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From wild plant to functional ingredient: phytochemical insights and neuroprotective activity of *Melissa officinalis* subsp. *altissima*

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Melissa officinalis (lemon balm) is a culinary and medicinal herb traditionally used across diverse cultural systems for its cognitive-enhancing and neuroprotective effects. In light of its historical use in herbal teas, dietary supplements, and functional foods, this study investigates the bioactive potential of *Melissa officinalis* subsp. *altissima*, a wild subspecies of growing interest in the food and nutraceutical sectors. Wild specimens collected in Corsica (France) were investigated through an integrated metabolomic approach, combining a comprehensive phytochemical characterization of different extractive fractions and essential oils with a targeted biological evaluation performed on a hydroalcoholic extract selected as a representative model of the whole phytocomplex relevant to functional nutrition. Phytochemical profiling using high-resolution NMR spectroscopy identified a range of metabolites, including rosmarinic, chlorogenic, caffeic, and caftaric acids. GC-MS analyses were performed in parallel on the hydrodistilled essential oils and the volatiles obtained via HS-SPME, revealing a rich profile of bioactive volatile compounds such as germacrene-D, α , τ -cadinol, β -caryophyllene, and β -ocimene, which are known for their antioxidant and antimicrobial activities. Biological assays of the hydroalcoholic extracts demonstrated significant antioxidant capacity against hydrogen peroxide-induced oxidative stress in rat hypothalamic cells, as well as anti-inflammatory and neuroprotective effects in *ex vivo* murine assays. Notably, the extracts modulated the gene expression of key mediators such as TNF- α , NOS-2, IL-6, BDNF, and AChE suggesting a potential role in supporting brain health. These findings reinforce the potential of this lemon balm subspecies as a valuable natural ingredient for the formulation of functional foods and nutraceuticals with antioxidant, neuroprotective, and preservative properties. Moreover, the study underscores the broader significance of native aromatic plants as sustainable resources for health-oriented food innovation.

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1. Introduction

Lemon balm, also known as *Melissa officinalis*, is a perennial shrubby plant from the Lamiaceae family. While it grows worldwide, its exact origins are not well-defined, though it's believed to have originated in the Eastern Mediterranean, Western Asia, Southern Europe, Caucasus, and Northern Iran. Italy and France are the major producers of lemon balm.¹ In Europe, two main subspecies of lemon balm have been identi-

fied: *Melissa officinalis* subsp. *officinalis* and *Melissa officinalis* subsp. *altissima* (Sm.), which are distinguished by the indumentum of the leaves and the shape of the fruiting calyx.² The *Melissa officinalis* subsp. *altissima* is also referred to as *Melissa romana* MILLER or wild lemon balm.³

The Lamiaceae family is important as a source of medicinal, aromatic, and edible plants. Indeed, the lemon balm plant is rich in biologically active components, including terpenes, phenolic acids, and flavonoids, and has been cited for significant pharmacological activities, as antibacterial, antifungal, antiviral, anti-inflammatory, and antioxidant properties.⁴

The growing preference for a healthy lifestyle and nutrition has led to increased consumption of natural and functional food products. As a result, there has been a significant expansion in the use of plants and their derivatives in the food industry and for nutraceutical applications.⁵ The use of lemon

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balm leaf products for mild stress symptoms, as sleep aid, and for mild digestive disorders has been approved by the HMPC (Committee on Herbal Medicinal Products) of the EMA (European Medicines Agency) based on traditional use. Although there aren't enough clinical studies, the efficacy of these herbal medicines is well-founded, and evidence shows their safe use in these ways for at least thirty years. Ethnopharmacological studies have reported that lemon balm is used in many countries to treat various disorders, mainly in the pharmaceutical forms of essential oil, infusion, and decoction.⁶

In addition to this, there are studies in the literature discussing the potential positive effects of this medicinal plant on memory formation and neuroprotection.⁷ Through mechanisms involving rosmarinic acid, its primary polyphenol, lemon balm appears to inhibit the formation of β -amyloid fibrils ($A\beta$ 1-40 and $A\beta$ 1-42).⁸⁻¹⁰

In this framework, plant metabolomics—dedicated to detecting bioactive chemicals responsible for therapeutic outcomes—has become increasingly prominent.¹¹ The vegetable metabolome consists of primary and secondary metabolites with different physicochemical properties, including some that are species-specific. Therefore, it is essential to gather as much information as possible about the composition of an aromatic plant, and thus the metabolites extracted, the characterization of the volatile component, and the biological properties that are related to it.

In our previous study,¹² metabolomic profiling *via* NMR was performed on three officinal plant species, including *Melissa officinalis* subsp. *officinalis*. The investigation evaluated the influence of both pedoclimatic factors and cultivation practices by comparing three ecotypes: Land Spontaneous Ecotype (LSE), Mountain Spontaneous Ecotype (MSE), and Organic Ecotype (OE). The goal was to map their metabolic diversity and assess how altitude, growing conditions (wild *vs.* organic cultivation), and ecotype typology affect the abundance and identity of metabolites.

In the present study, *Melissa officinalis* subsp. *altissima* (MOA), a wild-growing species from Corsica, was analysed using a multi-methodological analytical protocol to obtain a complete metabolomic profile. In particular, high-resolution Nuclear Magnetic Resonance (NMR) spectroscopy was applied to investigate the hydroalcoholic and organic metabolites. Moreover, the chemical compositions of the essential oils obtained from the vegetative parts of the plant were analysed by Gas Chromatography coupled to Mass Spectrometry (GC-MS).

The aim of this research was to determine the phytochemical composition of extracts and essential oils in relation to their antioxidant and antimicrobial activities, respectively. In addition, the effects on general inflammation and neuroinflammation were evaluated by biological assays. In this framework, the metabolomic characterisation of the non-polar fraction was performed exclusively for chemical profiling purposes, to provide a comprehensive description of the phytochemical composition of *Melissa officinalis* subsp. *altissima*.

No biological assays were conducted on the non-polar extract, and therefore no direct functional conclusions can be drawn for this fraction. Specifically, the biocompatibility of hydroalcoholic MOA extracts was assessed in rat hypothalamic cells (HypoE22). The same cell line was tested to evaluate the antioxidant activity exerted by MOA hydroalcoholic extracts against cell damage induced by hydrogen peroxide (H_2O_2). Then, the gene expression of inflammatory and neurotrophic mediators such as TNF- α (Tumour necrosis factor), NOS-2 (Nitric oxide synthase-2), and BDNF (Brain-derived neurotrophic factor) in brain samples from C57/BL6 mice (prefrontal cortex and hypothalamus) treated with MOA hydroalcoholic extracts was evaluated. The cortical tissues of mice were also exposed to β -amyloid to subsequently assess the expression of IL-6 (Interleukin-6) and AChE (Acetylcholinesterase) genes during treatment with hydroalcoholic extracts of MOA.

An effective strategy to enhance specific activities—such as antioxidant, antimicrobial, anti-inflammatory, or neuroprotective effects—is to combine compounds that possess the desired properties. The resulting synergistic effect can significantly increase the targeted activity, even at low concentrations.

2. Materials and methods

2.1. Plant material

Melissa officinalis subsp. *altissima* aerial parts were harvested in central Corsica (Corte, France, 42°17'56.5" N, 9°10'13.0" E), during the flowering stage in July 2023. The botanical determination of the plants was carried out according to the characteristics summarised in Flora Corsica.¹³

2.2. Chemicals and reagents

Ethanol CH_3CH_2OH (HPLC-grade), methanol CH_3OH (HPLC-grade), chloroform $CHCl_3$ (HPLC-grade), and diethyl ether ($C_2H_5)_2O$ were obtained from Merck (Milan, Italy).

Double-distilled water was purchased using a Millipore Milli-Q Plus water treatment system (Millipore Bedford Corp., Bedford, MA, USA).

EDTA deuterated was purchased from Cambridge Isotope laboratories, Inc. (Andover, USA). Monobasic potassium phosphate (KH_2PO_4) and dibasic potassium phosphate (K_2HPO_4) were purchased from Aldrich-Fluka-Sigma S.r.l. (Milan, Italy). Deuterated water (D_2O), methanol-D4 (CD_3OD), and chloroform-D ($CDCl_3$) were purchased from Euriso-Top (Saclay, France).

3-(Trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP) and Dimethyl sulfoxide (DMSO) were acquired from Merck (Milan, Italy). TRI reagent was obtained from Sigma-Aldrich (St Louis, MO, USA). All murine genes detailed below, PCR primers, TaqMan probes, and High-Capacity cDNA Reverse Transcription Kit were sourced from Thermo Fisher Scientific (Waltham, Massachusetts, USA). The genes included: GAPDH, TNF- α , NOS-2, IL-6, AChE and BDNF. β -Amyloid (1-42), (rat/mouse) (TFA) (95%) was acquired from



MedChemTronica (The European Branch of MedChemExpress LLC, Sweden).

