**Pilot-scale produced fermented lentil protects against *t*-BHP-triggered
oxidative stress by activation of Nrf2 dependent on SAPK/JNK
phosphorilation**

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ABSTRACT

Fermented lentil (FL) produced using *L. plantarum* and Savinase in alkaline conditions relieves metabolic alterations and oxidative stress in Zucker rats with metabolic syndrome. Here, we investigated the effect of up scaling the fermentation process on chemical composition and biological activity of FL. Moreover, we studied the molecular mechanisms by which FL exert a cytoprotective effect against oxidative stress in *tert*-butyl hydroperoxide (*t*-BHP)-challenged RAW264.7 macrophages. Up-scale production reduced overall biological effectiveness of FL with the exception of inhibition of intracellular ROS generation. FL prevented *t*-BHP-induced cytotoxicity and intracellular accumulation of reactive oxygen species through activation of catalase expression via SAPK/JNK phosphorylation and Nrf2 nuclear translocation. Different oligopeptides, phenolic acids and flavonols were identified as contributors of the observed effects. To the best of our knowledge, we reported for the first time that FL attenuates oxidative stress cellular damage via activation of SAPK/JNK phosphorylation, Nrf2 nuclear translocation and antioxidant enzymes expression.

Keywords: Nrf2, lentil, digestion, peptides, phenolic compounds, oxidative stress

Introduction

The exponential increase of people affected by metabolic syndrome (MetS) has led the scientific community to explore new lifestyle interventions that effectively revert all components of this disease including central obesity, dyslipemia, elevated plasma glucose, blood pressure and insulin resistance (Grundy, 2016). Patients with MetS are more prone to develop type 2 diabetes mellitus, cardiovascular diseases and non-alcoholic fat liver disease (NAFLD). Impaired antioxidant status and consequent development of oxidative stress are also intimately related to MetS and its associated comorbidities (Zaki et al., 2018). Reactive oxygen species (ROS) mediate various signaling networks exerting cytotoxic effects, such as peroxidation of lipids and phospholipids and oxidative damage to proteins and DNA, leading to tissue destruction (Zaki et al., 2018).

Legume interventions become a subject of increasing interest since they have shown beneficial properties related to the management of MetS (Martínez, López-Jurado, Wandenberghe, Sanz-Valero, Porres, & Kapravelou, 2016). Legume intake has positive effects including glycemia and lipemia decrease and improvement of insulin sensitivity, weight management and satiety perception (Clark & Duncan, 2017; Mollard, Luhovyy, Panahi, Nunez, Hanley, & Anderson, 2012). Beside fiber and oligosaccharides, other bioactive compounds such as phenolic compounds and peptides have emerged as contributors to the positive effects of legume intake on MetS (Padhi, Liu, Hernandez, Tsao, & Ramdath, 2017; Padhi & Ramdath, 2017; Reverri, Randolph, Kappagoda, Park, Edirisinghe, & Burton-Freeman, 2017). Health promoting properties of phenolic compounds and bioactive peptides of legumes come from their physiological effects such as modulation of blood pressure as well as glucose and lipid homeostasis given that they inhibit the activity of angiotensin converting enzyme I, intestinal α -glucosidase, pancreatic α -amylase and lipase (Iwaniak, Darewicz, & Minkiewicz, 2018; Zhang

et al., 2015). They may also act as antioxidants providing protection against oxidative stress (García-Mora et al., 2017; Padhi, Liu, Hernandez, Tsao, & Ramdath, 2017).

Different technological strategies including germination and fermentation have been employed to exploit legumes as functional foods for the management of MetS (Bautista-Expósito, Peñas, Silván, Frias, & Martínez-Villaluenga, 2018a; Kapravelou et al., 2017). We have recently developed a laboratory scale fermentation in alkaline conditions with *Lactobacillus plantarum* CECT 748 and the enzymatic preparation Savinase 16 L to increase the bioavailability of bioactive peptides and phenolics while enhancing the antioxidant, hypoglucemic, hypolipidemic, anti-inflammatory and anti-hypertensive activities of lentil (*Lens culinaris* Medik. var. Castellana) (Bautista-Expósito et al. 2018a). We demonstrated the individual effect of Savinase, mainly producing bioactive peptides, and *L. plantarum* responsible for the increased concentrations of *p*-hydroxybenzoic acid, vanillic acid and kaempferol glucosides in the end-product (Bautista-Expósito, Peñas, Dueñas, Silván, Frias, & Martínez-Villaluenga, 2018b). Due to this lentil fermentation procedure is intended to be used for obtaining a new functional ingredient against MetS, we have studied herein the effect of up scaling the process on bioactive peptides, phenolics and bioactivity of the end-product.

The beneficial effects of fermented lentil (FL) developed by our research group were recently validated in the obese Zucker rat experimental model of MetS and NAFLD (Martinez et al., 2018). Administration of FL effectively reduced steatosis through the expression of genes involved in hepatic β -oxidation. Moreover, FL improved glucose and lipid metabolism and reduced lipid peroxidation in the liver of Zucker rats through increased gene expression of nuclear factor-erythroid-2-related transcription factor-2 (Nrf2). This transcription factor controls the expression of genes whose protein products are involved in the detoxication and elimination of reactive oxidants and electrophilic agents (Nguyen, Nioi, & Pickett, 2009). Since regulatory mechanisms of Nrf2 activation by FL have not been elucidated yet, in this study, we

hypothesized that bioactive peptides and phenolic compounds present in FL activate redox signaling networks, induce Nrf2 translocation to the nucleus and subsequently the expression of antioxidant enzymes that would contribute to reduction of intracellular ROS levels and cell survival. To test this hypothesis, we performed *in vitro* experiments in which we simulated the gastrointestinal digestion of FL in physiological conditions and subsequently studied the antioxidant effects and mechanism of action of FL digest (FLD) using *tert*-butyl hydroperoxide-challenged RAW 264.7 cells as a model of oxidative stress.

The aims of this study were: i) to scale-up fermentation process to determine the feasibility of producing enough quantities of FL to be included as an ingredient in foods; ii) to evaluate the protective role of FLD on *tert*-butyl hydroperoxide (*t*-BHP) induced oxidative stress in RAW 264.7 macrophages; iii) to elucidate the molecular mechanisms involved in the protective effect of FLD against oxidative stress; iv) to characterize the peptide and phenolic profiles of FLD.

