Protective effects of Manuka honey on LPS-treated RAW 264.7 macrophages. Part 2: Control of oxidative stress induced damage, increase of antioxidant enzyme activities and attenuation of inflammation

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Abstract: The redox-system is altered by oxidative stress that is initiated by oxidative agents such as lipopolysaccharides (LPS) and reactive oxygen species (ROS), which are strongly involved in chronic inflammation. Even if Manuka honey (MH) is a good source of polyphenol rich antioxidants, its antioxidant and anti-inflammatory effects are still elusive. The aim of the present work was to explore the protective effects of MH against E.coli LPS stimulated oxidative stress and inflammatory condition and the underlying mechanisms on murine RAW 264.7 macrophages. Pre-treatment with MH markedly inhibited LPS induced ROS and nitrite accumulation and increased the protection against cellular biomolecules such as lipids, proteins, and DNA. Stimulation by LPS suppressed both antioxidant enzyme activities and expressions which were significantly ($p < 0.05$) increased in the presence of MH. The pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, and other inflammatory mediators (iNOS) were enhanced after LPS treatment, whereas MH suppressed the expression of these inflammatory markers. Moreover, MH also inhibited the expression of TLR4/NF-κB via IκB phosphorylation in LPS-stressed RAW 264.7 macrophages. In conclusion, MH acted as a natural agent for preventing oxidative and inflammatory-related diseases.

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Ancona, July 19th, 2018

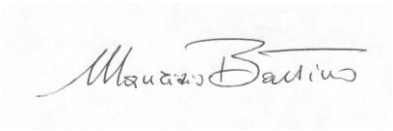
Dear Dr Domingo,

as agreed last week, my coworkers and I are submitting the second part of the research concerning "**Protective effects of Manuka honey on LPS-treated RAW 264.7 macrophages**" to be considered for publication in *Food and Chemical Toxicology*, being the full title "**Protective effects of Manuka honey on LPS-treated RAW 264.7 macrophages. Part 2: Control of oxidative stress induced damage, increase of antioxidant enzyme activities and attenuation of inflammation**".

The manuscript has not been published and is not under consideration for publication elsewhere. All the authors have read the manuscript and have approved this submission. The authors report no conflicts of interest.

Hoping that our paper could be suitable for publication I look forward to hearing from you.

Sincerely yours,



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Protective effects of Manuka honey on LPS-treated RAW 264.7 macrophages. Part 2: Control of oxidative stress induced damage, increase of antioxidant enzyme activities and attenuation of inflammation

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Abstract

The redox-system is altered by oxidative stress that is initiated by oxidative agents such as lipopolysaccharides (LPS) and reactive oxygen species (ROS), which are strongly involved in chronic inflammation. Even if Manuka honey (MH) is a good source of polyphenol rich antioxidants, its antioxidant and anti-inflammatory effects are still elusive. The aim of the present work was to explore the protective effects of MH against *E.coli* LPS stimulated oxidative stress and inflammatory condition and the underlying mechanisms on murine RAW 264.7 macrophages. Pre-treatment with MH markedly inhibited LPS induced ROS and nitrite accumulation and increased the protection against cellular biomolecules such as lipids, proteins, and DNA. Stimulation by LPS suppressed both antioxidant enzyme activities and expressions which were significantly ($p < 0.05$) increased in the presence of MH. The pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, and other inflammatory mediators (iNOS) were enhanced after LPS treatment, whereas MH suppressed the expression of these inflammatory markers. Moreover, MH also inhibited the expression of TLR4/NF-κB via IκB phosphorylation in LPS-stressed RAW 264.7 macrophages. In conclusion, MH acted as a natural agent for preventing oxidative and inflammatory-related diseases.

Keywords: Manuka honey; lipopolysaccharides; reactive oxygen species; anti-oxidative effects; anti-inflammatory effects.

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Keywords: Manuka honey; lipopolysaccharides; reactive oxygen species; anti-oxidative effects; anti-inflammatory effects.

Highlights

- Manuka honey protected RAW 264.7 macrophages against LPS induces oxidative stress.
- Manuka honey improved antioxidant enzyme activities and expressions.
- Manuka honey enhanced oxidative defense by activation of Keap1-Nrf2 signaling.
- Manuka honey suppressed LPS stimulated inflammatory mediators.
- Manuka honey suppressed LPS induced TLR4/NF-κB signaling.

1. Introduction

Inflammation is the defensive response in the human body to infection caused by microbial pathogens, chemical irritants or damaged cells (Martinon et al., 2009). Several studies have reported that chronic inflammation is the main link for the pathogenesis of various diseases, such as arthritis, age-related diseases, atherosclerosis and cancer (Medzhitov, 2008). In the inflammatory processes, uncontrolled reactive species destruct lipids in cell membranes, affecting their permeability and the structure and function of proteins; they lead to DNA damage and mutations, which have high impact on cellular oxidative state, and decrease antioxidant defense mechanisms (Nogueira and Hay, 2013). Inflammation is also promoted by genetic alterations in the intestinal tract because of the former production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), inflammatory chemokine and cytokine (Munkholm, 2003).

Macrophages are the main immune cells, which are associated with the progression of various inflammatory diseases, and are activated by cytokines, mitogens, radiation or bacterial lipopolysaccharides (LPS); at the same time, activated macrophage release various inflammatory mediators (Laskin et al., 2011). LPS activation of toll like receptor (TLR4) complexes on the outer surface of macrophages triggers intracellular nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathways, which lead to transcriptional activation of several genes, including inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2), prostaglandin E2 (PGE2), tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, and IL-6 (Laskin et al., 2011).

Although efficient anti-inflammation drugs are currently accessible, it is still a challenge to discover less toxic and effectual agents for treating the range of acute and chronic inflammatory

diseases. From the ancient time, honey has been used as conventional medicine in various cultural populations mainly for its wound healing and anti-bacterial properties (Alvarez-Suarez et al., 2013; Alvarez-Suarez et al., 2014). Recently, several reports have pointed out multiple activities of honey in enhancing anti-inflammatory (Alvarez-Suarez et al., 2010), anti-cancer (Afrin et al., 2018a; Afrin et al., 2018b), immune response (Al-Waili and Haq, 2004) and anti-cardiovascular (Alvarez-Suarez et al., 2013) effects. Manuka honey (MH), obtained from the nectar of honey bees (*Apis mellifera*), comes from New Zealand Manuka tree (*Leptospermum scoparium,* Family: Myrtaceae) and has been largely studied for its health beneficial effects (Alvarez-Suarez et al., 2014). It is a complex mixture of various pharmacological and biologically active compounds (Alvarez-Suarez et al., 2016; Afrin et al., 2018a; Afrin et al., 2017) and those compounds are thought to be involved in its anti-microbial (Alvarez-Suarez et al., 2014), wound healing, anti-oxidative (Alvarez-Suarez et al., 2016) and chemopreventive effects (Afrin et al., 2018a; Afrin et al., 2018b), but the anti-inflammatory properties remain to be unclear in spite of its high nutritional values.

The current study is aimed to investigate the antioxidant and anti-inflammatory effects of MH on RAW 264.7 macrophages. Therefore, in part 1 of our work we reported the nutritional and phytochemical characterization of MH (Afrin et al., 2018c). After that, ROS and nitrite accumulation and the activity and the expression of the antioxidant enzymes were estimated. Particular attention has been given on the molecular mechanisms by which MH exhibits its anti-inflammatory effects that were evaluated in by *E.coli* LPS activated RAW 264.7 macrophages.