2.3. NMR analysis

2.3.1. Extraction procedure. Fresh plant leaves were freeze-dried (Lyovapor L-200, $-55\text{ }^{\circ}\text{C}$, 0.200 mbar) and ground into powder for NMR extractions following the Bligh–Dyer protocol.^{12,14} A 0.2 g ground lyophilized sample was extracted with a methanol/water (2 : 1, v/v) mixture, sonicated (thermostat ultrasonic bath ARGOLAB DU-100 (Rome, Italy)) for 10 minutes at room temperature, then partitioned with CHCl_3 and water to form a biphasic system. After centrifugation (Eppendorf Centrifuge 5430 R (Milan, Italy)), the hydroalcoholic and organic phases were separated. The extraction was repeated twice on the pellet, and combined extracts were dried under nitrogen. Triplicate analyses were performed.

2.3.2. NMR experiments. The dried hydroalcoholic phase was dissolved in 0.7 mL of 100 mM phosphate buffer/ D_2O , containing TSP (0.4 mM) as internal standard. The CHCl_3 -dried fraction was reconstituted+ in 0.7 mL of $\text{CDCl}_3/\text{CD}_3\text{OD}$ mixture (2 : 1 v/v).

NMR analyses were conducted using a Jeol JNM-ECZ 600R instrument operating at a proton frequency of 600.17 MHz and equipped with a Jeol 5 mm FG/RO DIGITAL AUTOTUNE probe. Spectra processing and signal integration were performed using JEOL Delta software version 5.3.1. Additionally, two-dimensional experiments (^1H – ^1H TOCSY, ^1H – ^{13}C HMBC, and ^1H – ^{13}C HSQC), and the quantifications of the hydroalcoholic and organic portions were performed following the experimental protocols previously reported.¹²

2.4. GC-MS analysis

2.4.1. Distillation and extraction process. The aerial parts of the plant were air-dried (200 g) and subjected to hydro-distillation (5 h) using a Clevenger-type apparatus, according to the method recommended by the European Pharmacopoeia (Council of Europe, 1997). Hydrodistillation produced a yellow essential oil (EO) with a yield of 0.04% (w/dw)

The hydrosol was obtained by collecting the initial 300 mL (co-coating) from the Clevenger device during hydrodistillation. Then, the hydrosol underwent a liquid–liquid extraction procedure. A total of 300 mL was extracted in three portions of 50 mL each using diethyl ether. The organic phase was washed with 50 mL of water saturated with NaCl, dried over anhydrous Na_2SO_4 , and filtered. The resulting product was concentrated to yield the hydrosol extract (HYD). The obtained HYD was colorless, with a yield of 0.06% (w/dw).

2.4.2. HS-SPME conditions. Fresh aerial parts were finely chopped and subjected to headspace solid-phase microextraction (HS-SPME) using a divinylbenzene/carboxene/polydimethylsiloxane (DVB/CAR/PDMS, 30 μm) coated fiber (Supelco). Specifically, 0.7 g of plant material was placed in a 20 mL SPME vial and extracted under controlled conditions: equilibration time of 60 minutes, extraction time of 30 minutes, and extraction temperature of $70\text{ }^{\circ}\text{C}$.

2.4.3. GC-FID analysis. GC analysis was performed using a PerkinElmer Clarus 600 system (Waltham, MA, USA), equipped with a single injector, two flame ionization detectors (FIDs), and two fused silica capillary columns (60 m \times 0.22 mm i.d., film thickness 0.25 μm ; Restek, Bellefonte, PA, USA) with different stationary phase polarities. The oven temperature was programmed from $60\text{ }^{\circ}\text{C}$ to $230\text{ }^{\circ}\text{C}$ at $2\text{ }^{\circ}\text{C min}^{-1}$ and held isothermal at $230\text{ }^{\circ}\text{C}$ for 30 min. The injector and detector temperatures were maintained at $280\text{ }^{\circ}\text{C}$. Hydrogen was used as the carrier gas (0.7 mL min^{-1}). Retention indices (RIs) were calculated using a homologous series of n-alkanes and the Van den Dool and Kratz equation.¹⁵

2.4.4. GC-MS analysis. Plant extracts were analysed with a PerkinElmer TurboMass quadrupole detector directly connected to a PerkinElmer SQ8 (Walton, MA, USA), equipped with the same two fused silica capillary columns mentioned above. Both columns were used with the same quadrupole MS detector. Analyses were conducted sequentially using non-polar and polar column for each sample, resulting in two reconstructed ion chromatograms (RIC) for each sample. GC conditions remained constant and MS parameters were as follows: ion source temperature at $150\text{ }^{\circ}\text{C}$, ionisation energy at 70 eV and electron ionisation mass spectra acquired in a mass range of 35–350 amu during a scan time of 1 second. The injection volumes were 0.1 μL .

2.4.5. Compound identification and quantification. Identification of individual components in plant extracts was based on a methodology of GC retention indices and GC-MS in EI mode. The identification of individual components was based (i) on the comparison of the retention indices (RIs) determined on the polar and nonpolar columns with those of authentic compounds or literature data;^{16,17} (ii) on computer matching of the mass spectra with commercial MS libraries and the mass spectra with those listed in our homemade MS library of authentic compounds or literature data.^{18,19} The compound quantification was carried out using peak normalization, including FID response factors (RFs) rel. to tridecane (0.7 g per 100 g) used as internal standard and expressed as normalized contents (% abundances).

2.5. Biological assays

For biological assays, phytocomplex-related biological effects were evaluated using a green hydroalcoholic extraction protocol previously described.²⁰ In particular, 100 mg of lyophilized sample was added to 2 mL of a $\text{H}_2\text{O}/\text{EtOH}$ mixture (30 : 70% v/v) with a 1 : 20 ratio of plant matrix to extraction solvent. The obtained system was sonicated (ARGOLAB DU-100 ultrasonic thermostat bath (Rome, Italy)) at $30\text{ }^{\circ}\text{C}$ for 10 min and then centrifuged (Eppendorf 5430 R centrifuge (Milan, Italy)) for 5 min ($30\text{ }^{\circ}\text{C}$, 7745g). The hydroalcoholic extract was separated, and the residual pellet was extracted twice using the same protocol. The collected supernatants were pooled and filtered through 0.45 μm syringe filters and then brought to dryness under a constant flow of nitrogen at room temperature. The dried hydroalcoholic extract was dissolved in a DMSO/Milli-Q water mixture using a total solvent volume of 6 mL to maintain



the appropriate matrix/solvent ratio. In addition, to avoid the potential toxicity of MOA extract, the maximum phytochemical concentration tested was $200 \mu\text{g mL}^{-1}$, with less than 1% DMSO. Therefore, the stock solution for cell treatment or tissue incubation was prepared by adding a 50:50% v/v DMSO/H₂O mixture to the dried hydroalcoholic extract. The system was sonicated for 10 min at room temperature and filtered through $0.22 \mu\text{m}$ syringe filters to obtain a sterile solution. As shown in Table 1, the stock solution was used to create subsequent dilutions for *in vitro* and *ex vivo* assays.

2.5.1. *In vitro* studies. Rat HypoE22 were cultured in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 1% (v/v) penicillin G/streptomycin in 75 cm^2 cell culture flasks. The cultured cells were maintained in a humidified incubator with 5% CO₂ at 37 °C. When confluence reached 80%, a viability assay was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to evaluate the basal cytotoxicity and antioxidant activity post-H₂O₂ treatment of the MOA extract under investigation. For this assay, cells were seeded (3×10^3 cells per well) on 96-well flat-bottom culture plates and incubated overnight. After 24 hours, the cells were treated with vehicle or MOA extract at different concentrations (50, 100, 200, and $500 \mu\text{g mL}^{-1}$) and incubated overnight. In a second set of experiments, the cells were subsequently treated with $300 \mu\text{M}$ pro-oxidant stimuli (hydrogen peroxide, H₂O₂) for 3 hours. After this time, a total of $20 \mu\text{L}$ of MTT (5 mg mL^{-1} in PBS) was added to each well and incubated for 3 hours at 37 °C. The formazan dye formed was solubilized with dimethyl sulfoxide, and the plate was incubated in the dark room on a plate shaker at 200 rpm for 3 h (37 °C). The absorbance was recorded at 540 nm to determine cell survival. The effects on cell viability were evaluated against the control (Ctrl: untreated cells) and expressed as a percentage of the control culture. Each condition was run in triplicate, including an untreated control and a cell-free blank control. For statistical analysis one-way ANOVA, followed by Tukey's multiple comparison test, was applied to underline, in terms of HypoE22 viability, significant differences for each MOA hydroalcoholic extract con-

Table 1 Stock solution and its dilutions of dried hydroalcoholic extract of MOA. All concentrations were tested on cells for *in vitro* assays, but only 100 and $200 \mu\text{g mL}^{-1}$ were used to incubate cortical and hypothalamic tissues in *ex vivo* assays

	Phytochemical concentration	DMSO %
80×	16 mg mL^{-1}	50
10×	2 mg mL^{-1}	6.25
Highest tested	$500 \mu\text{g mL}^{-1}$	1.56 ^a
Higher Int. tested	$200 \mu\text{g mL}^{-1}$	0.625
Lower Int. tested	$100 \mu\text{g mL}^{-1}$	0.312
Lowest tested	$50 \mu\text{g mL}^{-1}$	0.156

^aThis percentage is higher than that tolerated by the cells, which is why the effects on cell viability were evaluated in comparison to two control lines (Ctrl) in 96-well culture plates (one line was at this DMSO concentration).

