of black rice, cyanidin-3-O-β-d-glycoside, and its metabolites, cyanidin and protocatechuic acid. *International Immunopharmacology, 10*(8), 959-966.

Montoya-Rodríguez, A., de Mejía, E. G., Dia, V. P., Reyes-Moreno, C., & Milán-Carrillo, J. (2014). Extrusion improved the anti-inflammatory effect of amaranth (Amaranthus hypochondriacus) hydrolysates in LPS-induced human THP-1 macrophage-like and mouse RAW 264.7 macrophages by preventing activation of NF-κB signaling. *Molecular Nutrition & Food Research, 58*(5), 1028-1041.

Montoya, T., Castejón, M. L., Sánchez-Hidalgo, M., González-Benjumea, A., Fernández-Bolaños, J. G., & Alarcón de-la-Lastra, C. (2019). Oleocanthal Modulates LPS-Induced Murine Peritoneal Macrophages Activation via Regulation of Inflammasome, Nrf-2/HO-1, and MAPKs Signaling Pathways. *Journal of Agricultural and Food Chemistry, 67*(19), 5552-5559.

Mora-Rochin, S., Gutiérrez-Uribe, J. A., Serna-Saldivar, S. O., Sánchez-Peña, P., Reyes-Moreno, C., & Milán-Carrillo, J. (2010). Phenolic content and antioxidant

672 activity of tortillas produced from pigmented maize processed by conventional
673 nixtamalization or extrusion cooking. *Journal Of Cereal Science*, *52*(3), 502-508.

674 Oh, K.-S., Patel, H., Gottschalk, R., Lee, W., Baek, S., Fraser, I., Hager, G., & Sung,
675 M.-H. (2017). Anti-Inflammatory Chromatinscape Suggests Alternative
676 Mechanisms of Glucocorticoid Receptor Action. *Immunity, 47*.

677 Oh, Y. S., Lee, J. H., Yoon, S. H., Oh, C. H., Choi, D. S., Choe, E., & Jung, M. Y.
678 (2008). Characterization and Quantification of Anthocyanins in Grape Juices
679 Obtained from the Grapes Cultivated in Korea by HPLC/DAD, HPLC/MS, and
680 HPLC/MS/MS. *Journal Of Food Science*, *73*(5), C378-C389.

681 Pan, Y., Deng, Z. Y., Zheng, S. L., Chen, X., Zhang, B., & Li, H. Y. (2018). Daily
682 Dietary Antioxidant Interactions Are Due to Not Only the Quantity but Also the
683 Ratios of Hydrophilic and Lipophilic Phytochemicals. *Journal of Agricultural*
684 *and Food Chemistry*, 66(34), 9107-9120.

685 Pang, Y., Ahmed, S., Xu, Y., Beta, T., Zhu, Z., Shao, Y., & Bao, J. (2018). Bound
686 phenolic compounds and antioxidant properties of whole grain and bran of white,
687 red and black rice. *Food Chemistry, 240*, 212-221.

688 Pedro, A. C., Granato, D., & Rosso, N. D. (2016). Extraction of anthocyanins and
689 polyphenols from black rice (Oryza sativa L.) by modeling and assessing their
690 reversibility and stability. *Food Chemistry, 191*, 12-20.

691 Peng, Y., Yan, Y., Wan, P., Chen, D., Ding, Y., Ran, L., Mi, J., Lu, L., Zhang, Z., & Li,

X. (2019). Gut microbiota modulation and anti-inflammatory properties of anthocyanins from the fruits of Lycium ruthenicum Murray in dextran sodium sulfate-induced colitis in mice. *Free Radical Biology Medicine And Science In Sports And Exercise*.

Pereira-Caro, G., Watanabe, S., Crozier, A., Fujimura, T., Yokota, T., & Ashihara, H. (2013). Phytochemical profile of a Japanese black–purple rice. *Food Chemistry, 141*(3), 2821-2827.

Rainsford, K. (2003). Discovery, mechanisms of action and safety of ibuprofen. *International journal of clinical practice. Supplement*, 3-8.

Ren, J., Li, L., Wang, Y., Zhai, J., Chen, G., & Hu, K. (2019). Gambogic acid induces heme oxygenase-1 through Nrf2 signaling pathway and inhibits NF-κB and MAPK activation to reduce inflammation in LPS-activated RAW264.7 cells. *Biomedicine & Pharmacotherapy, 109*, 555-562.

Riaz, M. N., Asif, M., & Ali, R. (2009). Stability of Vitamins during Extrusion. *Crit Rev Food Sci Nutr, 49*(4), 361-368.

Roager, H. M., Vogt, J., Kristensen, M., Hansen, L., Ibrügger, S., Mærkedahl, R., Bahl, M., Lind, M., Nielsen, R., Frokiaer, H., Gøbel, R., Landberg, R., Ross, A., Brix, S., Holck, J., Meyer, A., Sparholt, M., Christensen, A., Carvalho, V., & Licht, T. (2017). Whole grain-rich diet reduces body weight and systemic low-grade inflammation without inducing major changes of the gut microbiome: A randomised cross-over trial. *Gut, 68*, gutjnl-2017.

Journal Pre-proof

713 Ruiz-Gutiérrez, G. M., Amaya-Guerra, A. C., Quintero-Ramos, A., Pérez-Carrillo, E.,
714 Ruiz-Anchondo, D. T., Báez-González, G. J., & Meléndez-Pizarro, O. C. (2015).
715 Effect of Extrusion Cooking on Bioactive Compounds in Encapsulated Red
716 Cactus Pear Powder. *Molecules, 20*(5).

