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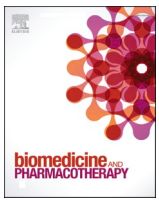

Olive leaf extract (OLE) reduces mast cell-mediated allergic inflammation

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ABSTRACT

Mast cell-mediated reactions promote various allergic disease, including anaphylaxis, allergic rhinitis, asthma, and atopic dermatitis. Different data demonstrated an intricate relationship between the use of antihistaminic drugs, the onset of side effects, and the development of resistance, underscoring the importance to find novel therapeutic approaches to treat allergic diseases. Olive leaf extract (OLE), is a by-product of the olive tree rich in bioactive compounds, known for its numerous therapeutic properties, including antioxidant, anti-tumoral and antidiabetic effects. In this study, we investigated the effect of OLE on the mast-cell-mediated allergic inflammation using human mast cells HMC-1.2. OLE reduced histamine and β-Hexosaminidase release from HMC cells activated by phorbol 12-myristate 13-acetate and calcium ionophore A23187 (PMACI) through modulation of calcium signal. Moreover, OLE decreased the PMACI-stimulated gene expression of proinflammatory cytokines such as tumor necrosis factor-a (TNF-α), interleukin-8 (IL-8) and interleukin-6 (IL-6) in human mast cells. This result was confirmed by multiplex assay in which the pre-treatment with OLE reduced the effective secretion of TNF-α, IL-6 and IL-8. These effects were correlated to ROS reduction and modulation of both mitochondrial mass and membrane potential. Finally, the inhibitory effect of OLE was nuclear factor (NF)-kB dependent as demonstrated by both activity assay and Western Blot analysis. Taken together, our results demonstrated that OLE inhibits mast-cell-derived allergic inflammation modulating mast cells degranulation, proinflammatory cytokines release and NF-kB activation. Therefore, OLE could represent a novel potential therapeutic approach for the treatment of mast cell–associated disorders.

1. Introduction

Over the past few decades, there has been a marked increase in the prevalence of allergic diseases such as atopic dermatitis, allergic asthma, and allergic rhinitis, posing significant challenges to patients' well-being and affecting their quality of life [1]. In this context, mast cells are key players promoting the initiation and progression of allergic reactions and inflammation [2–4]. Upon activation, these cells rapidly release a multitude of preformed mediators such as histamine and proteases, which are stored in their secretory cytoplasmic granules [2]. Additionally, they generate *de novo* multiple pro-inflammatory chemokines and cytokines, including IL-6, IL-8, and TNF-α, which are known to drive allergies [5–7]. These mediators play a significant role in the pathogenesis of allergic responses, enhancing susceptibility to allergic diseases and exacerbating symptoms. Allergies are immune-mediated diseases and are included in the type I hypersensitivity reactions [8]. From a molecular perspective, they are primarily mediated by the interaction of an allergen with immunoglobulin E (IgE), leading to the release of mediators from mast cells and basophils into the bloodstream [8]. The allergic reaction can be divided into two distinct phases [9,10]. The early phase starts when the allergen binds to the mast cell via its specific IgE receptors, triggering mast cell degranulation. This results in the release of key molecular mediators responsible for typical immune response symptoms, such as histamines, proteases, proteoglycans, and TNF-α [9]. The late phase usually starts 4–6 hours post allergen exposure and involves the further release of additional inflammatory modulators, including IL-6, IL-8, and TNF-α [11]. In mast cells, the secretion of cytokines is regulated by intracellular reactive oxygen species (ROS), mitogen-activated protein kinases (MAPK), and NF-kB signaling pathways [12,13] exacerbating allergic diseases. [13]. The most effective

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pharmacological agents for managing allergic diseases encompass antihistamines, corticosteroids, leukotriene receptor antagonists, short-acting beta-agonists, biological therapies, and allergen-specific immunotherapy [14]. Although these therapeutic approaches demonstrate efficacy, their use is associated with significant challenges, including adverse side effects, the emergence of drug resistance, and inconsistent efficacy among diverse patient populations risks [15]. These issues underscore the critical need for the discovery and development of novel therapeutic agents. Among these, plant-derived compounds have demonstrated the ability to suppress IgE production, cytokines, and eosinophils, while also inhibiting histamine release [16]. Here, we evaluated the potential effects of olive leaf extract (OLE) that already demonstrated multiple beneficial effects in different inflammatory-mediated diseases [17–20]. We focused on the mast cells-mediated inflammatory response by analysing the effect of OLE on human mast cells degranulation, cytokine release and mitochondrial activity. Our findings demonstrated that OLE effectively modulates mast cell activation, addressing the limitations of current antiallergic therapies and paving the way for more effective and safer treatment options in managing allergic disorders.

2. Materials and methods

2.1. Extract preparation of OLE

Olive leaf extract was obtained by Olea europea L cultivar Ravece. OLE preparation and characterization has been previously described [21,22].

2.2. Cell culture

Human mast cell line (HMC-1.2) was purchased from Sigma-Aldrich (Milan, Italy) and it was cultured in at standard condition in incubator at 37°C and with an atmosphere containing 5 % CO2 in 75 cm2 flasks (Thermo Fisher Scientific) considering a number equal to 200.000 cells/mL as recommended by the supplier. Cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10 % fetal bovine serum (FBS), 1 % penicillin/streptomycin antibiotics, 1,15 % L-glutamine and 0,08 % 1-Thioglycerol, 50 μM β-mercaptoethanol.