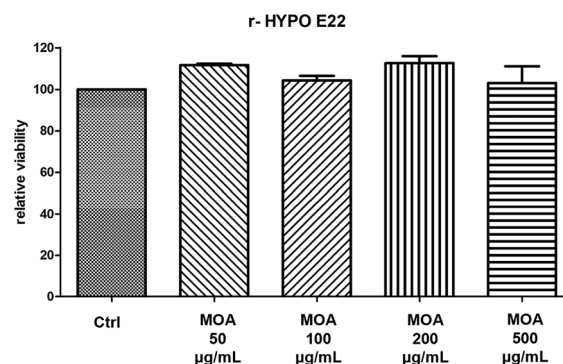


Fig. 1 Effects of MOA extracts ($50\text{--}200 \mu\text{g mL}^{-1}$) on HypoE22 cell viability under basal conditions. One-way ANOVA followed by Tukey's multiple comparison test revealed no significant differences in cell viability for any MOA extract concentration compared with the control. Data are expressed as means \pm SD from three independent experiments, each performed in triplicate.

centration vs. Ctrl, which is under basal conditions in Fig. 1 and pro-oxidant stimulus in Fig. 2. Statistical analysis was performed using GraphPad Prism™ (Version 4.00) software (GraphPad Software, Inc., San Diego, California, USA).

2.5.2. *Ex vivo* studies. Adult C57/BL6 mice ($n = 13$) were housed in Plexiglas cages ($55 \text{ cm} \times 33 \text{ cm} \times 19 \text{ cm}$) and maintained under standard laboratory conditions ($21 \pm 2 \text{ }^\circ\text{C}$; $55 \pm 5\%$ humidity) with a 14/10-hour light/dark cycle, with drinks and food *ad libitum*. Housing conditions and experimental procedures were strictly in accordance with European Community ethical standards (EU Directive No. 26/2014) regarding the care and use of animals for scientific research. Tissue collection was approved by the local ethics committee

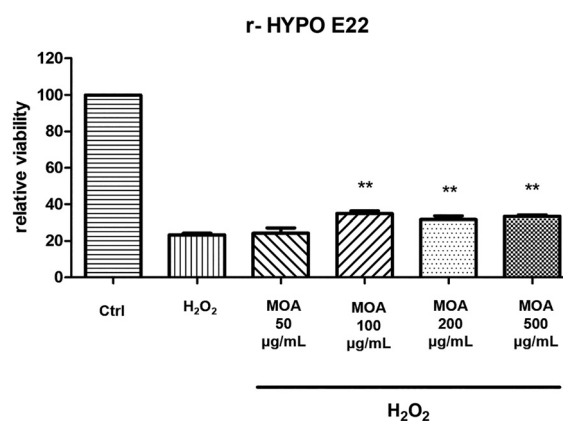


Fig. 2 Effects of MOA extracts ($50\text{--}500 \mu\text{g mL}^{-1}$) on HypoE22 cell viability under hydrogen peroxide (H₂O₂) stimulation. Significant differences in cell viability for each extract concentration vs the positive control (H₂O₂ – treated group) were assessed by one-way ANOVA followed by Tukey's multiple comparison test (** $p < 0.001$). The negative control corresponds to experiments performed under basal conditions. Data are expressed as means \pm SD from three independent experiments, each performed in triplicate.



(“G. d’Annunzio” University, Chieti, Italy) and the Italian Health Ministry (Project no. F4738.N.5QP). Rodents were sacrificed by CO₂ inhalation (100% CO₂ at a flow rate of 20% of the chamber volume per minute) for brain retrieval.

For the LPS stimulus, brain samples were cut to obtain sections of the prefrontal cortex (split in two, $n = 16$) and the hypothalamus ($n = 8$). Next, brain tissue samples were placed in 6-well plates in RPMI buffer by adding bacterial LPS (*E. coli*) (50 $\mu\text{g mL}^{-1}$) to each well, except for the control (Ctrl). The prefrontal cortex samples were treated with hydroalcoholic extract of MOA at concentrations of 100 and 200 $\mu\text{g mL}^{-1}$. The hypothalamic sections were treated only at the highest concentrations of MOA extract, 200 $\mu\text{g mL}^{-1}$.

For stimulation with β -amyloid, brain samples were cut to obtain prefrontal cortex sections divided into two, with $n = 10$. Thus, the brain tissue samples were placed in 10-well plates containing RPMI buffer, two of which were used as controls (Ctrl). Prefrontal cortex samples were treated with hydroalcoholic extract of basal MOA at concentrations of 50, 100, and 200 $\mu\text{g mL}^{-1}$, and with hydroalcoholic MOA extract (50, 100, and 200 $\mu\text{g mL}^{-1}$) after stimulation with β -amyloid ($C = 0.05 \text{ mg mL}^{-1}$). Two prefrontal cortex samples were subjected to β -amyloid ($C = 0.05 \text{ mg mL}^{-1}$) stimulus treatment alone.

After 4 h of incubation at 37 °C, all samples were collected and stored at $-80 \text{ }^\circ\text{C}$ until RNA extraction.

Total RNA was extracted from the brain tissues using TRI reagent, according to the manufacturer’s protocol, and 1 μg of total RNA from each sample in a 20 μL reaction volume was reverse transcribed using High-Capacity cDNA Reverse

Transcription Kit. Gene expression of TNF- α , BDNF, NOS-2, IL-6, and AChE was evaluated by quantitative real-time PCR using TaqMan probe-based chemistry, as previously reported.²¹ GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) was used as the housekeeping gene. Real-time PCR was performed in triplicate for each cDNA sample with respect to the selected genes. The elaboration of data was carried out with the Sequence Detection System (SDS) software version 2.3 (Thermo Fischer Scientific). Relative quantification of gene expression was performed by the comparative $2^{-\Delta\Delta\text{Ct}}$ method.²²

For statistical analysis one-way ANOVA, followed by Tukey’s multiple comparison test, was applied to underline, among genes expression, significant differences for each MOA hydroalcoholic extract concentration vs. LPS (Fig. 3) or vs. β -amyloid (Fig. 4) stimuli.

3. Results and discussion

3.1. NMR-based detection of hydrophilic metabolites

Assignments of ¹H NMR spectra of water-soluble MOA extracts in D₂O/phosphate buffer, Table 2, were based on 2D NMR experiments (¹H-¹H TOCSY, ¹H-¹³C HSQC, and ¹H-¹³C HMBC) and a previous NMR study.¹²

Five sugars (galactose, glucose, fructose, sucrose, and inulin), a polyol (myo-inositol), eight organic acids (acetate, citrate, lactate, quinate, formate, malate, succinate, and tartrate), ten amino acids (alanine, GABA, glutamate, glutamine, isoleucine, leucine, proline, threonine, tyrosine, and valine),