717 Sangkitikomol, W., Tencomnao, T., & Rocejanasaroj, A. (2010). Effects of Thai black
718 sticky rice extract on oxidative stress and lipid metabolism gene expression in
719 HepG2 cells. *Genetics and molecular research : GMR, 9*, 2086-2095.

720 Sarawong, C., Schoenlechner, R., Sekiguchi, K., Berghofer, E., & Ng, P. K. W. (2014).
721 Effect of extrusion cooking on the physicochemical properties, resistant starch,
722 phenolic content and antioxidant capacities of green banana flour. *Food*
723 *Chemistry, 143*, 33-39.

724 Shao, Y., Hu, Z., Yu, Y., Mou, R., Zhu, Z., & Beta, T. (2018). Phenolic acids,
725 anthocyanins, proanthocyanidins, antioxidant activity, minerals and their
726 correlations in non-pigmented, red, and black rice. *Food Chemistry, 239*,
727 733-741.

728 Shao, Y., Xu, F., Sun, X., Bao, J., & Beta, T. (2014). Identification and quantification
729 of phenolic acids and anthocyanins as antioxidants in bran, embryo and
730 endosperm of white, red and black rice kernels (Oryza sativa L.). *Journal Of*
731 *Cereal Science, 59*(2), 211-218.

732 Sirilun, S., Sivamaruthi, B., Pengkumsri, N., Keapai, W., Kesika, P., Saelee, M.,
733 Tojing, P., Sirilun, S., Chaiyasut, K., Peerajan, S., & Lailerd, N. (2016).

Journal Pre-proof

734 Germinated Thai Black Rice Extract Protects Experimental Diabetic Rats from
735 Oxidative Stress and Other Diabetes-Related Consequences. *Pharmaceuticals, 10*, 3.

737 Soison, B., Jangchud, K., Jangchud, A., Harnsilawat, T., Piyachomkwan, K.,
738 Charunuch, C., & Prinyawiwatkul, W. (2014). Physico-functional and
739 antioxidant properties of purple-flesh sweet potato flours as affected by extrusion
740 and drum-drying treatments. *International Journal of Food Science &*
741 *Technology, 49*(9), 2067-2075.

742 Sompong, R., Siebenhandl-Ehn, S., Linsberger-Martin, G., & Berghofer, E. (2011).
743 Physicochemical and antioxidative properties of red and black rice varieties from
744 Thailand, China and Sri Lanka. *Food Chemistry, 124*(1), 132-140.

745 Sumczynski, D., Kotásková, E., Družbíková, H., & Mlček, J. (2016). Determination
746 of contents and antioxidant activity of free and bound phenolics compounds and
747 in vitro digestibility of commercial black and red rice (Oryza sativa L.) varieties.
748 *Food Chemistry, 211*, 339-346.

749 Sun, Y., Qin, Y., Li, H., Peng, H., Chen, H., Xie, H.-r., & Deng, Z. (2015). Rapid
750 characterization of chemical constituents in Radix Tetrastigma, a functional
751 herbal mixture, before and after metabolism and their
752 antioxidant/antiproliferative activities. *Journal Of Functional Foods, 18*,
753 300-318.

754 Sun, Y., Tsao, R., Chen, F., Li, H., Wang, J., Peng, H., Zhang, K., & Deng, Z. (2017).

The phytochemical composition, metabolites, bioavailability and in vivo antioxidant activity of Tetrastigma hemsleyanum leaves in rats. *Journal Of Functional Foods, 30*, 179-193.

Surh, J., & Koh, E. (2014). Effects of Four Different Cooking on Anthocyanins, Total Phenolics, and Antioxidant Activity of Black Rice. *Journal Of the Science Of Food And Agriculture, 94*.

Teng, H., Fang, T., Lin, Q., Song, H., Liu, B., & Chen, L. (2017). Red raspberry and its anthocyanins: Bioactivity beyond antioxidant capacity. *Trends In Food Science & Technology, 66*, 153-165.

Ti, H., Zhang, R., Zhang, M., Wei, Z., Chi, J., Deng, Y., & Zhang, Y. (2015). Effect of extrusion on phytochemical profiles in milled fractions of black rice. *Food Chemistry, 178*, 186-194.

Vendrame, S., & Klimis-Zacas, D. (2015). Anti-inflammatory effect of anthocyanins via modulation of nuclear factor- B and mitogen-activated protein kinase signaling cascades. *Nutrition Reviews, 73*, 348-358.

Wang, H., Zhang, L., Xu, S., Pan, J., Zhang, Q., & Lu, R. (2018). Surface-Layer Protein from Lactobacillus acidophilus NCFM Inhibits Lipopolysaccharide-Induced Inflammation through MAPK and NF-κB Signaling Pathways in RAW264.7 Cells. *Journal of Agricultural and Food Chemistry, 66*(29), 7655-7662.

Journal Pre-proof

775 Wang, Y., Liu, Y., Xiao, C., Liu, L., Hao, M., Wang, J., & Liu, X. (2014).
776 Simultaneous Determination of 15 Phenolic Constituents of Chinese Black Rice
777 Wine by HPLC-MS/MS with SPE. *Journal Of Food Science, 79*(6),
778 C1100-C1105.

779 Wu, T., Guo, X., Zhang, M., Yang, L., Liu, R., & Yin, J. (2017). Anthocyanin in black
780 rice, soybean and purple corn increase fecal butyric acid and prevent liver
781 inflammation in high fat diet-induced obese mice. *Food Funct., 8*.