2. Materials and methods

2.1. Chemicals and reagents

MH samples were supplied by EfitSrl (Italy) which and reserved at 4 °C until analysis. All chemicals and reagents were used for the experimental analysis were purchased from Sigma-Aldrich (Milan, Italy). Media for cell culture were obtained from Carlo Erba Reagents (Milan, Italy). The primary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA), while secondary antibody was purchased from Sigma-Aldrich (Milan, Italy).

2.2. RAW 264.7 macrophages cell culture and treatments

Murine RAW 264.7 macrophages cells were purchased from the American Type Culture Collection (ATCC-TIB71). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin and 100 IU/mL streptomycin. Cells were preserved in Hera Cell CO_2 incubator at 37°C with 95% air and 5% CO_2. All the experiments were conducted between the passages 4^{th} to 6^{th}. In part 1 of our work, we performed the viability assay for choosing the subsequent concentration of MH and LPS (Afrin et al., 2018c). Cells were treated for 24 h with different condition: only DMEM (control), MH (3 mg/mL and 8 mg/mL), LPS (1 µg/mL), and MH (3 mg/mL)+LPS and MH (8 mg/mL)+LPS) combination.

2.3. Determination of intracellular ROS levels

CellROX® Oxidative Stress kit (Invitrogen™, Life Techonoliges, Milan, Italy) was used for determination of intracellular ROS generation (Giampieri et al., 2018). The cells were seeded in a 6 well plate at a density of 1.5 X 10^5 and treated for 24 h with different treatments as specified earlier. At the end of the treatment, the CellROX® orange reagents (2 µL/mL) were added and incubated for 30 min at 37°C. After the incubation, the cells were centrifuged for removing the

excess dye and medium, re-suspended the cells with 100 µL PBS and analyzed by using a Tali® Image-Based cytometer (Invitrogen™, Life Techonoliges, Milan, Italy) and the results were expressed as the fold change compared to control.

2.4. Determination of nitrite accumulation

Nitrite production was demonstrated by the Griess method (Pekarova et al., 2009). The cells were seeded in a T75 flask at a density of 1 X 10^6 and treated for 24 h with different treatments as specified earlier. At the end of the treatment, cells supernatants were mixed with equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthyl-ethylenediamine in 5% phosphoric acid) and incubated 10 min in the dark at room temperature. The absorbance was measured on a microplate reader (ThermoScientific Multiskan EX) at 540 nm. The nitrite concentration was determined by using a sodium nitrite standard curve (0.1 to 6.25 µM). Each experiment was carried out in three replicates and the results were expressed as nmol nitrite /mg protein, while the final results were expressed as the fold change compared to control.

2.5. Determination of biomarkers of oxidative stress and antioxidant enzyme activities

RAW 264.7 macrophages were treated for 24 h as previously described. The cellular lysates were prepared by using the RIPA buffer (Sigma-Aldrich, Milan, Italy) for determination of glutathione (GSH), lipid peroxidation (thiobarbituric acid-reactive substance, TBARS), protein carbonyl content and antioxidant enzyme activities (glutathione peroxidase (GPx), glutathione reductase (GR), glutathione transferase (GST), superoxide dismutase (SOD) and catalase (CAT)) following to the earlier described method (Giampieri et al., 2016; Gasparrini et al., 2017). The results of GSH, TBARS, and carbonyl content were expressed as nmol /mg protein, the results of GPx and GR were expressed as nmole NADPH oxidized/mg protein/min, the results of GST

were expressed as nmole 1-chloro-2,4-dinitrobenzene (CDNB)-reduced GSH conjugate/mg protein/min and the results of SOD and CAT were expressed as U/mg protein/min. The final results were expressed as the fold change compared to control.

2.6. Isolation of RNA and RT-PCR analysis

RAW 264.7 macrophages were treated for 24 h as previously described. By using PureLink® RNA Mini Kit (Invitrogen, Carlsbad, CA, USA), the total RNA content of the cells was determined. Microplate spectrophotometer system (BioTek Synergy HT, Winooski, USA) were used for checking the RNA concentration and purity. 5× All-In-One RT MasterMix kit (Applied Biological Materials Inc. Canada) were used for the synthesized cDNA from 75 ng RNA according to reverse transcription. Real-time PCR (RT-PCR) amplification (Corbett Life Science, Rotor-Gene 3000, Mortlake, Australia) was performed by using EvaGreen 2X qPCR MasterMix (EvaGreen 2X qPCR MasterMix kit, Applied Biological Materials Inc. Canada) of forward and reverse primers (Table 1). Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used for normalizing quantitative data and the results were calculated by the $2^{-\Delta\Delta ct}$ method, while the final results were expressed as the fold change compared to control.

2.7. Immunoblotting analysis

RAW 264.7 macrophages were treated for 24 h as previously described. Cell lysis buffer (120 mmol/L NaCl, 40 mmol/L Tris [pH 8], 0.1% NP40 with protease inhibitor cocktails (Sigma)) were used for the lysis of protein from cellular pellets. Proteins from cellular supernatants were separated on 8 to 10% polyacrylamide gel and transferred into a nitrocellulose membrane by using the trans-blot SD semidry electrophoretic transfer cell (Bio-Rad, Hercules, CA, USA). Tris

HCl buffered saline with Tween 20 (TBST) with 5% non-fat-milk were used for blocking the membrane. The primary antibodies 8-Oxoguanine glycosylase (OGG1), nuclear factor E2-related factor 2 (Nrf2), Kelch-like ECH-associated protein 1 (Keap1), SOD, CAT, heme oxygenase 1 (HO-1), TLR4, NF-κB, phosphorylated nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (p-IκBα), iNOS, TNF-α, IL-1β, IL-6, IL-10 and GADPH (1:500 dilutions) were used after all night incubation (4 °C). TBST were used for washing the membrane and incubated with secondary antibodies (1:80 000) for another 1 h. Immunolabeled proteins were recognized by using a chemiluminescence method (C-DiGit Blot Scanner, LICOR, Bad Homburg, Germany) and bands were quantified by using studio digits software 3.1 (C-DiGit Blot Scanner, LICOR, Bad Homburg, Germany).

2.8. Statistical analysis

The statistical analysis was performed by using STATISTICA software (Statsoft Inc., Tulsa, OK, USA) and the results are expressed as the mean values with the standard deviation of three independent repetitions. One-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) post hoc test ($p < 0.05$) were used for the significant differences and symbolized by letters.

3. Results and discussion

3.1. MH treatment decreases LPS induced intracellular ROS and nitrite accumulation

ROS play a vital role in modulating multiple molecular targets which are related to acute and chronic inflammation, growth differentiation, proliferation and apoptosis (Nogueira and Hay, 2013). In the present study, we first investigated whether MH treatment could induce any effects

on ROS accumulation in RAW 264.7 macrophages. The cells were exposed to 3 mg/mL and 8 mg/mL of MH for 24 h. As shown in Fig. 1a no significant differences ($p < 0.05$) were observed after the treatment of MH compared to control. Furthermore, ROS production increased nearly 2.5 fold after LPS treatment, while exposure of MH significantly (p-value < 0.05) decreased LPS induced ROS generation up to 1.8 to 1.5 fold (Fig. 1a). In agreement with our results, MH treatment significantly reduced the ROS levels in 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) stressed human dermal fibroblast (HDF) cells (Alvarez-Suarez et al., 2016). Decreased ROS generation was also observed in RAW 264.7 and HDF cells by polyphenol-rich food which improved endotoxin induced inflammatory stress (Choi et al., 2007; Gasparrini et al., 2017; Gasparrini et al., 2018).