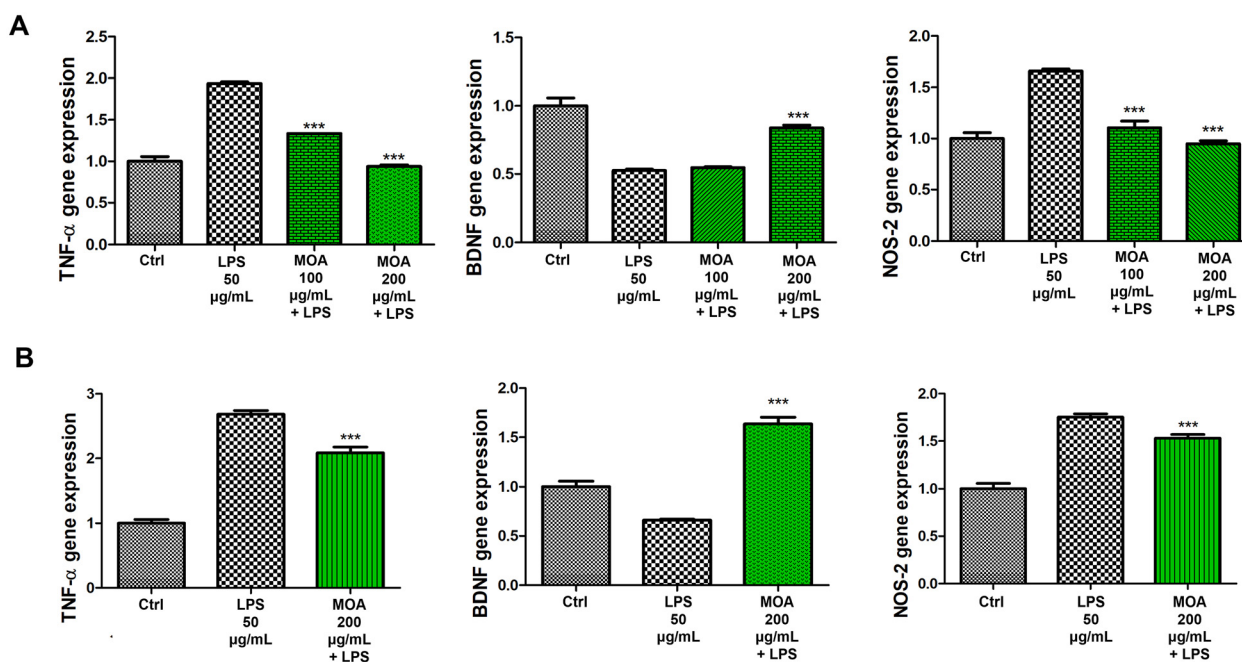


Fig. 3 Effects of MOA hydroalcoholic extract on LPS-induced and basal TNF- α , NOS-2 and BDNF gene expression in cortical (A) and hypothalamic (B) tissues of mice. Significant differences among gene expression levels for each extract concentration vs LPS were assessed by one-way ANOVA followed by Tukey’s multiple comparison test (** $p < 0.001$). Data are presented as means \pm SEM from three independent experiments, each performed in triplicate.



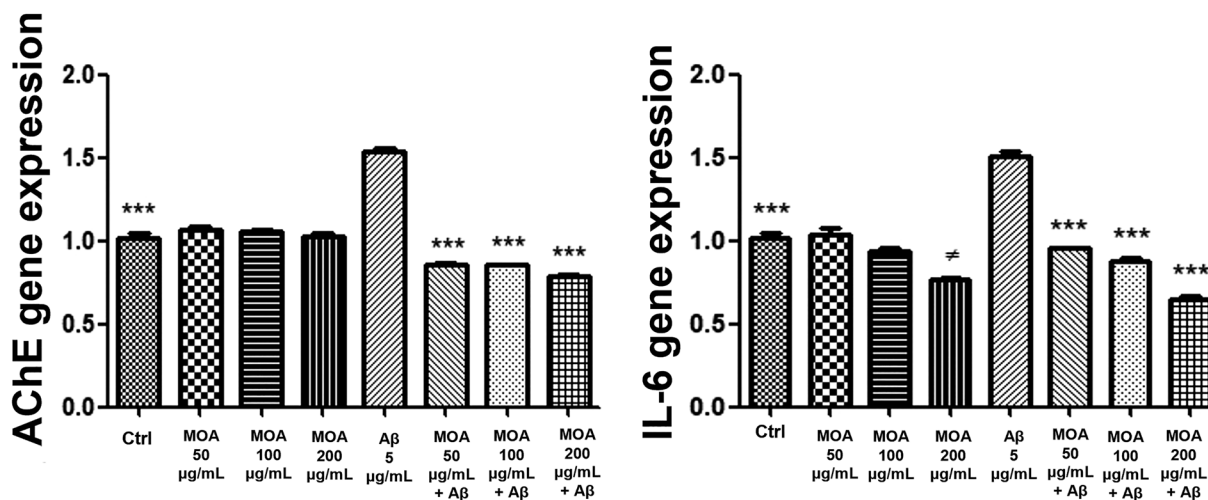


Fig. 4 Effects of the MOA hydroalcoholic extract on β -amyloid-induced and basal AChE and IL-6 gene expression in cortical tissues of mice. Significant differences among gene expression levels for each extract concentration vs A β were evaluated by one-way ANOVA followed by Tukey's multiple comparison test (***) $p < 0.001$). Data are presented as means \pm SEM from three independent experiments, each performed in triplicate.

and four polyphenols (caftaric, chlorogenic, caffeic, and rosmarinic acids) were identified.

Metabolic diversity is a key indicator of the physiological and adaptive flexibility of plants, often influenced by genetic and ecological differentiation at the subspecies level.²³ Subspecies may show distinct metabolic footprints due to variations in gene expression and environmental pressures. This biochemical heterogeneity affects both primary metabolites, which were essential for growth, and secondary compounds involved in stress response and ecological interactions.²⁴

To the best of our knowledge, the assignment and quantification of a ¹H NMR spectrum of an extract of *Melissa officinalis* subsp. *altissima* has not been reported previously.

Here, the metabolites identified in the MOA samples were quantified by integrating specific ¹H NMR signals and the results are shown in Table 2.

The following sections provide a separate discussion for each class of compounds, reflecting increased metabolic activity and indicating specific physiological states of the plant.

3.1.1. Sugars. Sugars represented the most abundant class of metabolites detected in the MOA aqueous extract, with sucrose levels being relatively high compared to the other sugars (Table 2). Previous studies have demonstrated that elevated sucrose content is associated with a marked increase in metabolites involved in glycolysis and related metabolic pathways, including glucose, fructose, maltose, glucose-6-phosphate, fructose-6-phosphate, raffinose, myo-inositol, and erythritol.²⁵ These changes are also reflected in an increased production of bioactive metabolites, including flavonoids and polyphenols, in the plants.^{26,27} However, the concentrations (Table 2) of glucose, galactose, inulin, fructose, and myo-inositol in the MOA hydroalcoholic extract were consistent with those reported in our previous study on the *officinalis* subspecies.¹² Comparison with literature data confirms that sucrose

is the predominant sugar in *Melissa officinalis*, typically exceeding glucose, fructose, and galactose.^{25,28} Reported sucrose levels (1500–1800 $\mu\text{g mL}^{-1}$) are similar to the $1736.28 \pm 4.20 \mu\text{g mL}^{-1}$ measured in our extract, while glucose and fructose are present at lower concentrations (α -glucose $362.84 \pm 2.90 \mu\text{g mL}^{-1}$), consistent with literature.²⁸ Inulin and myo-inositol occur at moderate to high levels, supporting their role in plant metabolism and bioactive compound biosynthesis, whereas β -galactose is minor ($51.38 \pm 1.15 \mu\text{g mL}^{-1}$), in agreement with previous reports.²⁸

3.1.2. Organic acids. The total content of organic acids measured in MOA was nearly half of that found in *Melissa officinalis* ecotypes.¹² Notably, this result aligns with findings reported for other wild or less domesticated plant species. For instance, in *Sanguisorba minor*, a marked reduction in total organic acid content was observed in wild samples compared to cultivated ones.²⁹ A similar trend was noted in *Centaurea raphanina* subsp. *mixta*, where wild populations exhibited lower levels of total organic acids than their cultivated counterparts.³⁰ However, some individual acids show interesting patterns of comparison. For example, malate was more abundant in the hydroalcoholic MOA extract compared to that of the cultivated subspecies.¹² Citrate levels (63.80 mg g^{-1}), see Table 2, were consistent with those observed in the mountain ecotype from our previous study.²⁹ Here, the most abundant organic acid was tartrate, followed by malate and quinate, whereas lactate and acetate were present in the lowest concentrations. However, from a quantitative perspective, MOA has much higher levels of malic, quinic, and tartaric acids than those typically found in cultivated lemon balm, where these acids are present in lower or negligible amounts.³¹ This higher relative abundance of malic, quinic, and tartaric acids in MOA aligns with the review evidence suggesting that wild or undomesticated matrices, while containing the same principal acids, can exhibit markedly different quantitative profiles.³²



Table 2 List of water-soluble metabolites identified in the 600 MHz ^1H NMR spectra of MOA extracts (the hydroalcoholic ones by Bigh-Dyer) in D_2O /phosphate buffer. For each compound, the signal assignment, proton chemical shift (^1H , ppm), multiplicity with coupling constant [J (Hz)], carbon chemical shift (^{13}C , ppm), and the mean concentration \pm standard deviation ($\text{mg } 100 \text{ g}^{-1}$ of dry weight) are reported. The asterisk (*) indicates the selected proton signal used for quantification