2. Material and Methods

2.1. Materials

Lentil seeds (*Lens culinaris* Medik. var. Castellana) were obtained from Semillas Iglesias S.A. (Salamanca, Spain). A commercial food grade protease Savinase® 16 L (16 KNPU/g), an enzymatic preparation of alkaline-serine protease from *Bacillus* spp. was provided by Novozymes (Bagsvaerd, Denmark). *L. plantarum* CECT 748 was purchased from the Spanish Type Culture Collection (Valencia, Spain).

2.2. Chemicals

Angiotensin I converting enzyme (ACE, EC 3.4.15.1), rat intestine α -glucosidase (EC 3.2.1.20), porcine pancreatic lipase type II (EC 3.1.1.3), pepsin from porcine gastric mucosa (EC 3.4.23.1), pancreatin from porcine pancreas (EC 232-468-9), 2,7-dichlorofluorescein diacetate (DCFDA)

and *t*-BHP were purchased from Sigma-Aldrich (Madrid, Spain). Murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Rockville, MD, USA). High-glucose Dulbecco's Modified Eagle's Medium (DMEM) and penicillin/streptomycin (10,000 U/mL) were purchased from Lonza Group (Madrid, Spain). Fetal bovine serum (FBS) was obtained from Hyclone (GE Healthcare, Logan, UT, USA). Primary antibodies for Cu-Zn superoxide dismutase (SOD-1), catalase and β -actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), rabbit transcriptional activator Nrf2, Lamin B1, stress-activated protein kinases (SAPK)/Jun amino-terminal kinases (JNK) and phosphorylated SAPK/JNK in Thr183/Tyr185 were from Cell Signaling Technologies (Danvers, MA, USA). Secondary antibodies [horseradish peroxidase (HRP)-conjugate recombinant mouse immunoglobulin G kappa (m-IgG κ) light chain binding protein and HRP-conjugate goat anti-rabbit IgG (heavy and light chain)] were obtained from Santa Cruz Biotechnology and Cell Signaling, respectively.

2.3. Scale-up of lentil flour fermentation

To scale-up production, in this study FL was produced in a 30 L final volume using a 50 L BIOSTAT[®] D bioreactor (Sartorius, Barcelona Spain). Lentil flour was suspended in autoclaved water (142.7 g flour/L), inoculated with *Lactobacillus plantarum* CECT 748 (2×10^8 CFU/L) and simultaneously treated with Savinase 16 L (361 mg/L lentil flour suspension). Treatment was performed at 37 °C and pH 8.0 (adjusted using 20% KOH), continuous stirring speed of 300 rpm, without aeration for 15 h. Flour suspension was centrifuged and the obtained soluble fraction was frozen at -80 °C and freeze-dried using FreeZone 12 L freeze-dryer (Labconco, Kansas, MO, USA).

2.4. Gastrointestinal digestion

FL was digested following an *in vitro* harmonized protocol that reached an international consensual agreement (Minekus et al., 2014). Briefly, freeze-dried lentil samples were dissolved in water (700 mg/5 mL) and then, diluted with simulated gastric fluid containing pepsin (2000 U/mL in the final mixture, ratio of 50:50, v/v). The mixture was incubated at 37 °C in an orbital shaker at 150 rpm for 120 min. The enzymatic reaction was stopped by adjusting the pH at 7.0 with 1 M NaOH. Gastric phase was mixed with simulated intestinal fluid containing pancreatin (100 U trypsin activity/mL of final mixture, ratio 50:50, v/v) and porcine bile extract (10 mM in the final mixture). Intestinal digestion phase was carried out at 37 °C and 150 rpm for 120 min. The enzymatic reaction was stopped at 85 °C for 10 min. Digestion was performed in duplicate. FLD was freeze dried and kept at -20 °C in vacuum sealed plastic bags until further analysis.

2.5. Determination of total peptides and phenolic compounds

Peptide concentration was measured by Pierce Quantitative Colorimetric Peptide Assay kit (Fisher Scientific, Madrid, Spain) in permeates obtained by ultrafiltration through cellulose membranes of 10 kDa pore size (Millipore, Billerica, MA). Results were expressed as mg of peptides/g of soluble fraction. Total phenolic compounds were determined using the Folin-Ciocalteu method as previously described (Garcia-Mora et al., 2015). The absorbance was read at 690 nm using a Synergy HT multi-well plate reader (BioTek, Winooski, VT, USA) and the results were expressed as mg gallic acid equivalents (GAE)/g of soluble fraction.

2.6. Peptides identification

A peptidomic analysis was performed to identify the peptides present in FLD. Twenty mg of freeze-dried sample were suspended in 1 mL of 0.1% trifluoroacetic acid (TFA) using and ultrasound bath. An aliquot (5 µL) was 5-fold diluted in 0.1% TFA. From this sample solution 2.5 µL were purified using a reverse phase C18 ZipTip (Millipore Corporation, MA, USA). Eluate was resuspended in 0.1% formic acid (FA)/2% acetonitrile (ACN) and injected (4 µL) in

an Easy liquid chromatography 1000 nano system (Thermo Fisher Scientific, Madrid, Spain) coupled to an ion source with nanoelectrospray LTQ-Orbitrap Velos (Thermo Fisher Scientific, Madrid, Spain). For each analysis, the sample was loaded into a precolumn Acclaim PepMap 100 (Thermo Fisher Scientific, Madrid, Spain) and eluted in an AcclaimPepMap 100 C18 (250 mm long, 75 μ m inner diameter and 3 μ m particle size; Thermo Fisher Scientific, Madrid, Spain). The mobile phase flow rate was 250 nL/min using 0.1% FA in water (solvent A) and 0.1% FA in ACN (solvent B). The gradient profile was set as follows: 0%-35% solvent B in 90 min, 35%-45% solvent B in 10 min, 45%-95% solvent B in 5 min; 95%-100% solvent B in 1 min, 100% solvent B in 4 min.

