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ARTICLE

# Cytotoxicity Thresholds and Limited Modulation of LPS-Induced Microglial Activation by Croatian Olive Leaf Extracts: A Preliminary Study in a BV-2 Washout Model

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**ABSTRACT: Objectives:** Neuroinflammation, largely mediated by microglial activation, plays a central role in the pathogenesis of neurodegenerative diseases. In this study, we aimed to evaluate olive leaf extracts (OLEs) from three Croatian cultivars (Buža, Oblica, and Leccino) in the context of lipopolysaccharide (LPS)-induced activation of BV-2 microglia. **Methods:** Extracts were prepared by aqueous maceration and ethanol extraction under conditions compatible with downstream cell-culture use. Total phenolic and flavonoid contents were determined for both extract types, alongside targeted liquid chromatography–mass spectrometry (LC–MS/MS) quantification of major phenolic compounds. Based on compositional analysis, ethanolic extracts were selected for further evaluation, including antioxidant capacity assays [2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)] and assessment of BV-2 microglial responses to LPS, i.e., measurements of cell viability, activation markers (CD86, CD206, iNOS), and selected markers of inflammatory (phospho-p65, Sirt1) and cytoprotective pathways (Nrf2, HSP70). **Results:** Ethanolic extracts exhibited higher total phenolic and flavonoid contents than aqueous macerates, supported by LC–MS/MS analysis showing increased levels of key constituents such as oleuropein and other flavonoids. Antioxidant activity varied depending on the assay: DPPH activity was comparable across cultivars ($p = 0.858$), whereas ABTS activity was highest for the Oblica extract ($p = 0.0002$). Ethanolic extracts were non-cytotoxic at 10 μg/mL, while higher concentrations induced dose-dependent cytotoxicity ($p = 0.0001$). In BV-2 cells, LPS (1 μg/mL, 3 h) increased CD86 ($p = 0.017$) and CD206 expression ($p < 0.0001$), with no change in iNOS levels ($p = 0.0697$). Short-term, non-cytotoxic pretreatment with ethanolic extracts (10 μg/mL, 3 h) did not significantly alter microglial activation markers or the examined inflammatory and cytoprotective pathways. **Conclusion:** These findings define the experimental limits under the applied conditions and indicate that compositional richness alone does not necessarily translate into measurable biological effects, supporting further studies with extended exposure and refined dosing.

**KEYWORDS:** Olive leaf extracts; BV-2 microglia; microglial activation; lipopolysaccharide; Croatian olive cultivars

## 1 Introduction

Olive tree (*Olea europaea* L.) with its by-products, olive fruit and olive leaves, has been used for centuries in medicine due to known health benefits [1]. Olive leaves were used by ancient Egyptians and as a traditional remedy for fever; in the 19th century, British colonists utilized them to treat malaria in tropical regions, and by the mid-20th century, their blood pressure-lowering effects were identified,

leading to growing scientific investigation into their therapeutic potential [2]. In contemporary nutrition science, extra-virgin olive oil (EVOO) is a widely consumed pillar of the Mediterranean diet supported by robust epidemiologic and randomized-trial evidence for cardiometabolic protection, whereas olive-leaf preparations are not staple foods but are used as herbal infusions and nutraceutical extracts with a more limited, emerging clinical evidence base [3]. In modern cultivation, leaves are routinely removed during pruning and harvesting, generating abundant biomass amenable to valorization as teas or standardized extracts [4]. Notably, during olive-oil production, most phenolic compounds from the fruit partition into by-products (pomace/pulp, skin, vegetation water), with only a minor fraction transferred to the oil, making leaves and processing residues particularly rich sources of recoverable bioactive constituents [5]. Beyond leaves, olive-processing side streams are increasingly repurposed: olive pomace is traditionally incorporated into livestock feed [6], and olive pâté, obtained from the fruit solids remaining after EVOO production, is being dried and formulated as a polyphenol-enriching ingredient in human foods [7]. Together, these streams show that there is a circular bioeconomy opportunity where olive leaves and processing residues are rich, cheap sources of phenylethanoids and secoiridoids that have antioxidant, anti-inflammatory, and immunomodulatory effects that are important in cardiometabolic, infectious, and neuroinflammatory contexts [3]. Olive leaf extracts (OLEs) are rich in phenolic compounds, predominantly secoiridoids such as oleuropein, along with phenolic alcohols, flavonoids, and phenolic acids [8,9]. These compounds are synthesized as part of the plant defense system and are responsible for the antioxidant, anti-inflammatory, and other health-promoting properties attributed to olive-derived products [8]. The phenolic composition of olive leaves is strongly influenced by cultivar genetics and is further shaped by processing and extraction conditions, resulting in distinct bioactive profiles [4,10,11]. Consequently, differences in phenolic content and composition among cultivars translate into variability in the biological activity and health relevance of OLEs, underscoring the importance of cultivar selection and extraction strategy.

Neuroinflammation is considered a defining feature of neurodegenerative diseases, especially Alzheimer's (AD) and Parkinson's diseases (PD) [12]. Brain neuroinflammation primarily includes microglial activation in response to various stimuli such as protein aggregates (Aβ in AD, α-synuclein in PD), neuronal damage, and peripheral immune signals [13]. Microglia initially play protective roles by phagocytosing and clearing pathological protein aggregates and cellular debris. However, if excessively or chronically activated, microglia go into a dysfunctional state with impaired phagocytic capacity, sustained release of neurotoxic inflammatory mediators, such as reactive oxygen species (ROS), nitric oxide (NO), reactive nitrogen species (RNS), and cytokines, exacerbating neuroinflammation and contributing to disease progression [13,14]. Given this central role, gently rebalancing microglial activation is a plausible strategy to limit inflammatory amplification in neurodegeneration.

Oleuropein and its metabolite hydroxytyrosol (HT), major phenolic constituents of olive leaf extracts, have been associated with neuroprotective effects through enhancement of mitophagy, mitochondrial function, and antioxidant defenses. In AD models, they reduce amyloid-β toxicity, support insulin signaling, and improve memory, whereas in PD experimental models, they protect dopaminergic neurons, stabilize α-synuclein, and improve motor function [15,16]. EVOO, oleic acid, and HT have also been shown to reduce oxidative stress and inflammation in multiple sclerosis preclinical studies [17], as well as to mitigate mitochondrial dysfunction and striatal degeneration in experimental Huntington's disease [18]. Overall, these compounds support neuronal survival and function across diverse neurodegenerative disorders through mitochondrial, anti-apoptotic, and antioxidant pathways. Because these targets (mitochondrial resilience, redox balance, and inflammatory signaling) are tightly coupled to microglial state, olive-leaf–derived biophenols are therefore of interest in the context of microglial responses.

Here, we examined olive-leaf extracts in a tractable inflammatory model of BV-2 microglia. We prepared two cell-compatible formulations per cultivar: a phenolic-enriched ethanolic extract (mechanism-oriented) and a hot-water macerate (consumer-relevant format) from three phytochemically distinct cultivars: Buža (Istria, Croatia), Oblica (Dalmatia, Croatia), and Leccino (Italy). In order to induce a TLR4-driven response, we first established a non-cytotoxic dosing window using BV-2 microglia that were challenged with lipopolysaccharide (LPS). Subsequently, we investigated whether short pretreatment (3 h) followed by a strict media washout protocol with affects canonical activation readouts (CD86, CD206, iNOS) and upstream signaling targets (SIRT1, phospho-p65, Nrf2, and HSP70). We posited that the effects of chemically defined olive-leaf extracts, particularly ethanolic preparations, may depend on cultivar and extraction format under cell-compatible conditions. We aimed to establish precise baseline dosing parameters and determine whether brief intracellular exposure is sufficient to alter microglial activation under high-stringency experimental constraints.

## 2 Materials and Methods

### *2.1 Olive Leaves (Olea europaea L.) Collection and Preparation*

Olive leaves (*Olea europaea* L.) were collected in December 2024 from three different varieties: Leccino, Buža, and Oblica. Leccino and Buža were collected in the Sovinjak region, Istria County, Croatia (45°22′49″ N, 13°54′41″ E). Oblica was collected in the Solin region, Split-Dalmatia County, Croatia (43°32′19″ N, 16°28′22″ E). Upon collection, olive leaves were stored at room temperature in the dark for approximately one month, followed by air-drying for one week. No visible signs of microbial growth or degradation (e.g., mold, discoloration, or odor changes) were observed during this period.

Given the high content of phenolic compounds in olive leaves, which exhibit antimicrobial and antioxidative properties, the samples remained stable under these conditions. Moreover, all samples were handled identically, ensuring consistency across the dataset.