782 Xie, C., Li, X., Zhu, J., Wu, J., Geng, S., & Zhong, C. (2019). Magnesium
783 isoglycyrrhizinate suppresses LPS-induced inflammation and oxidative stress
784 through inhibiting NF-κB and MAPK pathways in RAW264.7 cells. *Bioorganic*
785 *& Medicinal Chemistry, 27*(3), 516-524.

786 Zhang, H., Shao, Y., Bao, J., & Beta, T. (2015). Phenolic compounds and antioxidant
787 properties of breeding lines between the white and black rice. *Food Chemistry,*
788 *172*, 630-639.

789 Zhang, Q., Luna-Vital, D., & Gonzalez de Mejia, E. (2019a). Anthocyanins from
790 colored maize ameliorated the inflammatory paracrine interplay between
791 macrophages and adipocytes through regulation of NF-κB and JNK-dependent
792 MAPK pathways. *Journal Of Functional Foods*.

793 Zhang, Q., Luna-Vital, D., & Gonzalez de Mejia, E. (2019b). Anthocyanins from
794 colored maize ameliorated the inflammatory paracrine interplay between
795 macrophages and adipocytes through regulation of NF-κB and JNK-dependent

Journal Pre-proof

MAPK pathways. *Journal Of Functional Foods, 54*, 175-186.

Zhao, L., Zhang, Y., Liu, G., Hao, S., Wang, C., & Wang, Y. (2018). Black rice anthocyanin-rich extract and rosmarinic acid, alone and in combination, protect against DSS-induced colitis in mice. *Food & Function, 9*.

Zhu, Y., Sun, H., He, S., Lou, Q., Yu, M., Tang, M., & Tu, L. (2018a). Metabolism and prebiotics activity of anthocyanins from black rice (Oryza sativa L.) in vitro. *Plos One, 13*(4), e0195754.

Zhu, Y., Sun, H., He, S., Lou, Q., Yu, M., Tang, M., & Tu, L. (2018b). Metabolism and prebiotics activity of anthocyanins from black rice (Oryza sativa L.) in vitro. *Plos One, 13*, e0195754.

Journal Pre-proof

1 **Table 1.** Primer sequences of the genes used in RT-PCR.

Primer name	Forward (5'-3')	Reverse (5'-3')
β-actin	ATC ACT ATT GGC AAC GAG CG	TCA GCA ATG CCT GGG TAC AT
iNOS	CCC TCC TGA TCT TGT GTT GGA	TCA ACC CGA GCT CCT GGA A
COX-2	TGC TGT ACA AGC AGT GGC AA	GCA GCC ATT TCC TTC TCT CC
IL-6	TAG TCC TTC CTA CCC CAA TTT CC	TTG GTC CTT AGC CAC TCC TTC
IL-1β	CAA CCA ACA AGT GAT ATT CTC CAT G	GAT CCA CAC TCT CCA GCT GCA
TNF-α	TGT CTA CTC CTC AGA GCC CC	TGA GTC CTT GAT GGT GGT GC

2

Journal Pre-proof

Journal Pre-proof

Table 2. Characterization of chemical constituents of BRAEs by UPLC-LTQ-Orbitrap-MS^2

No.	t_R (min)	Formula	[M-H]⁻ ions (*m/z*)	Fragment ions (*m/z*)	Identification	Concentration (mg/g DW)		
						RAW	Drum-dried	Extruded
1	2.53	$C_9H_{10}O_5$	197.81	153.92 [M-H-COO]⁻, 135.88[M-H-H_2O-COO]⁻, 152.99, 170.0	Syringic acid	1.95±0.14 [c]	3.24±0.07 [b]	3.89±0.06 [a]
2	3.48	$C_7H_5O_4$	153.02	124.94, 109.01 [M-H-COO]⁻	Protocatechuic acid	2.31±0.08 [c]	2.64±0.12 [b]	9.50±0.10 [a]
3	7.95	$C_{27}H_{29}O_{16}$	609.15	284.99 [M-H-2hexose]⁻, 563.36 [M-H-HCOOH]⁻, 447.08[M-H-hexose]⁻, 499.12	Cyanidin 3,5-diglucoside	\	1.21±0.11 [a]	1.39±0.14 [a]
4	8.33	$C_{21}H_{19}O_{11}$	447.09	285.01[M-H-hexose]⁻, 279.21	Cyanidin-3-glucoside	27.45±0.38 [a]	17.70±0.41 [b]	7.45±0.12 [c]
5	9.07	$C_{22}H_{21}O_{11}$	461.11	299.05[M-H-hexose]⁻, 279.22	Peonidin-3-glucoside	3.22±0.26 [a]	2.12±0.03 [b]	1.32±0.01 [b]
6	9.83	$C_{15}H_9O_6$	285.04	257.03, 241.04	Cyanidin	\	1.13±0.03 [a]	0.36±0.01 [b]
7	10.12	$C_8H_7O_4$	167.04	123.00[M-H-COO]⁻, 151.96,108.01	Vanillic acid	2.52±0.23 [a]	1.53±0.08 [b]	1.85±0.12 [b]
8	10.57	$C_{21}H_{19}O_{12}$	463.09	301.01[M-H-hexose]⁻, 281.23, 395.24	Delphinidin-3-glucoside	1.37±0.04 [a]	0.62±0.01 [b]	0.70±0.01 [b]
9	10.83	$C_{34}H_{60}O_{16}$	723.50	677.46[M-H-HCOOH]⁻, 397.17	Gingerglycolipid B	\	\	\

Journal Pre-proof

Table 3. Main Antioxidant Components and Antioxidant Activities in Black Rice Extracts