Mass spectrometry (MS) analysis was performed using a LTQ-Orbitrap Velos working in the positive mode. For ionization, 2000 V of liquid junction voltage and 270 °C capillary temperature was used. The full scan method employed a m/z 400–2000 mass selection, an Orbitrap resolution of 60,000 (at m/z 200), a target automatic gain control (AGC) value of $3e^6$, and maximum injection times of 100 ms. After the survey scan, the 15 most intense precursor ions were selected for ion fragmentation. Fragmentation was performed by collision-induced dissociation in the ionic trap using a normalized collision energy of 35%. MS/MS scans were acquired using a starting mass of m/z 100, AGC target of $2e^5$, resolution of 17,500 (at m/z 200), intensity threshold of $8e^3$, isolation window of 2 m/z units and maximum injection time of 100 ms. Charge state screening was enabled to reject unassigned, singly charged, and equal or more than seven protonated ions. A dynamic exclusion time of 45 s was used to discriminate against previously selected ions.

MS data were analyzed with Proteome Discoverer (version 1.4.1.14) (Thermo Fisher Scientific, Madrid, Spain) using standardized workflows. Mass spectra raw files were searched against *Lens culinaris* database files (NCBI, 427 sequences protein entries) using SEQUEST search engine. Precursor and fragment mass tolerance were set to 10 ppm and 0.5 Da, respectively. In the search

parameters carbamidomethylation of cysteines and methionine oxidation were established as fixed modifications. Identified peptides were filtered using Percolator algorithm with a q-value threshold of 0.01.

2.7. Quantification and identification of phenolic compounds

Freeze-dried FLD soluble fraction (50 mg) was dissolved in 1 mL methanol:water (50:50, v/v). Sample solution was vortexed during 10 min and subsequently, centrifuged at 10,000 x g and 5 °C for 10 min. The supernatant was filtered through 0.45 µm polytetrafluoroethylene membrane (Agilent, Santa Clara, CA, USA). Chromatographic analyses were performed in an Agilent high performance liquid chromatography (HPLC) 1100 series equipped with a G1312A binary pump, a G1313 A autosampler, G1315B photodiode array detector, controlled by the Agilent software v. A.08.03 and G1322A degasser. Briefly, sample (20 µL) elution was carried out at a flow rate of 1 mL/min, on a Kinetex column (5 µm, C18, 250 × 4.6 mm; Phenomenex, Macclesfield, UK), with 1% FA in water (solvent A) and ACN (solvent B), starting with 5% B and installing a gradient to obtain 60% B at 37 min, 98% B at 40 min and 98% B at 42 min. HPLC was coupled to an ion-trap mass spectrometer equipped with an electrospray ionization system (ESI). The nebulizer pressure and flow rate of nitrogen were 65.0 psi and 11 L/min, respectively. The heated capillary and voltage were maintained at 350 °C and 3.5 kV, respectively. Mass scan and MS/MS daughter spectra were measured from m/z 100 to 1200. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, and the collision energy was set at 50%. MS data were acquired in the negative ionization mode. The phenolics compounds in FLD were identified according to their UV spectrum, molecular mass, daughter ions, and fragmentation pattern. For quantitative analysis, calibration curves were prepared by injection of known concentrations of different standard compounds obtained from Extrasynthese (Genay Cedex, France). Samples were analysed in duplicate. Data were expressed as µg/g of soluble fraction.

2.8. Angiotensin I converting enzyme inhibitory activity

Angiotensin I converting enzyme (ACE) inhibitory activity was measured following a previously described method (Garcia-Mora, et al., 2015). Fluorescence was read every minute for 30 min at emission and excitation wavelengths of 335 and 405 nm, respectively, in a microplate reader Synergy HT (BioTek, Winooski, VT, USA). The results were expressed as percent inhibition relative to the negative control having 100% enzymatic activity. All samples were analyzed in duplicate. IC₅₀ values (concentration of sample in mg/mL that inhibits 50% of the ACE activity) were calculated plotting the non-linear regression sigmoidal dose-response curves in GraphPad Prism 4.00 (GraphPad Software Inc., San Diego, CA, USA).

2.9. α -Glucosidase inhibitory activity

α -glucosidase sucrase and maltase inhibitory activities of FL (1.7 mg/mL final concentration) was determined following a previously described method (Bautista-Expósito, et al., 2018a). Glucose concentration in the reaction mixture was determined using the Amplex[®] Red glucose/glucose oxidase assay kit (Invitrogen, Carlsbad, CA, USA). Absorbance was measured using a Synergy HT plate reader (BioTek, Winooski, VT, USA) at 560 nm. Glucose concentration was calculated using a linear standard curve (0-200 μ M) from a freshly prepared 400 mM stock solution. All samples were analyzed in duplicate. Percent inhibition of sucrase and maltase activity of intestinal α -glucosidase in the presence of lentil samples was calculated relative to the negative control having 100% enzyme activity.

2.10. Lipase inhibitory activity

The ability of FL (final concentration of 2 mg/mL) to inhibit porcine pancreatic lipase was evaluated using a previously reported method (Bautista-Expósito et al. 2018a). Lipase activity was determined by measuring the hydrolysis of *p*-nitrophenyl butyrate to *p*-nitrophenol at 405 nm using a microplate reader Synergy HT (BioTek, Winooski, VT, USA). All samples

were analyzed in duplicate. Percent inhibition of lipase activity in the presence of lentil samples was calculated relative to the negative control having 100% enzyme activity.

2.11. Oxygen radical absorbance capacity

Radical scavenging activity was evaluated by fluorescence following the oxygen radical absorbance capacity (ORAC) assay described previously (Bautista-Expósito et al., 2018a). Fluorescence was measured in a Synergy HT microplate reader (BioTek, Winooski, VT, USA) at emission and excitation wavelengths of 520 nm and 485 nm, respectively. A Trolox standard curve with a linear concentration range (0-160 µM) was prepared from a freshly made 1 mM stock solution. All samples were analyzed in duplicate. Results were expressed as mM of Trolox Equivalents (TE)/g of soluble fraction.

2.12. Cell culture

In a previous in vivo study, it was demonstrated that fermented lentil intervention was able to reduce lipid peroxidation in the liver of obese Zucker rats with non-alcoholic fatty liver disease (NAFLD) by increased expression of Nfr2 (Martínez et al. 2018). To get a deeper insight on the antioxidant mechanism of action of fermented lentil described in the obese Zucker rat we decided to use a murine cell model of oxidative stress. We selected a murine macrophage cell line, as pathophysiology of NAFLD is characterized by infiltration of macrophages to the hepatic tissue increasing oxidative stress and inflammation (Dongiovanni, Lanti, Riso, & Valenti, 2016).