While alternative preservation methods such as freezing or lyophilisation may further improve stability, air-drying in the dark is a commonly applied approach in phytochemical studies. Prior to use, leaves were washed twice (tap water), air-dried, then blotted and left to air-dry at room temperature for 1 week. Dried material was stored in a dry and dark place at room temperature until extraction.

### *2.2 Olive Leaf Extracts Preparation*

Olive Leaf Macerates (OLMs) were prepared according to the procedure described by Peršurić et al. [9], with the following steps briefly outlined. Simulating household preparation conditions, dried olive leaves were coarsely ground in a mortar (0.4 g) and macerated in 40 mL of boiled distilled water (100°C) for 15 min. OLM was filtered through 0.45 μm microfilters into sterile tubes. This procedure was performed separately for each of the three olive varieties. The resulting extracts were aliquoted and stored at −20°C until further use.

For the preparation of olive leaf ethanol extracts (OLEEs), dried olive leaves were manually ground using a mortar and pestle to obtain a coarse powder. No mechanical grinder was used in this study. The resulting material was not further sieved but used directly for extraction. Powdered olive leaves (0.2 g) were extracted with 80 mL of 50% (v/v) ethanol (ethanol: water, 1:1, v/v), in duplicate. The samples were extracted for 30 min in round-bottom flasks with a reflux condenser on a boiling water bath. After cooling to room temperature, the extracts were filtered into a 100 mL volumetric flask. The filter paper (Whatman No. 1 filter paper, pore size 11 μm) was rinsed with 10 mL 50% (v/v) ethanol, and the combined filtrate was brought to volume in the 100 mL volumetric flask. OLEEs were then concentrated using a rotary evaporator (Heidolph Laborota 4002; Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) for 3 h at 100 mbar

and 40°C to a final volume of 50 mL. The concentrated extracts were aliquoted and stored at +4°C until further analysis.

### *2.3 Determination of Total Phenolic and Total Flavonoid Content*

The total phenolic content (TPC) from OLEEs and OLMs was determined using a modified Folin-Ciocalteu method as described by Singleton et al. [19]. Following a preliminary trial, the assay was optimized by diluting the initially prepared extract 1:2 to ensure that absorbance values fell within the calibration curve. Accordingly, 100 µL of the diluted extract was mixed with 100 µL of Folin & Ciocalteu reagent (Sigma-Aldrich, Darmstadt, Germany) and 900 µL of 7.5% (w/v) $Na_2CO_3$, followed by vortexing for 10 s. The reaction mixture was incubated in the dark at room temperature for 20 min. Subsequently, 200 µL of the reaction mixture was transferred to a 96-well plate in triplicate, with deionized water serving as the blank. The calibration curve was prepared using gallic acid standards (≥98% purity, Cayman Chemical, Ann Arbor, MI, USA; Item No. 11846) (dissolved in deionized water) at five concentrations (0, 25, 50, 100, and 200 mg/L). Absorbance was measured at 760 nm using the TECAN Monochromator Infinite M200 Pro (Tecan Group Ltd., Männedorf, Switzerland), and results were expressed as milligrams of gallic acid equivalents per gram of leaves' dry weight (mg GAE/G DW), as well as per millilitre of extract (mg GAE/mL extract).

The total flavonoid content (TFC) of OLEEs and OLMs was determined using a modified method of Arvouet-Grand et al. [20]. Briefly, each OLE sample (100 µL) was mixed with an equal volume (100 µL) of 80% (v/v) methanol–$AlCl_3$ solution (2 g/100 mL) and vortexed. The mixtures were incubated at room temperature for 10 min, after which 200 µL of each mixture was transferred into the wells of a 96-well plate in triplicate. Blanks were prepared by replacing the sample with the corresponding solvent and used for background correction. A calibration curve was prepared using quercetin standards at five concentrations (0, 12.5, 25, 50, and 75 mg/L). Absorbance was measured at 415 nm using a TECAN Monochromator Infinite M200 Pro, and results were expressed as milligrams of quercetin equivalents per gram of leaves' dry weight (mg QUE/g DW), as well as per millilitre of extract (mg QUE/mL extract).

#### *Preparation of OLEE for Cell Treatment Based on Total Phenolic Content*

The OLEE, following ethanol removal by rotary evaporation, were directly reconstituted in the culture medium. The concentrations applied in cell experiments were expressed as µg/mL of TPC. Thus, the reported concentrations represent the estimated phenolic content of the extract in the culture medium, rather than the total extract mass or flavonoid content. Before adding to cells, the reconstituted extract was added to the completed cell culture media in appropriate dilutions and sterilized by 0.22 µm filtration.

### *2.4 LC–MS/MS Analysis*

Targeted liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis was performed using an Agilent 1260 series HPLC system coupled to an Agilent 6460 triple quadrupole mass spectrometer equipped with an AJS ESI source (Agilent Technologies, Palo Alto, CA, USA), following a previously developed and validated method [21]. Chromatographic separation was carried out on a Purospher STAR RP-18 Hibar HR column (50 × 2.1 mm, 1.7 µm; Merck, Darmstadt, Germany), consistent with our subsequent application [22]. The mobile phases consisted of 0.1% formic acid in water (A) and acetonitrile (B), with gradient elution and mass spectrometric conditions as previously described [21]. Data acquisition and processing were performed using MassHunter Workstation software (Agilent Technologies). Quantification was performed using external calibration with analytically pure standards, applying 1/x weighting. Linearity, limits of detection (LOD) and limits of quantification (LOQ) were determined according to International

Council on Harmonization (ICH) guidelines [23] (Supplementary Table S1). All analytical standards (2,5-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, gallic acid, hydroxytyrosol, tyrosol, *p*-hydroxybenzoic acid, *p*-coumaric acid, oleuropein, apigenin, luteolin, and quercetin) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). The corresponding catalogue numbers and purity specifications for each standard are provided in the Supplementary Materials (Supplementary Table S1).

Prior to LC–MS/MS analysis, samples were filtered using Chromafil® cellulose acetate microfilters (0.45 μm, 25 mm; Macherey-Nagel GmbH & Co. KG, Düren, Germany) and directly injected without further processing, in order to preserve the original extract composition used in subsequent cell-based experiments.

### *2.5 Determination of Antioxidant Activity by ABTS and DPPH Radical Scavenging Assays*

The antioxidant activity of the OLEE samples was evaluated directly in the extracts using the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assays. Samples were diluted 5-fold (1:5) using the corresponding solvent (methanol for DPPH and water for ABTS) prior to analysis.

A 7 mM solution of ABTS and a 2.4 mM solution of potassium persulfate in water were combined in a 1:1 (v/v) ratio for the ABTS assay. The mixture was incubated at room temperature for 14 h in the dark. The prepared ABTS radical solution was diluted in water to an absorbance of approximately 0.7 prior to each measurement. 40 μL of each sample in dilutions was mixed with 160 μL of ABTS solution, and absorbance readings were performed at a 734 nm wavelength after 7 min of incubation in the dark using a TECAN Monochromator Infinite M200 Pro (Tecan Group Ltd., Männedorf, Switzerland). Deionized water was used as a blank (control), prepared by replacing the sample with solvent, measured under the same conditions, and recorded at time 0 for the calculation of radical scavenging activity. Results were expressed as millimolar Trolox equivalents (mM TE), based on a calibration curve constructed using Trolox standard solutions in water in the concentration range of 0.03–0.21 mM (seven points).

For the DPPH assay, a 0.1 mM solution of DPPH in methanol was prepared. 40 μL of each sample in dilutions and 160 μL of the DPPH solution were mixed, and absorbance was measured at a 517 nm wavelength after 30 min of incubation in the dark using a TECAN Monochromator Infinite M200 Pro. Methanol was used as a blank (control), prepared by replacing the sample with solvent, measured under the same conditions, and recorded at time 0 for the calculation of radical scavenging activity. Results were expressed as millimolar Trolox equivalents (mM TE), based on a calibration curve constructed using Trolox standard solutions in methanol in the concentration range of 0.03–0.21 mM (seven points).

All measurements were performed in technical triplicate, and results are expressed as the mean of three measurements.

