

Research Article

The Hydroalcoholic Extract Obtained from *Mentha piperita* L. Leaves Attenuates Oxidative Stress and Improves Survival in Lipopolysaccharide-Treated Macrophages

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Mentha piperita L. (peppermint) possesses antimicrobial properties, but little is known of its ability to modulate macrophages. Macrophages are essential in bacterial infection control due to their antimicrobial functions and ability to link the innate and adaptive immune responses. We evaluated the effects of the peppermint leaf hydroalcoholic extract (LHAE) on cultured murine peritoneal macrophages stimulated or not with lipopolysaccharide (LPS) *in vitro*. Vehicle-treated cells were used as controls. The constituents of the extract were also identified. Epicatechin was the major compound detected in the LHAE. LPS-induced macrophage death was reversed by incubation with LHAE (1–30 µg/ml). Higher concentrations of the extract (≥100 µg/ml) decreased macrophage viability (49–57%) in the absence of LPS. LHAE (1–300 µg/ml) attenuated H₂O₂ (34.6–53.4%) but not nitric oxide production by these cells. At similar concentrations, the extract increased the activity of superoxide dismutase (15.3–63.5-fold) and glutathione peroxidase (34.4–73.6-fold) in LPS-treated macrophages. Only LPS-unstimulated macrophages presented enhanced phagocytosis (3.6–6.6-fold increase) when incubated with LHAE (3–30 µg/ml). Overall, the LHAE obtained from peppermint modulates macrophage-mediated inflammatory responses, by stimulating the antioxidant pathway in these cells. These effects may be beneficial when the excessive activation of macrophages contributes to tissue damage during infectious disease.

1. Introduction

Macrophages are on the first line of the host's immune response to bacterial infection. Indeed, these cells play detrimental roles in pathogen recognition, bacterial killing, and antigen presentation, leading to further activation of adaptive immune responses (see for review [1–3]). Gram-negative

bacterial strains are major pathogens causatives of severe infectious diseases in humans, associated with high mortality rates [4, 5]. This is due not only to their ability to become resistant to the available antimicrobials [4] but also depends on an effective macrophage response to these pathogens [6].

The production of oxidant species by macrophages is a hallmark of the inflammatory response to infection (see for

review [7, 8]). Oxidant species such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) are produced following phagocytosis of the pathogen by these cells as part of their machinery to respond to harmful insults [9]. Alongside an excessive nitric oxide (NO) production, increased levels of prooxidant species may lead to damage and poor perfusion of vital organs of the host, contributing to multiple organ failure; thus, to counteract this response, antioxidant pathways are activated [10].

Natural antioxidants including phenolic compounds have been identified in a variety of plants. Additionally, antimicrobial properties have been attributed to these compounds, suggesting them to be potential therapies for bacterial infections. *Mentha piperita* L., a member of the family Lamiaceae and popularly known as peppermint, is native to the Mediterranean region and has been spread worldwide due to its medicinal properties, taste, and aroma [11]. Its medicinal properties include antitumor, antimicrobial, and antioxidant actions and have been reported especially for its essential oil [12–17]. Of importance, *M. piperita* essential oil was previously shown to be effective against Gram-negative and Gram-positive bacteria and to act as a potential antioxidant *in vitro* [12]. This essential oil was also shown to reduce the numbers of leukocytes in a murine model of skin inflammation [18] and modulate cytokine production *in vivo* [19]. However, the underlying mechanisms of the effects of *M. piperita* on macrophages remain unclear. Considering peppermint antioxidant and anti-inflammatory potentials, we hypothesized whether its leaf hydroalcoholic extract (LHAE) is able to modulate macrophage-mediated inflammatory responses. Therefore, the aim of this study was to investigate the effects of the peppermint leaf hydroalcoholic extract (LHAE) on cultured murine peritoneal macrophages *in vitro*.

2. Material and Methods

2.1. Plant. The leaves of *M. piperita* were collected in September at Santa Luzia, Maranhão, Brazil ($4^{\circ}48'S$, $45^{\circ}41'24''W$). A voucher specimen (number 01275) was deposited in the herbarium Ático Seabra of the Federal University of Maranhão, São Luís, Brazil.

2.2. Preparation of the Crude Hydroalcoholic Extract. The collected leaves were washed in running water before being dried under forced air circulation at $45^{\circ}C$. The dried leaves were triturated, and the resulting powder was macerated for 10 days in 70% ethyl alcohol (Sigma-Aldrich, St. Louis, MO, USA) at room temperature. The mixture was filtered through cellulose filter paper (Whatman No. 4, GE Healthcare UK, Amersham, UK) and evaporated to dryness under reduced pressure using a rotary evaporator (Eyela N-1200BV-W, Tokyo, Japan) at $40^{\circ}C$. The residual solvent was removed in a vacuum centrifuge at $40^{\circ}C$ to yield crude ethanol extracts of leaves.