Nitric oxide is a vital bioregulatory molecule in the immune systems and it modulates various pathophysiological conditions inside the cells. Acute and chronic inflammation is induced by overproduction of nitric oxide (Blaise et al., 2005), and therefore more awareness is being paid to the development of natural potent inhibitor of its production. In RAW 264.7 macrophages, no significant differences were found in nitrite generation after the treatment with MH compared to control (Fig. 1b). After incubation with LPS, nitrite production increased nearly 1.9 fold compared to control, while MH treatments significantly ($p < 0.05$) decreased LPS induced nitrite accumulation up to 1.5 to 1.4 fold (Fig. 1b). Inhibitory effects were also observed in LPS stimulated RAW 264.7 macrophages after the treatment with other phenolic compounds (Wang and Mazza, 2002). The authors reported that all the flavonoids decreased nitric oxide production and that the decrease was strongly correlated to the concentration of total polyphenols.

3.2. MH improves GSH levels against oxidative damage

GSH is a prototype antioxidant, which is able to protect the cellular components from the excessive oxidative stress (free radicals, peroxides, and heavy metals), acting directly or as a cofactor (Pompella et al., 2003). Increased GSH levels were observed after MH treatment in RAW 264.7 macrophages (Fig. 2a). LPS treated cells exhibited higher oxidative stress by significantly ($p < 0.05$) lowering GSH values (0.48 fold) compared to control (1.00 fold) and also compared to MH (1.34 fold) (Fig. 2a). Interestingly, pre-treatment with MH protected the LPS-induced stressed cells by restoring the GHS values up to 0.69 to 0.91 fold in the dose-dependent manner (Fig. 2a). These results were consistent with previous reports that highlighted increased GSH levels exerted by other food matrix or isolated compounds after LPS treatment in murine RAW 264.7 macrophages (Gasparrini et al., 2017; Li et al., 2018).

3.3. MH treatment decreases LPS stimulated lipid, protein and DNA damage

Disproportion in the redox system increases ROS or RNS production, damages immune system and may influence injuries to proteins, lipids, and DNA (Nogueira and Hay, 2013). ROS commences lipid peroxidation by a series of reaction that generates free radicals and additional substances (hydroperoxides, malondialdehydes, lipoperoxides and toxic aldehydes), which affect membrane permeability and augment inflammation (Mena et al., 2009).

To determine whether the oxidative stress was related to the damage to main biomolecules, we evaluated the markers of lipid (TBARS), protein (carbonyl content) and DNA damage (OGG1 expression) after the treatment of MH alone and in the presence of the oxidative agent (LPS) (Fig. 2b-2d). We observed that the highest concentration of MH (8 mg/mL) significantly ($p < 0.05$) decreased TBARS levels (0.41 fold) and carbonyl content (0.44 fold) in RAW 264.7

macrophages compared to untreated cells (1.00 fold) (Fig. 2b and 2c). After the incubation of LPS, the levels of lipid (1.85 fold) and protein (1.48 fold) damage were significantly higher compared to control group (Fig. 2b and 2c), while MH treatment significantly ($p < 0.05$) protected lipid and protein damage in LPS stressed cells by decreasing TBARS and protein carbonyl content up to 1.21 fold and 1.12 fold (Fig. 2b and 2c). In agreement with our results, MH has also shown a significant reduction of TBARS levels and protein carbonyl content triggered by AAPH in HDF cells (Alvarez-Suarez et al., 2016). Moreover, polyphenol-containing food demonstrated similar effects by reducing LPS-induced overproduction of TBARS and protein carbonyl content in RAW 264.7 macrophages (Gasparrini et al., 2017).

OGG1, a bifunctional glycosylase, represents a DNA damage marker that is capable to break the DNA backbone strand and split the glycosidic bond in the mutagenic area (Jacobs and Schär, 2012). Several studies exposed that the polyphenol-containing food have the ability to suppress the OGG1 expression in a stressed condition both *in vitro* and *in vivo* models (Giampieri et al., 2016; Gasparrini et al., 2017). In the present study, no significant differences were observed in the expression of OGG1 after the treatment of MH compared to control, whereas after LPS treatment the OGG1 expression was significantly increased compared to the control (Fig. 2d). Pre-incubation with MH lead to significantly reduced OGG1 expression against LPS generated damaged cells.

3.4. MH treatment improve antioxidant enzyme activity and expression against oxidative damage

The antioxidant enzyme GPS, GR, GST, SOD and CAT activities are showed in Fig. 3. On the one hand, the antioxidant system upholds the redox system by scavenging cellular ROS and decreasing the ratio of oxidized glutathione (GSH/GSSG) or $NADPH/NADP^+$. On the other

hand, the protective effects of the antioxidant enzyme are hampered due to excess ROS or other oxidants (Nogueira and Hay, 2013). Several studies reported that the natural compounds have the ability to protect the cells from oxidative damage condition by increasing the endogenous antioxidant enzyme activity and expression for restoring the normal condition of the cells (Lobo et al., 2010).

Therefore, we evaluated the endogenous antioxidant enzyme activity and expression after the treatment with MH alone or in the presence of LPS. The activity of GPx, GR and GST were dose-dependently increased after the treatment of MH compared to control group in RAW 264.7 cells (Fig. 3a). Furthermore, when the cells were incubated with LPS a significant ($p < 0.05$) reduction of above antioxidant enzyme activities were observed. MH was able to protect cells from this damage condition in a dose-dependent manner by improving the activity of GPx and GR, while GST activity was improved only for the highest concentration (8 mg/mL of MH) (Fig. 3a). No significant differences were found in SOD activity compared to control after both concentration of MH, while the CAT activity significantly ($p < 0.05$) increased in the presence of both doses of MH (Fig. 3b). Treatment of LPS decreased the activity of SOD and CAT, while pre-treatment of MH significantly increased the SOD (3 and 8 mg/mL of MH) and CAT activity (8 mg/mL of MH) (Fig. 3b). In a recent report by Alvarez-Suarez *et al.* MH showed the ability of MH to protect AAPH stimulated HDF cells by increasing antioxidant enzyme SOD and CAT activity (Alvarez-Suarez et al., 2016). Concomitantly, Gelam honey protected human diploid fibroblast against gamma radiation by improving the activity of SOD, CAT and GPx (Ahmad et al., 2013).

The transcription factors Nrf2 plays a vital role in cellular defense mechanism by stimulating various antioxidant and detoxification enzymes at the transcriptional level through the

antioxidant response element (ARE). Under quiescent conditions, Nrf2 is sequestered by Keap1 in the cytoplasm, while during oxidative stress, Nrf2 is separated from Keap1 and translocates to the nucleus, thus stimulating genes transcription having an ARE (Battino et al., 2018).