To test the cytoprotective effect of FLD in stressful conditions, a model of oxidative stress induced by a potent pro-oxidant was established based on previous studies (Lv, Liu, Zhou, Tan, Deng, & Ci, 2017). RAW 264.7 murine macrophages were cultured using DMEM supplemented with 10% FBS, 1% penicillin/streptomycin. Cells were maintained at a subconfluent density in 75 cm² tissue culture flasks and incubated in a humidified 5% CO₂/95%

air incubator at 37 °C. The culture medium was changed every 2 days. In all experiments, cells were allowed to acclimate for 24 h before treatments.

2.13. Cell viability

RAW 264.7 cells were grown in 96-well plates (5×10^4 cells/well) for 24 h and stimulated with *t*-BHP (0.05, 0.1, 1.0 and 2.5 mM) for an additional 3 h. Additional experiments consisted of cell treatment with FLD (50, 75 and 100 µg/mL) for 18 h or FLD (50, 75 and 100 µg/mL) for 18 h and with 2.5 mM *t*-BHP for an additional 3 h. Each treatment was performed in triplicate to confirm reproducibility. Control cells incubated in serum-free medium served as negative control. Cell viability was determined using the Cell Titer 96® AQueous One Solution Proliferation Assay kit from Promega (Madison, WI, USA). Medium was removed, cells were washed with phosphate buffer saline (PBS) and 100 µL of serum-free medium were added. Thereafter, 20 µL of Cell Titer 96® were added to each well for the quantification of the living metabolically active cells. After 45 min of incubation, absorbance was read at 515 nm employing a PowerWave™ XS microplate reader (BioTek, Winooski, VT, USA). The viability was calculated considering negative control (non-treated cells) as 100% viable.

2.14. Detection of intracellular ROS

Intracellular ROS were quantified by the dichlorofluorescein (DCF) assay. Macrophages (2×10^5 cells/well) were grown in clear bottom black 24 well-plates for 24 h. Cells were washed with PBS and incubated with 20 µM DCFDA for 30 min. After that, cells were washed with PBS and treated with *t*-BHP (0.05, 0.1, 1.0 and 2.5 mM) for an additional 3 h. Additional experiments consisted of cell treatment with FLD (50, 75 and 100 µg/mL) for 18 h or FLD (50, 75 and 100 µg/mL) for 18 h and 2.5 mM *t*-BHP for an additional 3 h. Each treatment was performed in triplicate to confirm reproducibility. Control cells incubated in serum-free medium served as negative control. ROS production was monitored for 3 h by fluorescence at an excitation and

emission wavelengths of 485 nm and 530 nm, respectively, using a Synergy HT plate reader (BioTek, Winooski, VT, USA).

2.15. Whole cell lysates preparation

Upon completion of treatments, cells were washed with 500 μ L of ice cold PBS to remove cellular debris. Cells were lysed with 100 μ L RIPA lysis buffer containing protease and phosphatase inhibitors cocktail (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and incubated for 5 min at 4 °C. The cells were dislodged using cell scrapers and vortexed for 5 min at 4 °C followed by centrifugation at 14,000 x g for 10 min at 4 °C. The pellet was discarded and the cell supernatant was transferred into pre-chilled tubes. The protein concentration of the whole cell lysates was measured using the Detergent Compatible Protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Whole cell lysates were stored at -80°C until use.

2.16. Preparation of cytosolic and nuclear fractions

For Nrf2 and Lamin B1 Western blot analysis, nuclear proteins were extracted after 18 h of FLD treatment (50, 75 and 100 μ g/mL). For Western blot analysis of phosphorylated (P)-SAPK/JNK and total (T)-SAPK/JNK, cytoplasmic proteins were extracted after 6 h of FLD treatment (50, 75 and 100 μ g/mL). Upon completion of treatments, cells were subsequently washed with 500 μ L of ice cold PBS to remove cellular debris. To separate cytoplasmic and nuclear fractions, NE-PER nuclear extraction kit (Thermo Fisher Scientific, Madrid, Spain) was used following manufacturer's instructions. All steps were carried out on ice or at 4 °C. The protein concentration of nuclear and cytosolic fractions was measured using the Detergent Compatible Protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Nuclear or cytoplasmic fractions were stored at -80°C until use.

2.17. Western blot

Expressions of SOD-1, catalase and β -actin were measured by western blot in the whole cell lysates. Nrf2 and Lamin B1 were measured in the nuclear fraction while P-SAPK/JNK and T-SAPK/JNK were measured in cytosolic fraction. Sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was run under denaturing conditions with 20 μ g of protein loaded in 4–20% on NuPAGE[®] Novex 4-12% Bis-Tris Gels (Invitrogen, Madrid, Spain). Gels were placed in XCell-sure lock Mini-Cell and run under reducing conditions at 200 V. NuPAGE[®] MES-SDS and NuPAGE[®] LDS (Invitrogen, Madrid, Spain) were used as running and sample buffers, respectively. After protein separation by SDS-PAGE and transfer, polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Logan, UT, USA) were blocked with 5% non-fat dry milk for 1 h at room temperature and washed with Tris buffer saline Tween 20 (TBST), three times for 10 min each. PVDF membranes were incubated in primary antibodies against SOD-1, catalase and β -actin, Nrf2 and Lamin B1, P-SAPK/JNK and T-SAPK/JNK overnight at 4 °C. After washing, secondary antibodies were added and incubated at room temperature for 2 h. After incubation and repeated washing, the PVDF membranes were prepared for detection using a 1:1 mixture of chemiluminescent reagent A (luminol solution) and B (peroxide solution) (GE Healthcare, Logan, UT, USA). The membrane pictures were taken on a ChemDoc XRS+ Imaging System (Bio-Rad Laboratories, Hercules, CA, USA). The relative amount of each target protein was normalized to β -actin. For the detection of Nrf2, equal amount of protein (15 μ g) from nuclear extracts was normalized to Lamin B1. All Western blot procedures were determined in at least three independent trials.

2.18. Statistical analysis

Experimental data represent the mean and standard deviation of three replicates analyzed in duplicate. Statistical significance of the data was tested by one-way analysis of variance, followed by the Duncan test to compare the means that showed significant variation ($p < 0.05$);

all of the statistical analyses were performed using Statgraphics Plus software, version 5.1 (Statistical Graphics Corporation, Rockville, MD, USA).