2.3. Chemical Characterization by High-Performance Liquid Chromatography (HPLC). For HPLC analysis, the peppermint LHAE was dissolved in methanol and water to a final

concentration of approximately 5 mg/ml and filtered through a $0.22\ \mu m$ nylon filter. An HPLC (Surveyor Plus/Finnigan) coupled to an ultraviolet-visible detector (HPLC-UV-Vis), with an ACE 5 C18 reverse phase analytical column ($250 \times 4.60\ mm$, $5\ \mu m$, ACE) protected by a C18 precolumn ($4 \times 3\ mm$, $5\ \mu m$, Gemini, Phenomenex), was used for the analysis. Compounds were separated at room temperature using an elution gradient at a flow rate of 0.6 ml/min. Mobile phases consisted of purified water containing 0.1% acetic acid (A) and methanol (B). The following gradient was used: 0–2 min, 5% B; 2–10 min, 25–40% B; 10–20 min, 40–50% B, 20–30 min, 50–60% B; 30–40 min, 60–70% B; and 40–50 min, 70–80% B. Injection volume was $10\ \mu l$ and UV-Vis detection was performed at 254 nm. Ursolic acid, epicatechin, caffeic acid, rutin, quercetin, naringenin, and kaempferol standards were diluted and analyzed under the same conditions.

2.4. Macrophage Assays

2.4.1. Animals. Nonfasted outbred male Swiss mice (2–3 months old) were used. Mice were obtained from the animal's facility of the Universidade CEUMA (UNICEUMA). Mice were kept in a climatically controlled environment (room temperature of $22 \pm 2^{\circ}C$ and humidity of around 60%) under 12:12 h light-dark cycle (lights on 07:00 h). All procedures were approved by the Ethics Committee of UNICEUMA and carried out in accordance with the Brazilian Society for Animal Welfare (SBCAL).

2.4.2. Macrophage Culture and Viability. Peritoneal cells were collected from animals injected intraperitoneally with 1 ml of phosphate-buffered saline (PBS, Sigma-Aldrich, Brazil) containing 1% oyster glycogen (Sigma-Aldrich, Brazil). Briefly, 18 h following injection, the peritoneal cavity was washed with 10 ml of cold PBS and the peritoneal cells were harvested, centrifuged (10 min, $4^{\circ}C$), and resuspended (final concentration of 2×10^6 cells/ml) in DMEM-Glutamax[®] (Life Technologies, Brazil) containing 10% FCS (v/v, Life Technologies, Brazil) and 1x penicillin-streptomycin (Sigma-Aldrich, Brazil). Cells (6×10^5 /well) were incubated in 96-well plates, at $37^{\circ}C$ under 5% CO_2 , and after 2 h, nonadherent cells were removed and the adherent cells (macrophages) were incubated with either peppermint LHAE (1–300 $\mu g/ml$) or vehicle (1% dimethyl sulfoxide (DMSO, Sigma-Aldrich, Brazil) in PBS), and after 15 min, stimulated with *K. pneumoniae* lipopolysaccharide (LPS, 100 ng/ml in PBS, Sigma-Aldrich, Brazil) or PBS for 24 h. After this period, the supernatant was collected and stored at $-80^{\circ}C$ for further analysis of NO_2^-/NO_3^- (NO end products) and H_2O_2 concentrations. For analysis of macrophage viability, the remaining cells were incubated with PrestoBlue[®] reagent (1:10, Life Technologies, Brazil) for 90 min; and then, the absorbances were read at 550 and 650 nm. Results were calculated according with the manufacturer's instructions and are expressed as absorbance in percentage (%) of cell viability in relation to vehicle/PBS-treated cells.

2.4.3. Phagocytosis. In a separate series of experiments, peritoneal macrophages were obtained and cultured (6×10^5 /

well) as described above in eight chamber culture slides (BD Falcon). Just after removal of nonadherent cells, macrophages were incubated with 2 μm fluorescent latex beads (1:100; 5 μl /well; Sigma-Aldrich, Brazil), for 24 h as described by Fernandes et al. [20]. After the incubation period, the cell culture medium was removed and each well was washed three times with PBS. Wells were fixed in 2% paraformaldehyde for 10 min and washed three times with PBS for the removal of excessive paraformaldehyde. Then, 10 μl PBS were added per well and slides were covered with a glass slip. Slides were analyzed in a fluorescence microscope (Zeiss Axio Image Z2, German, $\times 40$ objective, bright field). Two lots of 100 cells were counted for each well, and the average for each well was considered as an n number. Results are expressed as percentage of cells containing beads and number of phagocytosed beads per 100 cells.