Extracts	TPC[B] (mg GAE/g DW)	TFC (mg CAE/g DW)	TAC (mg C3G/100 g DW)	DPPH assay (mM trolox/g DW)	ABTS assay (mM trolox/g DW)	FRAP assay (mM $FeSO_4$/g DW)
BRAE[A]	138.82±4.21[a]	45.97±3.45[b]	121.79±8.28[a]	0.29±0.10[a]	3.27±0.36[a]	1.02±0.16[a]
D-BRAE	59.74±2.05[c]	10.30±0.18[c]	19.62±0.89[c]	0.05±0.01[c]	0.30±0.03[c]	0.24±0.02[b]
E-BRAE	113.75±6.24[b]	68.27±2.78[a]	33.68±1.95[b]	0.19±0.07[b]	1.44±0.02[b]	0.40±0.03[b]

A: BRAE, D-BRAE and E-BRAE represent Raw, Drum-dried and Extruded Black Rice Extracts, respectively.

B: TPC, total phenolic acid contents; TFC, total flavonoid acid contents; TAC, total anthocyanins contents;

Figure captions

Figure 1. HPLC chromatogram of the BRAE, D-BRAE and E-BRAE detected at 280 nm.

Figure 2. MS^2 spectra and the possible fragmentation patterns. (A) Protocatechuic acid, (B) Cyanidin 3,5-diglucoside, (C) Cyanidin-3-glucoside, (D) Peonidin-3-glucoside.

Figure 3. Effect of BRAE, D-BRAE and E-BRAE on cell viability of RAW264.7 cells. Cells were treated with different concentrations (25-500 μg/mL) of BRAE, D-BRAE and E-BRAE for 24 h, cell cytotoxicity was analyzed by MTT assay. Data are expressed as mean ± S.D. from three independent experiments.

Figure 4. Effect of BRAE, D-BRAE and E-BRAE on NO/PGE2 production, iNOS/COX-2 mRNA and protein expression in LPS-stimulated RAW264.7 cells. (A) NO production. (B) PGE2 production. (C) iNOS mRNA expressions. (D) COX-2 mRNA expressions. (E, F) iNOS and COX-2 protein expressions. Cells were pretreated with various concentrations of BRAEs for 1 h before stimulation of LPS (1 μg/mL) for 24 h. The mRNA levels were detected with real-time PCR. The protein expression was analyzed by Western blot. β-actin served as protein control. Data are expressed as mean ± SD. Bars with different letters in the same group indicate statistical difference compare with LPS group ($p < 0.05$); * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compare with the control group.

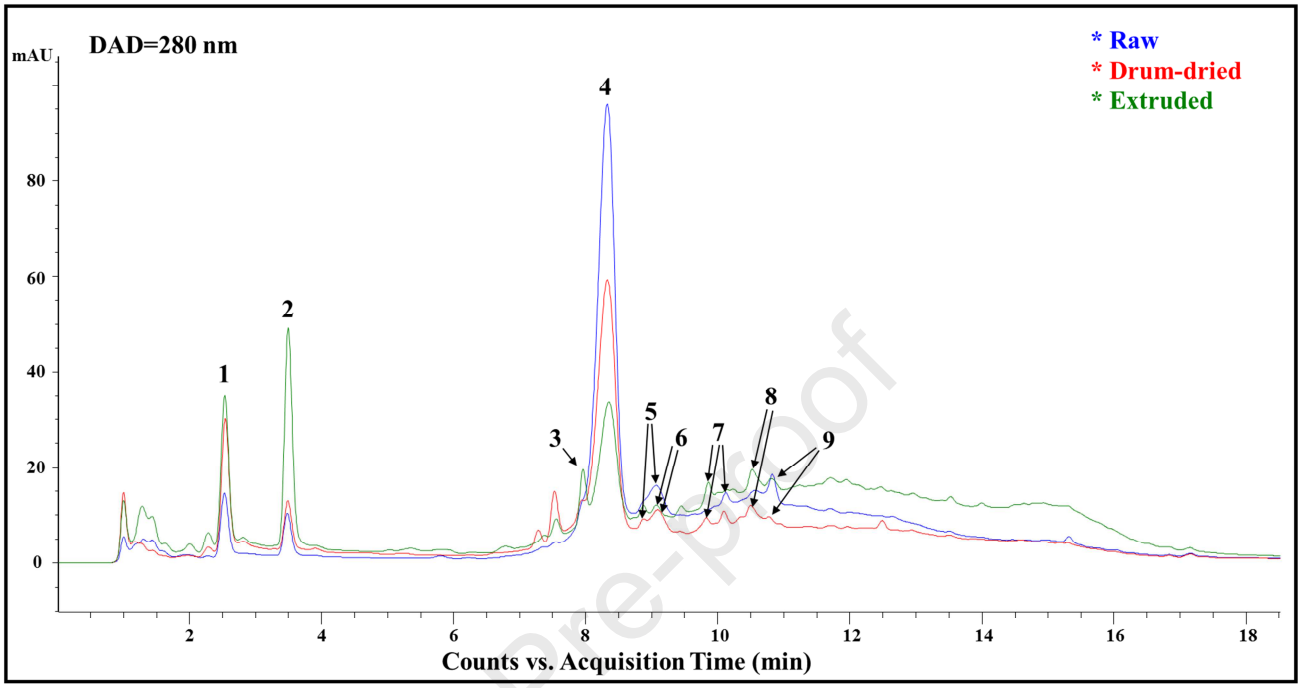
Figure 5. Effect of BRAE, D-BRAE and E-BRAE on production and mRNA expressions of pro-inflammatory cytokines. Cells were pre-treated with different

concentration of BRAE, D-BRAE or E-BRAE for 1 h. The secretion of TNF-α (A), IL-6 (C), and IL-1β (E) in the culture media were detected by ELISA after LPS (1 μg/mL) stimulated for 24 h. (B, D, F) The mRNA levels were detected with real-time PCR. Data are expressed as mean ± S.D. Bars with different letters in the same group indicate statistical difference compare with LPS group ($p<0.05$); * $p<0.05$, ** $p<0.01$, *** $p<0.001$compare with the control group.