Compound	Assignment	^1H (ppm)	Multiplicity [J (Hz)]	^{13}C (ppm)	Mean \pm SD (mg 100 per g)
Sugars					
α -D-Fructofuranose	C-2			105.9	
	CH-3	4.11		82.3	
β -D-Fructofuranose	C-2			102.6	167.61 \pm 1.10
	CH-4	4.12*		75.6	
β -D-Fructopyranose	C-2			99.3	
β -Galactose	CH-1	4.60*	d [7.9]	97.3	51.38 \pm 1.15
	CH-2	3.51			
	CH-3	3.67			
	CH-4	3.95			
	CH-5	4.05			
	CH-6	3.78			
α -Glucose	CH-1	5.23*	d [3.8]	93.3	362.84 \pm 2.90
	CH-2	3.54		72.6	
	CH-3	3.72		73.3	
	CH-4	3.41		70.8	
	CH-5	3.84		72.5	
β -Glucose	CH-1	4.66*	d [7.9]	97.0	229.40 \pm 1.83
	CH-2	3.25		75.3	
Inulin	CH-1 (Glc)	5.44*		93.9	738.19 \pm 3.88
	CH-2	3.57		72.1	
	CH-3	3.78		73.6	
	CH-4	3.48		70.2	
	CH-5	3.85		73.4	
	CH ₂ -6	3.83		61.1	
	CH ₂ -1' (Fru)	3.75; 3.89			
	CH-3'a	4.21		77.5	
	CH-3'b	4.26			
	CH-4'a	4.05		74.9	
	CH-5'	3.89		82.2	
Myo-inositol	CH-2,5	3.56			
	CH-3,6	3.65			
Sucrose	CH-4	3.28*		75.0	628.52 \pm 2.06
	CH-1 (Glc)	5.42*	d [3.9]	93.1	1736.28 \pm 4.20
	CH-2	3.53		71.8	
	CH-3	3.72		73.6	
	CH-4	3.42		70.2	
	CH-5	3.84		73.5	
	C2' (Fru)			104.8	
	CH-3'	4.23		77.4	
	CH-5'	3.92		82.4	
	CH-4'	3.83		61.2	
Organic acids					
Acetate	CH ₃	1.92*	s	24.4	13.22 \pm 1.12
Citrate	COOH			184.4	
	α,γ -CH	2.56*	d [16.0]	46.0	63.80 \pm 1.64
	α',γ' -CH	2.68		46.0	
	β -C			73.2	
	1,5-COOH			180.2	
	6-COOH			183.0	
Formate	HCOOH	8.46*	s		
Lactate	β -CH ₃	1.34	d [7.1]	21.4	4.66 \pm 0.28
Malate	α -CH	4.31	dd [9.8; 3.3]	71.0	470.89 \pm 1.41
	β -CH	2.68	dd [15.5; 3.3]	43.5	
	β' -CH	2.41	dd [15.5; 9.8]	43.5	
	CH ₂ -2,2'	1.89, 2.06*	dd [10.8; 13.4], m	41.8	365.84 \pm 1.30
Quinate	CH-3	4.16		71.5	
	CH-4	3.56		76.3	
	CH-5	4.03		68.0	
	α,β -CH ₂	2.42*	s	35.2	95.34 \pm 1.87
Tartrate	CH(OH)COOH	4.37*	s	75.3	287.50 \pm 2.75
Amino acids					
Alanine	α -CH	3.80		51.0	23.61 \pm 0.08
	β -CH ₃	1.49*	d [7.2]	17.3	
	COOH			178.6	



Table 2 (Contd.)

Compound	Assignment	¹ H (ppm)	Multiplicity [J (Hz)]	¹³ C (ppm)	Mean ± SD (mg 100 per g)
GABA	α-CH ₂	2.30*	t [7.4]	35.4	38.48 ± 0.00
	β-CH ₂	1.90		24.9	
	γ-CH ₂	3.01	t [7.5]	40.4	
Glutamate	α-CH	3.78		55.6	117.37 ± 1.51
	β,β'-CH ₂	2.07; 2.14*		28.0	
	γ-CH ₂	2.36	m	34.8	
Glutamine	α-CH	3.76		55.3	
	β,β'-CH ₂	2.14	m	27.5	
	γ-CH	2.45*	m	31.9	
Isoleucine	α-CH	1.97		38.0	43.43 ± 1.53
	β-CH	1.27		29.4	
	γ-CH ₃	1.01*	d [7.1]	15.7	
Leucine	δ-CH ₃	0.89	d [7.4]		13.10 ± 0.87
	β-CH ₂	1.74		41.0	
	γ-CH	1.71			
	δ-CH ₃	0.97*	d [6.4]	23.1	
	δ'-CH ₃	0.96		22.1	
	CH-4	7.38			
Proline	CH-3,5	7.43	m	130.6	251.67 ± 2.18
	α-CH	4.13		62.5	
	β,β'-CH ₂	2.07, 2.33		30.0	
	γ-CH ₃	2.01*	m	25.0	
	δ,δ'-CH ₃	3.33, 3.41		47.4	
	α-CH	3.59		62.3	
Threonine	γ-CH ₃	1.33*	d [6.7]	20.71	21.40 ± 0.53
	CH-2, 6 ring	7.22*	d [8.2]	132.0	
Tyrosine	CH-3, 5 ring	6.96	d [8.5]	117.0	728.15 ± 2.60
	α-CH	3.60			
Valine	β-CH	2.27			20.00 ± 0.15
	γ-CH ₃	1.10	d [7.1]	17.7	
	γ'-CH ₃	1.04*	d [7.1]	19.0	
Polyphenols					
Caffeic acid	α-CH=CH	6.36*	d [16.00]	116.0	145.07 ± 1.47
	β-CH=CH	7.29	d [16.00]	147.2	
Caftaric acid	CH-10	5.33	d [2.2]		454.49 ± 12.62
	CH-11	4.57	d [2.2]		
	CH-2	6.49*	d [16.0]	115.4	
	CH-3	7.72	d [16.0]	148.0	
	CH-8 ring	6.96	d [8.2]	117.6	
	CH-9 ring	7.17	dd [2.0; 8.2]	124.2	
	CH-5 ring	7.25	d [2.0]	117.5	
	COOH			171.4	
Chlorogenic acid	CH-2	6.39*	d [16.0]	115.8	525.78 ± 3.0
	CH-3	7.37	d [16.0]	146.8	
	CH ₂ -2'	1.89; 2.10	m	41.7	
	CH-4'	3.90	m	70.8	
	CH ₂ -6'	1.99; 2.06	m	38.6	
Rosmarinic acid	CH-2	6.22	d [16.0]	115.8	1352.01 ± 11.86
	CH-3	7.37	d [16.0]	146.8	
	CH-1a	5.01	m	77.5	
	CH ₂ -2a	2.85; 2.93	m	37.9	

3.1.3. Amino acids. Amino acids represented the least abundant class of metabolites in the MOA extracts, see Table 2. Among them, tyrosine was the most prevalent in the hydroalcoholic extracts, reaching a concentration of 728.15 mg g⁻¹. This amino acid plays a central role as a precursor in the biosynthesis of tocopherols, plastoquinone, and ubiquinone—compounds essential for plant survival.³³ Tyrosine is therefore a key entry point into the production of phenolic compounds and antioxidants, whose synthesis is often enhanced under abiotic stress conditions. Branched-chain amino acids (BCAAs) showed varying levels when com-

pared to previously analyzed ecotypes. Leucine concentrations were higher in the MOA samples than in the mountain and lowland ecotypes, and comparable to those observed in the organic ecotype.¹² Valine followed a similar trend. Notably, isoleucine levels in MOA were approximately five, three, and seven times higher than those measured in LSE, OE, and MSE samples, respectively.¹² Recent research on BCAA biosynthesis in plants has demonstrated that both genetic and environmental factors tightly regulate their accumulation.³⁴ Therefore, the elevated isoleucine content in MOA may be attributed to differences in enzyme activity involved in BCAA



biosynthesis, influenced by ecotypic variation and environmental conditions. The concentrations of threonine and alanine were comparable to those found in the lowland and mountain ecotypes (LSE, MSE).¹² In contrast, GABA levels were nearly five times lower in MOA compared to the cultivated subspecies. GABA is known to accumulate under abiotic stress, including drought, salinity, and oxidative conditions, but multiple environmental and developmental factors regulate its metabolism.³⁵ For this reason, the low GABA content in these samples cannot be used as direct evidence for the absence of abiotic stress. Instead, it may indicate a basal physiological state in which GABA biosynthesis has not been induced. Conversely, proline concentrations were notably higher in the MOA extracts. Proline accumulation is frequently associated with plant responses to water deficit and has been implicated in drought tolerance in several species, including *Melissa officinalis*.³⁶ However, given the complex regulation of proline metabolism and the lack of controlled stress experiments in this study, these data suggest a possible adaptation to drier conditions.

