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Protein hydrolysates from *Hermetia illucens* trigger cellular responses to cope with LPS-induced inflammation and oxidative stress in L-929 cells

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ABSTRACT

Insect protein hydrolysates (PH) are emerging as valuable compounds with biological activity. The aim of the present study was to assess the potential cytoprotective effects of PH from the Black Soldier Fly (BPH, in the range 0.1–0.5 mg/mL) against inflammatory conditions and oxidative stress in LPS-challenged L-929 cells. BPH was effective in inhibiting LPS-induced ROS and nitrite production and in reducing the protein and transcript levels of remarkable inflammatory markers, such as TNF-α, IL-6, IL-1α, and IL-1β, as determined by ELISA and/or qPCR. Moreover, the BPH antioxidant and anti-inflammatory activities rely on the induction of selected genes and proteins involved in the antioxidant response (i.e. *Cu/ZnSod, MnSod*, *Gpx*, *HO-1*) through *Nrf2*, as well as on the inhibition of the activation of NF-κB, a key player in inflammation. These findings suggest that BPH represents effective bioactive compounds with therapeutic potential for mitigating oxidative stress and inflammation in vitro, thus deserving further investigation into the underlying mechanisms before BPH application as novel drugs in the near future.

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1. Introduction

The increase in world population and the consequent protein shortage imposes the urgent need to find new sustainable protein sources to meet global demand. Among the possible alternatives, insects have received particular attention for their valuable nutritional content and for their remarkable sustainability. Indeed, insect farming produces a lower environmental impact than common farm animals due to the reduced use of land and water, and fewer greenhouse gas emissions (Van Huis and Oonincx 2017). Beyond their great potential as sustainable sources of high-value protein with a balanced amino acid content, valuable fatty acids and relevant microelements, insects have been recognized as an important source of bioactive peptides (Liceaga et al. 2018) whose positive impact on animal health is well acknowledged (Apostolopoulos et al. 2022). In the last years, research has demonstrated that these peptides can play an effective role as bioactive molecules with low toxicity in several cellular conditions including but not limited to oxidative stress, cancer, inflammation and immunomodulation (Quah et al. 2023). Indeed, increasing evidence showed that bioactive peptides, regardless of their source, represent a promising alternative to conventional drugs for health issues associated with inflammation and oxidative stress. During the inflammatory process, oxidative stress and reduced cellular antioxidant activity occur that in turn lead to the overproduction of reactive oxygen species triggering multiple signaling pathways to cope with cellular imbalance between free radicals and antioxidants common to several pathological conditions. The biological activities of diverse food-derived molecules have been emerging as valuable nutraceuticals in the management and prevention of pathological conditions where oxidative stress and inflammation are key players. Two different transcription factors are pivotal players in suppressing oxidative stress (Nrf2) or inducing inflammation (NF-kB) and their interplay is associated with many diseases.

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The Nrf2/NF-κB signaling pathway is known to intervene as one of the mechanisms of action of many natural compounds leading to the activation of the antioxidant cellular responses and the inflammatory responses. Nrf2 is a master regulator of antioxidant genes including *superoxide dismutase* (*SOD*) and *heme oxygenase 1* (*HO-1*) while NF-κB is a master regulator of inflammatory responses playing a key role in the control of the expression of genes encoding *tumor necrosis factor-α* (*TNF-α*) and interleukins, such as *IL-1β* and *IL-6*. Natural compounds are able to modulate the crosstalk between Nrf2 and NF-κB leading to cellular response against oxidative stress and inflammation through either the stimulation of antioxidant defenses by Nrf2 that in turn reduces the ROS-mediated NF-κB activation and/or the inhibition of NF-κB activation via HO-1 (Wu et al. 2022) but these complex molecular mechanisms still need to be further elucidated (Gao et al. 2022).

In the last years, several protein hydrolysates have been produced from insect protein, mainly via enzymatic hydrolysis by using different proteases such as papain, bromelin, alcalase, etc. (Firmansyah and Abduh 2019; Leni et al. 2020). The obtained oligopeptides and free amino acids have been characterized and assessed for their potential biological activity and beneficial role in animal health. Indeed, in addition to being used as a health promoting ingredient in animal feed (Hoffmann et al. 2020; Cho et al. 2020; Mikołajczak et al. 2020), the protein hydrolysates showed the ability to reduce allergenic risk and significant antioxidant and ACE inhibitor activities (Firmansyah and Abduh 2019; Leni et al. 2020b; Zhu et al. 2020; Dai et al. 2013). Moreover, insect protein hydrolysates from cricket (*Gryllodes sigillatus*), mealworm (*Tenebrio molitor*) and locusts (*Schistocerca gregaria*) have been shown anti-inflammatory activity (Zielińska et al. 2017). Peptides obtained from *Musca domestica* larvae by different proteases have been shown to exert relevant antioxidant properties by scavenging superoxide anion radicals (Zhang et al. 2016).

Despite the rising interest in exploring the biological effects exerted by the insects' bioactive peptides, the precise cellular mechanisms of action are still far from being elucidated.

Among the insects that have been successfully used to produce protein hydrolysates, *Hermetia illucens*, also known as Black Soldier Fly (BSF) is gaining major relevance for its role in waste valorization within the framework of the circular economy resulting from larvae ability to feed several organic matrices thus bio-converting waste into valuable larval biomass to be used for different applications. Recently, the BSF proteins hydrolysates (BPH) have been used as valid alternative protein sources in aquatic animals such as fish (*Oncorhynchus mykiss*) (Roques et al. 2020) or Pacific white shrimp (*Litopenaeus vannamei*) improving feed palatability (Terrey et al. 2021). Apart from the nutritional value and beneficial effects on animal wellness in different animal feeding trials, polypeptides and free amino acids in the BPH showed several biological activities. Mouithys-Mickalad (Mouithys-Mickalad et al. 2021) evaluated the antiarthritic potential of protein hydrolysates in HL-60 cells showing that BPH can prevent the development of arthritis by suppressing the reactive oxygen species (ROS) production. BPH showed also a cytoprotective effect in murine fibroblast L-929 cells where treatment with BPH in the range 0.01–1.5 mg/mL determined a dose-dependent reduction of ROS and the Nrf2 (Nuclear factor erythroid 2-related factor 2) translocation to the nucleus, that in turn can mediate the transcriptional response of cells to oxidative stress (Riolo et al. 2023).