2.3. MTT assay

The viability of HMC-1.2 cells was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded at a density of 10^3 cells per well in a 96-well plate. On the subsequent day, the HMC-1.2 cells were incubated with increased concentrations (ranging from 0.1 to 100 μg/mL) of OLE extracts. After a 24-hour interval post-treatment, 25 μL of MTT (Sigma-Aldrich, Milan, Italy), with a concentration of 5 mg/mL in saline, was administered to each well. The plate was then incubated at a temperature of 37 °C for a duration of 3 hours. The resultant blue formazan crystals were dissolved using a DMSO solution. The absorbance of each well was quantified at a wavelength of 545 nm using a microplate spectrophotometer reader (Multiskan FC, Thermo Scientific™, Waltham, MA, USA).

2.4. Quantitative real-time PCR

HMC-1.2 cell lines were seeded at a density of 1×10^6 cells per dish and subjected to treatment with OLE at a concentration of 10 μg/mL. Following an hour, the cells were stimulated with 50 nM of Phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich, Milan, Italy) and 1 μM of ionophore (Sigma-Aldrich, Milan, Italy) (PMACI), for additional 6 hours. Next, total RNA was isolated using TRI-Reagent (Sigma-Aldrich, Milan, Italy) according to the manufacturer's instructions and, for each sample, the RNA concentration obtained was quantified by Nanodrop (ThermoFisher Waltham, MA, USA.) Thereafter, 1 μg of the total RNA was reversed transcribed to cDNA using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Milan, Italy). Following the reverse transcription process, the cDNA was amplified through PCR cycles in the Bio-Rad CFX384 real-time PCR detection system (Bio-Rad, Milan, Italy) with the use of the subsequent primers:

S16 Gene ID (6217):
Forward Sequence: GGAGCGATTTGCTGGTGTAGAC
Reverse Sequence: CTCCTTCTTGGAAGCCTCATCC
IL-6 Gene ID (3569):
Forward Sequence: AGACAGCCACTCACCTCTTCAG
Reverse Sequence: TTCTGCCAGTGCCTCTTTGCTG
IL-8 Gene ID (3576):
Forward Sequence: GAGAGTGATTGAGAGTGGACCAC
Reverse Sequence: CACAACCCTCTGCACCCAGTTT
TNF-α Gene ID (7124):
Forward Sequence: CTCTTCTGCCTGCTGCACTTTG
Reverse Sequence: ATGGGCTACAGGCTTGTCACTC
GCLC Gene ID (2729):
Forward Sequence: GGAAGTGGATGTGGACACCAGA
Reverse Sequence: GCTTGTAGTCAGGATGGTTTGCG
GCLM Gene ID (2730)
Forward Sequence: TCTTGCCTCCTGCTGTGTGATG
Reverse Sequence: TTGGAAACTTGCTTCAGAAAGCAG
CMA1 Gene ID (1215)
Forward Sequence: TGTGGGCAATCCCAGGAAGACA
Reverse Sequence: GACCGTCCATAGGATACGATGC
TPSAB1 Gene ID (7177)
Forward Sequence: GTGACGCAAAATACCACCTTGGC
Reverse Sequence: CCATTCACCTTGCACACCAGGG

2.5. Cytokine secretion assay

HMC-secreted IL-6 and TNF-α were measured in supernatants with BioLegend's LEGENDplexTM (BioLegend, San Diego, CA, USA) bead-based immunoassays using HU Th Cytokine Panel (12-plex). The assay and the analysis were performed according to the manufacturer's instructions.

2.6. ELISA

IL-8 levels were measured in cell culture supernatants using ELISA kits according to the manufacturer's instruction (DuoSet ELISA, R&D systems).

2.7. Flow cytometry analysis

For live vs. dead comparison, OLE-treated HMC1.2 (10 μg/mL for 48 h) was stained using the Zombie Green Fixable Viability Kit (BioLegend, San Diego, CA, USA) according to the manufacturer's instruction. Cells were stained with 200 μl of Zombie, incubated for 20 minutes at 4°, washed with PBS and acquired to the flow cytometer.

To assess the impact on ROS production and mitochondrial activity, HMC-1.2 cells were treated with 10 μg/mL of OLE and subsequently stimulated with 50 nm of PMA and 1 μM of ionophore, as described above. After 24 hours, the cells were stained with DCFH-DA (D399 Thermo Fisher Waltham, MA, USA), MitoTracker Green (M7514, ThermoFisher Waltham, MA, USA), MitoTracker Deep Red (M22426, ThermoFisher Waltham, MA, USA), and Tetramethylrhodamine, methyl ester (T668, ThermoFisher Waltham, MA, USA). The staining was performed according to the manufacturer's instructions.

To investigate the effects of OLE on calcium influx, the cells were alternatively labeled with Fluo-3 probe. Cells were stained with 200 μl of the fluorescent probes, incubated at 37° for 30 minutes, washed with PBS and acquired to the flow cytometer.

The samples were acquired (10^5 events per samples) using BriCyte E6 flow cytometer (Mindray Medical Italy S.r.l., Milan, Italy), and the

data were analyzed using FlowJo software (TreeStar V.10).

Representative gating strategy used for HMC cells identification and doublet exclusion is reported in Supplementary Figure 1.