2.4.4. NO End Product (Nitrate NO_3^- plus Nitrite NO_2^-) Measurement. The $\text{NO}_2^-/\text{NO}_3^-$ content was measured by the Griess reaction assay as an indicator of NO production in supernatant samples as previously described [21]. NO_3^- was reduced to nitrite (NO_2^-) by incubating 80 μl of the sample with 20 μl of 1 U/ml nitrate reductase and 10 μl of 1 mM NADPH for 30 min at 37°C in a 96-well plate. Next, 100 μl Griess reagent (5% v/v H_3PO_4 containing 1% sulfanilic acid and 0.1% N-1-naphthylethylenediamine) was added and incubated for 15 min at 37°C. Absorbance at 550 nm was immediately measured using a spectrophotometer (Plate reader MB-580; Heales, Shenzhen, China). After subtraction of background readings, the absorbance in each sample was compared with that obtained from a sodium nitrite (0–100 μM) standard curve and expressed as NO^x concentrations (μM).

2.4.5. Measurement of H_2O_2 Concentrations. H_2O_2 production by macrophages was measured by using a H_2O_2 /peroxidase assay kit (Amplex Red H_2O_2 /Peroxidase assay kit, Invitrogen, Brazil), as described by Mendes et al. [21]. Briefly, 50 μl of the supernatants were incubated with 50 μl of a 0.05 M NaPO_4 (pH 7.4) solution containing 0.2 U/ml horseradish peroxidase (HRP) and 25.7 mg/ml Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) for 2 h, at 37°C. Samples incubated with 0.05 M NaPO_4 only were used as controls. After incubation, the absorbance was read at 560 nm. After subtraction of background readings, the absorbance in each sample was compared with that obtained from a H_2O_2 standard curve (0–40 μM). H_2O_2 concentrations are expressed in μM .

2.4.6. Antioxidant Enzyme Activities

(1) Sample Preparation. In another series of experiments, macrophages were obtained, isolated, cultured (6×10^5 /well) in 24-well plates, and stimulated as described above. Following incubation with LPS (24 h), the supernatant was removed and 500 μl of 0.05 M NaPO_4 (pH 7.4) (containing ethylenediaminetetraacetic acid (EDTA), 1 mM) was added to each well. Plates were placed on ice for 15 min. Then, cells were scraped from each well, transferred to tubes, and lysed by three snap freezing/defrosting times. Tubes were centrifuged

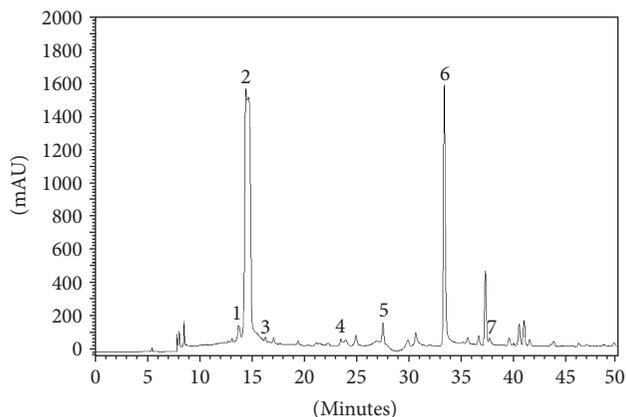


FIGURE 1: HPLC analysis of the peppermint LHAE. Peaks are numbered 1–7 and were shown to coelute with (1) ursolic acid, (2) epicatechin, (3) caffeic acid, (4) rutin, (5) quercetin, (6) naringenin, and (7) kaempferol.

TABLE 1: Registered retention times obtained by HPLC analysis of the peppermint LHAE. Retention times in minutes were registered for each peak.

Peak number	Retention time in min	Compound
1	13.8	Ursolic acid
2	14.5	Epicatechin
3	16.3	Caffeic acid
4	23.6	Rutin
5	27.6	Quercetin
6	33.5	Naringenin
7	37.8	Kaempferol

at 10,000 $\times g$ for 10 min at 4°C, and the supernatants were used for the enzyme activity assays.