3.1.4. Polyphenols. The analysis of polyphenols in *Melissa officinalis* subsp. *officinalis* has been the focus of several studies.³⁷ In our previous study on cultivated lemon balm species, chlorogenic, caffeic, and rosmarinic acids were identified but not quantified due to signal overlap.¹² Here, NMR spectroscopy enabled the assignment of caffeic, chlorogenic, rosmarinic, and caftaric acids in hydroalcoholic extracts of MOA (Table 2). The presence of these phenolic compounds in the plant has been widely demonstrated.^{38–40} MOA, like other species of the Lamiaceae family, had a high content of phenolic acids, particularly caffeic acid derivatives such as rosmarinic acid. Rosmarinic acid was the most abundant polyphenol, reaching concentrations of 1.352 mg per 100 g of dry weight (Table 2). This compound is well known in aromatic plants for its strong antioxidant and anti-inflammatory properties, which contribute to the reduction of oxidative stress.⁴¹ Rosmarinic acid has been shown to effectively reduce oxidative damage by eliminating a wide range of ROS generated in ascorbic acid/Cu²⁺ systems.⁴² Chlorogenic acid was found to be the second predominant polyphenolic compound (Table 2). This metabolite is widely found in the plant kingdom, with particularly high concentrations in green coffee.⁴³ It plays a central role in the redox homeostasis of plants and in defence mechanisms.⁴⁴ In particular, thanks to its structural characteristics, the chlorogenic acid exhibits a marked antioxidant capacity. *In vitro* and *in vivo* studies have reported a broad spectrum of biological activities associated with high levels of chlorogenic acid, including antioxidant, anti-inflammatory, and antibacterial effects.^{45–48} Furthermore, it has been shown to modulate glucose and lipid metabolism and exert neuroprotective properties,^{49,50} positioning it as a compound of considerable interest for nutraceutical applications and functional foods.⁵¹ Among the quantified phenolic constituents, caffeic acid and caftaric acid were also present at appreciable levels. Specifically, caffeic acid reached a concentration (145.07 ± 1.47 mg per 100 g),

whereas caftaric acid was found in even higher amounts (454.49 ± 12.62 mg per 100 g), (Table 2). In the current investigation, the concentrations of caffeic and caftaric acids exceeded those reported in the literature.^{4,52,53}

3.2. NMR-based detection of lipophilic metabolites

The identification of lipophilic metabolites was based on a combination of 1D and 2D NMR experiments, comparing literature data on complex lipid mixtures,^{12,54} and is reported in Table 3.

In line with an untargeted NMR-based metabolomic approach, the analysis allowed the comprehensive detection of the main classes of lipophilic constituents present in the organic extracts, including fatty acids, sterols, chlorophyll derivatives, phospholipids and galactolipids. Quantitative evaluation was subsequently focused on selected representative metabolites showing well-resolved and diagnostic NMR signals, while additional minor components were annotated at the qualitative level to define the overall lipophilic fingerprint. Quantitative analysis of the lipophilic portion revealed that MOA contained significantly lower amounts of total fatty acids and unsaturated fatty acids than *M. o. officinalis*, whereas presenting an exceptionally high percentage of β -sitosterol (91.07 ± 5.37 mg per 100 g),¹² Table 3. These values suggest an active biosynthetic pathway for phytosterol and a possible role in membrane stabilization under environmental stress conditions, as reported for other plant species.^{55,56} High levels of β -sitosterol have been linked to increased tolerance to abiotic stress and to the health properties of plant extracts.⁵⁷ Among sterols, although stigmasterol was found in the previously found,¹² it was undetected in MOA. Besides sterols and fatty acids, the untargeted NMR analysis also revealed the presence of chlorophyll and pheophytin, detected through their characteristic downfield resonances (δ ~11.1 to 11.2 ppm), which are commonly observed in plant lipophilic fractions and reflect the photosynthetic pigment pool. Although present at low concentrations, these compounds contribute to defining the lipophilic metabolomic signature of MOA and are indicative of chloroplast-derived components. MOA has a significantly reduced lipid profile compared to the other study,¹² Table 3. In all fatty acid classes, the levels detected in MOA were drastically lower than those reported for the *officinalis* subspecies. DUFAs occurred at roughly one-twenty-third of the levels

Table 3 Compounds and relative selected signals (ppm) for quantitative analysis in the organic extracts

	ppm	Group	Metabolite	Mean ± SD (mg 100 per g)
$I_{\beta\text{-Sit}}$	0.65	CH ₃ -18	β -Sitosterol	91.07 ± 5.37
I_{FA}	2.30	CH ₂ -11	Totally fatty acids	8.15 ± 1.0
I_{DUFA}	2.73	CH ₂ -11	Linoleic acid	0.37 ± 0.05
I_{TUFA}	2.77	CH ₂ -11, ¹⁴	Linolenic acid	1.58 ± 0.38
I_{PCG}	3.23	N(CH ₃) ₃	Glyceroyl-phosphatidylcholine	0.10 ± 0.00
I_{DGG}	4.87	CH-1	Glyceroyl-digalactose	0.54 ± 0.01
I_{PHEO}	11.15	CH-5	Pheophytin	0.10 ± 0.00
I_{CHL}	11.13	CH-5	Chlorophyll	0.05 ± 0.01



reported by Ambroselli *et al.* (2024),¹² whereas TUFAs and MUFAs reached only about one-seventeenth of those values, see Table 3. Even SFAs reached only about a quarter of the concentrations previously detected. Signals attributable to glycerol-based lipids, such as glycerophosphocholine and digalactosyldiacylglycerols, were also observed in the 2D NMR spectra, supporting the presence of membrane-related lipid components, although their low abundance prevented an extensive quantitative evaluation. Total fatty acids in MOA amount to about one-tenth and total unsaturated fatty acids to about one-eighteenth of the levels found in *officinalis* ecotypes.¹² This widespread depletion is particularly pronounced in the unsaturated fractions, showing a fundamentally different membrane lipid composition.⁵⁸ Compared with published data on *Melissa o. officinalis*, this divergence is even more evident. Studies on Tunisian and Moroccan populations of this subspecies consistently report lipid fractions dominated by unsaturated fatty acids—especially linoleic acid, which typically accounts for 60–75% of total fatty acids—accompanied by moderate amounts of saturated fatty acids.^{53,59} These works also identify a sterol profile composed of β -sitosterol, stigmasterol, and campesterol, confirming the presence of multiple sterol species within the lipophilic matrix of *Melissa o. officinalis*. In contrast, MOA exhibits a drastic depletion across all fatty-acid classes and a near-exclusive accumulation of β -sitosterol, with stigmasterol completely absent. Furthermore, comprehensive GC/MS analyses such as those reported by Ieri *et al.* describe *Melissa o. officinalis* as chemically rich in lipophilic components, reinforcing that a lipid-poor and sterol-skewed profile such as that observed in MOA is not typical of the *officinalis* subspecies.⁶⁰

3.3. GC-MS analysis of volatile compounds

The chemical compositions of the essential oil and the volatile fraction are listed in Table 4. In the essential oil MOA, 38 components were identified, constituting 90.5% of the oil. In the volatile fraction 28 components were identified. The main components of the oil were hydrocarbon compounds (70.7%), with germacrene D (44.2%) as the dominant sesquiterpene. The oxygen compounds included 11 sesquiterpenes (17.3%), one diterpene (0.7%), one monoterpene (0.4%) and one non-terpenic compound (1.2%). Of the 54 individual compounds identified from the essential oil and volatile fraction, 51 were matched using EI-mass spectra and library retention indices. The main oil components besides germacrene D were α -cadinol (7.4%), β -caryophyllene (6.9%) and τ -cadinol (5.9%). Few studies on the volatile component of MOA have been found in the literature. The chemical characterization of essential oil from this subspecies (formerly known as *M. officinalis* L. var. *romana*) has been reported by Dawson *et al.*⁶¹ In a study,³ the composition of MOA samples from different stations in Sardinia (Italy) and in different growing seasons was analysed. Subsequently, Miceli *et al.* reported the essential oil composition of samples collected in southern Italy.⁶² A comprehensive survey of essential oils extracted from *Melissa officinalis* L. ssp. *altissima* collected in Tarquinia (Italy) was