The protein expression of Nrf2-Keap1 signaling and other antioxidant enzyme SOD, CAT and HO-1 were determined after the treatment of MH alone or in the presence of LPS in RAW 264.7 macrophages. We found that MH treatment increased the expression of Nrf2 and similar trend was observed after LPS incubation, while co-treatment with MH significantly ($p < 0.05$) increased the activities Nrf2 compared to control (Fig. 4). The protein levels of Keap1 were increased after the exposure to MH or LPS (Fig. 4), consequently, the MH+LPS treatment induced a significant upregulation of Keap1 in the RAW 264.7 cells (Fig. 4) compared to control group. The expression of other antioxidant enzyme SOD, CAT and HO-1 were upregulated after the MH treatment in a dose-dependent manner. In addition, in case of LPS, the expression of SOD and HO-1 increased more compared to MH treatment while for CAT it induced similar effects like MH (Fig. 4). At the same time, MH+LPS combination led to a significant (p-value < 0.05) higher increase in the expression of these genes compared to control and also other treatments (Fig. 4). Similar trends were observed in AAPH stressed HDF cells, where MH treatment increased the expression of Nrf2, SOD, and CAT for protecting the oxidative damage condition (Alvarez-Suarez et al., 2016). Other natural compounds such as epigallocatechin-3-gallate protected TNF-α induced NF-κB activation by increasing Nrf2-Keap1 pathway in macrophage foam cells (Jiang et al., 2012), whereas fisetin induced anti-inflammatory effects demonstrated by HO-1 activation in LPS stimulated RAW 264.7 macrophages (Li et al., 2018). Furthermore, polyphenol-rich foodstuff acted against oxidative damage through increasing both

SOD and CAT activity and expression in RAW macrophages and HDF cells (Gasparrini et al., 2017; Gasparrini et al., 2018).

3.5. MH treatment suppresses key inflammatory biomarkers induced by LPS

Since chronic inflammation has been associated with the development of cancer, atherosclerosis, and aging-related diseases, we evaluated the anti-inflammatory activity of MH (Fig. 5 and Fig. 6) (Medzhitov, 2008). The transcription factor NFκB acts as a key role in inflammation by modulating numerous genes responsible for the generation of inflammatory mediators. In presence of multiple pro-inflammatory stimuli, inhibitor of kappa B (IκB) is rapidly degraded and NFκB subunits translocated into the nucleus to stimulate the transcription of various genes encoding chemokines, cytokines, anti-apoptotic factors and growth factors (Lawrence, 2009). In the colonic epithelial barrier, TLR4 activation leads to increasing intracellular NF-κB signaling and other pro-inflammatory cytokine (IL-1β, IL-6, iNOS, COX-2, PGE2 etc.) which lead to progression of chronic inflammatory diseases (Foran et al., 2010).

In our work, we quantified the mRNA and protein expression of several inflammation markers to highlight if MH has a protective effect against the inflammation induced by LPS in RAW 264.7 macrophages. No significant differences were observed in the mRNA expression of NF-κB, iNOS, TNF-α, and IL-1β after the treatment of MH, while all the mRNA expression were significantly increased after the LPS incubation (Fig. 5). Interestingly, pre-treatment with MH at both concentrations were able to decrease LPS induced over activation of NF-κB, iNOS, TNF-α, and IL-1β and at the highest concentration of MH values similar to the control were found (Fig. 5). Our results are very similar to those obtained in RAW264.7 mouse macrophages in which

plant polyphenol treatment suppressed LPS stimulated iNOS, TNF-α, COX-2, and IL-6 mRNA expression (Kanno et al., 2006).

The anti-inflammatory effects were also confirmed by evaluating the protein expression of TLR4, NF-κB, p-IκBα, iNOS, TNF-α, IL-1β, IL-6 and IL-10 by Western Blot (Fig. 6). MH treatment significantly (p-value < 0.05) decreased the expression of the above inflammatory markers after 24 h treatment in RAW 264.7 cells (Fig. 6). Incubation with LPS significantly (p < 0.05) increased the protein levels of all the inflammatory markers, while in the presence of MH suppressed expressions were observed (Fig. 6). Earlier reports have suggested that stimulation by LPS induced the production of several inflammatory biomarkers, such as NF-κB, p-Iκα, TNF-α, iNOS, COX-2, IL-6 and IL-1β, whereas co-treatment with nutritionally rich foodstuff (Kim et al., 2016; Gasparrini et al., 2017; Limtrakul et al., 2015) or biologically active compounds from plants sources (Hou et al., 2015; Terra et al., 2007; Kanno et al., 2006) protected RAW 264.7 macrophages by suppressing the pro-inflammatory production. A different expression was observed of IL-10, which was significantly (p < 0.05) increased after the LPS treatments even in the presence of MH alone or in the combinations (Fig. 6). This result is quite similar to those obtained in RAW macrophages cells which found a greater amount of inflammatory cytokines stimulated by LPS, improved by the co-treatment of other plant polyphenols (Zong et al., 2012; Lee et al., 2013; Mo et al., 2014).

4. Conclusions

This study explored the anti-oxidative and anti-inflammatory effects of MH against oxidative damage in inflammation mediatory immune cells, macrophages. Anti-oxidative effects of MH were related to the suppression of ROS and nitrite production as well as the protection of lipid,

protein and DNA damage. This mechanism was accompanied by increased antioxidant enzyme activities, consequently to the enhanced Nrf2 expression and its synchronized enzymes SOD, CAT and HO-1 expression. Subsequent analysis confirmed that MH effectively suppressed the expression of inflammation mediators such as TNF-α, IL-1β, IL-6 and iNOS. Finally, we also found that MH effectively inhibited the expression of TLR4 and NFκB pathway via suppression of p-IκBα. Taken together, these promising findings may increase the potential efficacy of MH as a useful natural compound for inhibiting the inflammation via enhancing antioxidant activity and consequently suppressing key inflammation mediators. Therefore, future research is required to validate the anti-oxidative and anti-inflammatory approach *in* vivo models.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgment

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Abbreviations

AAPH, 2,2'-Azobis(2-amidinopropane) dihydrochloride; ARE, antioxidant responsive element; COX-2, cyclooxygenase 2; CAT, catalase; DMEM, dulbecco's modified eagle medium; GADPH, glyceraldehyde-3-phosphate dehydrogenase; GSH, glutathione; GST, glutathione transferase; GPx, glutathione peroxidase; GR, glutathione reductase; HDF, human dermal fibroblast, HO-1, heme oxygenase 1; IL-1b, interleukin 1-beta; IL-6, interlukin 6; IL-10, interlukin 10; iNOS, inducible nitric oxide synthase; Keap1, kelch-like ECH-associated protein

1; LPS, lipopolysaccharide; MH, Manuka honey; NF-kB, Nuclear factor kappa-light-chain-enhancer of activated B cells; Nrf2, nuclear factor E2-related factor 2; OGG1, 8-Oxoguanine glycosylase; PGE2, prostaglandin E2; p-IκBa, phosphorylated nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; RNS, reactive nitrogen species; ROS, reactive oxygen species; SD, standard deviation; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; TBST, Tris HCl buffered saline with Tween 20; TLR4, toll like receptor 4; TNF-a, tumor necrosis factor alpha.