### *2.6 In Vitro Cell Culture of BV-2 Mouse Microglial Cells*

The study was carried out in BV-2 murine microglial cells (a kind gift from Dr. J. Kriz via Prof Ivana Munitić; originally from ATCC). BV-2 murine microglial cells were authenticated by Short Tandem Repeat (STR) profiling. The cells were routinely tested and confirmed to be free of mycoplasma contamination. Cells were cultured in 75 mL (T75) and 25 mL (T25) flasks for adherent cells (Thermo Fisher Scientific, Loughborough, UK) and maintained in LEEC Culture Safe Touch 190 $CO_2$ incubator (LEEC Limited, Nottingham, UK) with 5% $CO_2$ at 37°C. The non-completed nutrient liquid medium that was used was Dulbecco's Modified Eagle Medium (DMEM) (PAN-Biotech GmbH, Aidenbach, Germany), composed of 4.5 g/L glucose, stable glutamine, sodium pyruvate, and 3.7 g/L $NaHCO_3$. The completed liquid medium

that was used implies an addition of 5% or 10% of fetal bovine serum (FBS) (PAN-Biotech GmbH, Aidenbach, Germany) and 1% penicillin/streptomycin (Sigma Aldrich, Darmstadt, Germany).

For experimental treatments, BV-2 cells were seeded at $1 \times 10^4$ cells/well in 96-well plates (for the cytotoxicity assays) or $1 \times 10^5$ cells/well in 6-well plates (for the Western blot analyses) and allowed to adhere overnight. Two sets of experiments were conducted. OLEEs dose-response: cells were treated with increasing concentrations (10 μg/mL, 50 μg/mL, and 100 μg/mL) of OLEEs obtained from three cultivars. Control wells contained untreated BV-2 cells cultured under identical conditions. LPS challenge: cells were stimulated with LPS (1 μg/mL) for 3 h or 6 h. Control wells contained untreated BV-2 cells cultured under identical conditions. LPS challenge ± OLEE pretreatment: cells were pretreated with a selected concentration of each OLEE or vehicle for 3 h prior to LPS stimulation, and then cells were stimulated with LPS (1 μg/mL) for the following 3 h. At the end of the experiments, cells were either harvested for downstream molecular analyses, or the media was collected for the cytotoxicity assays.

### *2.7 Measurement of Lactate Dehydrogenase Activity*

The activity of lactate dehydrogenase (LDH), a cytosolic enzyme released upon cell membrane disruption, was measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega Corporation, Madison, WI, USA). In this assay, LDH released from damaged cells catalyses the conversion of a tetrazolium salt into a red formazan product, the intensity of which is proportional to the number of damaged cells.

BV-2 microglial cells were seeded at $1 \times 10^4$ cells/well in 96-well plates in DMEM supplemented with 10% FBS. After 24 h, cells were treated in triplicate with OLEEs from three different cultivars at concentrations of 10, 50, or 100 μg/mL. Negative controls consisted of complete DMEM medium without cells, vehicle controls were seeded cells without treatment, and positive controls were seeded cells treated with the kit-provided 10× lysis solution.

OLEEs were applied for 6 h, after which 50 μL of culture medium from each well was transferred to a fresh 96-well plate. An equal volume of CytoTox 96® reagent was added to each well, and the plate was incubated in the dark for 30 min at room temperature. The reaction was stopped with 50 μL of 0.1 M acetic acid, and absorbance was measured at 490 nm using an Infinite M200 PRO microplate reader (Tecan, Männedorf, Switzerland).

The percentage of cytotoxicity was calculated using the following formula:

$$Cytotoxicity\ (\%) = \left( \frac{Experimental\ LDH\ release - Spontaneous\ LDH\ release}{Maximum\ LDH\ relase - Spontaneous\ LDH\ release} \right) \times 100$$

The results were then represented as cell viability (%) using the formula:

$$CellViability\ (\%) = 100\% - Cytotoxicity\ (\%)$$

where spontaneous LDH release was measured from vehicle-treated cells, and maximum LDH release was measured from cells treated with the kit-provided lysis solution.

### *2.8 Western Blot Analyses*

For the Western blot analyses, microglia were pre-treated for 3 h with OLEEs (10 μg/mL) and then exposed to LPS for an additional 3 h. At the end of the experiment, the medium was removed and 100 μL of radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X, 1% Na-deoxycholate, 0.1% SDS) was added to the cells, and the microglia were then scraped off the

wells. After 20 min on ice with occasional mixing, cell lysates were collected and centrifuged (14,000 rpm, 4°C, 10 min) (UNIVERSAL 320 R, Andreas Hettich GmbH, Germany). The separated supernatants were stored at −80°C, and the protein concentration was determined by the Quick Start™ Bradford assay kit (#5000205, Bio-Rad, Hercules, CA, USA) [24].

Proteins (~10 μg per lane) were added to a 10% polyacrylamide gel. After the electrophoresis at 200 V for 40 min, proteins were transferred from the gel to the nitrocellulose membrane using the semi-dry Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Hercules, CA, USA). Blocking was done with 5% milk or 5% bovine serum albumin solution in Tris-buffered saline and 0.1% Tween 20 (TBST) for 1 h, after which the membranes were incubated overnight at 4°C with primary antibodies (details listed in Supplementary Table S2). Then, the membranes were washed with TBST for 30 min and treated with biotin-conjugated secondary antibodies. After incubation for 1 h, the membranes were incubated for 30 mi with streptavidin and horseradish peroxidase (HRP) conjugate (1:10,000 dilution; cat. no. 434323; Invitrogen, Rockford, IL, USA). Protein visualization was performed using the chemiluminescent kit and Kodak Image Station 440 (Eastman Kodak, Rochester, NY, USA) with Kodak 1D analysis program (v.3.6.5, Kodak Scientific Imaging Systems, Rochester, NY, USA). To verify equal protein loading and efficient transfer across all lanes, we dyed the membranes for 5 min in the Ponceau S solution [0.1% Ponceau S (Fisher Scientific, Geel, Belgium) in 5% acetic acid] (images provided in Supplementary Figs. S1–S4).

Bands used for densitometric analysis were recorded and stored as .bip files, and contain raw data generated by the Kodak Image Analysis system for image processing. In this type of file, the full dynamic range is preserved and intensity levels can be scaled without loss of information. Densitometric analysis was performed on the original (unscaled) data, analyzed in the Kodak Image Analysis system for image processing.

### *2.9 Laboratory Data Collection and Statistical Analyses*

Data were collected using Microsoft Excel (Microsoft Corp., Redmond, WA, USA) and, when necessary, corrected for between-session variation as described previously [25]. Statistical analyses were performed using the GraphPad Prism 10 (GraphPad Inc., San Diego, CA, USA). Results are expressed as means ± standard deviations (SD). Statistical differences were evaluated using one-way or two-way analysis of variance (ANOVA), as appropriate. For these analyses, the F-test was employed to assess main effects and interactions, with degrees of freedom ($df$) determined by the number of experimental groups and total sample size for each comparison. For one-way ANOVA, post hoc analyses were performed using Tukey's multiple comparisons test or Dunnett's multiple comparisons test, while for two-way ANOVA, Šídák's multiple comparisons test was applied. In all the comparisons, $p < 0.05$ was considered statistically significant.

## 3 Results

### *3.1 Total Phenolic and Flavonoid Content in Olive Leaf Macerates and Ethanol Extracts*

TPC and TFC of OLMs from three Croatian olive cultivars Buža, Oblica, and Leccino, were determined using the Folin-Ciocalteu and AlCl3 colorimetric methods, respectively. While the extracts exhibited comparable total phenolic and flavonoid contents, normalization to dry weight (DW) demonstrated the superiority of ethanol extraction (Table 1). Results showed that Leccino had the highest value of total phenols with 16.42 ± 0.05 mg GAE/g DW, 30% more than Oblica and 15% more than Buža (Table 1). In contrast, Buža had the highest value of total flavonoids with 2.66 ± 0.01 mg QUE/g DW, 46% more than Leccino and 4% more than Oblica (Table 1).

**Table 1:** Total phenolic and flavonoid content in olive leaf macerates of Buža, Oblica, and Leccino.

| Olive Variety | Total Phenols | | Total Flavonoids | |
|---|---|---|---|---|
| | (mg GAE/mL Extract) | (mg GAE/g DW) | (mg GAE/mL Extract) | (mg QUE/g DW) |
| Buža | 0.1422 ± 0.0002 | 14.22 ± 0.02 | 0.0266 ± 0.0001 | 2.66 ± 0.01 |
| Oblica | 0.1169 ± 0.0020 | 11.69 ± 0.20 | 0.0255 ± 0.0002 | 2.55 ± 0.02 |
| Leccino | 0.1642 ± 0.0005 | 16.42 ± 0.05 | 0.0182 ± 0.0001 | 1.82 ± 0.01 |

Note: Expressed as mg gallic acid equivalents (GAE) and mg quercetin equivalents (QUE) per mL of extracts and g dry weight (DW); results are presented as mean ± SD.