The aim of this study was to assess the potential anti-inflammatory activity of BSF protein hydrolysates by evaluating the cellular responses in L-929 cells under lipopolysaccharide (LPS)-induced inflammatory conditions *in vitro*. Findings from functional assays together with the assessment of the expression of genes and proteins involved in the antioxidant (*Nrf2*, *Cu/ZnSOD, MnSOD, Gpx, HO-1*) and inflammatory responses (*IL-1α, IL-1β, IL-6, TNF-α, NF-kB, IkB-α*, *Ikk-γ*) can provide insights into the mechanisms underlying the biological activity of BPH and their potential for future applications as health-promoting ingredients.

2. Materials and methods

2.1. In vitro cell culture

Mouse fibroblast cells (L-929) obtained from American Type Culture Collection (ATCC) were grown in cell culture T75 flask in DMEM complete medium including 10% fetal bovine serum, streptomycin (100 µg/mL) and penicillin (100 µg/mL) (Sigma-Aldrich) at 37 °C with 5% of CO_2. L-929 cells were seeded into 96-well culture plates (5×10^4 cells per well) and incubated with different concentrations of BPH (0.5; 0.1 mg/mL) for 30 min and subsequently stimulated by LPS (10 µg/mL) for 24 h in presence (treatments) of BPH or not (control). The BPH concentrations used in this study were chosen based on our previous research describing the protein hydrolysates production and characterization from *H. illucens* larvae. Briefly, BSF larvae were provided by the established colony (www.progettohermetia.it, Gioia Tauro, Italy) and reared on

organic matrices composed of 65% vegetal; 5% meat/fish; 25% bread/pasta/rice; 5% other. Larvae were collected, pasteurized and dried at room temperature prior to storing at −80°C until further processing. To produce the protein hydrolysates (BPHs), BSF larvae were subjected to defatting with *n*-hexane and the protein fraction was subjected to enzymatic digestion by using an enzyme mix (flavourzyme/protamex/alkalase 1:1:1) for 10 h at 50°C (Riolo et al. 2023).

2.2. Nitrite and ROS quantification

The generation of intracellular ROS was determined by carboxy-H2DCFDA (Invitrogen, Thermo Fisher) assay according to the manufacturer's protocol; briefly, the culture medium was removed and cells were incubated with the probe for 60 min. Subsequently, the probe was removed and the cells were washed. Then fluorescence intensity signals were evaluated under a fluorescence microscope (Evos M5000, Thermo Fisher). The nitrite concentration was calculated by the Griess reaction from a nitrite standard curve as previously described (Kiemer and Vollmar 2001).

2.3. Enzyme-linked immunosorbent assay (ELISA)

TNF-*α* and IL-6 levels were evaluated in cell culture supernatant with commercial kits (ThermoFisher # 88-7324-88 and ThermoFisher # 88-7064-88 for TNF-*α* and IL-6, respectively) according to manufacturer's protocol. To evaluate the expression of proteins involved in oxidative stress responses and glutathione levels, cells were lysed with RIPA Lysis and Extraction Buffer (Catalog number 89901, Thermo Fisher Scientific). Then cell lysates were analysed with commercially available ELISA Kits for Mouse HO-1 (EEL141, Thermo Fisher Scientific), MnSOD (DYC3419-2, R&D system), according to the kit instructions.

2.4. Colorimetric assays

Cell lysates were analysed with Glutathione Peroxidase Assay Kit (MAK437, Merk), and Gluthatione colorimetric assay kit (ab239727, abcam) according to the kit instructions to determine the GPX activity and GSH levels, respectively.

2.5. Immunofluorescence

Cells were grown in 6-well tissue culture plate. Control and treated cells were fixed with 4% paraformaldehyde (pH 7.4) and then permeabilizated with 0.1% Triton X-100 in 1X PBS before incubating with primary anti NF-kB p65 antibody (Invitrogen, ThermoFisher # MA5-51360) and subsequently with specific secondary antibody Texas Red labeled (Invitrogen, ThermoFisher, # T-2767) and DAPI for nuclear counterstaining. Then the cells were washed and observed under fluorescence microscopy EVOS M5000 (ThermoFisher Scientific). At least 200 cells were counted from each experiment. The percentage of NF-κB–p65–positive cells was calculated as the number of cells showing nuclear staining divided by the total cell number showing DAPI nuclear blue staining × 100.

2.6. RNA extraction and cDNA synthesis

Total RNA was extracted from treated and control cells using the PureZOL™ RNA Isolation Reagent (BIO-RAD) according to manufacturer's protocol. Quality and quantity of RNA was evaluated by Nano Drop spectrophotometer (Thermo Fisher Scientific, Italy) and 1% agarose gel electrophoresis. After the removal of any possible contamination of genomic DNA, the cDNA synthesis was performed starting from 1 µg of total RNA using the ImProm-II™ Reverse Transcription System (Promega) following the manufacturer's protocol. All cDNA samples were stored at −20°C for the subsequent analyses.

2.7. Quantitative polymerase chain reaction (qPCR)

To evaluate the expression of the genes involved in the antioxidant and anti-inflammatory responses in cells, quantitative real-time PCRs were performed using the GoTaq® qPCR Master Mix (Promega) in a Rotor-Gene Q2 plex Hrm thermocycler (Qiagen). cDNAs were first 1:20 diluted and then amplified using specific qPCR primers for genes associated with different metabolic pathways, namely the antioxidant response (*Nrf2*, *Cu/ZnSOD, MnSOD, Gpx, HO-1*) and the inflammatory response (*IL-1α, IL-1β, IL-6, TNF-α*, *NF-kB*, *IkB-α*, *Ikk-γ*). *Gadph* and *36b4* were assessed as reference genes (Table 1). Primer sets were reported in previous studies (Riolo et al. 2023; Giannetto et al. 2020). After determining PCR efficiencies using a five-point standard curve of a 5-fold dilution series from pooled RNA, the raw target gene data were corrected using the normalization factor calculated by the geNorm software (http://medgen.ugent.be/~jvdesomp/genorm/). All experiments were conducted in triplicate.

2.8. Statistical analysis

Data from gene expression were subjected to analysis of variance followed by Student-Newman-Keuls post hoc tests to evaluate any differences in the expression

Table 1. Primer sequences of target and reference genes were used in this study.