Figure 6. BRAE, D-BRAE and E-BRAE inhibit LPS-induced inflammatory effects through MAPK and NF-κB pathways in RAW264.7 cells. (A) Phosphorylated JNK and ERK protein expression levels, (B) Phosphorylated IκB-α and p65 protein expression levels. (C) NF-κB p65 subunit nuclear translocation determined by Confocal laser-scanning microscopy. RAW264.7 cells were pretreated with 400 μg/mL of the BRAE, D-BRAE and E-BRAE for 1 h, followed by 1 μg/mL LPS stimulated 1 h for JNK/p-JNK and ERK/p-ERK, 2h for IκBα/p-IκBα and p65/p-p65. Data are expressed as mean ± S.D. Bars with different letters in the same group indicate statistical difference compare with LPS group ($p<0.05$); * $p<0.05$, ** $p<0.01$, *** $p<0.001$compare with the control group.

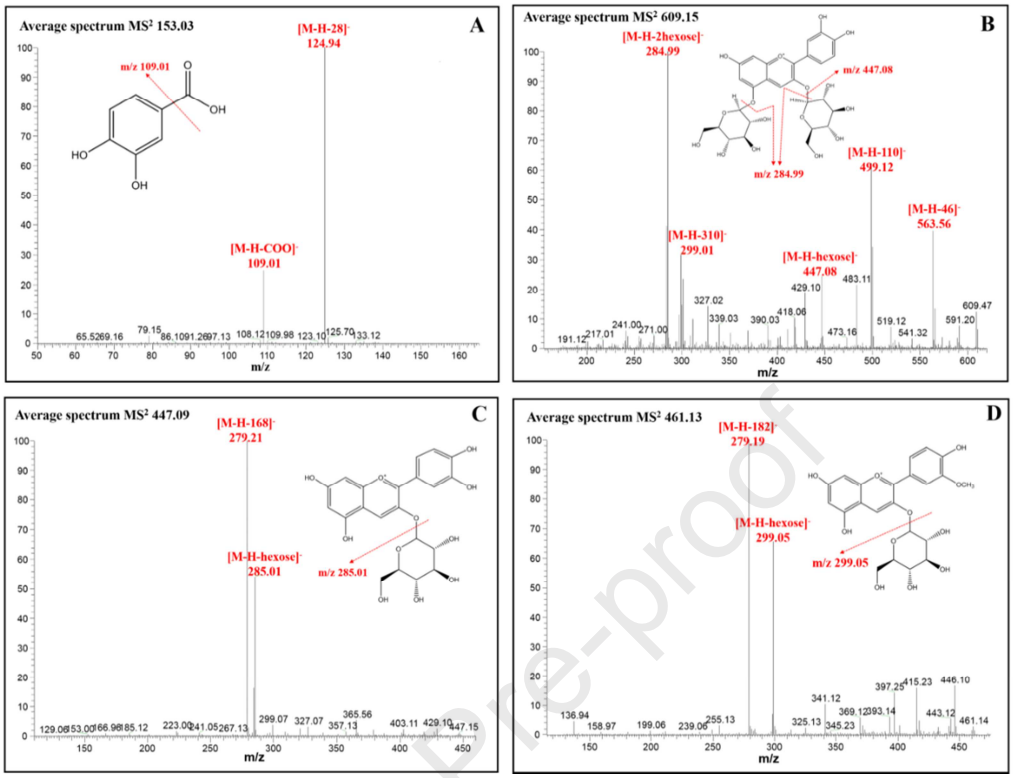
Journal Pre-proof

Figure 1.