In the OLEEs, TPC and TFC from three Croatian olive cultivars, Buža, Oblica, and Leccino were determined using the same methods. Results showed that Leccino leaves (DW) had the highest total phenol value (52.51 ± 0.64 mg GAE/g DW), 17% higher than Oblica, and 13% higher than Buža (Table 2). On the other hand, Leccino had the lowest total flavonoid content, with Oblica having a 27% greater value than Leccino, and 20% higher than Buža.

**Table 2:** Total phenolic and flavonoid content in olive leaf ethanol extracts of Buža, Oblica, and Leccino.

| Olive Variety | Total Phenols | | Total Flavonoids | |
|---|---|---|---|---|
| | (mg GAE/mL Extract) | (mg GAE/g DW) | (mg GAE/mL Extract) | (mg QUE/g DW) |
| Buža | 0.1140 ± 0.0022 | 45.60 ± 0.88 | 0.0186 ± 0.0003 | 7.45 ± 0.11 |
| Oblica | 0.1088 ± 0.0017 | 43.51 ± 0.67 | 0.0233 ± 0.0002 | 9.30 ± 0.08 |
| Leccino | 0.1313 ± 0.0016 | 52.51 ± 0.64 | 0.0169 ± 0.0006 | 6.77 ± 0.22 |

Note: Expressed as mg gallic acid equivalents (GAE) and mg quercetin equivalents (QUE) per g dry weight (DW); results are presented as mean ± SD.

### *3.2 Quantitative LC–MS/MS Analysis of Selected Bioactive Compounds*

The concentrations of selected bioactive compounds in olive leaf macerates and ethanol extracts obtained from Buža, Oblica, and Leccino cultivars were determined by LC–MS/MS and expressed relative to dry weight (DW) (Fig. 1). The analysis focused on key compounds relevant to biological activity, including flavonoids (apigenin, luteolin, quercetin), phenolic alcohols (hydroxytyrosol, tyrosol), and the secoiridoid oleuropein. The complete quantitative LC–MS/MS analysis results are provided in Supplementary Table S3.

Across all cultivars, ethanol extracts showed substantially higher levels of the analyzed compounds compared to macerates. Oleuropein was the dominant constituent in all samples and was approximately 3–10-fold higher in ethanol extracts, with the most pronounced increase observed in Leccino. Hydroxytyrosol levels were approximately 2–4-fold higher in ethanol extracts compared to macerates, while tyrosol was detected only in Buža and Oblica macerates (Supplementary Table S3). Flavonoid content showed the most marked extraction-dependent differences. Apigenin levels were approximately 20–40-fold higher in ethanol extracts compared to macerates, while luteolin showed an increase of approximately 10–50-fold, depending on the cultivar. Quercetin was low or not detected in macerates but was consistently present in ethanol extracts, with the highest levels observed in Oblica OLEE.

Comparison among ethanol extracts further demonstrated cultivar-specific phenolic profiles (Fig. 1 and Supplementary Table S3). Oblica OLEE was characterized by the highest apigenin and quercetin concentrations, whereas Buža OLEE contained the highest luteolin and hydroxytyrosol levels. In contrast, Leccino OLEE exhibited the highest oleuropein concentration and the highest total quantified phenolic content among the analyzed cultivars.

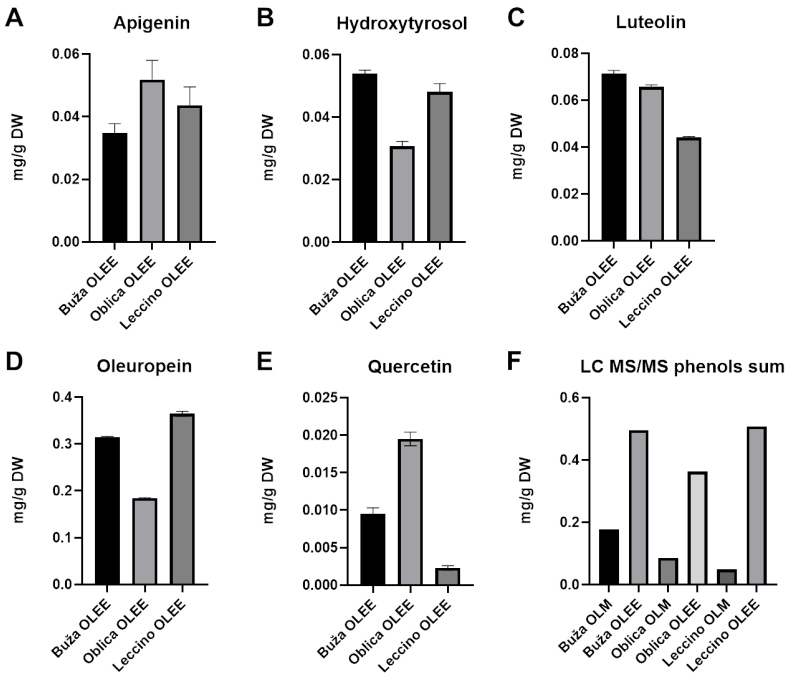


**Figure 1:** Targeted LC–QQQ quantitative analysis of individual phenolic compounds in olive leaf ethanolic extracts (OLEE) obtained from Buža, Oblica, and Leccino cultivars. Quantitative analysis was performed using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Panels show the concentrations of selected phenolic compounds: (**A**) apigenin, (**B**) hydroxytyrosol, (**C**) luteolin, (**D**) oleuropein, and (**E**) quercetin. (**F**) The cumulative concentration of quantified phenolic compounds in OLEEs and olive leaf macerates (OLM). All results are expressed as mean ± standard deviation (SD) in mg/g dry weight (DW), based on triplicate measurements.

### *3.3 Comparative in Vitro Antioxidant Activity of Olive Leaf Extracts from Buža, Oblica, and Leccino Cultivars*

The *in vitro* antioxidant activity of OLEEs from Buža, Oblica, and Leccino cultivars was evaluated using ABTS and DPPH radical scavenging assays (Fig. 2). In the ABTS assay (Fig. 2A), significant differences were observed among the cultivars (one-way ANOVA, $F(2, 6) = 51.49$, $p = 0.0002$). Post hoc analysis with Tukey's multiple comparisons test revealed that Oblica extracts exhibited significantly higher antioxidant activity compared to the other cultivars. In contrast, the DPPH assay (Fig. 2B) showed no significant differences among the cultivars (one-way ANOVA, $F(2, 6) = 0.1577$, $p = 0.858$), indicating similar hydrogen-donating antioxidant capacity in all samples.

### *3.4 Olive Leaves Extracts from Buža, Oblica, and Leccino Cultivars Have Concentration-Dependent Cytotoxic Effects on BV-2 Cells*

Since measurements of total phenolic and flavonoid concentrations showed that OLEEs contained approximately threefold higher levels of these compounds than OLMs, we chose to focus subsequent experiments on OLEEs to achieve the desired concentrations in the cell culture medium. Accordingly, the effects of OLEEs were evaluated in BV-2 microglia cells.

OLEEs of Buža, Oblica, and Leccino were added to the cells for 6 h to determine cytotoxicity of three different concentrations for each of the cultivars: 10 μg/mL, 50 μg/mL, and 100 μg/mL (Fig. 3). The highest concentration of 100 μg/mL showed to be the most cytotoxic for all three cultivars' OLEEs in comparison to the LDH activity measured in cell media samples from the control (untreated) cells (overall one-way ANOVA:

F(9, 20) = 7.439, $p = 0.0001$; Dunnett's multiple comparisons post-hoc test: $p < 0.001$ for all three cultivars at 100 μg/mL compared to control, vehicle-treated cells). OLEEs of Oblica cultivar at a concentration of 50 μg/mL were also shown to be significantly toxic compared to the control ($p = 0.017$).

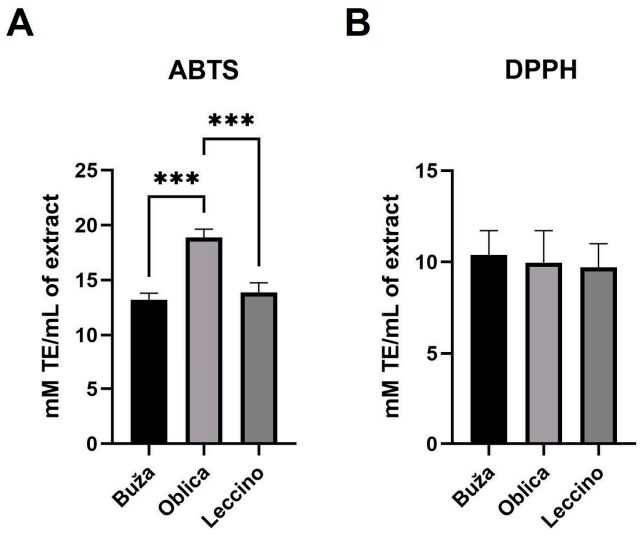


**Figure 2:** Antioxidant activity of olive leaf ethanol extracts (OLEEs) from Buža, Oblica, and Leccino cultivars. The antioxidant capacity was evaluated using two radical scavenging assays. (**A**) ABTS radical scavenging activity of OLEEs, expressed as Trolox equivalents (TE). (**B**) DPPH radical scavenging activity of OLEEs, expressed as Trolox equivalents (TE). Values are presented as mean ± SD of three independent measurements (n = 3). ***$p < 0.001$ indicates statistically significant differences among cultivars; one-way ANOVA with Tukey's multiple comparisons test.