Gene Name	Gene accession number	Forward (5′ – 3′) Reverse (5′ – 3′)	Size (bp)
Cu/ ZnSOD	NM_011434.2	GAAGAGAGGCATGTTGGAGACC TCTTGTTTCTCATGGACCACCA	143
MnSOD	NM_013671.3	ATCAAGCGTGACTTTGGGTCTT AAGCGACCTTGCTCCTTATTGA	116
Gpx	NM_001329527.1	GACACCAGAATGGCAAGAATGA TCTCACCATTCACTTCGCACTT	114
HO-1	NM_010442.2	GCACAGGGTGACAGAAGAGG GTGAGGACCCACTGGAGGAG	234
IL-1α	NM_010554.4	CCTACTCGTCGGGAGGAGAC GCAACTCCTTCAGCAACACG	114
IL-1β	NM_008361.4	TCGCAGCAGCACATCAACA GGTCCACGGGAAAGACACAG	96
IL-6	NM_001314054.1	GCCTTCTTGGGACTGATGCT CATTTCCACGATTTCCCAGAG	159
TNF-α	NM_001278601.1	CAACGGCATGGATCTCAAAG CTTGACGGCAGAGAGGAGGT	178
IkB-α	NM_010907.2	AGCATCTCCACTCCGTCCTG CGTGGATAGAGGCTAGGTGC	72
Ikk-γ	NM_001136067.2	AACAAGCACCCCTGGAAGAA ACAGCGTTCCCTCAGCATCT	225
NF-kB	NM_001410442.1	CATGGTGGTTGGCTTTGCAA CTGTCATCCGTGCTTCCAGT	83
Gadph	NM_001289726.2	TCCATGACAACTTTGGCATTG TCACGCCACAGCTTTCCA	103
36b4	NM_007475.5	GGACCCGAGAAGACCTCCTT GCACATCACTCAGAATTTCAATGG	85
Nrf2	NM_010902.5	TTTCAGCAGCATCCTCTCCA AGCCTTCAATAGTCCCGTCC	194

levels of the target genes between control and treated cells using SigmaPlot (Systat software). Significance levels were set at *P* values of less than 0.05.

3. Results

3.1. Effects of BPH on ROS and NO production in LPS-stimulated cells

The LPS exposure determined an increase in intracellular ROS in L-929 cells (Figure 1a,b). However, the treatment with BPH was able to mitigate this response suggesting that both the BPH concentrations (0.5 and 0.1 mg/mL) can effectively reduce the intracellular reactive oxygen species (ROS) production. Moreover, we investigated the impact of the BPH on nitric oxide (NO) production in response to lipopolysaccharide (LPS) by assessing nitrite concentration. As depicted in Figure 1c, following LPS stimulation, we observed a significant increase in nitrite levels. However, cells challenged with LPS and treated with BPH exhibited significantly lower nitrite levels compared to untreated cells.

3.2 Effects of BPH on cellular oxidative stress response

The effects of BPH in L-929 cells challenged with LPS were assessed by measuring the expression levels of key genes involved in the oxidative stress response. The treatment with BPH induced a modulation of antioxidant genes as well as the transcription factor *Nrf2* (Figure 2). *Nrf2*, together with the two *SOD* genes, *Cu/ZnSOD* and *MnSOD*, were upregulated in response to LPS stimulation. Both the BPH concentrations (0.5 and 0.1 mg/mL) were able to further increase the LPS-induced upregulation of gene expression. This increase in the transcript levels was BPH concentration-dependent, with the highest levels at 0.5 mg/mL for *Cu/ZnSOD* (Figure 2b), while no significant differences in *Nrf2* and *MnSOD* gene expression were observed between the two BPH concentrations (Figure 2a,c).

The glutathione peroxidase (*Gpx*) gene expression levels were increased in the LPS group with respect to the control. These high levels were maintained in both the BPH groups with no significant differences among LPS and BPH groups (Figure 2d).

HO-1 gene expression in the LPS group was comparable to the control. However, the treatment with BPH induced a strong increase in *HO-1* transcript levels (2.03 and 2.23 times for BPH 0.5 mg/mL and BPH 0.1 mg/mL vs LPS, respectively) (Figure 2e).

To provide insights into the mechanisms underlying the BPH antioxidant activity, and its ability to reduce the oxidative stress induced by LPS in challenged cells, the levels of HO-1, MnSOD, glutathione peroxidase (GPX) and glutathione (GSH) were measured by ELISA or colorimetric assay. Compared with the LPS group, both BPH treatments significantly increased MnSOD and HO-1 protein expression in a dose-dependent manner (Figure 2f,h). Moreover, glutathione peroxidase activity was reduced in the LPS group and increased following the treatment with BPH at both concentrations (Figure 2g). The assessment of glutathione levels in control and treated cells showed that GSH levels were reduced in the LPS group, while BPH increased significantly the GSH levels with values higher than control in the BPH 0.5 mg/mL group (Figure 2i).

3.3. Effects of BPH on pro-inflammatory cytokines production

To explore the influence of BPH in LPS-induced inflammatory response in L-929 cells, the secretion and the gene expression levels of major proinflammatory cytokines such as TNF-*α* and IL-6 were evaluated by ELISA and real-time PCR. After the LPS stimulation, a significant increase in TNF-*α* and IL-6 protein and gene expression levels was found (Figure 3). The groups treated with BPH showed significantly lower levels of TNF-*α* and IL-6 in response to LPS stimulation at both protein and gene expression levels. Furthermore, we confirmed the