carried out, showing a variability in the relative amounts of the main compounds depending on the extraction time and harvest period.⁶³ In particular, although some of the main compounds have already been reported, their relative quantities vary depending on when they are extracted, harvested and, most importantly, the place where the plant grows. In the first cited study,⁶¹ the major compound was β -cubebene (39%) and the minority β -caryophyllene (4%), totally disagreeing with Usai *et al.*,³ showing a high concentration of terpenes such as β -caryophyllene (17.3–27.5%), α -muurolene (17.5–38.8%) and caryophyllene oxide (3.3–7.9%). The EO obtained from the flowering tops of plants, in Italy, showed the predominance of caryophyllene (15.8%), cumene (14%), β -pinene (12%) and citral (12%), whereas other constituents presented in smaller quantities were menthol (6.5%), α -pinene (5.1%), spathulenol (4.8%) and γ -terpinene (4.0%).⁶² Comparing these results with the oils from Tarquinia, in particular with the August material, significant differences in the percentages of caryophyllene, β -pinene and spathulenol are observed.⁶³ Depending on the harvest period, chemical diversity was observed; GC/MS analysis showed that *cis*-caryophyllene and its oxide were the main constituents of EO. Other compounds were predominant only in certain months, such as germacrene D in July and September, and terpinene-4-ol in August and September. However, our results are in line with those from Sardinia.³ Variations in the amounts of the compounds reflect regional and seasonal differences, as some OE constituents are characteristic of specific phenological phases. In contrast, others are only associated with the reproductive period of the plant, as observed in the case of the samples from Italy. In the volatile fraction analyzed by HS-SPME, described for the first time, hydrocarbon compounds are dominant, accounting for 62.1% of the total composition, with 37.8% monoterpenes. The main components identified were (*E*)- β -ocimene (20%) and germacrene D (16.7%). Also detected was the presence of oxygenated molecules (1–11), mainly aliphatic aldehydes with relatively short linear chains, including isovaleraldehyde (4.4%), hexanal (5.2%) and 2-methylbutanal (3.3%), which were not found in the essential oil. Significant quantitative and qualitative differences were observed between OE and VF analysis for oxygenated and hydrocarbon compounds. Establishing a direct correlation between the HS-SMPE and hydrodistillation techniques proved difficult. The former is governed by phase equilibrium, whereas the latter involves the near-complete extraction of plant volatiles.

Thus, germacrene D and β -ocimene were the main volatile compounds identified in MOA. Germacrene D is a sesquiterpene, a volatile organic compound belonging to the terpene class, known to occur in numerous aromatic plants, derived from the mevalonate metabolic pathway common in terpenoid synthesis.⁶⁴ This compound is well-known for its antimicrobial, anti-inflammatory, and antioxidant properties.⁶⁵ It also plays a role in the defence of plants against pests and herbivores due to its ability to inhibit the development of pathogenic microorganisms. In cosmetics and pharmaceuticals, it is exploited for its skin-soothing and protective properties.



Table 4 Volatile components extracted by hydrodistillation (%EO) and by HS-SPME (% VF) of MOA

No. ^a	Compounds	RIalitt ^b	RIaexp ^c	RIpexp ^d	% Identification		Identification ^e
					EO	VF	
1	Butyraldehyde	605	607	880	—	1.1	MS, Ref
2	2-Methyl 1-propanol	605	605	1081	—	0.5	MS
3	Isovaleraldehyde	632	631	1082	—	4.3	MS
4	2-Methylbutanal	644	643	909	—	3.3	MS, Ref
5	Penten-3-ol	662	662	1144	—	1	MS
6	Furan-2-ethyl	691	690	960	—	0.5	MS, Ref
7	3-Methylbutanol	719	716	1177	—	2.1	MS
8	2-Methylbutanol	727	717	1178	—	1.8	MS
9	Hexanal	777	770	1308	—	5.2	MS
10	(<i>E</i>) Hex-3-en-1-ol	814	810	1360	—	1.3	MS
11	(<i>Z</i>)-Hex-3-en-1-ol	836	831	1380	—	2.8	MS
12	α-Thujene	922	922	1405	—	1.6	MS
13	Benzaldehyde	930	929	1536	0.2	—	MS
14	α-Pinene	931	931	1023	—	0.4	MS
15	Oct-1-en-3-ol	962	959	1447	1.2	3.5	MS
16	Sabinene	973	963	1124	0.4	3	MS
17	β-Pinene	978	969	1114	0.4	6.6	MS
18	Myrcene	978	976	1159	—	0.4	MS
19	P-Cymene	1015	1010	1272	0.1	0.8	MS
20	1,8-Cineole	1020	1020	1209	—	0.1	MS
21	(<i>Z</i>)-β-Ocimene	1029	1023	1234	0.7	5	MS
22	(<i>E</i>)-β-Ocimene	1040	1035	1252	3	20	MS
23	Terpinen-4-ol	1161	1161	1599	0.4	—	MS
24	α-Copaene	1379	1376	1490	1.4	—	MS
25	β-Bourbonene	1386	1379	1516	0.1	0.7	MS
26	β-Cubebene	1388	1383	1593	0.4	—	MS
27	β-Elementene	1388	1388	1590	2.6	1	MS
28	(<i>E</i>)-β-Caryophyllene	1421	1419	1597	6.9	3.2	MS
29	β-Copaene	1430	1426	1571	0.5	—	MS
30	α-Humulene	1455	1450	1666	1	—	MS
31	Alloaromadendrene	1462	1458	1642	1	1.8	MS
32	γ-Muurolole	1474	1471	1681	0.8	—	MS
33	Germacrene D	1479	1480	1716	44.2	16.7	MS
34	γ-Gurjunene	1472	1480	1704	—	0.3	MS
35	Ledene	1494	1492	1695	0.5	—	MS
36	α-Muurolole	1496	1488	1723	0.8	—	MS
37	Valencene	1494	1496	1719	0.2	—	MS
38	γ-Cadinene	1507	1507	1752	1.1	—	MS
39	<i>Cis/trans</i> calamenene	1512	1510	1816	0.2	—	MS
40	δ-Cadinene	1520	1516	1748	3.9	—	MS
41	<i>trans</i> -Cadina-1,4 Diene	1523	1524	1763	0.1	—	MS
42	α-Cadinene	1534	1530	1748	0.4	0.6	MS
43	<i>E</i> -Nerolidol	1553	1547	2038	0.5	—	MS
44	<i>trans</i> -Hydrate-sesquisabinene	1543	1550	1990	0.8	—	MS
45	Caryophyllenoxide	1578	1575	1975	0.4	—	MS
46	Zingiberenol	1606	1608	2108	0.7	—	MS
47	Zingiberen-2-ol	1613	1615	2192	0.4	—	MS
48	Epicubanol	1623	1622	2051	0.3	—	MS
49	τ-Cadinol	1633	1628	2185	5.9	—	MS
50	α-Cadinol	1643	1642	2227	7.4	—	MS
51	Oplopenol 1	1644	1644	2205	0.4	—	MS
52	14-Hydroxy-9- <i>epi-E</i> -caryophyllene	1658	1657	2300	0.2	—	MS
53	Eudesma-4(15)-7-dien-1-β-ol	1671	1666	2348	0.3	—	MS
54	<i>E</i> -Phytol	2096	2098	2605	0.7	—	MS
	Total identification %	90.5	89.6				
	Hydrocarbon compounds	70.7	62.1				
	Oxygenated compounds	19.8	27.5				
	Hydrocarbon monoterpenes	4.6	37.8				
	Oxygenated sesquiterpenes	17.3	0				
	Hydrocarbon sesquiterpenes	66.1	24.3				
	Other oxygenated compounds	2.5	27.5				

^a Compounds are listed in order of their elution from non-polar Rtx-1 column. ^b RIalitt: Retention indices for a non-polar column (R. P. Adams, 2007). ^c RIaexp: Retention indices determined experimentally on the non-polar Rtx-1 column. ^d RIpexp: Retention indices determined experimentally on the polar Rtx-wax column. ^e The contents were determined on the non-polar column Rtx-1 column. EO: essential oil, VF: volatiles sampled by SPME, Ref: compounds identified from commercial libraries.



β -Ocimene is a monoterpene, a volatile aromatic compound found in many plants, flowers, and fruits.⁶⁶ It imparts a fresh, floral aroma to many natural essences and displays antifungal, antimicrobial, and antioxidant activities, which support its application in aromatherapy and the formulation of natural fragrances. Moreover, this terpene contributes to plant signaling, enhancing pollinator attraction while deterring herbivorous insects.⁶⁶ In cosmetics and pharmaceuticals, β -ocimene is also valued for its soothing and protective qualities.