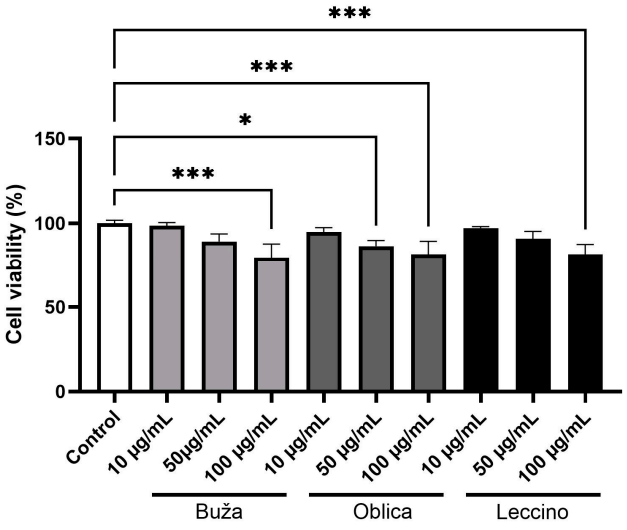


**Figure 3:** The effects of Buža, Oblica, and Leccino cultivar olive leaf ethanol extracts (OLEEs) on lactate dehydrogenase activity in the cell culture medium, used as the measurement of cell viability, on BV-2 microglial cells. Cells were incubated with OLEEs (10, 50, and 100 μg/mL) for 6 h. Data are presented as cell viability (%), calculated as 100% − toxicity (%) and expressed as mean ± SD (n = 3 biological replicates per group). *$p < 0.05$ vs. Control; ***$p < 0.001$ vs. Control; one-way ANOVA with Dunnett's multiple comparisons test.

### *3.5 Temporal Dynamics of Microglial Marker Expression Following LPS Stimulation*

To investigate the effects of inflammatory stimulation on microglial activation, BV-2 microglial cells were treated with 1 μg/mL LPS for 3 h or 6 h, and the expression of microglial activation markers CD206, CD86, and iNOS was evaluated by Western blot (Fig. 4).

Densitometric analysis of Western blot data, normalized to total protein using Ponceau S staining (Supplementary Fig. S1), revealed significant differences in the expression levels of all analyzed microglial activation markers (two-way ANOVA with Šídák's multiple comparisons test). After 3 h of LPS treatment, the expression of CD206, an anti-inflammatory marker, was significantly increased compared with control cells ($p < 0.0001$). CD86, a marker of classical microglial activation, was also significantly upregulated ($p = 0.017$). In contrast, iNOS, a pro-inflammatory marker, showed a trend toward increased expression but did not reach statistical significance ($p = 0.0697$).

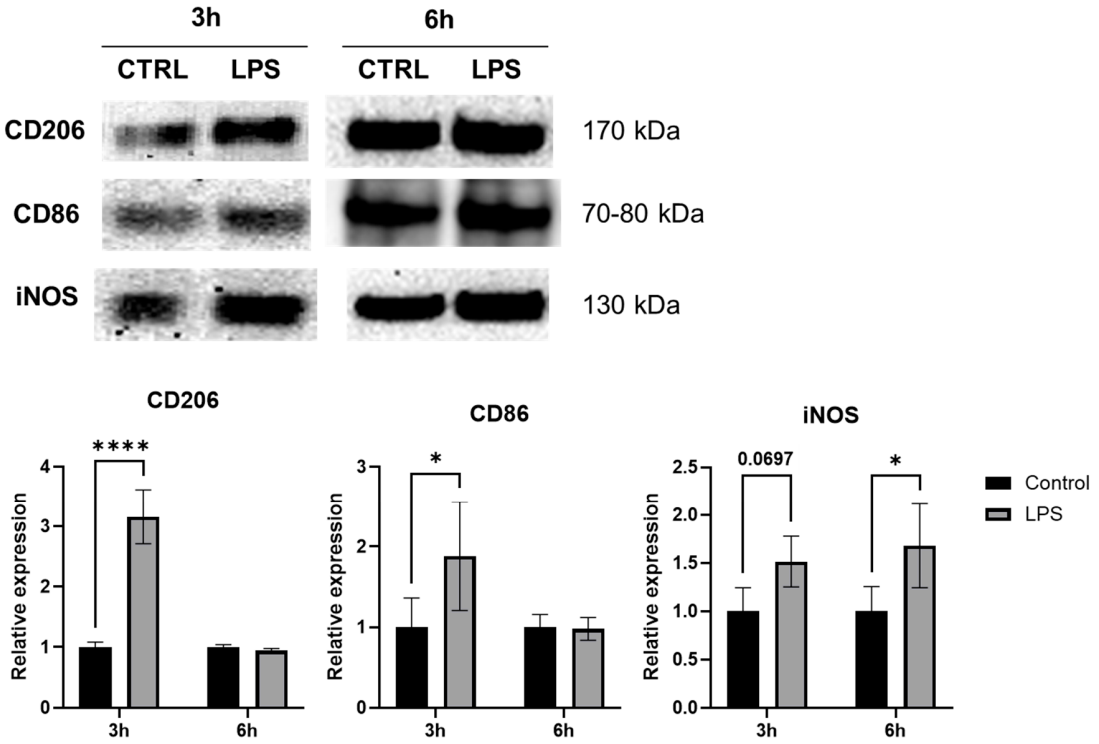


**Figure 4:** Effect of LPS (1 μg/mL) on the expression of BV-2 microglial activation protein markers at 3 h and 6 h. Representative Western blots (left) show the expression of CD206 (anti-inflammatory), CD86 (classical activation), and iNOS (pro-inflammatory). Graphs depict protein expression normalized to control cells. Protein loading was verified by Ponceau S staining (see Supplementary Fig. S1). Data are shown as means ± SD from n = 3–4 biological replicates. ****$p < 0.0001$; *$p < 0.05$ vs. control at the corresponding time point. Statistical analysis was performed using two-way ANOVA followed by Šídák's multiple comparisons test.

After 6 h of LPS treatment, no statistically significant changes were observed in CD206 ($p = 0.944$) or CD86 ($p = 0.996$), whereas iNOS expression was significantly increased at this time point ($p = 0.036$). These findings indicate that the early microglial activation response observed at 3 h does not persist at 6 h. Because changes in microglial activation markers were more pronounced at 3 h than at 6 h, the 3 h time point was selected for subsequent experiments to assess the effects of olive leaf extracts under early activation conditions.

### *3.6 Evaluation of Olive Leaf Extract Pretreatment on Microglial Activation, NF-κB Signaling, and Cytoprotective Pathways in LPS-Stimulated BV-2 Cells*

In order to assess the effects of OLEEs under LPS-stimulated conditions, we used LPS treatment to induce BV-2 cell activation. Cells were pre-treated for 3 h with OLEEs (10 μg/mL) from the three olive cultivars, followed by stimulation with 1 μg/mL LPS for an additional 3 h. We used the 10 μg/mL concentration for OLEEs as it was shown to be non-toxic for all three investigated olive cultivars.

Protein expression of the microglial activation markers CD206, CD86, and iNOS, as well as signaling and stress-related proteins SIRT1, phosphorylated NF-κB p65 (P-p65), NRF2, and HSP70, was evaluated by Western blot. Data were analyzed by one-way ANOVA, and none of the measured proteins showed statistically significant changes following OLEE pretreatment (CD206, $p = 0.385$; CD86, $p = 0.600$; iNOS, $p = 0.228$; P-p65, $p = 0.334$; SIRT1, $p = 0.504$; NRF2, $p = 0.593$; HSP70, $p = 0.944$). These results are presented in Figs. 5–7: CD206, CD86, and iNOS (Fig. 5); P-p65 and SIRT1 (Fig. 6); Nrf2 and HSP70 (Fig. 7).