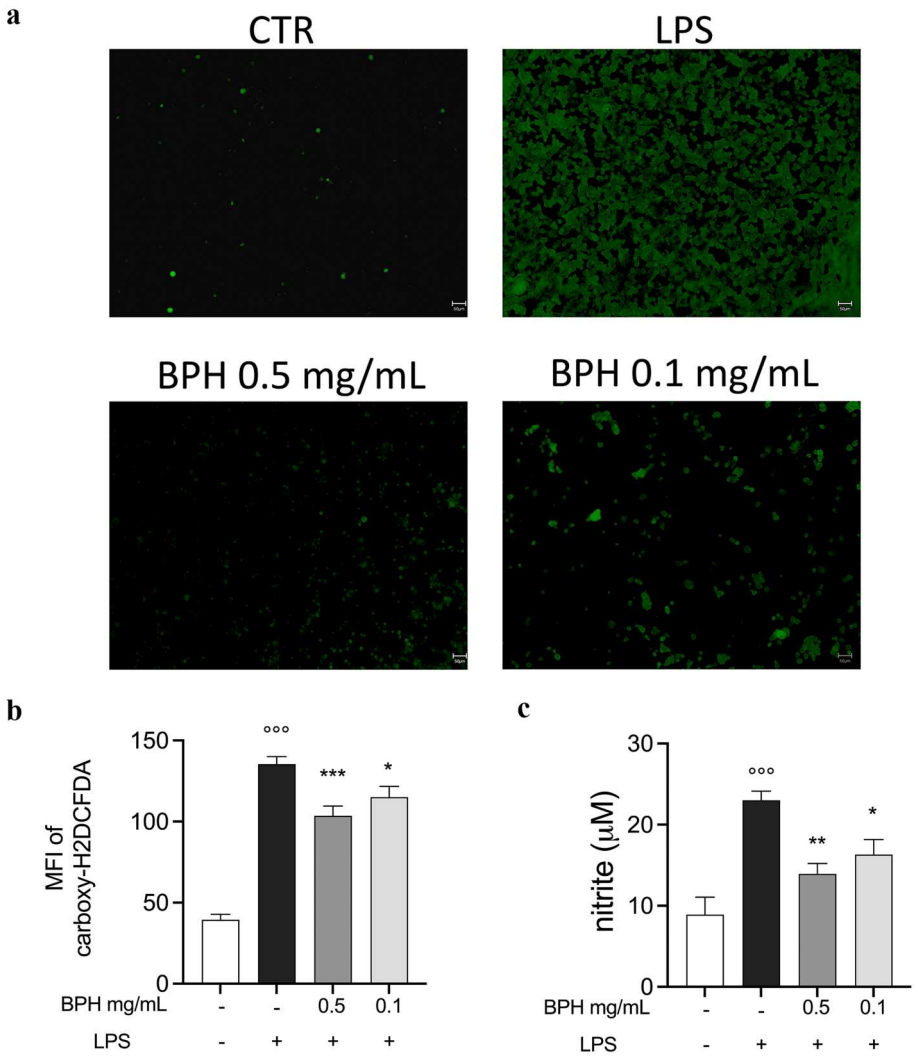

Figure 1. ROS and nitric oxide production in L-929 cells. (a,b) Representative images of ROS detection and quantification by carboxy-H2DCFDA probe. (**c**) Nitrite quantification by Griess reaction. Data are representative of at least three independent experiments and presented as means ± standard error of mean (SEM), °°° $p < 0.001$ versus CTR, *** $p < 0.001$ versus LPS; * $p < 0.5$ vs. LPS.

anti-inflammatory effect of BPH on L-929 stimulated cells through the expression analysis of the genes involved in the production of other inflammatory cytokines that were investigated by qPCR. The expression of *IL-1α* and *IL-1β* was significantly increased in LPS-treated cells as compared with the control (untreated cells). However, following treatment with BPH, the expression levels of both these inflammatory mediators were decreased as compared to LPS-treated cells; interestingly, the effects of BPH on these transcript levels were dose-dependent, with the highest BPH concentration showing the greatest decrease in these cytokines (Figure 3).

These data indicated that the BPHs play a protective role against cell inflammation induced by LPS-treatment by decreasing the levels of *TNF-α*, *IL-6*, *IL-1α* and *IL-1β*.

3.4. Effects of BPH on NF-kB pathway

To further investigate the mechanisms underlying the anti-inflammatory effects of BPH in LPS-stimulated cells, NF-kB and its negative regulatory subunits *IkB-α* e *IkB-γ* mRNA levels were measured in cells pre-treated with BPH at different concentrations followed by treatment with LPS. Results showed that *NF-kB* transcript levels were increased in cells treated with LPS compared to control; the treatment with the lowest BPH concentration (0.1 mg/mL) was not effective in reducing the observed *NF-kB* upregulation. On the contrary, the pretreatment with BPH 0.5 mg/mL caused a significant decrease in *NF-kB* transcript levels (Figure 4a). The *IkB-α* gene was upregulated 1.43 times in LPS group with respect to the control group, while in the cells pretreated with BPH (0.5 and 0.1 mg/mL) this gene was downregulated