(2) Superoxide Dismutase (SOD) Activity Assay. SOD activity was measured as described by Ukeda et al. [22], with modifications. Briefly, 20 μl of sample were incubated with 200 μl of a solution containing 2.5 ml sodium carbonate buffer (50 mM; pH 9.4) and 0.1 ml of a mixture containing xanthine (3 mM), EDTA (3 mM) and 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT, 153 mU/ml), in the presence and absence of SOD. Samples (200 μl /well) were added in 96-well plates and the absorbance was read at 470 nm for 20 min. Results are expressed as milli-units (mU) of SOD/mg of protein. One unit of SOD was defined as the amount of enzyme capable of dismutating 1 μmol of O_2^- /min.

(3) Glutathione Peroxidase (GPx) Activity Assay. GPx activity was determined as described by Paglia and Valentine [23]. For this, 30 μl of sample per well (diluted 1:3) was incubated for 5 min at 37°C, with 145 μl per well of 0.05 M phosphate buffer (pH 7.4) containing 0.1 M EDTA, 5 μl of glutathione (GSH, 80 mM), and 5 μl glutathione reductase (0.0096 U/ μl). After incubation, 5 μl of 0.46% *tert*-butyl

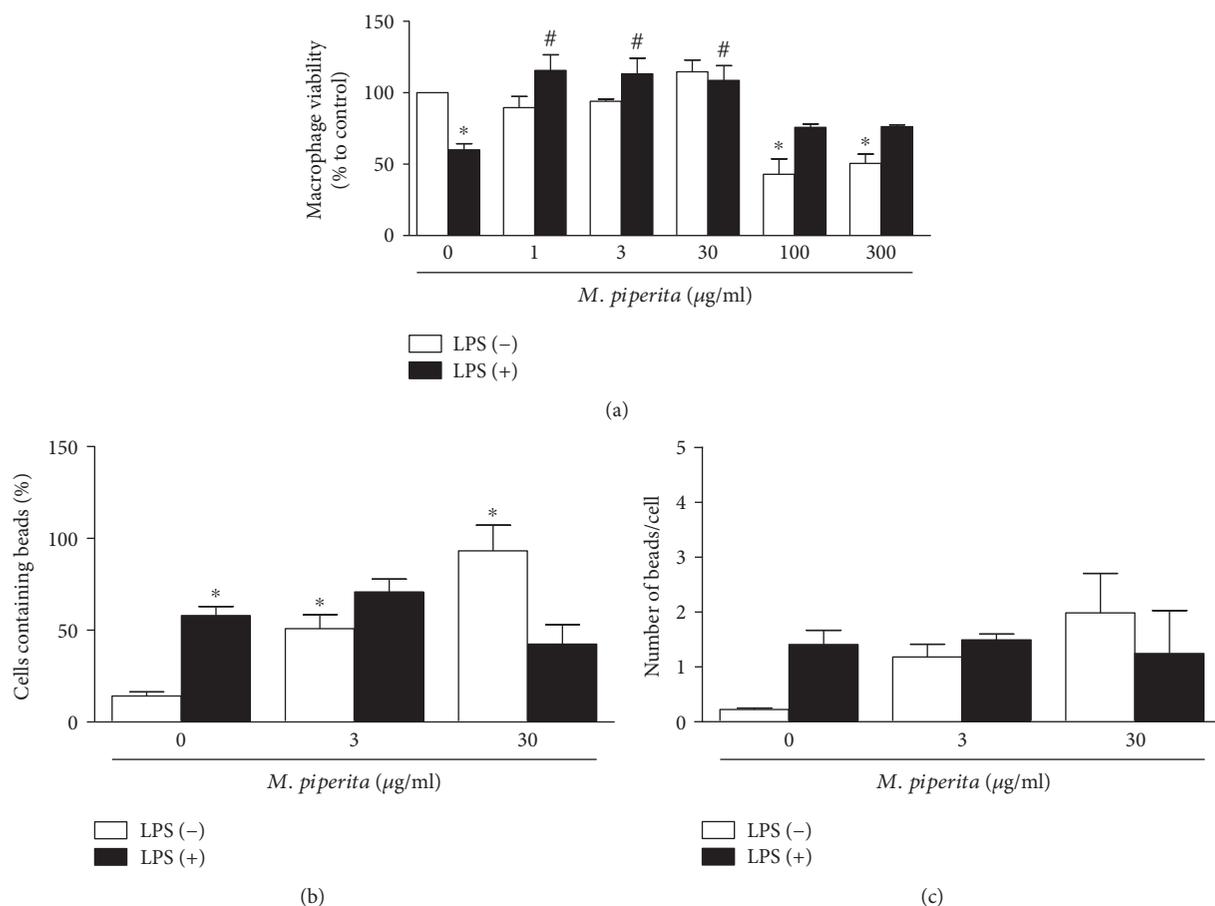


FIGURE 2: Effect of peppermint LHAE on peritoneal macrophage viability and phagocytosis. Cell viability (a), number of cells containing beads (b), and number of beads per cell (c) were quantified on peritoneal macrophages pretreated with peppermint LHAE (1–300 µg/ml, in 1% DMSO in PBS) and stimulated with LPS for 24 h. Vehicle-treated cells were used as controls. Data are expressed as mean ± SEM. * $p < 0.05$ compared with vehicle-treated cells; # $p < 0.05$ compared with LPS-treated cells.

hydroperoxide solution and 10 µl of 1.2 mM NADPH were added to each well. Absorbances were monitored at 340 nm for 10 min. The results are expressed as µmol of GSH/min/mg of protein.