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Tables

Table 1. Sequences of real-time PCR oligonucleotide primers.

Gene (symbol)	Direction	Sequence (5′-3′)
NF-κB p65	Forward	AGGCTTCTGGGCCTTATGTG
	Reverse	TGCTTCTCTCGCCAGGAATAC
iNOS	Forward	CCCTTCCGAAGTTTCTGGCAGC
	Reverse	GGCTGTCAGAGCCTCGTGGCTT
TNF-α	Forward	ATGAGCACAGAA AG CATGATC
	Reverse	TACAGGCTTGTCACTCGA ATT
IL-1β	Forward	TGCAGAGTTCCCCAACTGGTACATC
	Reverse	GTGCTGCCTAATGTCCCCTTGAATC
GADPH	Forward	CAC TCA CGG CAA ATT CAA CGG CAC
	Reverse	GAC TCC ACG ACA TAC TCA GCA

Figure captions

Fig. 1. Intracellular ROS and nitrite levels. RAW 264.7 macrophages treated with MH and LPS alone or in combination for 24 h. (a) Intracellular ROS levels were determined by using the Tali™ Image-based Cytometer assay. Representative fluorescence image of RAW 264.7 macrophages cells: non-fluorescent upon no ROS and red fluorescence upon oxidation by ROS. (b) Nitrite levels were determined by using the Griess method. Results are expressed as a fold change in comparison with control (no treatment). All data are indicated as the mean ± standard deviation (SD) (n=3). Different letters indicate significant differences (p-value < 0.05) between each treatment.

Fig. 2. Levels of glutatione (GSH) and biomarkers of oxidative damage (lipid, protein, and DNA). (a) GSH levels, (b) lipid damage levels (TBARS), (c) protein carbonyl levels and (d) protein expression of OGG1 (DNA damage) were determined in RAW 264.7 macrophages treated with MH and LPS alone or in combination for 24 h. Results are expressed as a fold change in comparison with control (no treatment). All data are indicated as the mean ± SD (n=3). Different letters indicate significant differences (p-value < 0.05) between each treatment.

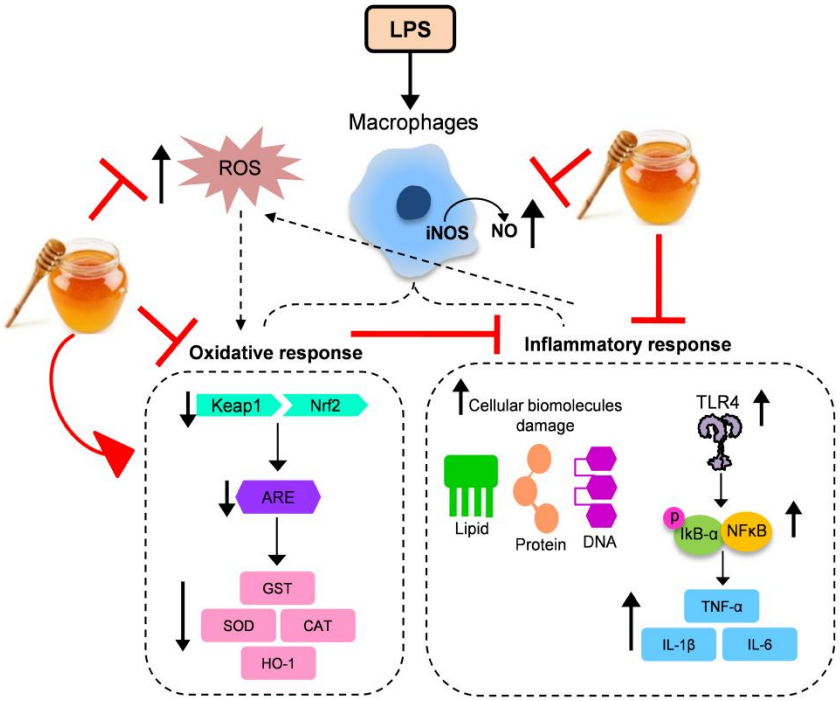
Fig. 3. Antioxidant enzyme activities. The activity of antioxidant enzymes (a) GPx, GR, GST and (b) SOD, CAT were determined in RAW 264.7 macrophages treated with MH and LPS alone or in combination for 24 h. Results are expressed as a fold change in comparison with control (no treatment). All data are indicated as the mean ± SD (n=3). Different letters indicate significant differences (p-value < 0.05) between each treatment.

Fig. 4. Protein expression levels of Nrf2-Keap1 and other antioxidant enzymes. RAW 264.7 macrophages treated with MH and LPS alone or in combination for 24 h. The protein expression of Keap1, Nrf2, SOD, CAT, and HO-1 were analyzed by western blotting. GADPH was utilized as a loading control. Results are expressed as a fold change in comparison with control (no treatment). All data are indicated as the mean ± SD (n=3). Different letters indicate significant differences (p-value < 0.05) between each treatment.

Fig. 5. mRNA levels of inflammation markers. RAW 264.7 macrophages treated with MH and LPS alone or in combination for 24 h. The mRNA expression of NF-κB, iNOS, TNF-α, and IL-1β were analyzed by RT-PCR. GADPH was utilized as a loading control. Results are expressed as a fold change in comparison with control (no treatment). All data are indicated as the mean ± SD (n=3). Different letters indicate significant differences (p-value < 0.05) between each treatment.

Fig. 6. Protein expression levels of inflammation markers. RAW 264.7 macrophages treated with MH and LPS alone or in combination for 24 h. The protein expression of (a) TLR4, NF-κB, p-IκBα, iNOS and (b) TNF-α, IL-1β, IL-6, IL-10 were analyzed by western blotting. GADPH was utilized as a loading control. Results are expressed as a fold change in comparison with control (no treatment). All data are indicated as the mean ± SD (n=3). Different letters indicate significant differences (p-value < 0.05) between each treatment.