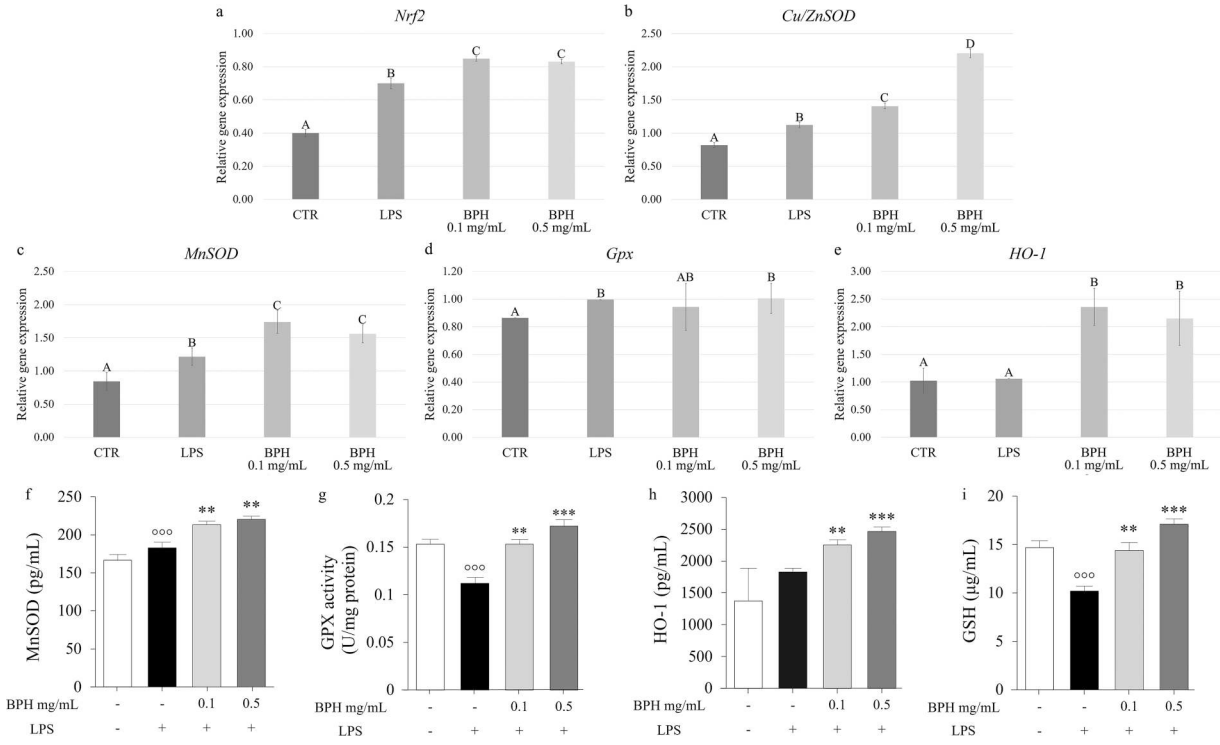

Figure 2. Oxidative stress response. Transcript levels of (a) *Nrf2*, (b) *Cu/ZnSOD,* (c) *MnSOD*, (d) *Gpx* and (e) *HO-1* as determined by qPCR. Relative mRNA levels were evaluated in L-929 cells from each experimental group. Data are expressed as mean ± standard deviation (S.D.) ($n = 6$); different letters above each column denote significant differences among experimental groups ($p < 0.05$). Protein levels of (f) MnSOD, (g) HO-1, enzymatic activity of (h) GPX and (i) GSH levels. Data are representative of at least three independent experiments and presented as means ± SEM. °°° $p < 0.001$ vs. CTR, *** $p < 0.001$ vs. LPS; **$p < 0.001$ vs. LPS. * $p < 0.5$ vs. LPS.

showing a dose-dependent effect (Figure 4b). In cells from LPS group the *Ikk-γ* transcript levels were lower compared to control; however, after the pretreatment with BPH 0.1 mg/mL, mRNA levels increased 1.9-fold compared to LPS group (Figure 4c).

Moreover, the NF-kB nuclear translocation and activation were evaluated by immunofluorescence and nuclear localization. As shown in Figure 5, we found a significative presence of NF-kB positive cells in LPS group detectable as a higher number of cells with nuclear expression of Nf-kB (red) in relation to nuclear counterstain DAPI (blue). While the treatment with the lowest BPH concentration (0.1 mg/mL) was not effective in reducing the NF-kB nuclear translocation into the nucleus, the treatment with the highest BPH concentration decreased the levels of NF-kB positive cells.

4. Discussion

To date, bioactive molecules from nature have contributed to the development of novel treatments for several pathological conditions (Kim et al. 2022). More recently, researchers have focused on the great potential of insect protein and their derivatives as nutraceuticals, health-promoting ingredients and new drugs with relevant biological properties including antioxidant, antimicrobial, and anti-inflammatory effects. Protein extracts from *H. illucens* have revealed remarkable biological properties in the feeding trials of several fish and aquatic species (Roques et al. 2020; Terrey et al. 2021; Mouithys-Mickalad et al. 2021). Studies on the effects of BPH on a cell model of H_2O_2-iduced oxidative stress have shown that the antioxidant activity of BSF protein hydrolysates mainly relies on the reduction of ROS levels together with the nuclear translocation of Nrf2, a master regulator of the antioxidant response (Riolo et al. 2023). To further investigate the possible biological activities of BPH previously obtained by enzymatic hydrolysis from BSF protein extracts, in this study L-929 cells were challenged with LPS to induce the inflammatory response and the potential mechanisms underlying the effects of BPH against LPS-induced oxidative and inflammatory stress were assessed.

Oxidative stress is a well-documented driver of cellular damage, and in our study, LPS exposure resulted in a notable increase in intracellular reactive oxygen species (ROS). However, BPH administration effectively counteracted this increase in ROS establishing its role as a valuable antioxidant in this context. The chemical