Overall, none of the observed changes in CD206, CD86, iNOS, phospho-p65, SIRT1, NRF2, or HSP70 reached statistical significance, indicating that short-term pretreatment with 10 μg/mL OLEEs from any of the tested olive cultivars did not significantly alter LPS-induced microglial activation or related inflammatory and cytoprotective signaling under these experimental conditions.

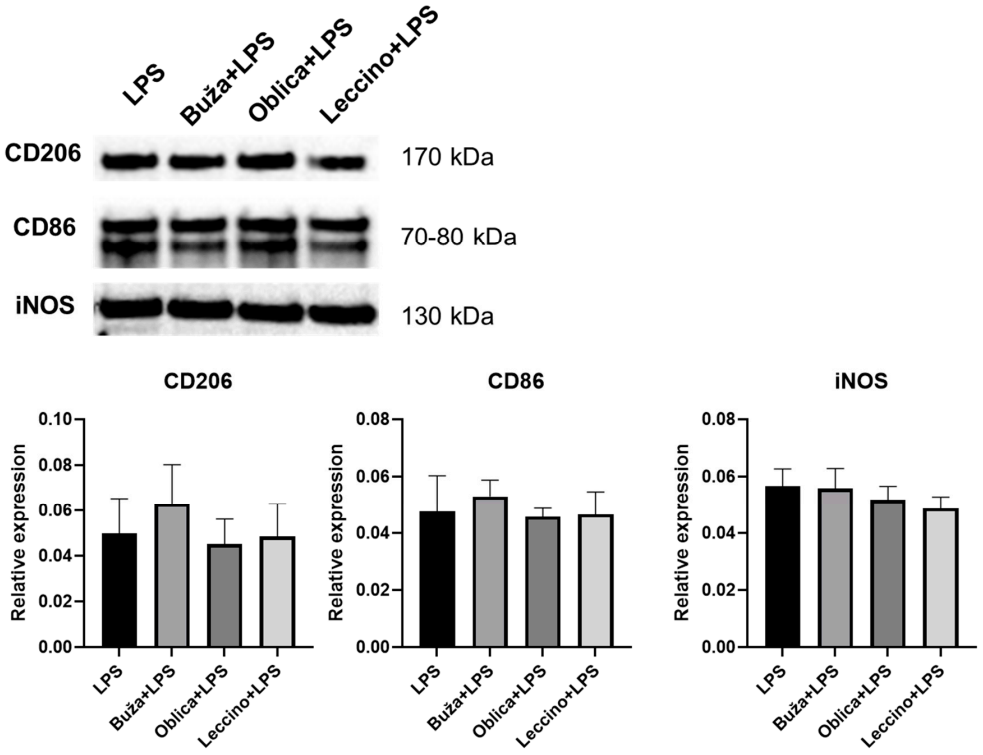


**Figure 5:** Effects of olive leaf ethanol extract (OLEE) pretreatment on microglial activation markers in LPS-stimulated BV-2 cells. BV-2 microglial cells were pretreated with OLEE for 3 h (10 μg/mL) prior to stimulation with LPS (1 μg/mL) for 3 h. Representative Western blots and the analyses of the protein expression levels of CD206, an anti-inflammatory marker, CD86, a classical activation marker, and iNOS, a pro-inflammatory marker, are shown. Protein expression levels were quantified by densitometry and normalized to total protein using Ponceau S staining (see Supplementary Fig. S2). Data are presented as the mean ± SD of n = 3–4 independent experiments. Statistical analysis was performed using one-way ANOVA.

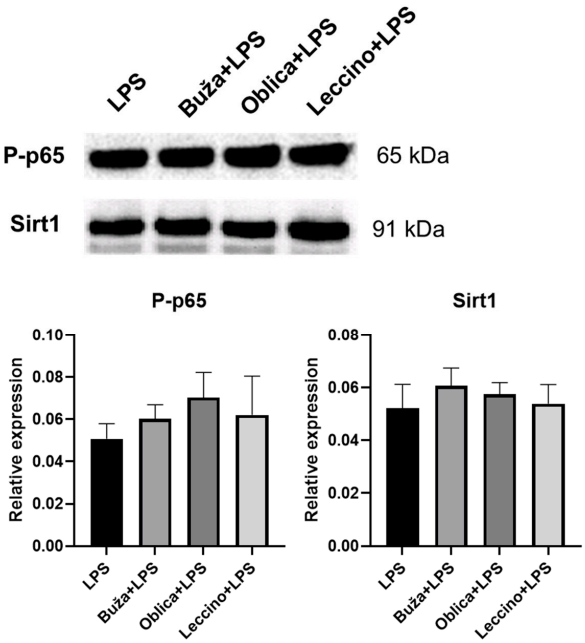


**Figure 6:** NF-κB and SIRT1 signaling in LPS-stimulated BV-2 microglial cells and the effects of olive leaf ethanol extract (OLEE) treatment. BV-2 microglial cells were pretreated with OLEEs (10 μg/mL) for 3 h followed by 3h LPS challenge (1 μg/mL). Representative Western blots and the results of the analysis of the expressions of phospho-p65 (P-p65), a key NF-κB activation marker and Sirt1, a negative regulator of NF-κB signalling, are presented. Protein expression levels were quantified by densitometry and normalized to total protein using Ponceau S staining (see Supplementary Fig. S3). Data are presented as mean ± SD from n = 3–4 biological replicates. Statistical analysis was performed using one-way ANOVA.

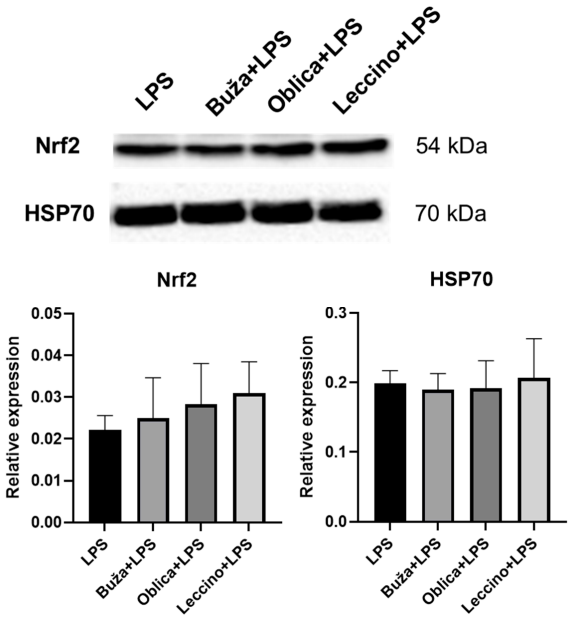


**Figure 7:** Antioxidant and stress-response markers in LPS-stimulated BV-2 microglial cells and the effects of olive leaf ethanol extract (OLEE) treatment. BV-2 microglial cells were pretreated with OLEEs (10 μg/mL) for 3 h followed by 3h LPS challenge (1 μg/mL). Representative Western blots and the results of the analysis of the expressions of Nrf2,

a master regulator of antioxidant responses and HSP70, a stress-response and cytoprotective protein, are presented. Protein expression levels were quantified by densitometry and normalized to total protein using Ponceau S staining (see Supplementary Fig. S4). Data are presented as mean ± SD from n = 3–4 biological replicates. Statistical analysis was performed using one-way ANOVA.

## 4 Discussion

### *4.1 Extract Selection Based on Phenolic and Flavonoid Content and LC–MS/MS Qualitative Analysis*

The selection of extraction procedures was guided by the underlying chemistry of phenolic compounds. Polyphenols, particularly those containing ortho-dihydroxyl (catechol) motifs, exhibit enhanced hydrogen-donating capacity and are more efficiently extracted in polar organic media than in water alone. Accordingly, a 50% (v/v) ethanol–water system was employed to balance extraction efficiency with broader solvation capacity and compatibility with downstream cell-based assays, as ethanol has been shown to outperform less polar solvents in recovering antioxidant-active phenolics [26,27]. In parallel, hot-water macerates were prepared as a consumer-relevant format and to maintain comparability with previously characterized Croatian cultivars [9].

As an initial assessment of extract composition, total phenolic and flavonoid contents were determined using Folin–Ciocalteu and $AlCl_3$ assays. Ethanol extracts yielded approximately threefold higher total phenolic and flavonoid contents compared to macerates, indicating more efficient recovery of phenolic constituents. Among cultivars, Leccino exhibited the highest total phenolic content, while flavonoid levels varied depending on both cultivar and extraction method, with Buža showing higher values in macerates and Oblica in ethanol extracts. These results highlight the combined influence of genotype and extraction conditions on extract composition [9]. At the same time, it should be noted that both assays provide only semi-quantitative estimates, as the Folin–Ciocalteu method is not fully phenolic-specific and the $AlCl_3$ assay shows variable sensitivity depending on flavonoid subclass [28].