Graphical Abstract



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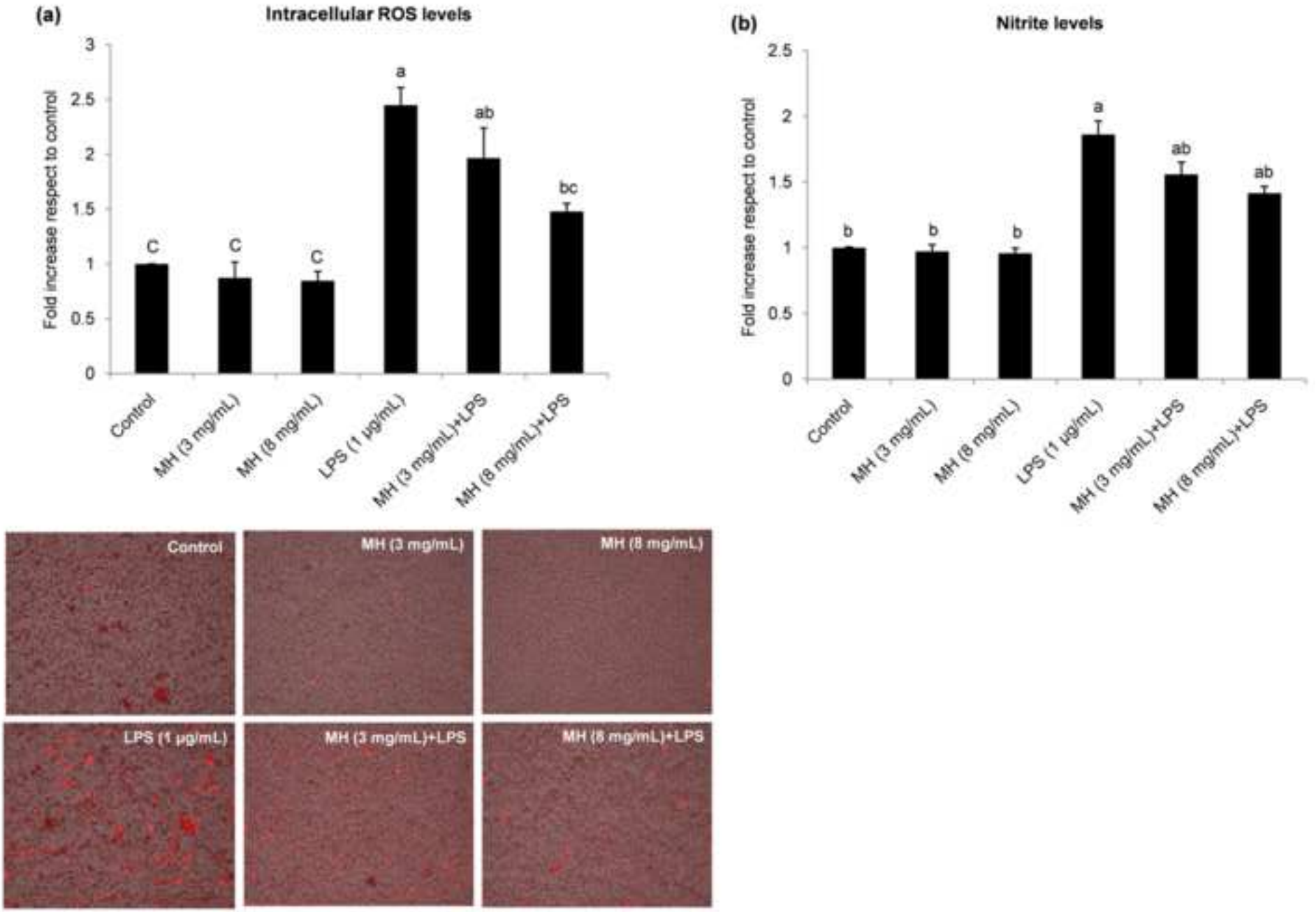

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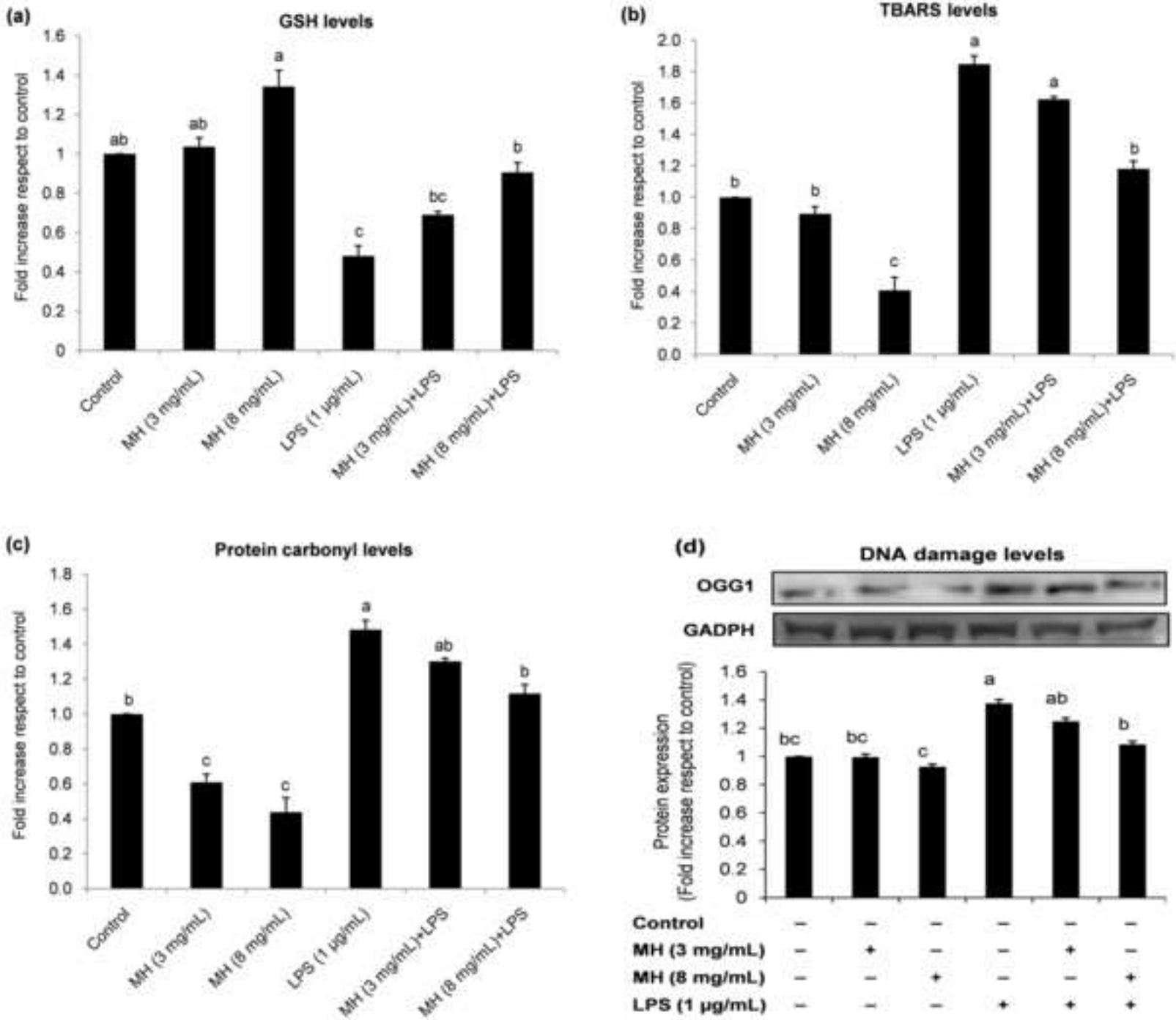