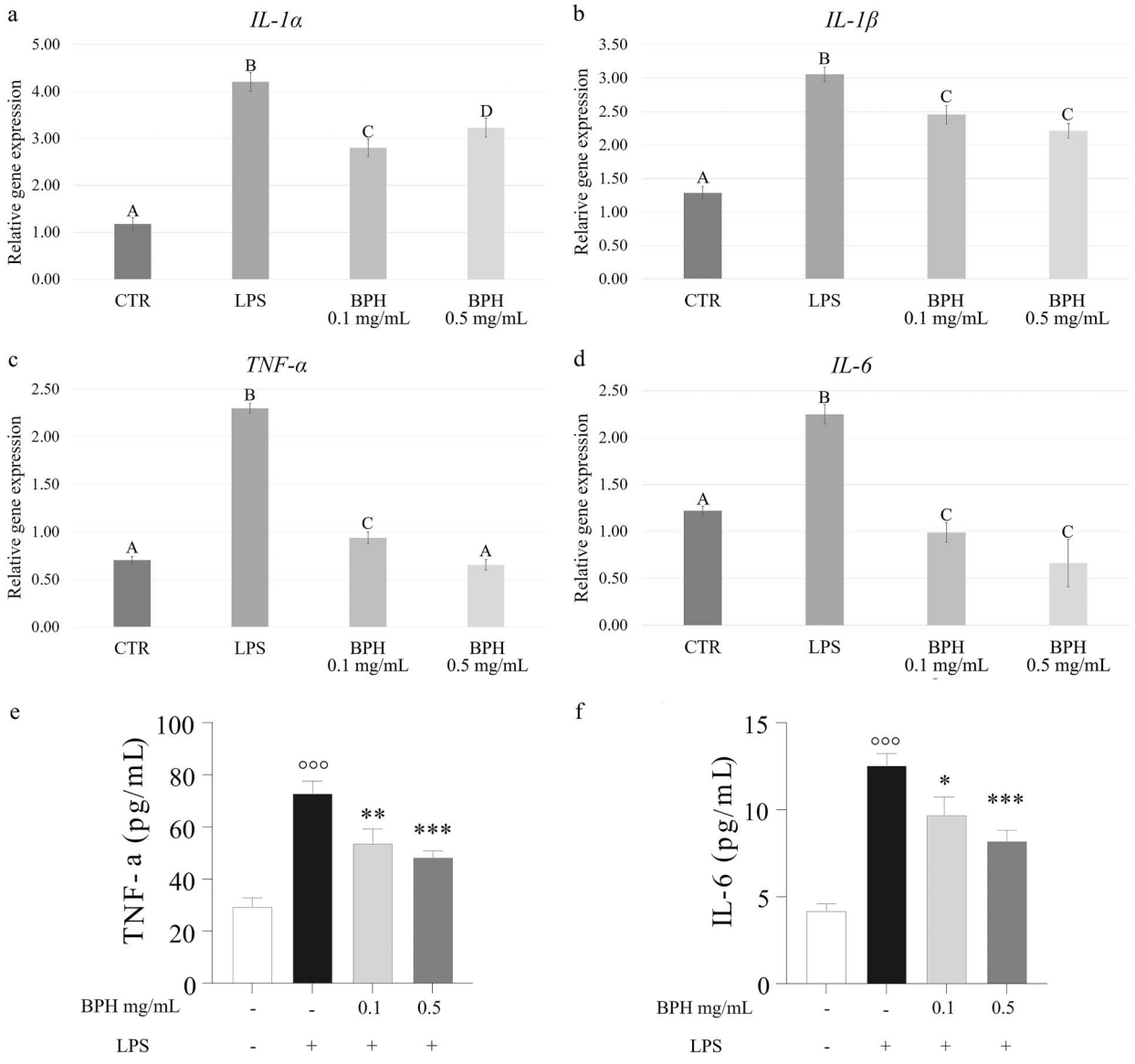

Figure 3. Expression profile of pro-inflammatory cytokines. Transcript levels of (a) *IL-1α*, (b) *IL-1β*, (c) *TNF-α* and (d) *IL-6* as determined by qPCR. Relative mRNA levels were evaluated in L-929 cells from each experimental group. Data are expressed as mean ± S.D. ($n = 6$); different letters above each column denote significant differences among experimental groups ($p < 0.05$). ELISA test for cytokines quantification. Protein levels of (e) TNF-*α* and (f) IL-6. Data are representative of at least three independent experiments and presented as means ± SEM. °°° $p < 0.001$ vs. CTR, *** $p < 0.001$ vs. LPS; **$p < 0.001$ vs. LPS. * $p < 0.5$ vs. LPS.

characterization of our BPH (Riolo et al. 2023) showed a metabolite profile with high levels of hydrophobic and aromatic amino acids that may contribute to the radical-scavenging activity according to previous data on housefly water extract (Li et al. 2017).

The reported antioxidant effects of BPH (Riolo et al. 2023) are particularly important as oxidative stress is a critical contributor to cellular damage and various inflammatory conditions that represent one of the major risk factors for the progression of several chronic diseases (Arulselvan et al. 2016).

Our investigation into the effect of BPH on NO production in response to LPS revealed a significant increase in NO levels upon LPS stimulation. Nevertheless, BPH treatment led to a considerable reduction in NO production, underscoring its potential as a regulator of NO, a key mediator in the inflammatory response.

The inhibitory effects of BPH on oxidative stress induced by LPS stimulation in this cell model involve the reduction of LPS-induced ROS production through the improvement of antioxidant endogenous systems. Interestingly, the significant increase in heme oxygenase-1 (HO-1) transcript and protein levels in both BPH groups, suggests its potential involvement in the Nrf2/HO-1 pathway, one of the mechanisms underlying the

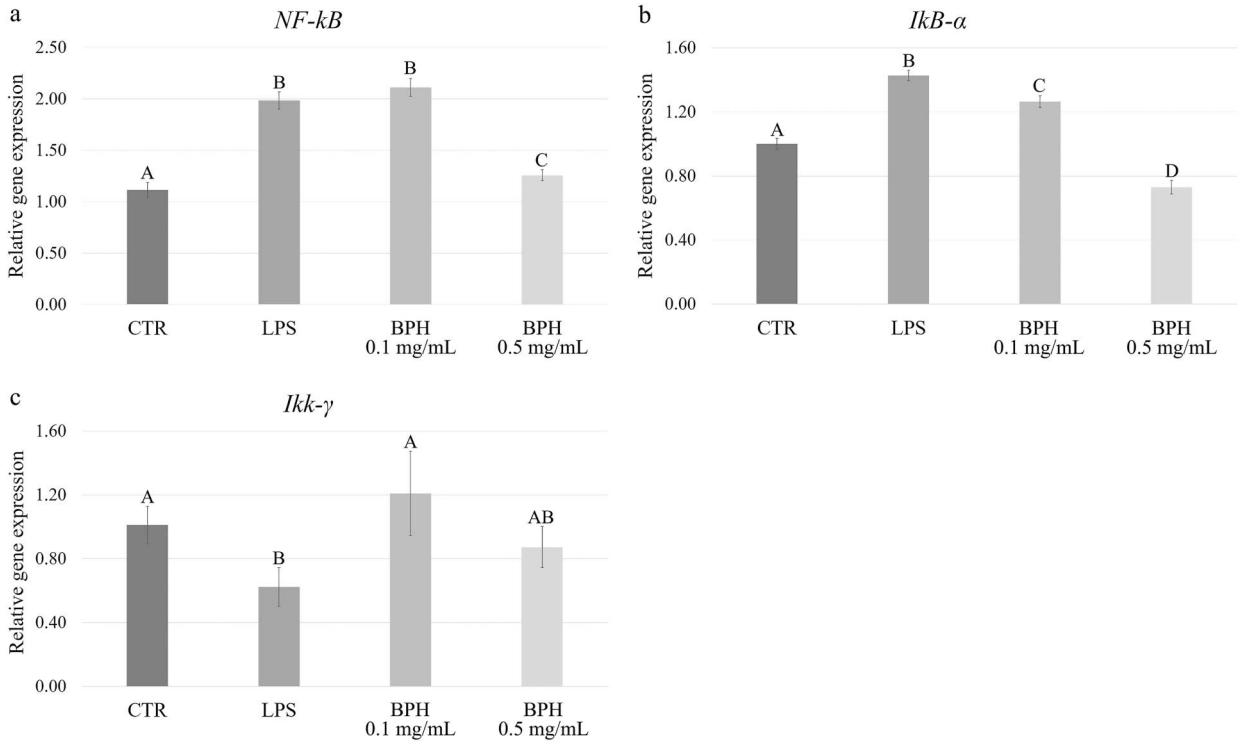

Figure 4. Expression profile of NF-kB pathway. Transcript levels of (a) *NF-kB*, (b) *IkB-α* and (c) *Ikk-γ* as determined by qPCR. Relative mRNA levels were evaluated in L-929 cells from each experimental group. Data are expressed as mean ± S.D. ($n = 6$); different letters above each column denote significant differences among experimental groups ($p < 0.05$).