To obtain compound-level resolution, targeted LC–MS/MS (LC-QQQ) analysis was performed. This confirmed that ethanol extraction resulted in substantially higher concentrations of key olive leaf constituents, particularly oleuropein and major flavonoids, while macerates contained lower overall levels. Importantly, LC–MS/MS analysis revealed distinct cultivar-specific profiles, with Leccino characterized by higher secoiridoid content and Buža by greater relative enrichment of flavonoids, whereas Oblica exhibited intermediate characteristics. These findings demonstrate that total phenolic and flavonoid contents represent aggregate measures and do not fully capture compositional differences between extracts.

Taken together, these results supported the selection of ethanol extracts for subsequent analyses, as they consistently yielded higher concentrations of phenolic compounds, including flavonoids, than aqueous macerates. Because flavonoid content normalized to dry weight provided a practical basis for standardized comparison across cultivars, this parameter was used for graphical presentation and extract comparison. In parallel, targeted LC–MS/MS analysis provided compound-level characterization of cultivar-specific differences in extract composition.

### *4.2 Assay-Dependent Differences in Antioxidant Activity of Olive Leaf Extracts*

In our experimental conditions, OLEEs from the Oblica cultivar exhibited significantly higher antioxidant activity compared to other cultivars in the ABTS assay, whereas no significant differences were observed in the DPPH assay. This discrepancy may be attributed to the different reaction mechanisms and sensitivities of the two assays [5,29]. The ABTS assay detects both hydrophilic and lipophilic antioxidants

and is based on electron transfer reactions, while the DPPH assay primarily reflects hydrogen-donating capacity and is less responsive to hydrophilic compounds [29,30]. Olive leaves were extracted using a 50:50 ethanol–water mixture, enabling the recovery of a broad spectrum of phenolic compounds. The observed differences between assays are therefore more likely related to assay-specific reactivity than to differences in extract composition alone.

Notably, Oblica had the highest total flavonoid content (9.30 mg QUE/g DW), even though its total phenol content (43.51 mg GAE/g DW) was slightly lower than that of Leccino (52.51 mg GAE/g DW). Given that flavonoids are among the major contributors to antioxidant activity, this may have contributed to the higher ABTS activity observed for the Oblica extract.

Taken together, these findings indicate that antioxidant readouts depend on both assay selection and extract composition and highlight flavonoid content as a more informative parameter for extract characterization than total phenolics alone. Accordingly, subsequent experimental design and extract comparison were guided by total phenolic and flavonoid content normalized to dry weight, rather than by single-assay antioxidant capacity.

### *4.3 Concentration-Dependent Cytotoxicity of Olive Leaves Ethanol Extracts on BV-2 Microglial Cells*

Although higher total phenolic content (TPC) is generally associated with increased antioxidant activity [30], in this study, extracts were normalized based on TPC to enable comparable evaluation across cultivars and experimental conditions. Therefore, prior to biological evaluation, it was necessary to define a concentration range that does not compromise cell viability.

Previous studies have reported concentration-dependent cytotoxic effects of olive leaf extracts across different cell models. For example, Jordan olive leaf extract at a concentration of 100 μg/mL showed cytotoxic effect measured by MTT assay after 72 h in breast cancer MCF7 and MB-MDA-231 cell lines [31]. Another study by Martinho et al. in human carcinoma cell lines showed growth inhibition in the concentration range of 209–844 μg/mL for fresh leaves from several Portuguese olive tree cultivars [32]. Zaïri et al. investigated the cytotoxic effect of aqueous olive leaf extracts from two Tunisian cultivars on murine oligodendrocytes (158N) using the MTT assay. The authors reported reduced cell viability at a concentration of 400 μg/mL [33].

In line with these observations, our results demonstrated a concentration-dependent cytotoxic effect of OLEEs in BV-2 microglial cells. The highest tested concentration (100 μg/mL) was the most cytotoxic across all three cultivars after 6 h of treatment, while 50 μg/mL showed a moderate, cultivar-dependent effect. In contrast, the lowest tested concentration (10 μg/mL) did not significantly affect cell viability under the same conditions. Accordingly, 10 μg/mL was selected for all subsequent experiments as a non-cytotoxic concentration that allows evaluation of extract effects without compromising cell viability.

### *4.4 Evaluation of Olive Leaf Extract Pre-Treatment on Microglial Inflammatory and Cytoprotective Responses*

In this study, the effects of OLEE pre-treatment were assessed in the LPS-induced BV-2 model. The aim was to examine whether short-term pre-treatment influences the microglial response prior to inflammatory challenge, within a defined, non-cytotoxic exposure window.

Microglial cells are macrophage-like immune cells that constitute the first line of defense in the central nervous system [34]. Although microglia express numerous surface receptors, including various Toll-like receptors (TLRs) and purinergic receptors such as P2Y6, accessory molecules—most notably cluster of differentiation 14 (CD14)—are required for effective LPS recognition and signalling [34,35]. As demonstrated

by Dai et al. [34], LPS stimulation of BV-2 cells enhances TLR4 and MyD88 activation, promotes NF-κB translocation, and subsequently drives the production of pro-inflammatory cytokines.

Sustained or chronic microglial activation is therefore expected to result in a phenotypic shift from a neuroprotective, anti-inflammatory/reparative state toward a neurotoxic, pro-inflammatory profile. Kumar et al. [36] observed significant induction of inducible iNOS following treatment of BV-2 cells with 500 ng/mL LPS. In line with these findings, our results demonstrated increased CD86 expression after a 3-h exposure to 1 μg/mL LPS.

Unexpectedly, we also observed an increase in CD206 expression, a marker commonly associated with the anti-inflammatory microglial phenotype, under the same experimental conditions. Previous studies have shown that LPS decreases CD206 expression within 24 h, with CD206 reduced after 24 h in monocytes and already significantly downregulated by 6 h in microglia [37,38]. However, emerging evidence suggests that BV-2 cells may transiently adopt a mixed activation state, characterized by the simultaneous expression of both pro- and anti-inflammatory markers, before committing to a dominant phenotype. This transient co-expression may reflect a compensatory regulatory phase at the 3-h time point, which is subsequently overridden as cells progress toward a fully pro-inflammatory, iNOS-positive profile at later stages, such as 6 h post-stimulation [39]. Indeed, at the 6-h time point, we observed increased iNOS expression, while no significant changes were detected in CD86 or CD206 levels following LPS challenge.

Based on these observations and available literature, a 3 h pretreatment followed by 3 h LPS exposure was selected as a working condition [40,41]. The present study was not designed as a comprehensive anti-inflammatory screening platform but rather as a preliminary investigation aimed at defining non-cytotoxic concentration ranges and evaluating whether biological effects remain detectable following extract removal in a BV-2 washout paradigm. Accordingly, the primary objective was to establish experimental boundaries for subsequent mechanistic investigations rather than to provide a comprehensive assessment of the anti-inflammatory potential of olive leaf extracts.

Under the applied conditions, no significant changes in the protein expression of CD206, CD86, iNOS, or the signaling and stress-related proteins SIRT1, phospho-p65, Nrf2, and HSP70 was observed. Several factors may contribute to this outcome. First, the timing of assessment may not have captured potential effects; while 3 h post-LPS corresponds to the early peak of some microglial activation markers, many signaling proteins (including transcription factors and stress response proteins) may require longer incubation to exhibit measurable changes [40–42]. Crucially, the lack of observed modulation on key regulatory targets like the NF-κB pathway is likely due to the structural constraints of our experimental design. Phytochemical complexes typically require extended, cumulative exposure or continuous co-incubation paradigms to fully interact with cell surface receptors or undergo sufficient cellular uptake. By utilizing a brief 3 h pretreatment followed by a strict media washout, our protocol isolated only persistent intracellular reprogramming and excluded these slower, cooperative mechanisms. Consequently, these outcomes should be interpreted strictly within these narrow experimental boundaries rather than as a general indicator of biological inactivity.