3. Results

3.1. Effect of scale up on peptide and phenolic yields and bioactivity profile of FL

In an earlier study, FL was produced at laboratory scale using *L. plantarum* CECT 748 along with Savinase 16 L at pH 8.5 for 15 h to enhance the multifunctional potential of lentil in relation to the management of hypertension, hyperglycemia, dislipemia and oxidative stress (Bautista-Expósito et al., 2018a). In the present study, we scale up FL production from 1.5 L to 30 L final volume with some minor modifications. As sodium may have a negative impact in blood pressure (Bazzano, Green, Harrison, & Reynolds, 2013), NaOH was substituted by KOH to adjust pH value during fermentation. Pilot scale fermentation was performed at pH 8.0 instead of pH 8.5, therefore, lower volume of alkali solution (20% KOH) were required to adjust the pH value.

Peptide and phenolic yields as well as bioactivity of FL produced at lab-scale and pilot-scale were compared as it is shown in **Table 1**. FL produced at pilot plant scale was comprised of 480.4 mg protein/g, 181.3 mg peptides/g and 49.2 mg GAE/g. As compared to lab-scale, FL production at 30 L scale significantly reduced protein content, peptide and phenolic yields ($p < 0.05$). Similarly, FL bioactivity was significantly lower when the fermentation process was scaled up to 30 L. Lower inhibition of ACE, sucrase activity of intestinal α -glucosidase and pancreatic lipase as well as ORAC values were observed for FL produced at pilot scale. On the contrary, scale up of fermentation process did not affect maltase activity of FL and noticeably increased the effectiveness of FL to protect RAW 264.7 macrophages against oxidative stress ($p > 0.05$; **Table 1**). Based on these results, to get a deeper insight in the protective effects of FL against oxidative stress we further performed an *in vitro* gastrointestinal digestion of FL to mimic

physiological processes that upon intake have an impact on chemical composition and bioactivity. This experimental approach allowed us to compare our results with those previously observed in Zucker rats with MetS (Martinez et al., 2018). Thus, subsequent experiments were performed to investigate the protective effect and mechanisms of action of FLD in *t*-BHP-induced RAW 264.7 macrophages.

3.2. FLD protect RAW264.7 cells against t-BHP-induced cytotoxicity

To test the cytoprotective effect of FLD, a model of oxidative stress induced by a potent pro-oxidant such as *t*-BHP was used based on previous studies (Lv, Liu, Zhou, Tan, Deng, & Ci, 2017). *t*-BHP can induce cell damage, inhibiting cell viability and inducing cell apoptosis. Different *t*-BHP concentrations (0.05, 0.1, 1 and 2.5 mM) were tested to assure that toxicity by oxidative stress on RAW 264.7 macrophages was produced. Results show that 0.1, 1.0 and 2.5 mM of *t*-BHP resulted in a remarkable decrease of cell viability (78%, 39% and 11% respectively, **Figure 1A**). Subsequently, macrophages were exposed to increasing concentrations of FLD (10, 50, 75 and 100 µg/mL) during 18 h and cell viability was measured. Cell viability of FLD-treated cells was not significantly different compared to control cells ($p>0.05$, **Figure 1B**), therefore, none of the tested concentrations of FLD induced cytotoxicity. To determine whether treatment with FLD has a cytoprotective effect against stressful oxidant conditions, cells were treated with FLD (10, 50, 75 and 100 µg/mL) for 18 h and subsequently exposed to 2.5 mM *t*-BHP for 3 h. Results showed that FLD at 75 and 100 µg/mL significantly inhibited *t*-BHP induced cell death (42% and 61%, respectively, **Figure 1C**).

3.3. FLD attenuated t-BHP-triggered ROS production in RAW 264.7 cells

t-BHP exposure to RAW 264.7 cells increased in a dose-dependent manner the production of ROS (**Figure 1D**). Levels of oxidant radicals in cells treated with increasing concentrations of FLD did not show significant differences compared to control untreated cells ($p>0.05$, **Figure**

1E). These results indicate that FLD alone did not induce oxidative stress to RAW 264.7 macrophages. Pretreatment of RAW 264.7 cells with FLD (10, 50, 75 and 100 µg/mL) for 18 h followed by 2.5 mM exposure of *t*-BHP for 3 h dose-dependently reduced the generation of oxidant radicals (3, 19, 32 and 67% inhibition, respectively, **Figure 1F**).

3.4. FLD induces catalase protein expression

To elucidate the possible molecular pathways of the anti-oxidant role of FLD, the protein expression of antioxidant enzymes (catalase and SOD-1) by Western blot was evaluated (**Figure 2A**). Cell exposure to 0.1 mM *t*-BHP increased catalase and SOD-1 levels compared to control untreated cells ($p < 0.05$, **Figure 2B and 2C**). The choice of *t*-BHP dose was based on higher cell viability (**Figure 1A**). Pretreatment of cells with FLD (50, 75 and 100 µg/mL) for 18 h and subsequently with 0.1 mM *t*-BHP for 3 h further enhanced catalase protein expression in a dose-dependent manner as compared with *t*-BHP exposed cells ($p < 0.05$, **Figure 2B**). Conversely, FLD did not increase SOD-1 expression induced by 0.1 mM pro-oxidant exposure ($p > 0.05$, **Figure 2C**).

3.5. FLD induces the nuclear translocation of Nrf2

We investigated the effect of FLD in the expression and nuclear translocation of the transcription factor Nrf2 by Western blot (**Figure 2E**). Accordingly, RAW 264.7 cells were pretreated with FLD (50, 75 and 100 µg/mL) for 18 h and subsequently with 0.1 mM *t*-BHP for 3 h and Nrf2 in nuclear fraction were measured by Western blot. **Figure 2F** shows a dose dependent increase of Nrf2 levels in nuclear fraction ($p < 0.05$).

3.6. FLD increases the phosphorylation of SAPK/JNK in RAW 264.7 macrophages

Because redox signaling mechanisms involve the activation of the stress-sensitive Ser/Thr systems (Evans, Goldfine, Maddux, & Grodsky, 2002), we examined the upstream signaling pathways involved in the activation of Nrf2 by determination of phosphorylation of SAPK/JNK.