anti-inflammatory and anti-oxidative effects of diverse biological compound in several cell models (Luo et al. 2018). Recently, the activation and translocation of Nrf2 induced by the treatment with BPH has been reported in our previous study (Riolo et al. 2023) where BPH showed remarkable antioxidant activity in an oxidative stress cellular model by counteracting ROS production and inducing the transcription of *Nrf2* and its entry into the nucleus. Interestingly, *Nrf2* gene expression analysis provided evidence that the protective effects of BPH against LPS-induced oxidative stress may be related to Nrf2 pathway that in turn can boost the transcription of the antioxidant genes thus counteracting the LPS-induced oxidative stress in L-929 cells. Notably, the transcriptional factor Nrf2 is known to play a relevant role in protecting the cells from oxidative stress and also inflammation-induced damage, mainly controlling the expression of the key components of the antioxidant system in the nucleus, as well as enzymes involved in ROS and xenobiotic detoxification, and heme metabolism (Tonelli et al. 2018; Wu et al. 2022). In this study, the treatment with BPH resulted in a modulation of the antioxidant response; both the cytosolic and mitochondrial *SOD* genes showed higher expression levels in BPH groups with respect to LPS and control groups demonstrating that protein hydrolysates potentially exert antioxidant activities by increasing cellular defence against oxidative stress; these data were supported by the high MnSOD protein levels as well as by the activation of glutathione peroxidase observed in cells treated with both BPH concentrations. Additionally, BPH was effective in restoring the glutathione levels to counteract its reduction in LPS-challenged cells confirming their remarkable antioxidant activity.

These findings suggested that BPH could contain molecules playing a role as effective Nrf2 activators counteracting inflammation and oxidative stress in L-929 cells. However, given the complexity of the potential bioactive molecules contained within BPH, we don't have a direct relationship with this compound on the NRF2 pathway. Therefore, this observed effect could be both a direct and an indirect effect, mitigating the cellular damage induced by the inflammatory response.

Several authors showed that the increase in *HO-1* expression plays an important role in cell protection against oxidative stress and inflammation (Ryter et al. 2006; Chung et al. 2008). In contrast with Chung et al. (Chung et al. 2008) who reported that HO-1 can be rapidly induced by various oxidative-inducing agents, including also LPS, our results showed that LPS challenge in L-929 cells does not lead to the increase in *HO-1* gene expression; however, BPH treatment was

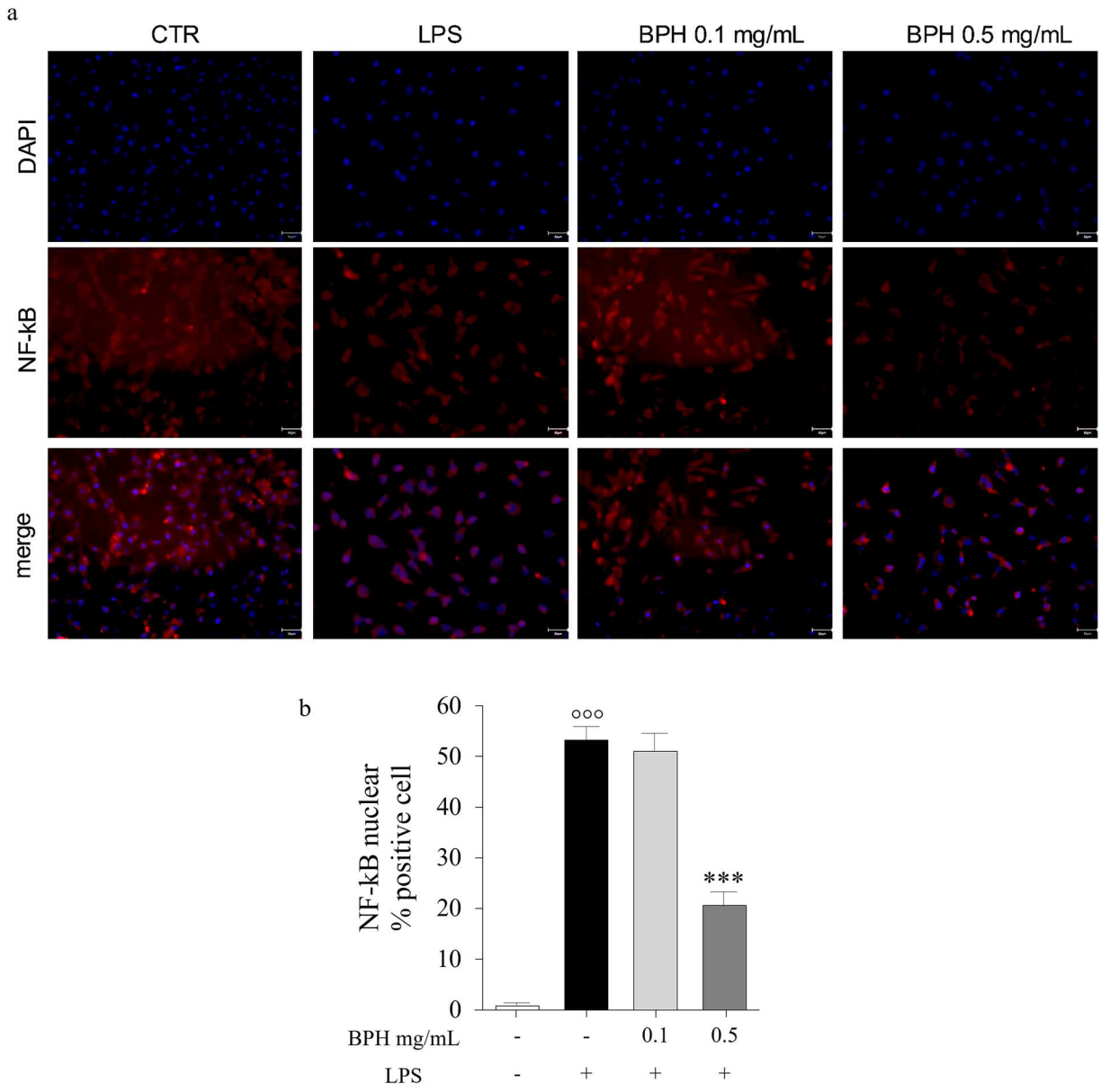

Figure 5. Immunolocalization of NF-kB. (a) Immunofluorescence staining of NF-kB (red) and DAPI (blue) in the different experimental groups. (b) Percentage of nuclei positive cells to NF-kB-p-65. The results are representative of at least three independent experiments and presented as means ± SEM. °°° $p < 0.001$ vs. CTR, *** $p < 0.001$ vs. LPS.

able to significantly increase the levels of *HO-1* transcript. These findings could suggest that BPH could have a role in the improvement of endogenous antioxidant pathway, even though we cannot pinpoint the exact pathway involved in this effect.