2.8. Histamine release

Histamine release was quantified in the supernatants of OLE-treated HMC after 24 hours by using a histamine Enzyme-Linked Immunosorbent Assay (ELISA) kit (Abcam, Cambridge, UK) in strict accordance with the guidelines provided by the manufacturer.

2.9. Quantification of β-hexoaminidase

HMC-1.2 cells were seeded in a 24-well plate at a density of 2×10^5cells per well and were subjected to pretreatment with OLE at a concentration of 10 μg/mL. After an hour, the cells were stimulated with PMA at a concentration of 50 nM and ionophore at a concentration of 1 μM for additional 2 hours. Next, 50 μL of the supernatant was transferred to a 96-well plate in the presence of β-hexosamine (1 mM dissolved in a citrate buffer, pH 4.5). Concurrently, the cell pellet was lysate with 1 % Triton X-100 and treated as supernatant. After an incubation period of 90 minutes at 37°C, the reaction was halted by the addition of carbonate buffer. The absorbance was then measured at 405 nm using a Multiskan GO microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

2.10. Western-blotting

The HMC1.2 cells (4×10^6 cells/well) were plated into dish and treated with OLE (10 μg/mL) for 1 h before the stimulation with PMA 50 nM and ionophore 1 μM for 6 h. Nuclear extracts were prepared as previously described [21]. Protein concentration was measured via the Bradford method (Bio-Rad). The protein extracts were separated via sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) filter membranes using a Trans-Blot Turbo Transfer Starter System (Bio-Rad). The membranes were blocked with 5 % low-fat milk in PBS with 0.1 % Tween 20 (PBST) at room temperature for 2 h and were incubated with the following primary antibodies: NF-κB p65 XP (#8242; Cell Signaling, Milan, Italy) and α-Tubulin (#3873; Cell Signaling, Milan, Italy) overnight at 4 °C. After three washes with PBST, the membranes were incubated with anti-mouse (Santa Cruz Biotechnology, Heidelberg, Germany) or anti-rabbit (Jackson ImmunoResearch, Milan, Italy) secondary antibody, and horseradish peroxidase (HRP) conjugate for 2 h at room temperature. The membranes were developed with the Chem-iDoc™ MP Imaging System (Bio-Rad, Milan, Italy) via the ECL chemiluminescence method.

2.11. Statistical analysis

Data are expressed as mean ± standard error of mean (SEM). All assays were performed at least in triplicate and data were analyzed with GrapdPad Prism 9.0 software program (GraphPad Software, Inc., San Diego, CA, United States). One-way analysis of variance (ANOVA) with post-hoc Bonferroni's test was performed to determine differences between groups. $P < 0.05$, $P < 0.01$ or $P < 0.001$ was considered statistically significant.

3. Results

3.1. OLE did not affect the proliferation and vitality of human mast cells

First, we evaluated the antiproliferative effects of OLE on human mast cells HMC1.2 (HMC). The cells were treated with increasing concentrations of OLE (0.1, 1, 10, and 100 μg/mL) for a period of 48 hours, followed by an assessment of cell proliferation using an MTT analysis. As shown in the Fig. 1A, OLE did not affect the proliferation of HMC cells at all the tested concentrations compared to the control cells. For further studies, we decided to use the concentration of 10 μg/mL that previously demonstrated to modulate the proinflammatory function of murine macrophages [22]. Therefore, to further confirm the non-cytotoxic effect of this concentration, we performed a live versus dead comparison by staining the cells with the Zombie Green Fixable Viability Kit. As demonstrated in Fig. 1B-C, the viability of HMC cells was not compromised by OLE treatment at 10 μg/mL.

3.2. OLE reduced histamine and β-hexosaminidase release in human mast cells

Next, we evaluated whether OLE was able to modulate mast cells activation and degranulation. In particular, we focused on the release of histamine and β-hexosaminidase, key mediators in the allergic response [23]. Therefore, we stimulated HMC cells with phorbol 12-myristate 13-acetate and A23187 (PMACI) a stimulator that activates the signalling process involved in mast cell degranulation and inflammatory response [24]. As expected, stimulation with PMACI increased histamine release in HMC cells. Conversely, this increase was significantly reduced when cells were pre-treated with OLE (Fig. 2A). Likewise, β-hexosaminidase release, another valuable tool for mast cell degranulation was significantly reduced when cells were pre-treated with OLE compared to PMACI-stimulated HMC (Fig. 2B). To corroborate our findings, we evaluated the mRNA expression levels of chymase 1 (CMA1) and tryptase beta 1 (TPSAB1), two key enzymes involved in mast cells degranulation [25]. As shown in Fig. 2C, the mRNA expression levels of both CMA1 and TPSAB1 were significantly increased upon stimulation with PMACI whilst pre-treatment with OLE effectively reduced their expression levels. Increased intracellular calcium is crucial for degranulation and cytokine secretion following mast cell stimulation [26]. To elucidate the mechanism by which OLE inhibits mast cell degranulation, we measured intracellular calcium levels by using the fluorescent indicator Fluo-3/AM. Stimulation with PMACI significantly elevated intracellular calcium levels in HMC cells, and pre-treatment with OLE effectively reduced these calcium elevations (Fig. 2D-E). Collectively, our data demonstrated that OLE effectively modulate mast cell activation and degranulation in allergic responses.