2.5. Statistical Analysis. Data are expressed as mean ± standard error (SEM). Differences between groups were analysed by two-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison tests, or paired *t*-test as appropriate. Percentages of inhibition were calculated as the mean of the inhibitions obtained for each individual experiment. *p* values < 0.05 were considered statistically significant.

3. Results

3.1. Chemical Analysis. HPLC analysis of peppermint LHAE detected the presence of seven peaks that coeluted with ursolic acid, epicatechin, caffeic acid, rutin, quercetin, naringenin, and kaempferol (Figure 1). Epicatechin and naringenin were the major compounds, with retention times of 14.5 min and 33.5 min, respectively (Table 1).

3.2. Peppermint LHAE Modulates Macrophage Viability. Peppermint LHAE effects were evaluated on macrophage

viability stimulated or not with LPS. As expected, LPS reduced macrophage viability by 40% (Figure 2(a)). LPS-induced macrophage death was reversed by incubation with LHAE (1–30 µg/ml; Figure 2(a)). At higher concentrations (≥100 µg/ml), the extract decreased (49–57%) the viability of macrophages cultured in the absence of LPS (Figure 2(a)).

3.3. Macrophage-Mediated Phagocytosis. LPS stimulated phagocytosis in comparison with vehicle-treated cells, as denoted by an increase in the percentage of cells containing beads (4.1-fold increase) and in the number of beads per cell (6.2-fold increase; Figures 2(b) and 2(c)). Peppermint LHAE potentiated the ability of macrophages to phagocytose in the absence but not in the presence of LPS (Figures 2(b) and 2(c)). This potentiation was as high as 6.6- and 8.8-fold for the percentage of macrophages containing beads and number of beads per cell, respectively (Figures 2(b) and 2(c)).

3.4. Peppermint LHAE Reduces H₂O₂ but Not NO Production. Figures 3(a) and 3(b) show the measured concentrations of H₂O₂ and NO, respectively, in supernatant samples from macrophages incubated or not with LPS and LHAE.