Moreover, Lin and colleagues (Lin et al. 2003), showed that the high expression of *HO-1* is correlated to the inhibition of NO production in LPS-challenged cells; the observed reduction of NO levels following BPH treatments in our study, together with the remarkable increase in the *HO-1* gene expression is in accordance with Lin et al (Lin et al. 2003) suggesting that *HO-1* can inhibit LPS-induced NO production in BPH-treated cells.

To delve into the molecular mechanisms underlying the biological effects of BPH, we examined the NF-kB response, a well-known signalling pathway stimulated by LPS. Upon LPS recognition by toll like receptor 4 (TLR4), NF-kB, which together with its regulator IkB-α is a pivotal factor in inflammatory and stress responses, is activated. When NF-kB is active, it migrates into the nucleus inducing the transcription of numerous genes including pro-inflammatory cytokines such as *TNF-α* and *IL-6*. Remarkably, we observed a significant increase in *NF-kB* expression in the LPS-stimulated cells. However, the pre-treatment with BPH 0.5 mg/mL led to a dose-dependent reduction in NF-kB activation, further

substantiating its anti-inflammatory properties. The NF-kB negative regulator *IkB-α* showed a similar expression pattern with increased mRNA levels following LPS stimulation that were decreased by the treatment with BPH in a dose-dependent manner. These observations could rely on the sophisticated mechanisms of NF-kB regulation where IkB-α production is also NF-kB dependent thus resulting in auto-regulatory feedback loops in the NF-kB response (Oeckinghaus and Ghosh 2009); however, further investigations on the protein levels are needed to clarify the mechanisms involved in the regulation of NF-kB by IkB-α in these experimental conditions.

Moreover, the NF-kB nuclear translocation and activation evaluated by immunofluorescence and nuclear localization showed that BPH treatments result in a significant and dose-dependent decrease of immune-positive cells with respect to what was observed in the LPS group confirming that BPH can act as an attenuator of the inflammatory cascade.

Previous studies demonstrated that Nrf2 inhibits NF-κB as a regulatory feedback loop and Nrf2 downstream gene *HO-1*, one of the centres of the crosstalk between Nrf2 and NF-κB, playing a vital role in regulating inflammatory responses by inhibiting the nuclear translocation of the NF-κB (Gao et al. 2022).

The high *Nrf2* gene expression levels observed in our study could account for the *NF-kB* inhibition in BPH groups, thus indicating that BPH triggers the cellular response against LPS stimulation. Moreover, *HO-1* can inhibit the LPS-induced *TNF-α* and *IL-1β* expression through suppression of NF-κB activation according to findings by Rushworth et al. (Rushworth et al. 2008).

In the regulation of the NF-kB pathway, the *IKK-γ* gene encoding for the regulatory subunit of the IκB kinase (IKK) complex is known to play a key role (Makris et al. 2000). IKK-γ is not a kinase per se but it is essential for NF-kB activation by multiple activators (Hiscott et al. 2001). The observed modulation of IKK-γ in LPS-challenged cells after treatment with BPH suggests the involvement of this regulatory subunit in the activation of NF-kB pathway by BSF protein hydrolysates; however, since the information on the molecular mechanisms underlying the role of IKK-γ in the NF-kB pathway regulation is still scarce, further studies are needed to better clarify the effects of BPH on the modulation of the *IKK-γ* gene expression levels. Future investigations on the levels of the protein associated with the NF-kB pathway activation in response to BPH can provide important insights into the complex mechanisms underlying the biological activities of BPH.

Moving beyond the intracellular responses, we explored the secretion of proinflammatory cytokines, specifically TNF-α and IL-6 besides their gene expression. Following LPS stimulation, a marked increase in the levels of these cytokines was observed. Yet, cells treated with BPH exhibited significantly lower levels of *TNF-α* and *IL-6*, emphasizing the potent anti-inflammatory effect of BPH. The production of pro-inflammatory and cytotoxic mediators, such as *TNF-α, IL-6, IL-1α, IL-1β* and ROS, is known to significantly increase following treatment with LPS as stimuli to induce inflammation in several in vitro cell models (Lv et al. 2016; Verma et al. 2012). Interestingly, pre-treatment with BPH of LPS-stimulated L-929 cells reduced the production of most pro-inflammatory and cytotoxic mediators. In particular, the expression levels of *IL-6*, a key component of the inflammatory processes, were significantly reduced by all tested doses of BPH, even with the lowest one (0.1 mg/mL). The simultaneous decrease in *TNF-α, IL-1α* and *IL-1β* playing a major role in inflammation, strongly suggests that protein hydrolysates from BSF are characterized by a remarkable anti-inflammatory potential involving the transcriptional modulation of the pro-inflammatory cytokine genes. The evidence for the potential protective anti-inflammatory effects of BPH herein reported for the first time, open a new scenario for the further characterization of specific components in the BSF protein hydrolysates showing relevant biological activities and for their future applications as new drugs and health-promoting ingredients.

Conclusions

In this study, we explored the impact of BPH on the inflammatory and oxidative stress responses induced by LPS in L-929 cells. Our results provide compelling evidence that BPH exerts a profound influence on mitigating oxidative stress and inflammatory responses in these cells, thus shedding light on its therapeutic potential. BPH anti-oxidative properties rely on the decrease in ROS and nitrite production together with a remarkable increase in antioxidant gene and protein expression. Moreover, BPH can effectively decrease the expression and protein levels of inflammation mediators together with the involvement of NF-kB pathway.

The mechanism of action appears to involve the inhibition of NF-kB activation, together with the induction of the antioxidant response. Further investigations to better understand the intricate molecular mechanisms through which BPH exerts its anti-inflammatory and antioxidant effects and its potential applications in diverse contexts of inflammatory conditions are still needed.

Disclosure statement

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