3.3. OLE reduced the expression of pro-inflammatory mediators in human mast cells

Pro-inflammatory cytokines play critical roles in various inflammatory responses, including immune cell recruitment, immune cell activation, and tissue remodelling during chronic inflammation [27]. To assess the influences of OLE on the expression of pro-inflammatory cytokines in human mast cells, qPCR was carried out to measure the RNA expression levels of TNF-α, IL-8 and IL-6. The stimulation with PMACI significantly increased the RNA expression levels of all analysed cytokines. However, pretreatment with OLE diminished the expression of IL-6, TNF-α and IL-8 (Fig. 3A-C). This observation was corroborated by the quantification of cytokines in the culture supernatants of HMC cells, with or without OLE pretreatment. Consistent with the qPCR analysis, we noted a significant decrease expression IL-6 (Fig. 3D) TNF-α (Fig. 3E) and IL-8 (Fig. 3F) in the culture medium of HMC cells treated. This further emphasizes the potential therapeutic value of OLE in managing inflammatory conditions.

3.4. OLE reduced ROS production and modulated mitochondrial functions in human mast cells

Mast Cell activation is associated with an increased production of Reactive Oxygen Species (ROS) and alteration in mitochondrial activity [28]. Therefore, we investigated the potential antioxidant effects of OLE on the HMC cells. Pretreatment with OLE inhibited the generation of

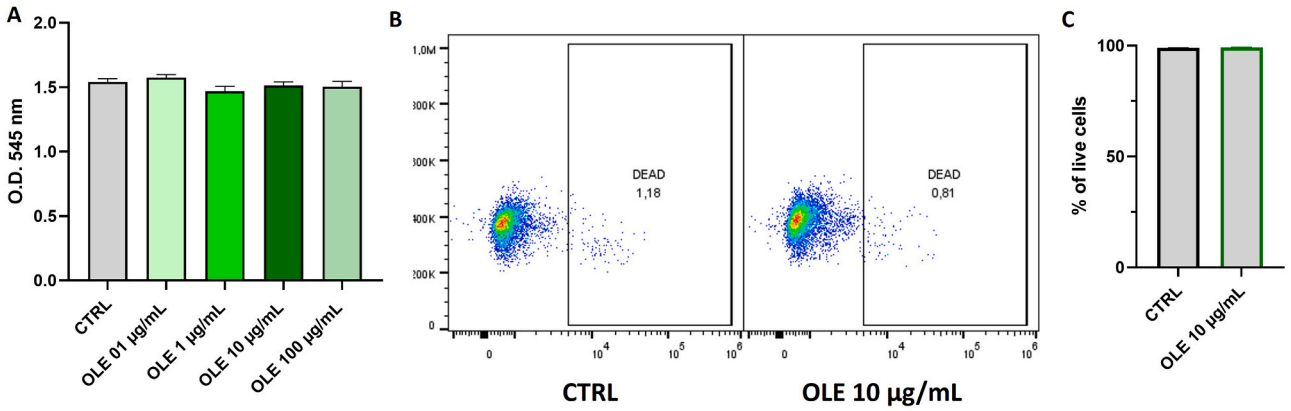

Fig. 1. OLE did not affect the proliferation and vitality of human mast cells. (**A**) HMC1.2 cells were treated with increasing concentrations of OLE (01–100 µg/mL). Cytotoxicity was evaluated via MTT assay 24 h after treatment. (**B**) Representative plot and (**C**) frequency of dead cells after treatment for 24 h with OLE 10 µg/mL. Values were expressed as mean ± SEM from at least three independent experiments.