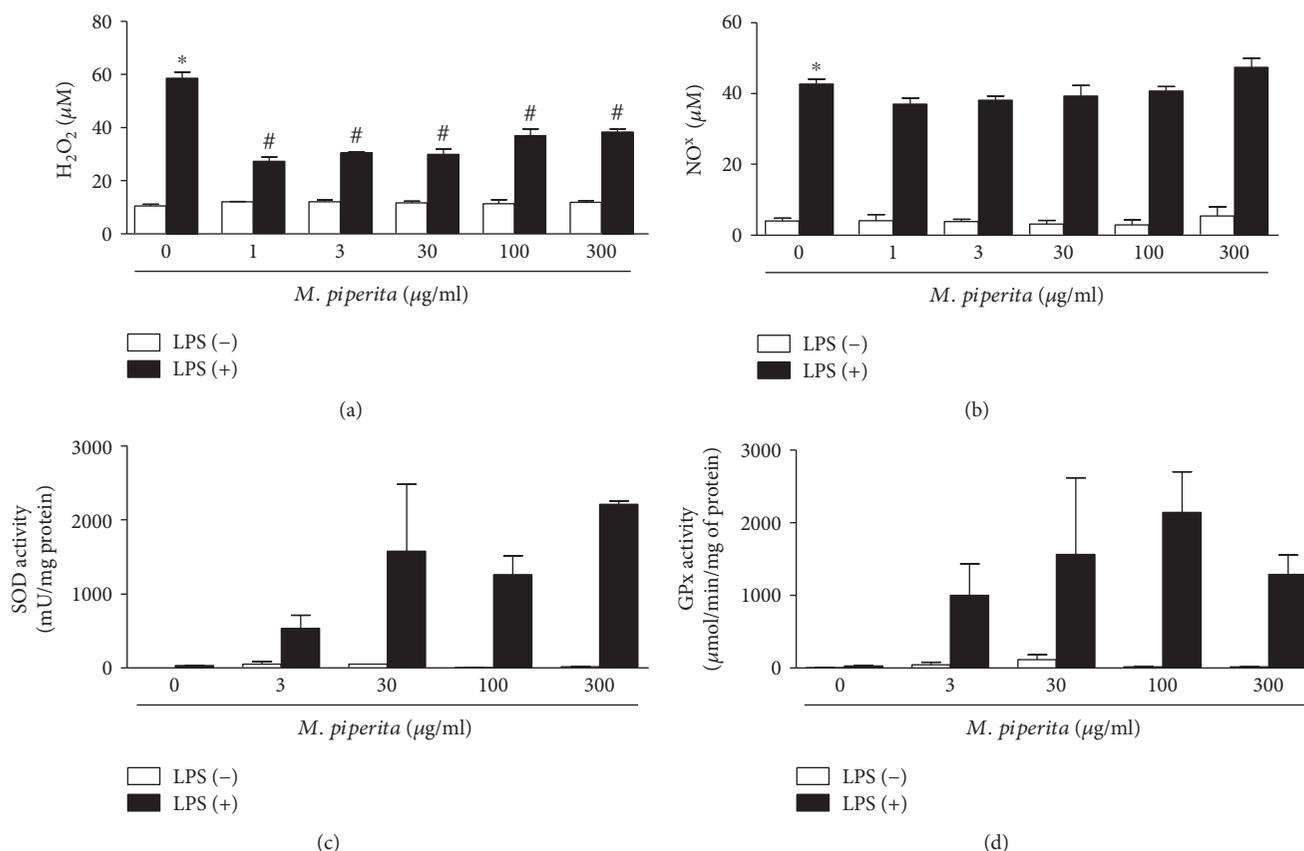


FIGURE 3: Effect of peppermint LHAE on H₂O₂ and NO release. H₂O₂ (a) and NO^x (b) concentrations in supernatant samples of cultured peritoneal macrophages. SOD (c) and GPx (d) activities in cultured peritoneal macrophages. Cells were pretreated with peppermint LHAE (1–300 µg/ml, in 1% DMSO in PBS) and stimulated with LPS for 24 h. Vehicle-treated cells were used as controls. Data are expressed as mean ± SEM. * $p < 0.05$ compared with vehicle-treated cells; # $p < 0.05$ compared with LPS-treated cells.

Incubation of macrophages with LPS triggered the release of both H₂O₂ and NO by these cells, with fold increases of 5.6 and 10.0, respectively, for LPS-treated cells in comparison with vehicle controls. H₂O₂ but not NO release was reduced (34.6–53.4%) in LHAE-treated macrophages.

3.5. SOD and GPx Activities Are Increased in LPS-Stimulated Macrophages Treated with Peppermint LHAE. Figures 3(c) and 3(d) show the measured activities of SOD and GPx in cultured macrophages. Peppermint LHAE increased the activation of both enzymes in LPS-treated macrophages in comparison with vehicle controls. SOD activity was increased by 15.3–63.5-fold (Figure 3(c)), whilst GPx activity was raised by 34.4–73.6-fold (Figure 3(d)).

4. Discussion

M. piperita was previously suggested to have antimicrobial activity against both Gram-negative and Gram-positive bacteria [12], in addition to presenting with antioxidant potential *in vitro* [12, 17, 24]. *In vivo* anti-inflammatory actions were also reported for this plant in murine models of infection and inflammation. However, little is known on the modulatory effects of this plant in inflammatory cells. Here, we investigated the effects of a peppermint LHAE on

cultured macrophages stimulated or not with LPS from *K. pneumoniae*. We found that this extract is able to modulate macrophage responses to LPS.