RAW 264.7 cells were treated with FLD (50, 75 and 100 µg/mL) for 6 h and Western blots were performed to detect T- and P-SAPK/JNK proteins (**Figure 3A**). FLD-treated cells showed a significant and dose-dependent increase of phosphorylation of SAPK/JNK that was evident after 6 h (**Figure 3B**). Conversely, no significant differences were observed in total levels of SAPK/JNK of FLD treated cells compared to control cells ($p>0.05$, **Figure 3C**). The ratio of phosphorylated versus total SAPK/JNK kinase increased with FLD treatment in a dose dependent manner ($p<0.05$, **Figure 3D**).

3.7. Characterization of FLD

Most abundant peptides in FLD are presented in **Table 2**. A total of 7 fragments with molecular masses within the range of 839 to 2768 Da were identified. These peptides derived from the major lentil proteins: convicilin, allergen Len c 1.0101 and vicilin type C. **Table 3** shows the phenolic profile of FLD acquired by HPLC-ESI-DAD-MSⁿ analysis in the negative-ion mode. A total of 9 unique mass signals were detected ranging from 258-741 Da. Of these, 8 compounds were tentatively identified based on spectral database matching as well as literature data (Dueñas et al., 2016). An unidentified compound with a mass/charge (m/z) ratio of 659 and mass fragments of 468 and 298 was the most abundant compound, reaching up to 846.5 µg/g in FLD. Phenolic acid derivatives such as feruloylquinic acid, gallic acid derivatives I and II and caffeic acid glucuronide were the greatest phenolic group identified in FLD, reaching up to 88.9, 72.1, 56.9 and 16.5 µg/g, respectively. The second most abundant phenolic compounds identified in FLD were the flavonols kaempferol rutinoside-rhamnoside and quercetin-glucuronide, which reached 51.0 and 32.3 µg/g, respectively.

4. Discussion

One of the purposes of this study was to scale up the production of FL and to investigate the impact of pilot scale production on peptide and phenolic contents as well as on bioactivity.

Experimental results indicated that fermentation scale up from 1.5 L to 30 L moderately reduced the peptide and phenolic content as well as bioactivity of FL. These losses could be partially associated to the fact that FL production at pilot scale was performed in milder alkaline conditions (pH 8.0) as compared to lab-scale production (pH 8.5). These small changes in pH value could have a negative impact on Savinase protease activity (optimum pH 8.5) and subsequently on the generation of bioactive peptides, resulting in moderate losses of biological effectiveness. The lower phenolic content found in the soluble fraction of fermented lentil produced at pilot scale could be related to differences in fermentation conditions which may have an impact in solubilization of bound phenolic fraction mediated by microbial metabolism, Savinase (with feruloyl esterase side activity) and alkaline hydrolysis (Bautista-Exposito et al., 2018). Regarding antioxidant activity, what is suggested from our results is that upscale production of fermented lentil slightly reduced the ability to scavenge peroxy radicals through hydrogen donation while improved inhibition of intracellular ROS production most likely through activation of cellular antioxidant response. Antioxidant activity assays such as ORAC and inhibition of *t*-BHP induced cellular oxidative stress provide different information about mechanisms of action of antioxidant compounds. The former only measures the ability of an antioxidant to quench free radicals (i.e. peroxy radical) by hydrogen donation stabilizing the peroxy radical by resonance (Granato et al., 2018). The latter evaluates the ability of compounds as modulators of cell signaling pathways related to activation of cellular antioxidant response (Granato et al., 2018). In order to test this hypothesis the effect of fermented lentil after gastrointestinal digestion on the activation of cellular antioxidant response was further examined.

Despite the moderate losses in bioactivity observed in the present work, a recent study demonstrated that FL produced in the same conditions described herein was efficient at ameliorating alterations in glucose and lipid metabolism and liver functionality in an experimental animal model of MetS and NAFLD (Martinez et al., 2018). In particular, FL was

effective at improving all the parameters and indices related to insulin resistance (blood glucose, area under the curve after an oral glucose overload and profile curve, triglyceride/glucose index), lipid metabolism (plasmatic triglycerides, phospholipids and high density lipoprotein cholesterol levels, atherogenic index), hepatic steatosis (fat content, fatty acid composition) and oxidative stress (hepatic lipid peroxidation) in obese Zucker rats. These health benefits of FL were consistent with the *in vitro* antioxidant, intestinal α -glucosidase and pancreatic lipase inhibitory activities observed in the present study for FL and it could be attributed to the presence of bioactive peptides and phenolic compounds.

Accumulating scientific evidence demonstrates that MetS is associated with an impaired antioxidant status due to a general decrease of hepatic antioxidant enzymes and subsequent increase of lipid peroxidation in the liver (Martinez et al., 2018). Lipid peroxidation is the ROS-triggered metabolic process that results in the destruction of membrane lipids and tissue damage. Martinez et al. (Martinez et al., 2018) found that daily FL administration (1g/kg) for 8 weeks reverted hepatic lipid peroxidation in the obese Zucker rat. With that knowledge, a second purpose of the current study was to investigate the protective effect of FL on *t*-BHP-induced oxidative stress in RAW 264.7 macrophages. Moreover, we determined whether these effects were associated to the activation of Nrf2-mediated antioxidant signaling pathways in RAW 264.7 macrophages. In this study, we demonstrated that FLD was able to counteract *t*-BHP-induced oxidative stress and cell death as well as to improve cellular antioxidant capacity of macrophages by increasing the activity of catalase via a mechanism that involves activation of SAPK/JNK and Nrf2 nuclear translocation. Thus, our results show for the first time the mechanisms of action of FLD to prevent oxidative damage in macrophages through the modulation of signaling pathways involved in antioxidant enzyme regulation.

We selected RAW 264.7 macrophages because it is well known that this cell line could own over-production of ROS following oxidant exposure (Zhang et al., 2018). Previous studies

have shown that *t*-BHP exposure to RAW 264.7 macrophages reduced cell viability and promoted cell apoptosis and necrosis resulting from intracellular ROS generation (Lv, Liu, Zhou, Tan, Deng, & Ci, 2017). Similarly, we found that RAW 264.7 cells exposed to *t*-BHP altered intracellular redox milieu and decreased cell viability even at a relatively low level of *t*-BHP (0.1 mM). We have shown herein that cell pretreatment with FLD prevented the increase in intracellular ROS and promoted cell viability and survival of RAW 264.7 macrophages in a strong oxidant environment (2.5 mM *t*-BHP). These results are in agreement with an earlier study showing that FL administration to obese Zucker rats decreases hepatic thiobarbituric reactive species, a marker of lipid peroxidation (Martinez et al., 2018).