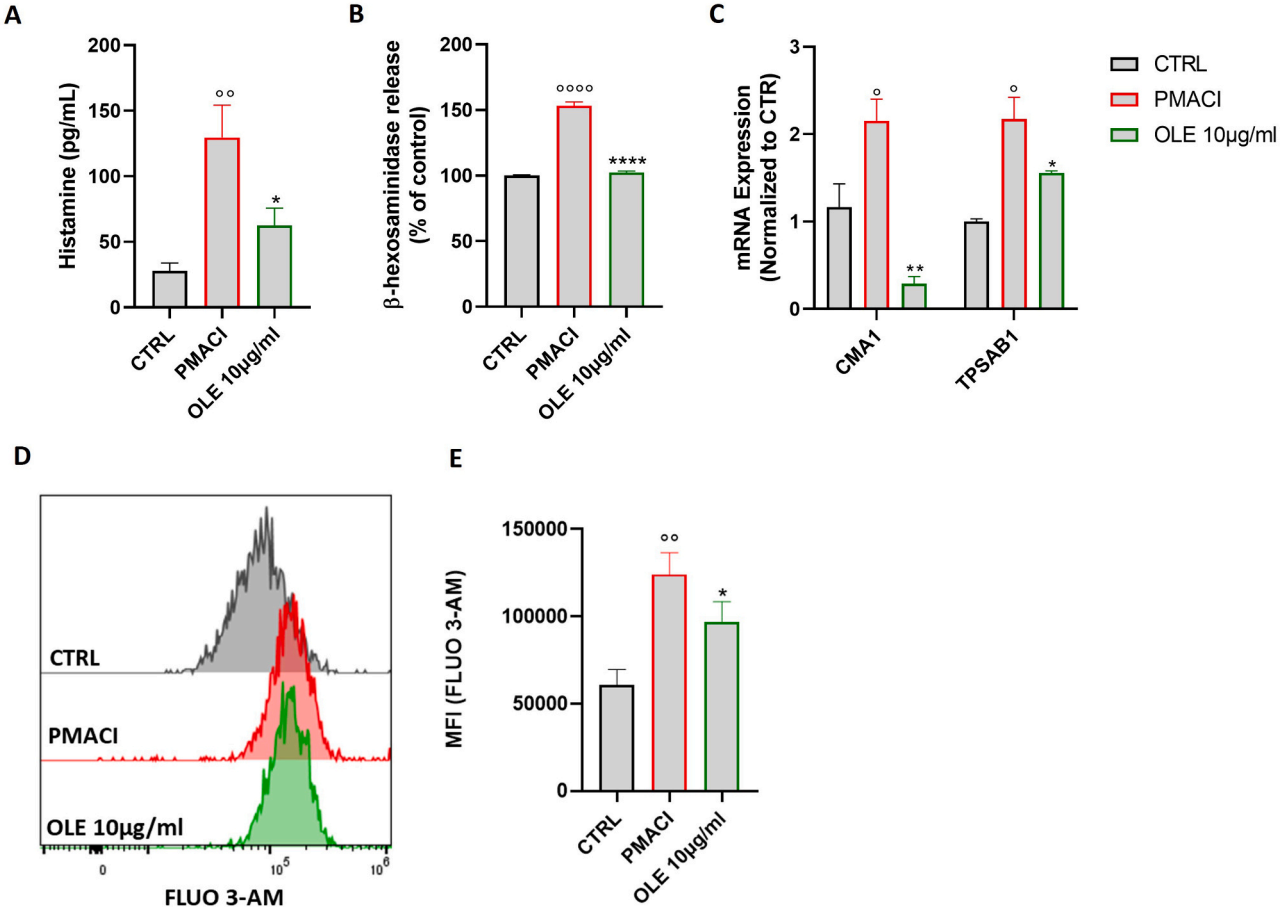

Fig. 2. OLE reduced histamine and β-hexosaminidase release in HMC cells. (A, B) Effect of OLE on Histamine (**A**) and β-hexosaminidase (**B**) release. (**C**) mRNA expression of CMA1 and TPSAB1 assessed by qPCR in HMC following pretreatment with OLE and stimulation with PMACI for 6 h. (**D**) Representative examples of flow cytometry analysis of calcium fluxes with the FLUO-3AM probe. (**E**) Quantitative analysis of FLUO-3AM in terms of Mean Fluorescence Intensity (MFI) in OLE-treated HMC. Data were expressed as mean ± SEM of at least three independent experiments (° $p < 0.05$, °° $p < 0.01$, °°°° $p < 0.0001$ indicate a significant effect of PMACI compared with unstimulated cells (CTRL); * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ indicated a significant effect of OLE compared with PMACI-stimulated cells.

ROS (Fig. 4A, B) and modulated mitochondrial functions reducing both mitochondrial mass and membrane potential (ΔΨm) (Fig. 4C-H) in PMACI-stimulated HMC cells. Next, we assessed the expression of Glutamate-Cysteine Ligase Catalytic Subunit (GCLC) and Glutamate-Cysteine Ligase Modifier Subunit (GLCM), the key antioxidant enzymes involved in the synthesis of glutathione [29,30]. As shown in Fig. 4I and J, qPCR analysis showed that pre-treatment with OLE notably enhanced the transcription of these two genes. These results indicate that OLE exerts a protective effect against oxidative stress in mast cells by reducing ROS production, preserving mitochondrial