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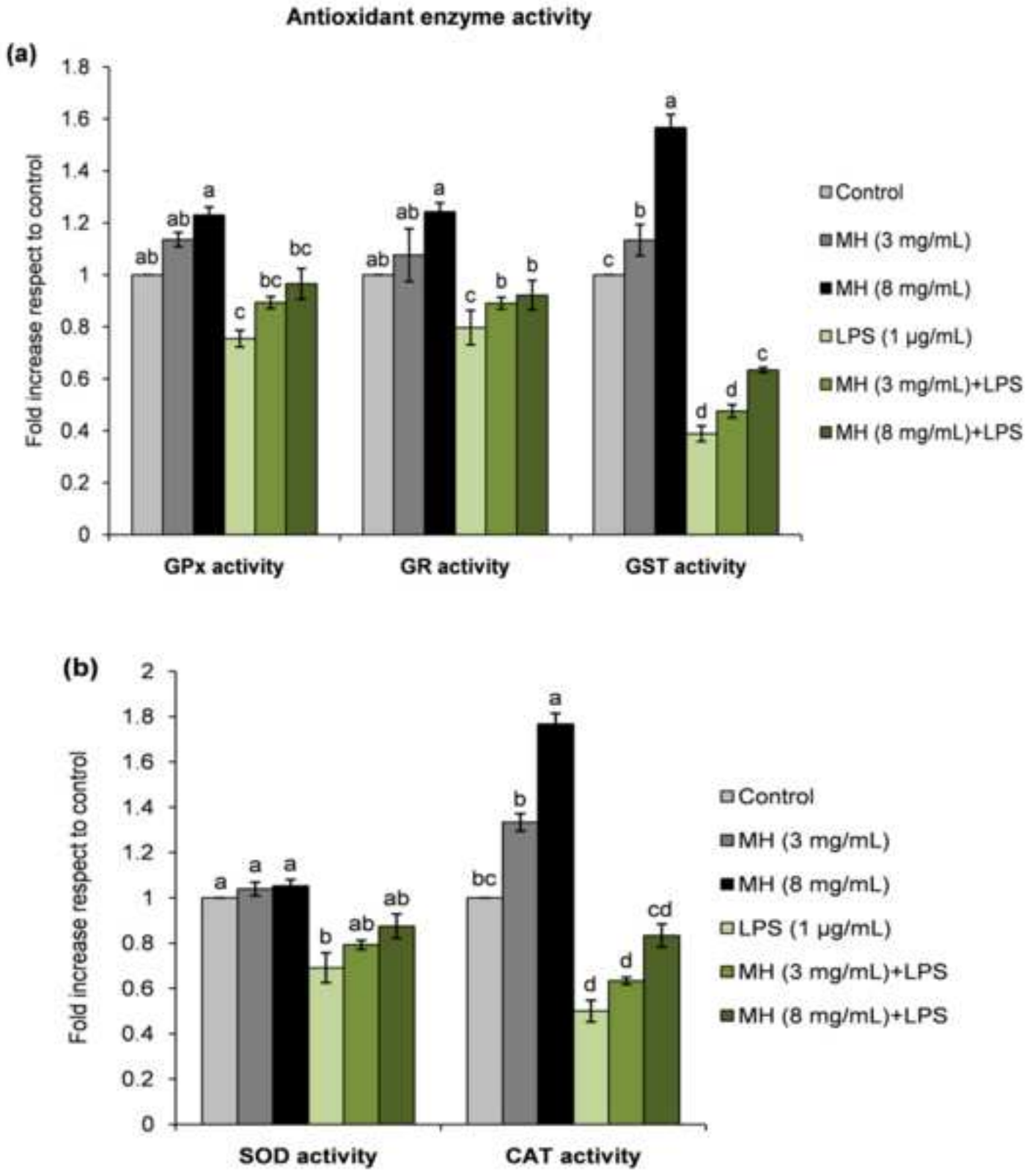

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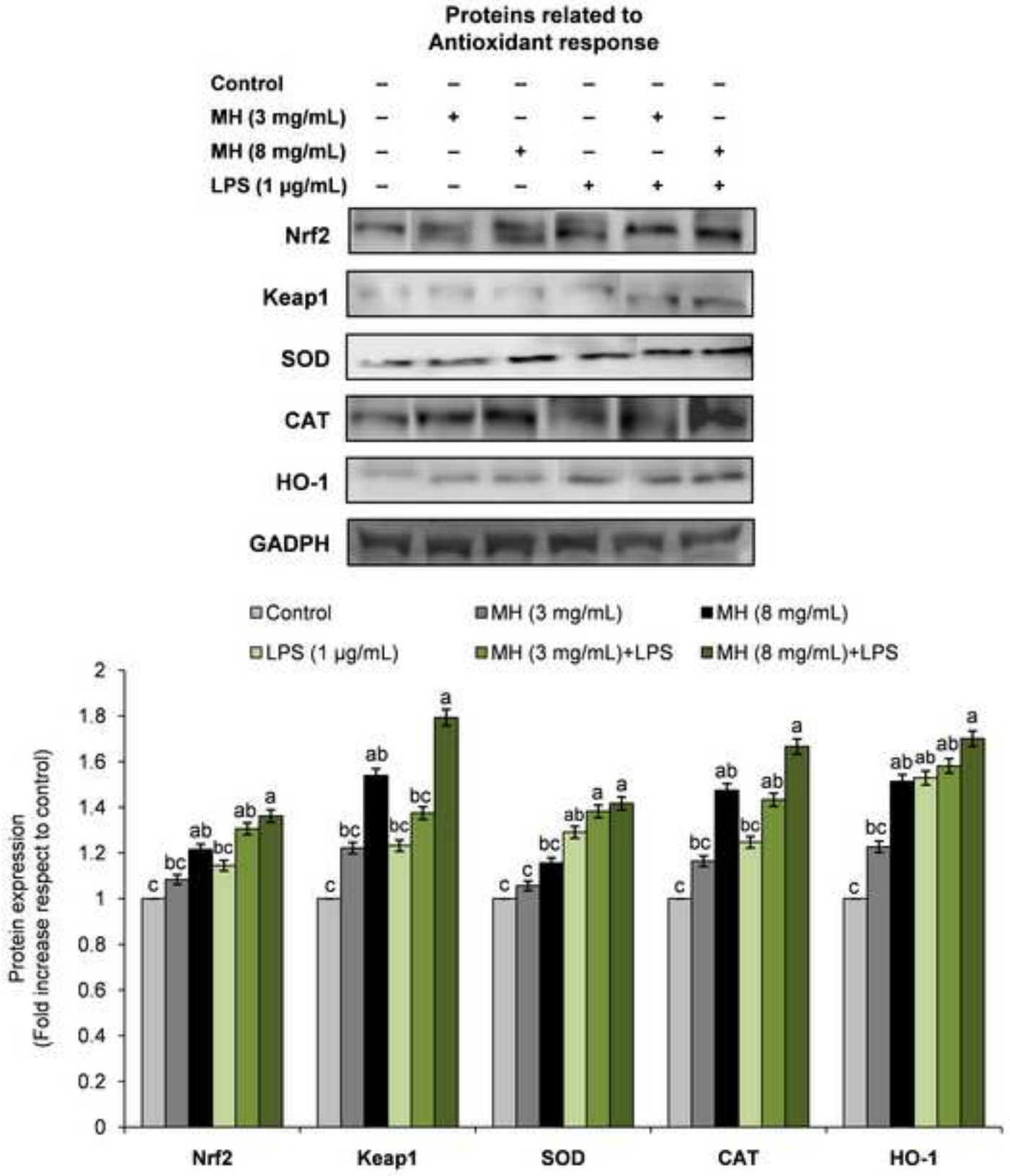