Mentha spp. effects on macrophage viability *in vitro* have been suggested to be concentration dependent. Indeed, RAW264.7 macrophage viability was previously shown not to be affected by treatment with *M. piperita* essential oil at concentrations as high as 100 µg/ml [16]. On the other hand, extracts from different *Mentha* species were found to be cytotoxic in both macrophage and monocyte cell lines when assessed at concentrations >200 µg/ml [25]. Thus, we initially evaluated the effects of LHAE on peritoneal macrophage viability. LPS-stimulated cells had their viability increased when incubated with LHAE in comparison with LPS controls. This was observed for the smallest concentrations tested and did not affect macrophage's ability to phagocytose when stimulated with LPS. On the other hand, at higher concentrations (≥100 µg/ml), LHAE caused cytotoxicity in cells not stimulated with this endotoxin. Additional effects were also observed for LPS-untreated cells, as they presented increased phagocytosis. To the best of our knowledge, we present here the first evidence on that *M. piperita* affects the viability and phagocytosis of LPS-stimulated murine peritoneal cells. This set of results allows us to suggest that *M. piperita* effects on macrophage may be not only

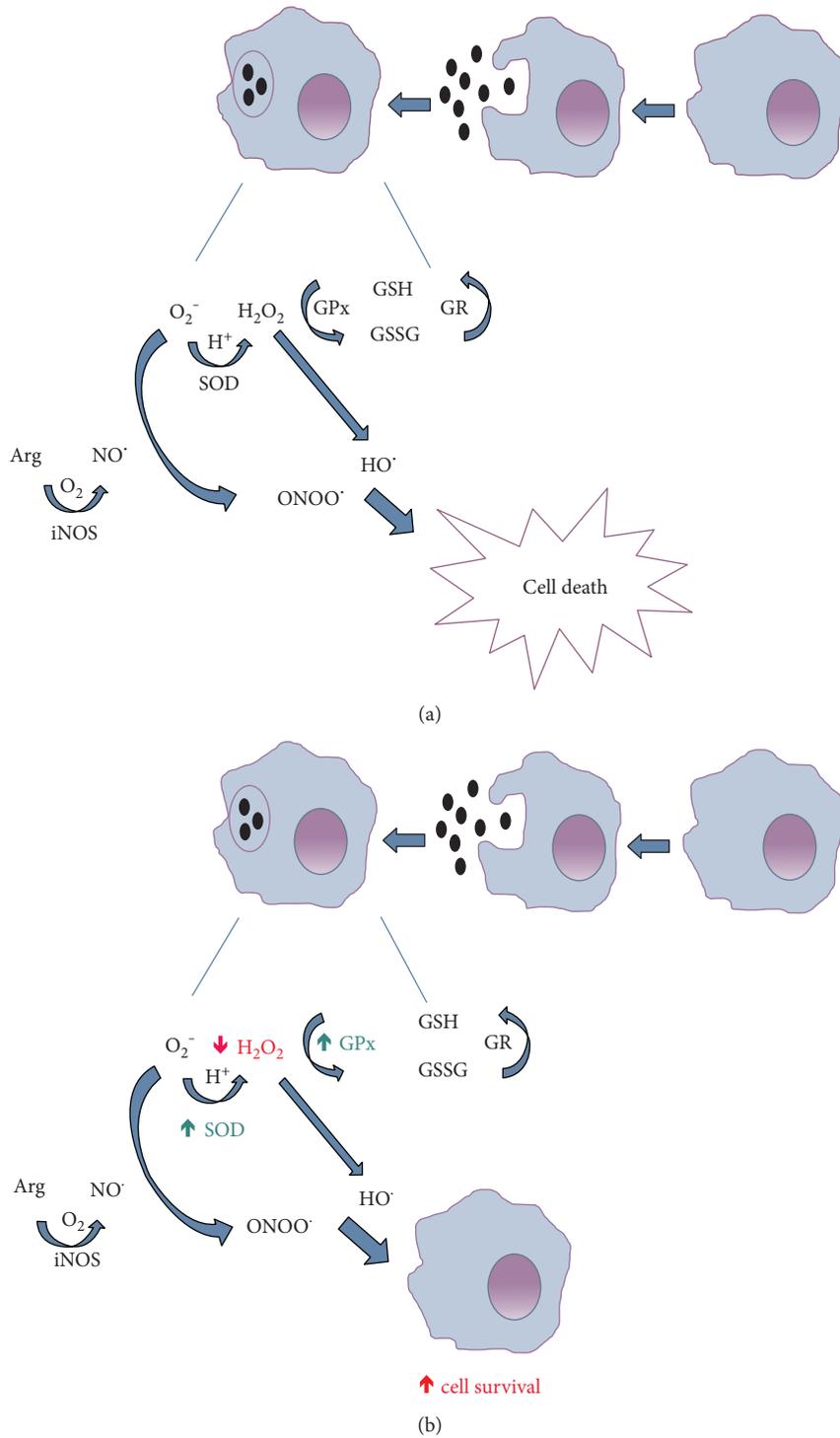


FIGURE 4: Mechanisms of action of LHAE on macrophage-mediated responses. (a) As part of the host's immune response during bacterial infections, macrophages are activated by bacteria-derived products, such as lipopolysaccharide (LPS); and as a result of this activation, reactive species are formed. Superoxide (O_2^-) anion produced by NADPH oxidase is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). H_2O_2 can, in turn, be further reduced to H_2O by glutathione peroxidase (GPx) or even render hydroxyl radical (HO^\bullet), a much more potent oxidant that can lead to diminished cell survival via peroxidation (and further breakdown) of lipids, as well as oxidation of protein and DNA bases. In parallel, nitric oxide (NO^\bullet) continuously produced by inducible NO synthase (iNOS) can react with O_2^- and form peroxynitrous acid (ONOOH) which, after homolytic breakdown, can also render HO^\bullet in addition to the highly reactive nitro (NO_2^\bullet) radical (a potent modifier of proteins and lipids), thus potentiating cell death. (b) The incubation of LPS-stimulated macrophages with LHAE does not affect NO formation but rather increases SOD and GPx activities (thus lowering O_2^- and H_2O_2 availability). As a consequence, OH and/or NO_2^\bullet formation is avoided, thus improving macrophage survival.

dependent on concentration but also on the culture conditions (presence versus absence of LPS).

In a recent study by Sun et al. [16], a peppermint essential oil reduced LPS-induced NO production by naïve RAW264.7 macrophages at similar concentrations to those tested for LHAEE herein. A similar result was observed for an aqueous extract from *Mentha haplocalyx* when incubated with LPS-stimulated macrophages [26]. These studies and others [12, 17, 24] also suggested an antioxidant potential for peppermint and other plants from the same genus. We found that H₂O₂, but not NO production, was decreased in LHAEE-treated cells stimulated with LPS at concentrations as low as 1 µg/ml. The