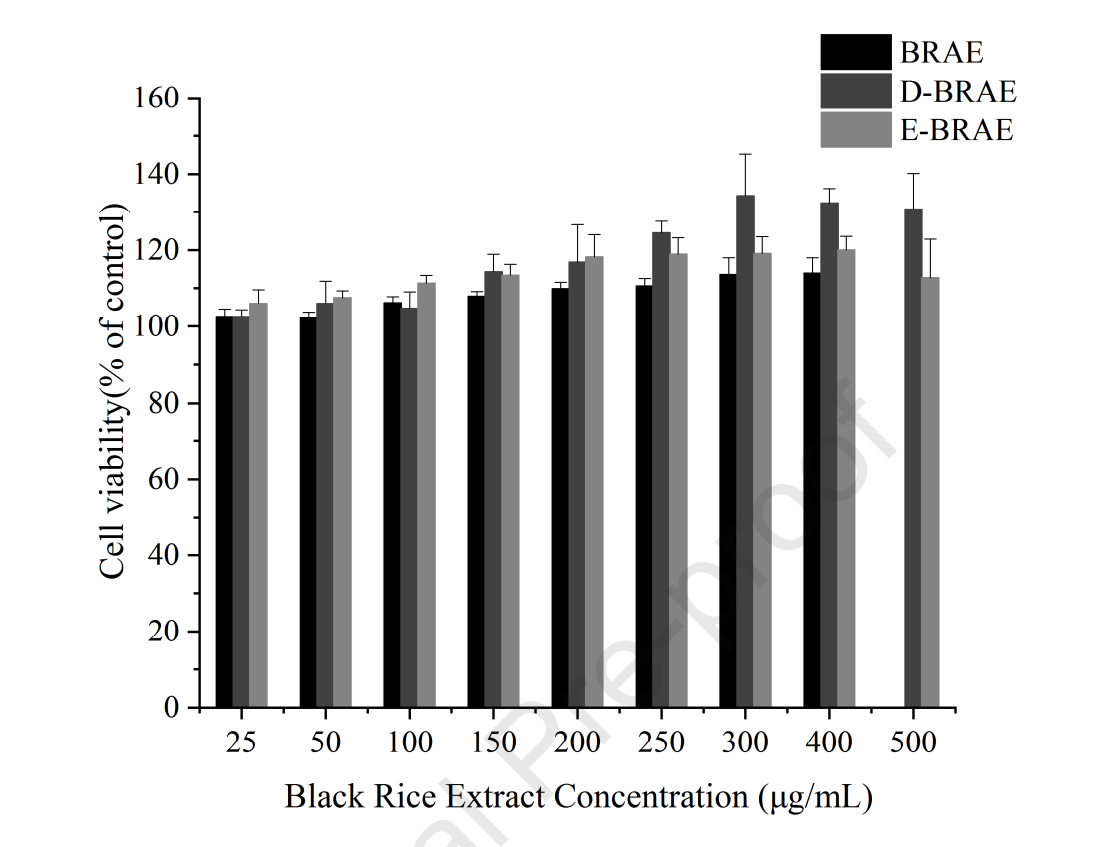
Journal Pre-proof

Figure 2.