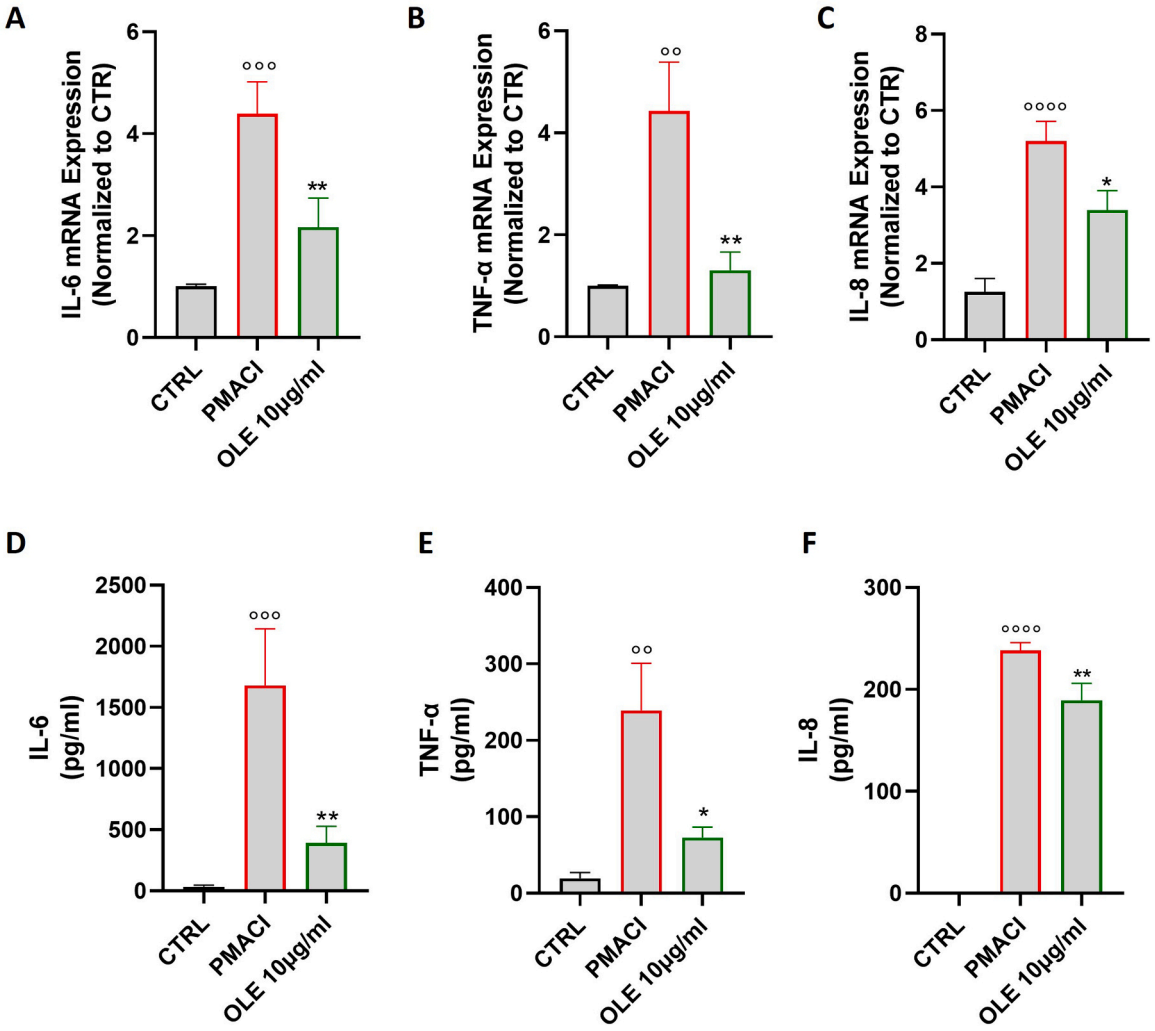

Fig. 3. OLE reduced the Expression of Pro-inflammatory Mediators in Human Mast Cells. (A-C) HMC 1.2 cells were treated with OLE (10 µg/mL) for 1 h before stimulation with PMACI for 6 h. Subsequently, mRNA levels of the pro-inflammatory cytokines IL-6 (**A**), TNF-α (**B**) and IL-8 (**C**). The levels of IL-6 (**D**) TNF-α (**E**) and IL-8 (**F**) were determined in the cell culture medium of HMC cells treated with OLE (10 µg/mL) for 1 h before the stimulation with PMACI for 24 h. Data were expressed as mean ± SEM of at least three independent experiments (°° $p < 0.01$, °°° $p < 0.001$, °°°° $p < 0.0001$ indicate a significant effect of PMACI compared with unstimulated cells (CTRL); * $p < 0.05$, ** $p < 0.01$ indicated a significant effect of OLE compared with PMACI-stimulated cells.

function, and upregulating critical antioxidant enzymes, thereby highlighting its potential as a valuable therapeutic agent for mitigating mast cell-related disorders.

3.5. OLE suppressed the activation of NF-kB in human mast cells

It is well recognized that the activation of mast cells and the consequent release of histamine and proinflammatory cytokines are induced by the nuclear translocation of nuclear factor (NF)-kB following degradation of IkBa [31,32]. Therefore, we assessed the impact of OLE on NF-kB activation by evaluating the nuclear translocation of the NF-κB p65 subunit by Western blot analysis. Upon stimulation with PMACI there was a significant increase in the nuclear levels of p65. However, pre-treatment with OLE significantly reduced the nuclear expression of p65 in HMC cells confirming the inhibitory effect of OLE on NF-κB activation (Fig. 5A and B). To corroborate this finding, we performed an NFKB activity assay on nuclear cell lysate confirming the significant inhibitory effect of OLE on the activation of this transcription factor (Fig. 5C). These observations underscore that OLE effectively attenuates mast cell activation by inhibiting the nuclear translocation and activity of NF-κB, thereby reducing the release of histamine and proinflammatory cytokines and highlighting its potential as a therapeutic agent in controlling inflammatory responses.

4. Discussion

Allergic diseases have seen a significant rise in terms of prevalence over recent decades, posing a global health challenge. These diseases are characterized by immune-mediated inflammatory reactions, primarily driven by the interaction of allergens with IgE. This interaction triggers the release of various mediators from mast cells and basophils, leading to typical symptoms of allergic reactions. The allergic response can be divided into two phases: the early phase, marked by mast cell degranulation and release of mediators like histamines and proteases, and the late phase, characterized by the release of additional inflammatory modulators such as IL-6, IL-8, and TNF-α. Targeting the regulatory mechanisms of mast cell activity presents an alternative and promising avenue for therapeutic intervention in allergic diseases. In the last few decades, various natural products, such as flavonoids, polyphenols, and plant extracts, have demonstrated the ability to modulate allergic responses by targeting mast cell activity, underscoring their potential as therapeutic agents in managing allergic diseases [33–36]. In this context, our study focuses on the potential of OLE as a natural remedy, investigating its anti-histaminergic and anti-inflammatory effects on