Accumulating evidence shows that cytoprotective effect of antioxidants may be linked to their ability to regulate the expression of antioxidant enzymes (Ramachandran & Saravanan, 2013). Expression of antioxidant enzymes is critical in the detoxication and elimination of ROS and electrophiles through conjugative reactions and by enhancing cellular antioxidant capacity. Our results revealed that pretreatment of RAW 264.7 macrophages with FLD and subsequent exposure to oxidant conditions enhanced the expression of catalase in RAW 264.7 macrophages as compared to non-stressed cells. Catalase catalyzes the conversion of hydrogen peroxide to water and oxygen (Finkel & Holbrook, 2000). Therefore, we proposed that the increased endogenous defense capacity evoked by FLD could be a critical determinant to protect against oxidative stress-induced injury. Similarly, early studies have found that legume (*Phaseolus vulgaris* L.) extracts displayed protective benefit to endogenous antioxidant enzymes such as SOD, catalase, glutathione peroxidase and glutathione reductase in human colonic carcinoma Caco-2 cells (Chen, Zhang, Marcone, Pauls, Liu, Tang, et al., 2017).

A major mechanism in the cellular defense against oxidative stress is activation of the Nrf2-antioxidant response element (ARE) signaling pathways (Nguyen, Nioi, & Pickett, 2009). Activation of Nrf2 is dependent on mechanisms that increase its stability, leading to its

475 accumulation in the cell. A well established mechanism that controls Nrf2 stability is mediated
476 by Keap1 (Nguyen, Sherratt, Nioi, Yang, & Pickett, 2005). Nrf2 is targeted for degradation by
477 Keap1 in the nucleus, a process that requires the transient shuttling of Keap1 into this
478 compartment. In cells under stress, stabilization of Nrf2 is thought to be dependent on
479 modifications of reactive cysteines in Keap 1 that reduce access of this protein to Nrf2
480 (Kobayashi et al., 2006). We investigated the mode of action of FLD to induce the antioxidant
481 defense via Nrf2 activation in RAW 264.7 macrophages. In the present study, we observed that
482 FLD pretreatment of cells clearly induces the nuclear translocation and dose-dependent
483 accumulation of Nrf2, a necessary event to exert its activity. These results were consistent with
484 the increase of catalase gene expression and suggest a clear regulatory mechanism promoting
485 Nrf2 stabilization in response to FLD and induction of ARE-mediated antioxidant enzymes gene
486 expression. Similarly, fermented powder of common bean (*Phaseolus vulgaris* L.) was able to
487 regulate antioxidant and detoxifying enzymes through the Nrf2 pathway, and reduce H₂O₂-
488 induced endoplasmic reticulum (ER) stress (La Marca et al., 2015).

489 Nrf2 activation by phosphorylation at specific serine and/or tyrosine residues has also
490 been reported to be mediated by upstream signaling pathways such as mitogen activated protein
491 kinases (MAPK), phosphatidylinositol-3-kinases/protein kinase B or protein kinase C δ (Lv, Liu,
492 Zhou, Tan, Deng, & Ci, 2017; Niture, Khatri, & Jaiswal, 2014). As with other members of the
493 MAPK family, JNK is activated upon dual phosphorylation on a Thr-Pro-Tyr motif, which is
494 activated by phosphorylation on serine and threonine residues by an upstream kinase (MKK4
495 and MKK7) (Davis, 1999). Therefore, to identify upstream regulatory mechanisms involved in
496 the nuclear accumulation of Nrf2 by FLD, the effect on the phosphorylated levels of SAPK/JNK
497 were also examined in the present study. We found that FLD induced the phosphorylation of
498 SAPK/JNK, therefore, these results suggest that the activation of this pathway could be linked
499 to Nrf2 activation. The present study demonstrates for the first time that FLD induces the

expression of antioxidant enzymes through a mechanism involving activation of Nrf2/MAPK pathways.

Lentil is a source of bioactive peptides and phenolic compounds with antioxidant activity, therefore, the last purpose of the present study was to establish a relationship between the cytoprotective effect of FLD against *t*-BHP induced oxidative stress in RAW 264.7 macrophages and FLD peptide and phenolic composition. HPLC-MS/MS analysis allowed the identification of 7 peptides in FLD derived from the main lentil proteins (convicilin, convicilin, allergen Len c 1.0101 and vicilin type C). Two of these sequences derived from convicilin (EQSPGQWRPSHGKEEDEEEK and EQSPGQWRPSHGKEEDEEEKEQK) were previously found in FL which was indicative of the resistance of these peptides to *in vitro* gastrointestinal digestion simulating physiological conditions (Bautista-Exposito et al., 2018b). Structure-activity relationship analysis revealed that amino acid sequences containing proline, histidine or tyrosine within the sequence and hydrophobic amino acids at the N-terminus and/or C-terminus are determinant for the antioxidant activity (Li & Li, 2013). Moreover, it has been shown that amino and carboxylic group of acidic (aspartic acid and glutamic acid) and basic (arginine, lysine and histidine) amino acids act as metal chelators (Díaz, Dunn, McClements, & Decker, 2003). These structural features were found in most of the peptides collected in **Table 2**. Therefore, it can be assumed that lentil derived peptides in FLD identified in the present study could have contributed to reduction of intracellular ROS levels in *t*-BHP-induced macrophages through their radical scavenging activity. In addition, there is accumulating evidence pointing out that legume derived oligopeptides could exert an indirect antioxidant mechanism through the activation of the cellular antioxidant system. In particular, the chickpea-derived peptide NRYHE upregulates Nrf2 expression and participates in the ARE-mediated activation of antioxidant enzymes including NADP(H):quinone oxidoreductase 1, heme oxygenase 1, γ -glutamyl cysteine synthetase in H₂O₂-treated HT-29 and Caco-2 cells (Guo, Zhang, Jiang, Miao, & Mu, 2014).