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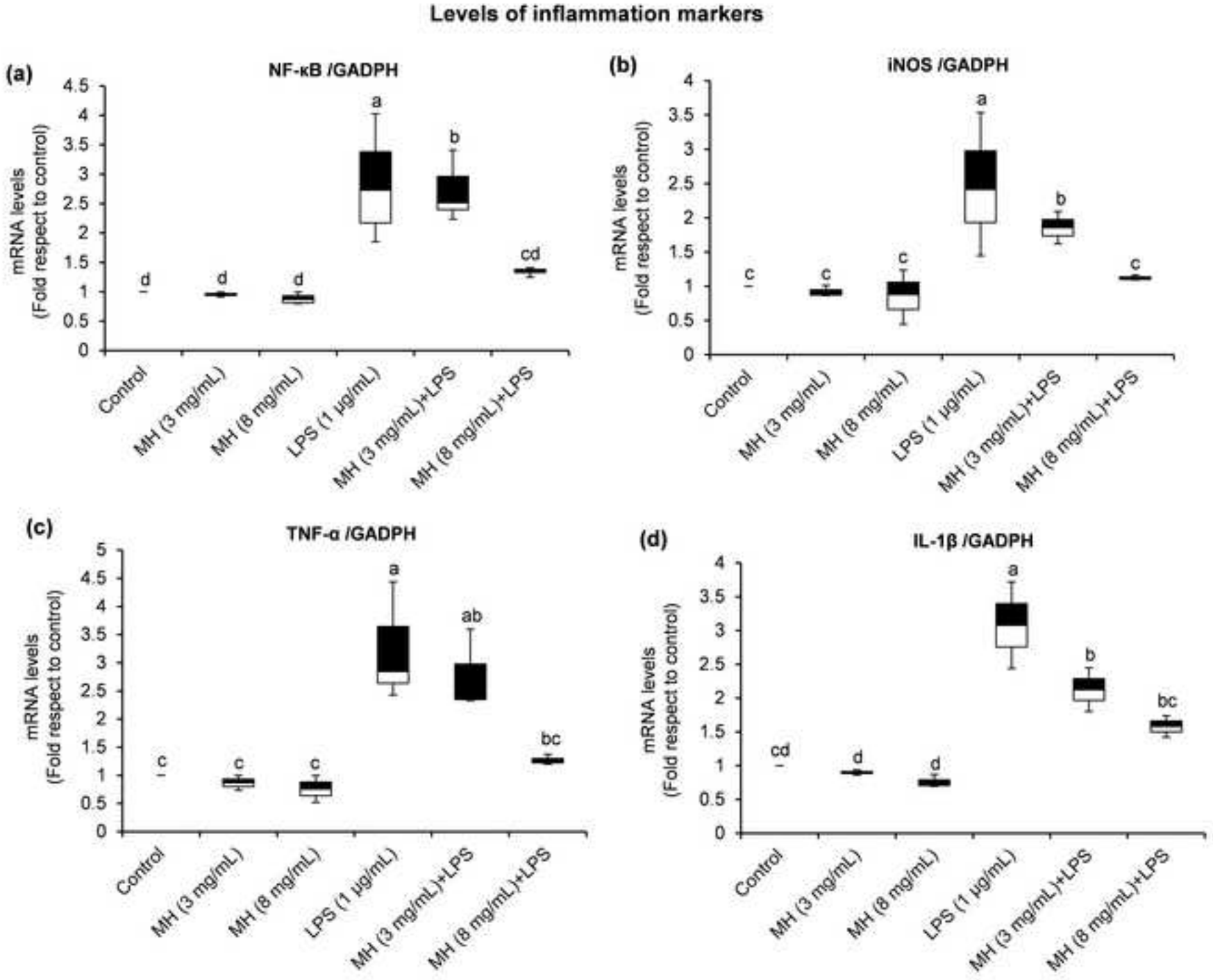

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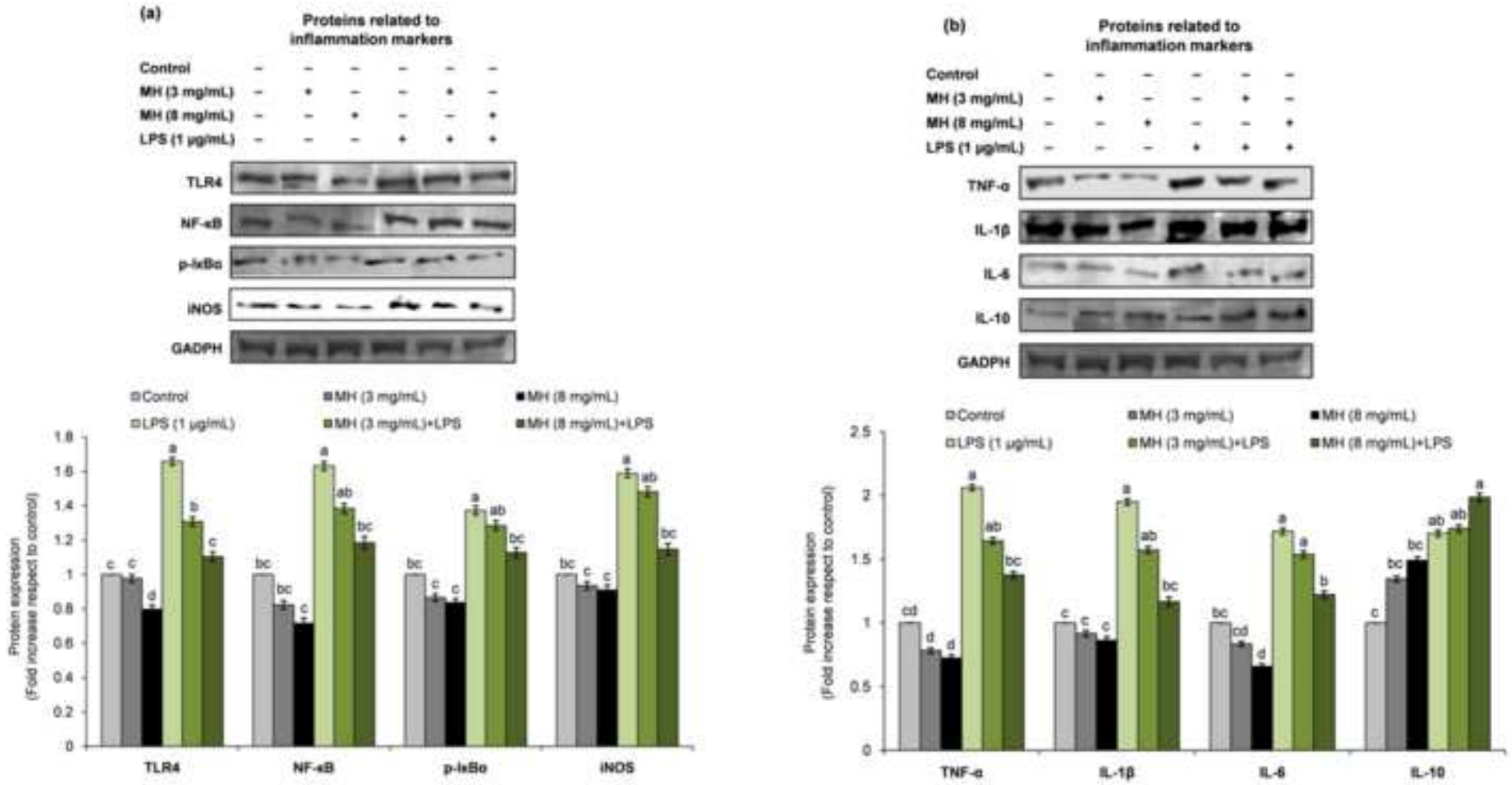

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