same cells presented increased SOD and GPx activities, as key antioxidant enzymes. Increased SOD activity was previously reported in mice treated with peppermint aqueous extract [27]. More recently, peppermint essential oil was shown to act as a scavenger of hydroxyl radicals and to be an antioxidant at concentrations ≥200 µg/ml [16]. These results allow us to suggest that peppermint antioxidant actions on macrophages may be due to increased activation of SOD and GPx, which in turn, leads to decreased H₂O₂ production by these cells. These evidences, in addition to recent reports on that peppermint LHAEE increases serum concentrations of anti-inflammatory cytokines in *Schistosoma mansoni*-induced infection [19], indicate an important anti-inflammatory action for *M. piperita*.

In regards to NO production, our results contradicted those described for *Mentha* spp. in the literature [28] as LHAEE did not affect its levels upon macrophage stimuli with LPS. However, the inhibitory effects of *Mentha* spp. on NO release by LPS-stimulated macrophages were shown for hexane and ethyl acetate fractions [28], in addition to aqueous extract [26], suggesting that compounds found in different fractions and extractions of *Mentha* spp. may present different actions on NO production.

Different compounds were detected in essential oils obtained from peppermint leaves in previous studies [12, 17, 24, 29]. *M. piperita* antioxidant actions were previously suggested to be due to the presence of phenolic constituents in its leaves including rosmarinic acid and different flavonoids such as rutin, naringin, eriocitrin, luteolin, and hesperidin [30–33]. Here, HPLC analysis of the peppermint LHAEE detected some peaks that coeluted with pure ursolic acid, epicatechin, caffeic acid, rutin, quercetin, naringenin, and kaempferol. These compounds were previously shown to act as anti-inflammatory and/or antioxidants [30, 33, 34]. It is possible that all these compounds contribute to the modulatory actions of LHAEE observed in our study. However, we observed an unexpected lack of effect for LHAEE on NO release by LPS-stimulated cells. This was rather surprising as its detected compounds are known as potent inhibitors of NO production [35–40]. On the other hand, ursolic acid effects on NO release by macrophages are controversial and may be concentration dependent. Indeed, some evidences suggest this compound increases NO production by both infected [41] and resting [42] macrophages, whilst others show ursolic acid inhibits NO release by LPS-stimulated cells [43]. We suggest that, although the different compounds detected in the LHAEE may contribute

synergistically to its antioxidant effects, it is possible they counteract each other's abilities to stimulate or inhibit NO production by macrophages depending on their bio-availability in the extract.

Overall, our data show that the peppermint LHAEE modulates macrophage-mediated inflammatory responses, by stimulating the antioxidant pathway in these cells (Figure 4). These effects may be beneficial when the excessive activation of macrophages contributes to tissue damage in diseases in which there is an unbalanced oxidative stress, such as those of infectious nature.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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