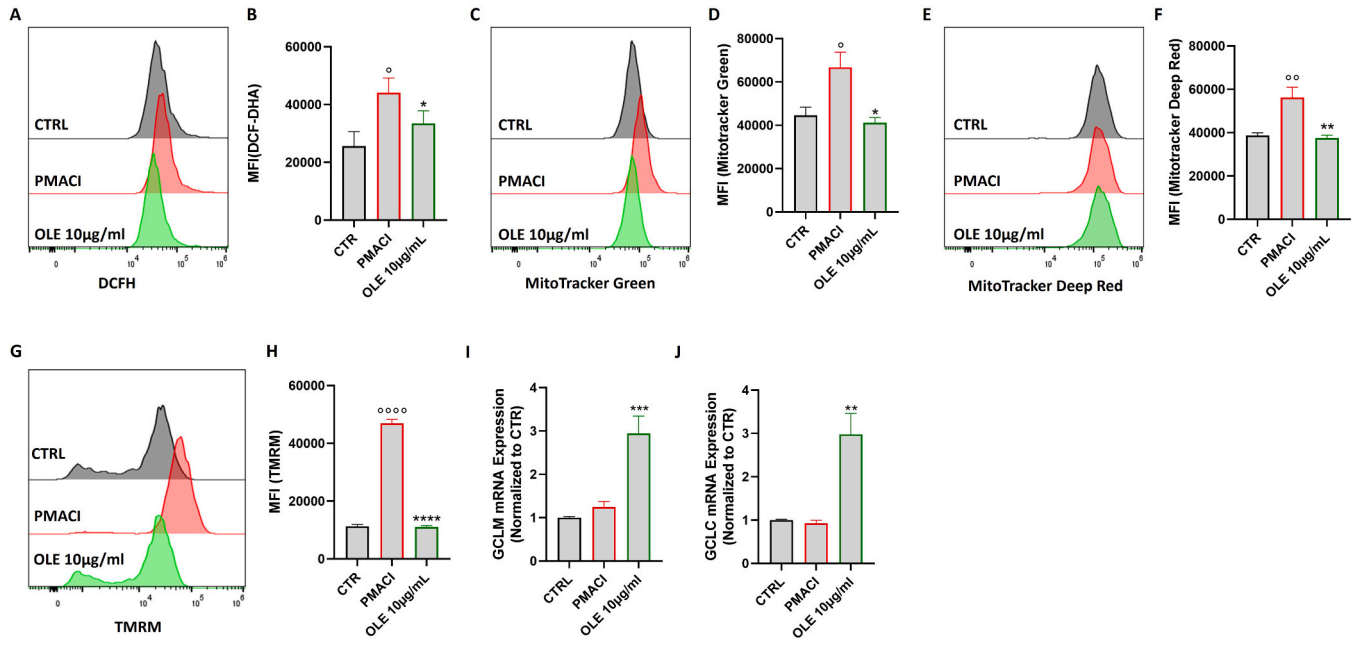

Fig. 4. OLE reduced ROS production and modulated mitochondrial functions in human mast cells. (A–H) Representative examples of flow cytometry analysis and quantification in terms of MFI of DCFH (**A, B**) MitoTracker Green **(C, D),** MitoTracker Deep Red **(E, F)** and TMRM **(G, H)** of HMC cells following pretreatment with OLE and stimulation with PMACI for 24 h. (**I, J**) mRNA expression of GCLM (**I**) and GCLC (**J**) assessed by qPCR in HMC following pretreatment with OLE and stimulation with PMACI for 6 h. Data were expressed as mean ± SEM of at least three independent experiments (° $p < 0.05$, °° $p < 0.01$, °°°° $p < 0.0001$ indicate a significant effect of PMACI compared with unstimulated cells (CTRL); * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ indicated a significant effect of OLE compared with PMACI-stimulated cells.

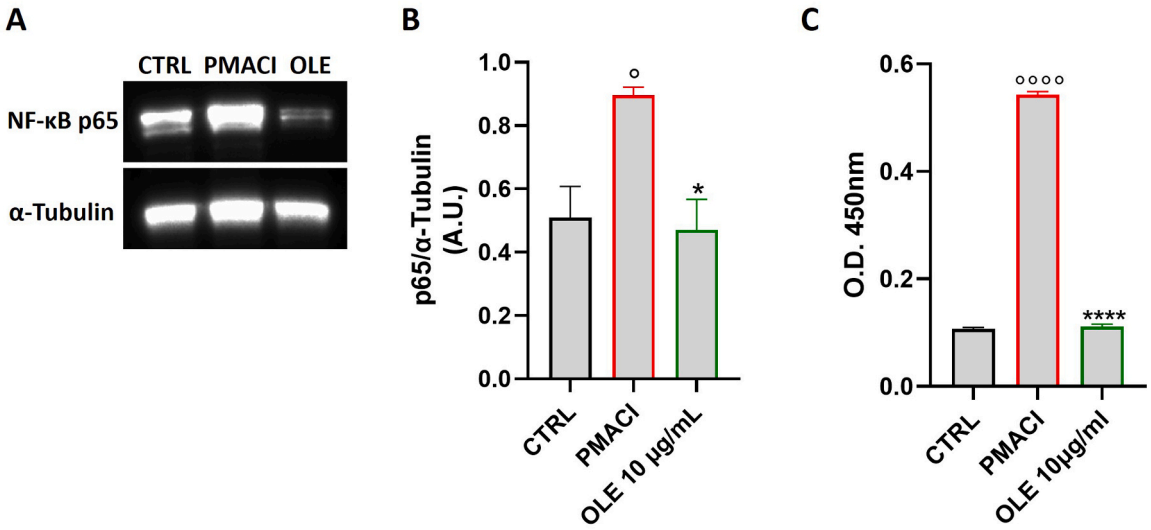

Fig. 5. OLE suppressed the activation of NF-kB in human mast cells (**A**) Representative images of p65 protein detected by Western blot analysis in nuclear extracts of OLE-treated HMC cells. α-tubulin was used as an internal control. (**B**) Relative quantification of NF-kB (p65) (**C**) NF-kB (p65) levels were assessed in nuclear extracts with NF-κB (p65) Transcription Factor Assay Kit. Data were expressed as mean ± SEM of at least three independent experiments (° $p < 0.05$, °°°° $p < 0.0001$ indicate a significant effect of PMACI compared with unstimulated cells (CTRL); * $p < 0.05$, **** $p < 0.0001$ indicated a significant effect of OLE compared with PMACI-stimulated cells.