Journal Pre-proof

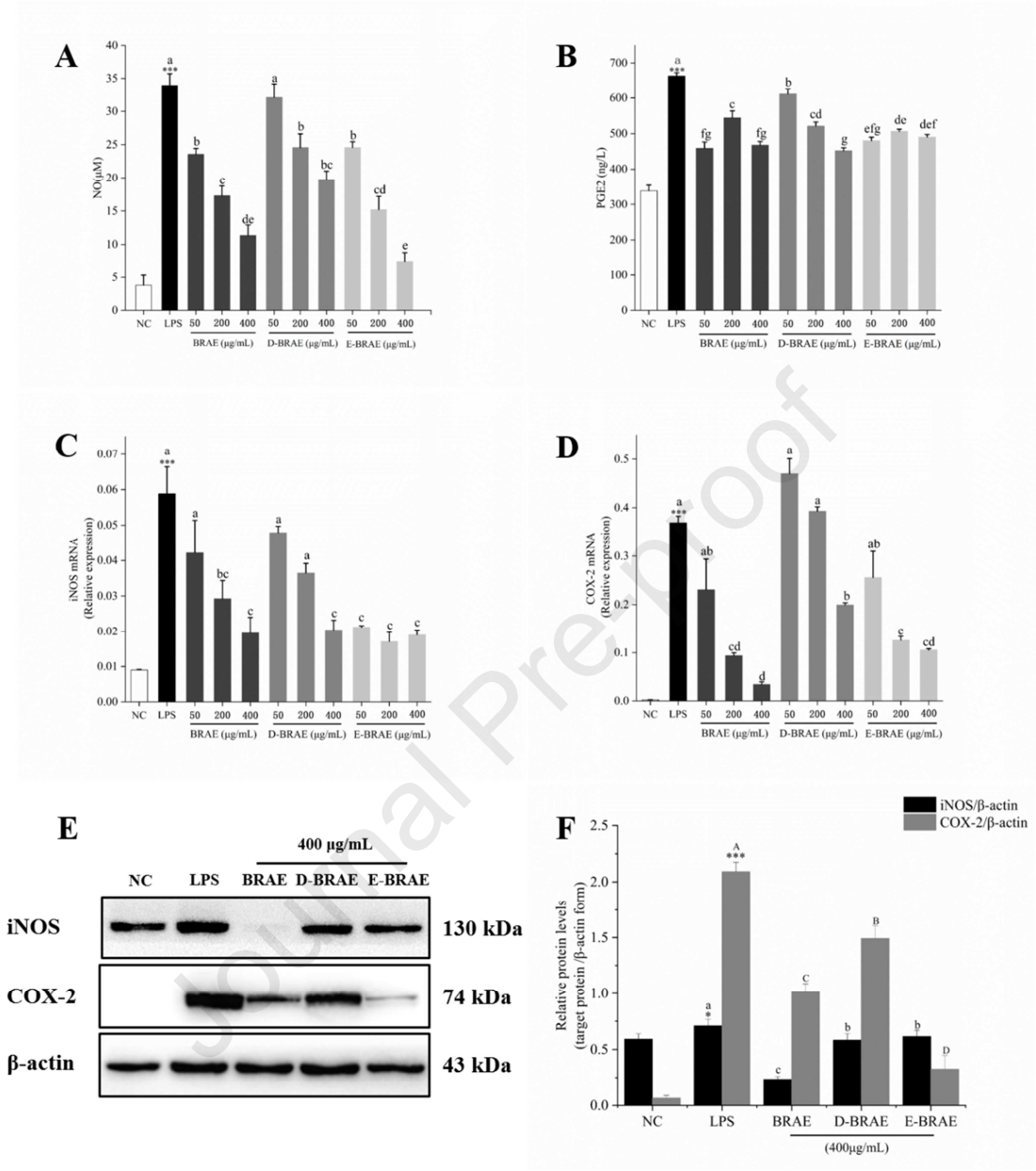
43 **Figure 3.**



44

Journal Pre-proof

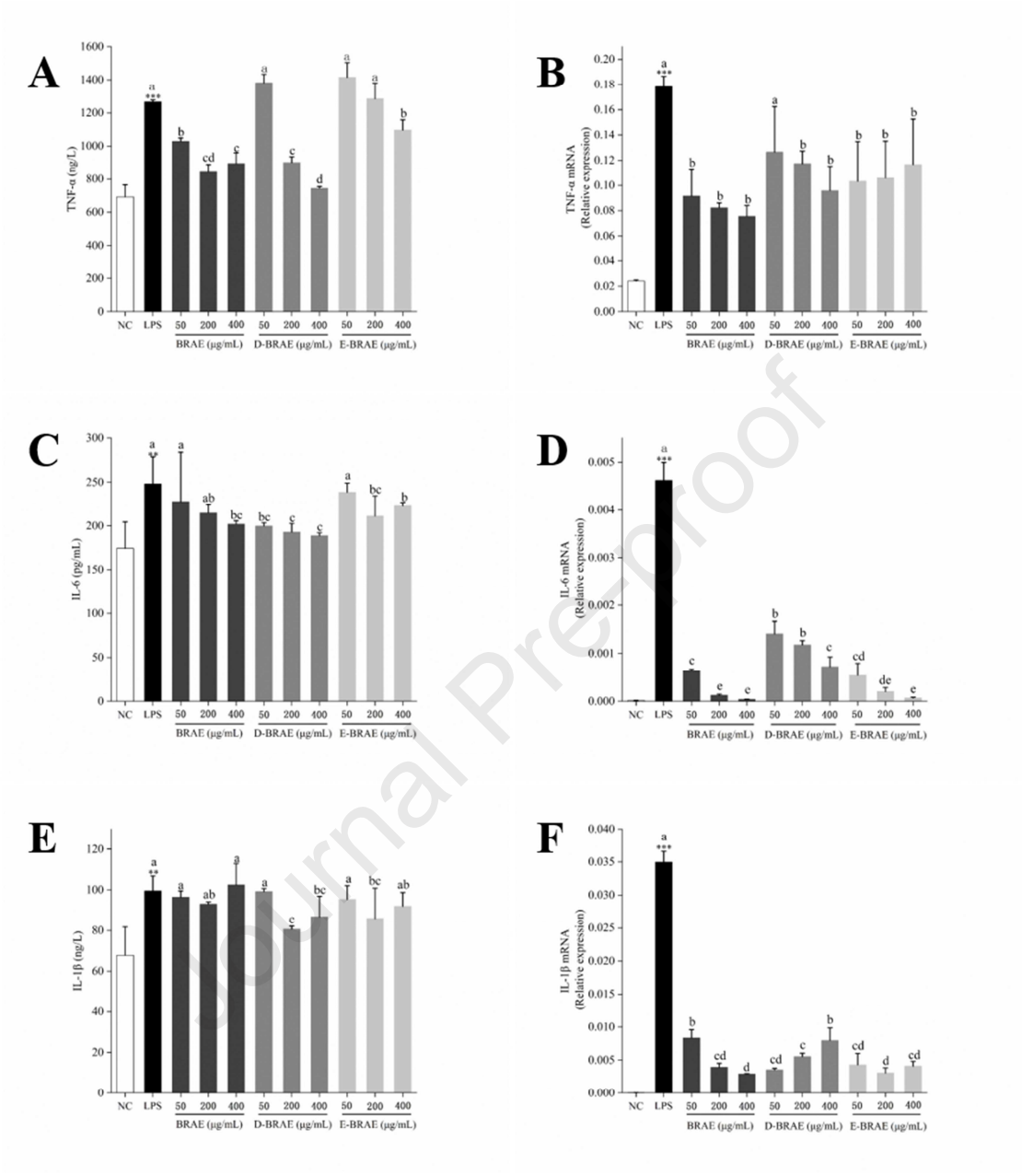
45 **Figure 4.**



46

Journal Pre-proof

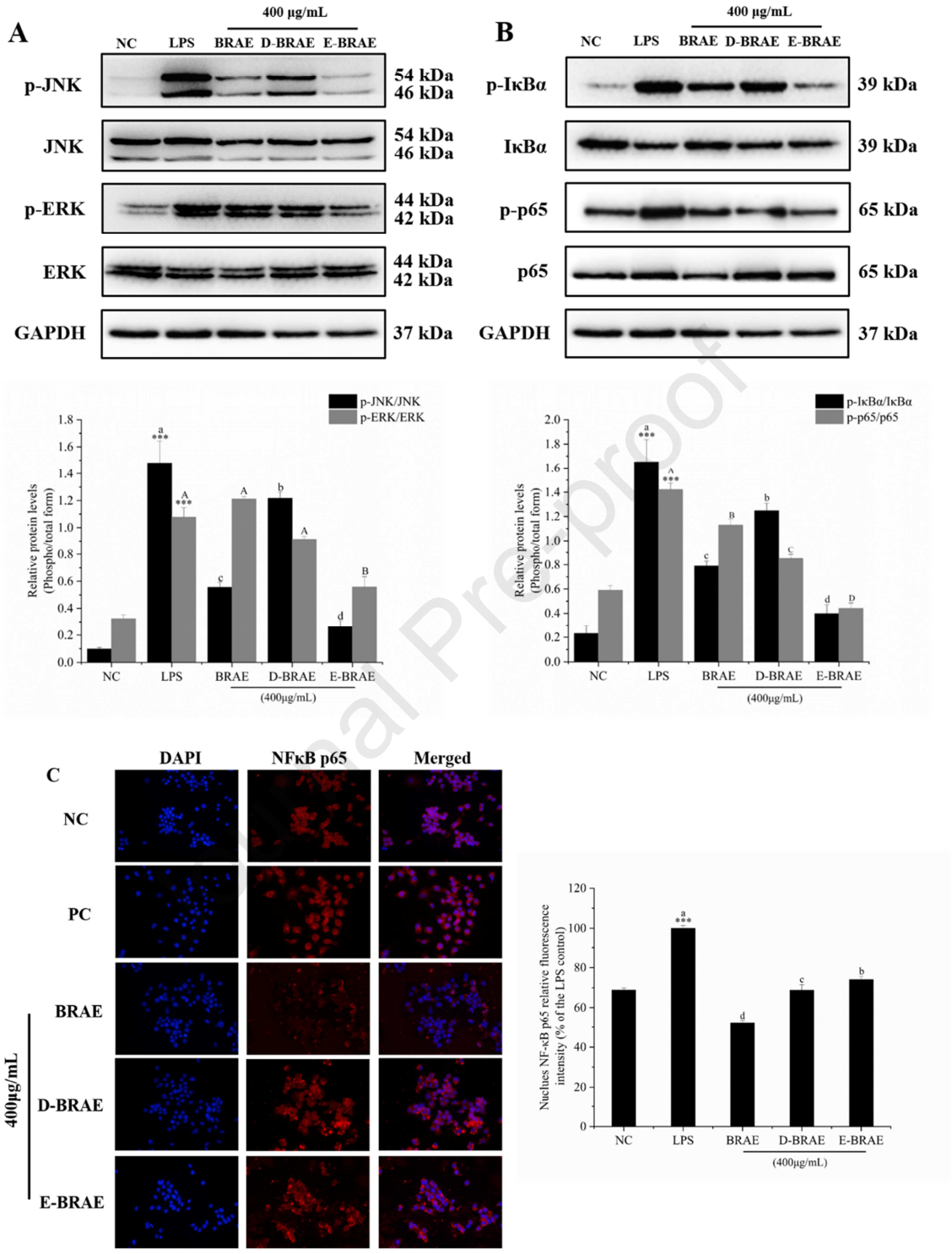
47 **Figure 5.**



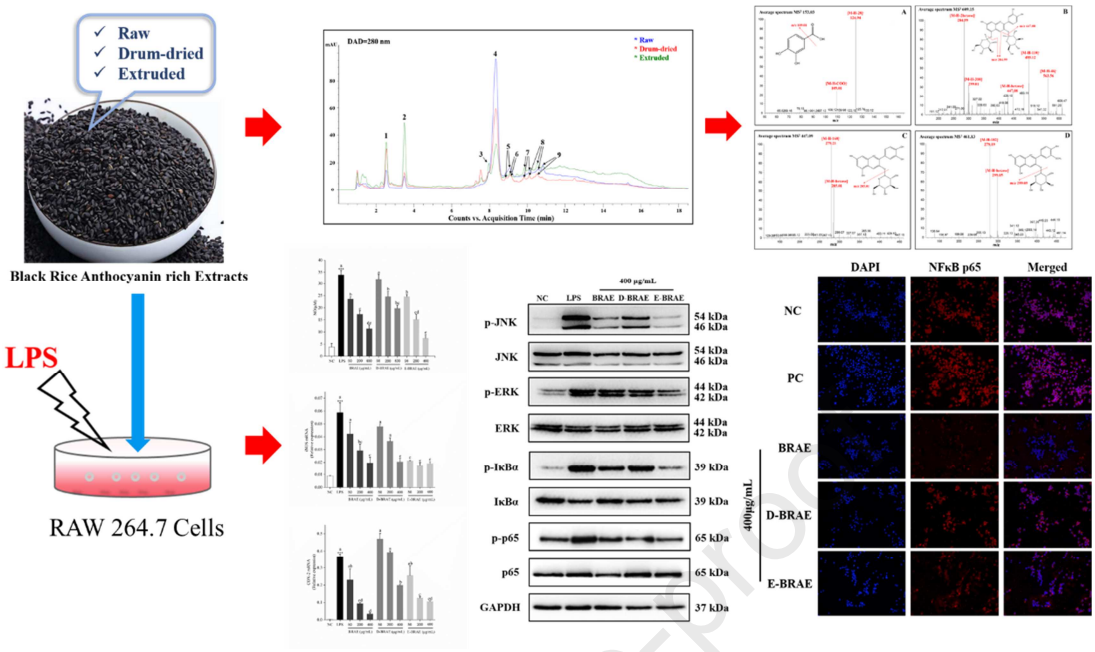
48

Journal Pre-proof

Figure 6.


A
400 μg/mL
NC
LPS
BRAE
D-BRAE
E-BRAE
p-JNK
JNK
p-ERK
ERK
GAPDH
54 kDa
46 kDa
44 kDa
42 kDa
37 kDa
B
p-IκBα
IκBα
p-p65
p65
39 kDa
65 kDa
p-JNK/JNK
p-ERK/ERK
Relative protein levels (Phospho/total form)
(400μg/mL)
p-IκBα/IκBα
p-p65/p65
C
DAPI
NFκB p65
Merged
400μg/mL
Nuclues NF-κB p65 relative fluorescence intensity (% of the LPS control)

The Abstract Figure



Journal Pre-proof

Journal Pre-proof

Conflict of interest

The authors declared that they have no conflicts of interest to this work.

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

2020.05.14

Journal Pre-proof