Second, the concentration and bioavailability of active components in OLEEs may have been insufficient relative to the applied LPS stimulus. Plant-derived compounds often have narrow effective ranges *in vitro*, and stability or cellular uptake issues can further limit their intracellular activity [3]. In the experiments reported by Silvestrini et al., the effect of 2-h pre-treatment with OLEs macerated in phosphate-buffered saline (PBS; 4 μg/mL according to TPC), oleacin (5 μM), and oleuropein-aglycone (5 μM) followed by 3-h treatment with LPS (500 ng/mL) on young human umbilical vein endothelial cells (yHUVECs) and human leukaemia monocytic cell line THP-1 was researched [3]. They have observed the decrease in pro-inflammatory markers IL-1b, TNF-α, IL-8, ICAM-1, VCAM, and IL-6 for yHUVECs pre-treated with PBS-

macerated OLEs, and oleacin compared to cells treated only with LPS. In the THP-1 cell line, Silvestrini et al. also showed an increase in IL-1b, TNF-α, IL-8, and IL-6. Therefore, to confirm or decline these conclusions, more research is needed on other immortalized cell lines, primary mouse glial cells, and human cell lines.

Third, the markers selected may not fully represent the pathways most responsive to OLEEs. Olive leaf polyphenols, such as oleuropein and HT, have been reported to influence oxidative stress, mitochondrial function, and autophagy, which may not directly affect the measured inflammatory and stress-related proteins within the tested timeframe. Finally, subtle protein expression changes may have been below the detection sensitivity of Western blotting, and more quantitative or sensitive techniques (e.g., ELISA, qPCR, or immunofluorescence) could reveal modest effects. Collectively, these considerations indicate that the lack of observable effects under the applied conditions may reflect time, dose-, exposure paradigm-, and pathway-dependent responses, warranting further optimization of experimental design and the inclusion of co-incubation models in future studies.

We acknowledge that the absence of functional readouts in this preliminary experiment (such as nitric oxide quantification, ROS measurement, or detection of the release of pro-inflammatory cytokines) limits the ability to fully characterize the inflammatory shift occurring in LPS-stimulated BV-2 microglia cells. Future studies should focus on these downstream functional mediators to determine if OLEEs can suppress the secretory phase of neuroinflammation even when surface marker expression remains largely unchanged.

Finally, while BV-2 cells served as an efficient model for mapping OLEE cytotoxicity and dosing windows, we acknowledge the limitations regarding their translational relevance. As an immortalized line, BV-2 cells often exhibit a more 'primed' baseline phenotype and a different TLR4 response magnitude compared to primary microglia. Furthermore, while a traditional phytochemical positive control (e.g., quercetin or curcumin) was not included to benchmark the washout efficacy, the system's dynamic range was fully validated by its rapid, classic pro-inflammatory response to the LPS challenge, confirming that the cells remained highly sensitive to stimulation. Consequently, the lack of effect observed under this stringent 3-h washout protocol should be interpreted strictly as a baseline for BV-2 cells. Future studies employing primary microglial cultures or iPSC-derived microglia are warranted to confirm whether these OLEEs might exhibit more robust immunomodulatory effects in a more physiologically sensitive system.

A central constraint of this study is the experimental design, which was highly conservative and may have biased the results toward negative outcomes. We employed a 'washout' (priming) model, where OLEEs were removed before the LPS challenge to specifically assess lasting intracellular reprogramming. While this avoids direct extracellular interference, it represents a high threshold for bioactivity. In contrast, a non-washout (co-incubation) model, where OLEEs remain present during LPS stimulation, is often more sensitive, as it allows for both intracellular effects and direct extracellular interactions, typically leading to stronger suppression of pro-inflammatory markers [43].

Furthermore, the biological replicates used in this study (n = 3–4) represent a standard exploratory framework for *in vitro* screening. However, we acknowledge that this sample size limits the statistical power to detect subtle or highly variable changes in protein expression. Consequently, the lack of statistically significant modulation of CD86, CD206, and inflammatory signaling pathways should be interpreted within this constraint. Rather than serving as a definitive functional assessment of microglial modulation, these null results are best understood as a preliminary baseline within a feasibility and boundary-setting framework. They map the precise cytotoxicity thresholds and immediate intracellular constraints of these specific Croatian olive leaf extracts under strict washout conditions. Consequently, while these findings establish clear operational boundaries for the current model, a broader experimental scope, incorporating extended

exposure paradigms, multi-dose relationships, and larger-scale validation, is necessary to comprehensively evaluate the presence or absence of bioactivity across a wider range of biological variability.

## 5 Conclusions

This study links olive leaf chemistry with extraction strategy and downstream biological evaluation. Using a dual-solvent approach, a 50% (v/v) ethanol-water system yielded approximately threefold higher total phenolic and flavonoid contents compared to hot–water maceration, confirming more efficient recovery of phenolic constituents under mixed solvent conditions. At the same time, targeted LC–MS/MS analysis revealed cultivar-specific compositional differences, with Leccino characterized by higher secoiridoid content and Buža by relatively higher flavonoid enrichment. In BV-2 assays, these compositional differences did not translate into measurable differences in microglial activation under the applied conditions. However, a clear concentration-dependent effect on cell viability was observed, with 10 μg/mL identified as a non-cytotoxic concentration across all cultivars. Under short, non-cytotoxic pretreatment conditions (10 μg/mL, 3 h), OLEEs did not significantly alter LPS-induced changes in CD206 and CD86 expression, nor in the analyzed signaling and stress-related proteins, indicating that brief exposure at conservative doses is insufficient to modulate canonical activation markers in this model.

Next steps in our investigative efforts should pair LC-MS profiling with constituent-normalized dosing and longer, sub-toxic pretreatments, including non-washout experiments, to test whether cultivar-specific chemotypes can achieve measurable attenuation of microglial activation.

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**Author Contributions:** Conceptualization, Kristina Pilipović and Lara Saftić Martinović; methodology, Kristina Pilipović, Lara Saftić Martinović, Lucia Fabijančić and Iva Kristić; software, Kristina Pilipović and Lara Saftić Martinović; formal analysis, Kristina Pilipović, Lara Saftić Martinović and Lucia Fabijančić; investigation, Kristina Pilipović, Lara Saftić Martinović, Lucia Fabijančić and Iva Kristić; resources, Kristina Pilipović and Lara Saftić Martinović; writing—original draft preparation, Kristina Pilipović, Lara Saftić Martinović and Lucia Fabijančić; writing—review and editing, Kristina Pilipović and Lara Saftić Martinović; visualization, Kristina Pilipović; supervision, Kristina Pilipović; project administration, Kristina Pilipović. All authors reviewed and approved the final version of the manuscript.

**Availability of Data and Materials:** The data that support the findings of this study are available from the Corresponding Author, Kristina Pilipović, upon reasonable request.

**Ethics Approval:** Not applicable.

**Conflicts of Interest:** The authors declare no conflicts of interest.

**Supplementary Materials:** The supplementary material is available online at https://www.techscience.com/doi/10.32604/biocell.2026.079918/s1.

## Abbreviations

| | |
|---|---|
| ABTS | 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) |
| AD | Alzheimer's disease |
| ANOVA | Analysis of variance |
| CD14 | Cluster of differentiation 14 |

| | |
|---|---|
| CD206 | Cluster of differentiation 206 |
| CD86 | Cluster of differentiation 86 |
| DMEM | Dulbecco's modified Eagle medium |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| DW | Dry weight |
| ELISA | Enzyme-linked immunosorbent assay |
| EVOO | Extra-virgin olive oil |
| GAE | Gallic acid equivalents |
| HSP70 | Heat shock protein 70 |
| HT | Hydroxytyrosol |
| ICAM-1 | Intercellular adhesion molecule 1 |
| IL-1β | Inteleukin-1β |
| IL-6 | Inteleukin-6 |
| IL-8 | Inteleukin-8 |
| iNOS | Inducible nitric oxide synthase |
| LPS | Lipopolysaccharide |
| MyD88 | Myeloid differentiation primary response 88 |
| NO | Nitric oxide |
| Nrf2 | Nuclear factor erythroid 2-related factor 2 |
| OLE | Olive leaf extracts |
| OLEE | Olive leaf ethanol extracts |
| OLM | Olive leaf macerates |
| PBS | Phosphate buffered saline |
| PD | Parkinson's disease |
| Phospho-p65 | Phosphorylated p65 subunit of the nuclear factor-kappa B |
| qPCR | Quantitative polymerase chain reaction |
| RIPA | Radioimmunoprecipitation Assay |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| Sirt1 | Silent mating type information regulation 2 homolog 1 (Sirtuin 1) |
| TBST | Tris-buffered saline with Tween 20 |
| THP-1 | Tohoku Hospital Pediatrics-1 |
| TLR4 | Toll-like receptor 4 |
| TNF-α | Tumor necrosis factor-alpha |
| TPC | Total phenolic content |
| VCAM | Vascular cell adhesion protein 1 |
| yHUVECs | Young human umbilical vein endothelial cells |

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