HMC1.2 human mast cells. Firstly, our data indicate that OLE does not affect the proliferation or viability of human mast cells HMC. This finding confirms that the anti-inflammatory effects of OLE are not due to cytotoxicity. This aligns with previous studies demonstrating the non-toxic nature of OLE in various cell types, reinforcing its potential safety for therapeutic use [22,37]. Importantly, OLE significantly reduced the release of histamine and β-hexosaminidase from PMACI-stimulated HMC cells. These mediators are crucial in allergic responses, and their reduction suggests a strong anti-allergic potential for OLE. The decrease in intracellular calcium levels following OLE treatment indicates that OLE interferes with calcium signalling, a key pathway for mast cell activation and degranulation [38]. In addition, OLE significantly reduced the expression and secretion of pro-inflammatory cytokines, such as TNF-α, IL-8, and IL-6. These cytokines play pivotal roles in propagating allergic inflammation and immune cell recruitment [39,40]. This aligns with previous research demonstrating that plant extracts with antioxidant properties can suppress cytokine production in inflammatory conditions, suggesting that

OLE could be beneficial in managing chronic inflammatory diseases, such as asthma and atopic dermatitis, which involve elevated cytokine levels [39,41–43]. Given the correlation between Mast Cell activation and an increase in the production of Reactive Oxygen Species (ROS) [28], we assessed the potential antioxidant effect of OLE on HMC cells. OLE enhanced the transcription of key antioxidant enzymes, GCLC and GCLM, reduces ROS production, and preserves mitochondrial functions. Similar antioxidant effects have been reported for other natural compounds, emphasizing the role of antioxidants in mitigating mast cell-related disorders [34,43–45]. NF-κB is a key transcription factor involved in the mast cell-mediated inflammatory response triggering the release of histamine and pro-inflammatory cytokines [46]. Therefore, we evaluated the impact of OLE on NF-kB activation. In particular, the nuclear translocation of the NF-κB p65 subunit was evaluated through both an NF-kB activity assay and Western blot analysis. The results indicated that OLE reduced the nuclear expression of p65 subunit in HMC, suggesting that the anti-inflammatory activity of OLE is elucidated through the inhibition of NF-kB and its downstream mediators. These results are in line with previous findings where OLE demonstrated to modulate NF-kB activation in activated murine macrophages highlighting the potential of NF-κB inhibitors in treating different immuno-mediated diseases [22]. In conclusion, our study demonstrates that OLE significantly inhibits mast cell-mediated allergic inflammation through multiple mechanisms, providing a strong foundation for its potential therapeutic application in treating mast cell-associated disorders. These findings suggest that OLE could offer a novel, non-toxic approach to managing allergic diseases and warrant further exploration in clinical settings. Nevertheless, it is important to note that the findings of this study are based on the use of a specific olive leaf cultivar (Ravece). While oleacin and oleuropein are generally present as principal bioactive components in both olive leaves and fruits, their concentrations may vary across different cultivars as also demonstrated for other plant extracts [42,43]. Therefore, future research should aim to validate and expand these results by investigating extracts from other olive varieties. Likewise, further studies are needed to better elucidate the mechanisms underlying the anti-inflammatory effects by performing in vivo studies and to explore the clinical relevance of OLE in freshly isolated mast cells from both human and murine tissues as well as in allergic disease models.

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CRediT authorship contribution statement

Daniela Claudia Maresca: Investigation, Data curation. **Benedetta Romano:** Methodology, Investigation, Data curation. **Fabio Somma:** Writing – original draft, Methodology, Investigation. **Giuseppe Ercolano:** Writing – review & editing, Validation, Supervision. **Angela Ianaro:** Project administration, Funding acquisition, Conceptualization. **Giancarlo Tenore:** Writing – review & editing. **Maria Maisto:** Writing – original draft, Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.117784.

Data availability

Data will be made available on request.

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Glossary

CMA1: chymase 1
ELISA: Enzyme-Linked Immunosorbent Assay
FBS: fetal bovine serum
GCLC: Glutamate-Cysteine Ligase Catalytic Subunit
GLCM: Glutamate-Cysteine Ligase Modifier Subunit
HMC: Human mast cell line
IgE: immunoglobulin E
IL-6: interleukin-6
IL-8: interleukin-8
IMDM: Iscove's Modified Dulbecco's Medium
MAPK: mitogen-activated protein kinases
MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
OLE: Olive leaf extract
PBST: PBS with 0.1 % Tween 20
PMACI: phorbol 12-myristate 13-acetate/calcium ionophore A23187
PVDF: polyvinylidene difluoride
ROS: Reactive Oxygen Species
TNF-α: tumor necrosis factor-a
TPSAB1: tryptase beta 1.