The antioxidant activity of lentil derived peptides can be potentiated by the variety of phenolic compounds identified in FLD. Phenolic acids were the most prominent phenolic compounds in FLD followed by flavonols. Our results contrast with the phenolic profile of non-digested FL that is mainly made up of flavonols (kaempferol aglycone and glucosides, isorhamnetin glucuronide), followed by flavan-3-ols [(+)-catechin-3-*O*-glucoside, prodelfinidin dimer and (+)-catechin] and minor amounts of hydroxycinnamic and hydroxybenzoic compounds (Bautista-Exposito et al., 2018b). The differences observed in the phenolic profile of FLD could be attributed to instability of flavonols and flavan-3-ols to pH changes during the different stages of gastrointestinal digestion (Bautista-Exposito et al., 2018b). Earlier studies have shown that phenolic compounds present in FLD are able to neutralize radicals and chelate metal ions through hydrogen, electron donating and mixed mechanisms (Ganesan & Xu, 2017). However, only some of them have been proven as Keap1-Nrf2 activators. For instance, kaempferol aglycone and glucosides have shown to exert protective effects against oxidative stress through activation of Nrf2-mediated defensive response in lung tissues (Kim, Im, Lee, & Chae, 2017; Kumar, Kim, More, Kim, & Choi, 2014). Similarly, gallic acid, quercetin and quercetin glucoside protects against oxidative stress-induced hepatotoxicity by activation of the Keap1-Nrf2 pathway (Feng, Wang, He, Yang, & Wan, 2017; Joko, Watanabe, Fuda, Takeda, Furukawa, Hui, et al., 2017; Kim, Im, Lee, & Chae, 2017). Structure-activity relationship analysis revealed that compounds bearing phenolic hydroxyl groups are bifunctional antioxidants that exert protective role by scavenging hazardous oxidants and inducing cytoprotective enzymes (Dinkova-Kostova & Talalay, 2008).

5. Conclusions

In summary, we have shown that FLD composed of antioxidant peptides and phenolic compounds is able to enhance the cellular antioxidant defense capacity, thereby, protecting macrophages from oxidative stress. FLD reverted t-BHP-induced cytotoxicity and ROS

production through activation of SAPK/JNK phosphorylation followed by nuclear accumulation and transactivation and the subsequent expression of catalase. All these events were essential to maintain cellular redox homeostasis and prevent oxidative stress cell damage.

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ABBREVIATION LIST

ACE: angiotensin I converting enzyme inhibitory activity; ACN: acetonitrile; ARE: antioxidant response element; *t*-BHP: *tert*-butyl hydroperoxide; DCF: dichlorofluorescein; DCFDA: dichlorofluorescein diacetate; DMEM: High-glucose Dulbecco’s Modified Eagle’s Medium; FA: formic acid; FBS: Fetal bovine serum; FL: fermented lentil; FLD: fermented lentil digest; HRP: horse radish peroxidase; JNK: Jun amino-terminal kinases; NAFLD: non-alcoholic fat liver disease; Nrf2: nuclear factor like 2; MetS: metabolic syndrome; m-IgGκ: mouse IgGκ light chain binding protein; ORAC: oxygen radical absorbance capacity; PBS: phosphate buffer saline; PVDF: Polyvinylidene fluoride; ROS: reactive oxygen species; SAPK-stress-activated protein kinases; SOD-1: Cu-Zn superoxide dismutase; TFA: trifluoroacetic acid; TBST: Tris buffer saline Tween 20

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706 **FIGURE CAPTIONS**

707 **Figure 1. FLD reduces *t*-BHP-induced cell death (A-C) and intracellular redox status (D-F) in**
708 **RAW 264.7 macrophages.** RAW 264.7 macrophages were exposed to *t*-BHP (0.05, 0.1, 1.0 and 2.5
709 mM) for 3 h (A and D) FLD (50, 75 and 100 µg/mL) for 18 h (B and E) and FLD (50, 75 and 100
710 µg/mL) for 18 h followed by treatment with 2.5 mM *t*-BHP for 3 h (C and F). Cell viability was
711 measured using a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-
712 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] and an electron coupling
713 reagent (phenazine ethosulfate). Cell viability was expressed as relative percentage of control cells.
714 Oxidant production was measured by DCF probe. Data were expressed relative percentage of
715 fluorescence intensity in control cells. All results are expressed as means ± standard deviation of six
716 independent experiments for each experimental condition. Different letters denote statistically
717 significant differences ($p < 0.05$, Duncan test).

718 **Figure 2. FLD activates catalase protein expression (A-C) and Nrf2 nuclear translocation (D**
719 **and E) in RAW 264.7 macrophages.** RAW 264.7 macrophages were treated with FLD (50, 75 and
720 100 µg/mL) for 18 h followed by 0.1 mM *t*-BHP for 3 h. Following treatment, protein was harvested
721 and immunoblotted for the indicated proteins. Representative Western blot of three different
722 experiments (A and D). Protein expression of catalase, SOD-1 and Nrf2 were relative to untreated
723 cells (B, C and F respectively). The intensities of catalase, SOD-1, Nrf2, Lamin B1 and β-actin was
724 quantified using Bio-Rad Image Lab software. All results were expressed as means ± standard
725 deviation of three independent experiments. Different letters denote statistically significant
726 differences ($p < 0.05$, Duncan test).

727 **Figure 3. FLD protective effect against oxidative stress involves MAPK activation.** Total (T) and
728 phosphorylated (P) SAPK/JNK were detected by Western blot in cytosolic lysates after RAW 264.7
729 macrophages treatment with FLD (50, 75 and 100 µg/mL) for 6 h. Following treatment, protein was
730 harvested and immunoblotted for the indicated proteins. Representative Western blot image of T-

731 SAPK/JNK and P-SAPK/JNK (A). T-SAPK/JNK and P-SAPK/JNK intensity relative to β -actin
732 expressed as percentage relative to control cells (B). Ratio of P-SAPK/JNK vs. T-SAPK/JNK (C).
733 The intensities of T-SAPK/JNK and P-SAPK/JNK and β -actin were quantified using Bio-Rad Image
734 Lab software. All results were expressed as means $\pm$ standard deviation of three independent
735 experiments. Different letters denote statistically significant differences ($p < 0.05$, Duncan test).

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