3.4. Antioxidant and neuroprotective activity of MOA hydroalcoholic extract

3.4.1. *In vitro* studies. In the present assay, the cytotoxic effects of hydroalcoholic extracts of MOA, in the concentration range 50–200 $\mu\text{g mL}^{-1}$, on the viability of the hypothalamic HypoE22 cell line under basal conditions were investigated. The cells were exposed to the MOA extract under basal conditions and were tolerated up to a concentration of 500 $\mu\text{g mL}^{-1}$, demonstrating good biocompatibility, see Fig. 1. In addition, hydroalcoholic extracts of MOA (50, 100, 200 and 500 $\mu\text{g mL}^{-1}$) were tested on the HypoE22 cell line, even in the presence of H_2O_2 , which is the reference pro-oxidant stimulus capable of reducing cell viability below the biocompatibility limit (70% viability compared to the control group), see Fig. 2. MOA extract effectively protected cells from cytotoxicity induced by 300 μM of hydrogen peroxide from a phytochemical concentration of 100 $\mu\text{g mL}^{-1}$, demonstrating good antioxidant activity. Only the lowest concentration (50 $\mu\text{g mL}^{-1}$) showed less protection from H_2O_2 cytotoxicity, due to the lower amount of the phytocomplex. On the other hand, all MOA extract concentrations (50, 100, 200 and 500 $\mu\text{g mL}^{-1}$) maintained the cell viability comparable with the control (Fig. 1). These findings are aligned with prior literature on *Melissa officinalis*, which reported a cytoprotective and antioxidant effect of its extracts in non-transformed cells exposed to oxidative stress. For example, in human umbilical vein endothelial cells (HUVECs), pre-treatment with a hydroalcoholic extract of lemon balm at 100–500 $\mu\text{g mL}^{-1}$ significantly improved viability after H_2O_2 challenge, concomitantly reducing hydroperoxides and increasing cellular antioxidant capacity (assessed by FRAP).⁶⁷ Similarly, an aqueous extract of *Melissa officinalis* was found to be non-cytotoxic to normal human keratinocytes (HaCaT) up to 1000 $\mu\text{g mL}^{-1}$, while selective cytotoxicity was observed in tumour cells (melanoma) only at the highest concentration tested, indicating a cell type-dependent effect of lemon balm extracts.⁶⁸ In various tumor cell lines, hydro-alcoholic or ethanolic extracts of *Melissa officinalis* have been reported to induce significant antiproliferative and cytotoxic effects, in some cases reducing cell viability to low percentages—though the magnitude of the effect strongly depends on cell type, extract preparation, and concentration/time of exposure.^{69–71}

3.4.2. *Ex vivo* studies. MOA extract (100 and 200 $\mu\text{g mL}^{-1}$) was tested in isolated samples of mouse cortex and hypothalamus. After excision, the mouse specimens were treated with LPS from *E. coli*, a pro-inflammatory stimulus, to assess their

potential protective effects on neuroinflammatory pathways. In this context, gene expression of TNF- α and NOS-2 was analysed. An LPS stimulus (50 $\mu\text{g mL}^{-1}$) induced their up-regulation in both the cortex and hypothalamus (Fig. 3). All tested extracts proved effective in reversing the increased expression of these two genes, suggesting potential protective effects on neuroinflammation. Furthermore, TNF- α and NOS-2 gene expression were even lower than in the control groups after administration of MOA extract at a concentration of 200 $\mu\text{g mL}^{-1}$ in cortical samples (Fig. 3A). TNF- α and NOS-2 are highly active pleotropic modulators of cell function that are generated in abundance during inflammation.⁷² These pro-inflammatory factors contribute to the accumulation of reactive oxygen species (ROS) and the dysregulation of oxidative stress, which in turn leads to neurodegenerative processes.⁷³ Particularly, TNF- α is identified as one of the most potent and early inflammatory mediators that can trigger a cascade response, including the transcription of other cytokines, such as IL-6, to apoptosis when overproduced. Therefore, intervening at an earlier stage of the inflammatory process could potentially improve the prevention of neurodegenerative and cognitive disorders.^{74,75} The effect of this aromatic plant could be related to its phenolic compounds, which appear to possess a dose-dependent protective effect by suppressing proinflammatory cytokines.⁷⁶ Furthermore, the effects of MOA extract on LPS-induced BDNF levels, a neurotrophic factor that is strongly reduced in proinflammatory states of brain tissue, were investigated. Treatment with the extract was effective in both tissues tested in counteracting LPS-induced down-regulation of the BDNF gene, demonstrating a neuroprotective effect. The higher concentration of MOA extract (200 $\mu\text{g mL}^{-1}$) reversed the LPS-induced decrease in BDNF gene expression. Indeed, BDNF levels were higher after MOA treatment than in vehicle-incubated hypothalamic tissue (Fig. 3). BDNF is a major neurotrophic factor that plays a crucial role in neuronal development and survival, as well as in the development of neuronal processes and synaptic plasticity. Low levels of BDNF expression are closely associated with the pathophysiology of several neurological disorders, including Alzheimer's, Parkinson's and Huntington's disease,^{77,78} and may be related to cognitive impairments.⁷⁹ The results of the present study indicate that MOA may have the potential to positively regulate BDNF expression levels in the brain and enhance neuronal plasticity. A previous study investigated the effect of a hydroalcoholic extract of *Melissa officinalis* on learning and memory.⁸⁰ It was observed that BDNF gene expression increased and was associated with the presence of polyphenols in the hydroalcoholic extract. In particular, this effect could be attributed to the high amount of rosmarinic acid, a characteristic of the Lamiaceae family, for which other studies have also demonstrated a capability to restore synaptogenesis and neurogenesis, typically compromised in AD progression.^{81,82} At the same time, MOA extracts (50, 100 and 200 $\mu\text{g mL}^{-1}$) were tested in isolated samples of pre-frontal cortex from mice; the cortical specimens were treated with β -amyloid. This pro-inflammatory stimulus was chosen to evaluate the potential ameliorative



activities exerted by MOA on specific neuroinflammatory pathways involved in certain neurodegenerative disorders such as Alzheimer's disease (AD). In this context, the presence of β -amyloid stimulus (0.05 mg mL^{-1}) increased the expression of AChE and IL-6 genes in cortical tissue (Fig. 4). All tested extracts proved effective in reversing the increased expression of these two genes, suggesting potential protective effects also on specific neuroinflammation. Gene expression of AChE, the primary target in AD, was even lower than that of the control group after administration of MOA extracts in cortical samples. A previous study evaluated the MOA extract using an acetylcholinesterase inhibition assay, which found that it inhibited the enzyme in a time- and dose-dependent manner.⁸³ In the assay of the present study, the MOA extracts at concentrations of 100 and $200 \mu\text{g mL}^{-1}$ also reversed the IL-6 gene expression, which was increased by β -amyloid, reaching values lower than those observed in the vehicle-incubated cortex. Treatment with the highest concentration of MOA extract ($200 \mu\text{g mL}^{-1}$) reduced IL-6 gene expression even under basal conditions. IL-6 represents a potential point of convergence between memory dysfunction and metabolic alterations in AD.⁸⁴ IL-6, together with TNF- α , is documented to increase AD-like tau phosphorylation.^{85–87} This scientific evidence suggests that inflammation mediated by inflammatory cytokines, such as IL-6 and TNF- α , is promoted from a bystander to a key role in the pathogenesis of AD. Thus, targeting pro-inflammatory IL-6 signalling may be a strategy to alleviate memory impairment and metabolic changes in AD. Consistent with our findings, Thingore *et al.* (2021) reported that rosmarinic acid—a major phenolic compound in MOA extract—exerts neuroprotective effects by downregulating IL-6 and TNF- α levels, as well as AChE activity.⁸⁷ In this context, the hydroalcoholic extract of MOA may exert neuroprotective effects by modulating oxidative stress-mediated inflammatory pathways and counteracting neurotoxic insults such as β -amyloid, a key factor in AD pathogenesis. This activity is likely supported by the extract's enhanced antioxidant capacity, which may be attributed to hydroxycinnamic acid derivatives such as rosmarinic, caffeic, caftaric, and chlorogenic acids. These compounds, derived from tyrosine, point to the involvement of the shikimate/phenylpropanoid pathway in MOA.²⁷ In plants, L-tyrosine acts as a key precursor of such phenolics, with its metabolic flux closely linked to stress responses. Notably, tyrosine metabolism is also associated with the activation of tyrosine kinase-mediated signalling, which in mammalian systems plays a crucial role in neuronal survival and synaptic plasticity.^{88–90} Importantly, several of these polyphenols are capable of crossing the blood-brain barrier (BBB) in either native or conjugated forms, enabling them to exert direct antioxidant and anti-inflammatory effects within the central nervous system.^{20,91} For instance, chlorogenic and caffeic acids have been shown to reach detectable brain concentrations following dietary intake and to attenuate both neuroinflammation and oxidative stress, supporting their contribution to the neuroprotective effects observed in this study.^{92,93} Such enhanced bioactivity is likely the result of synergistic interactions among the various poly-

phenolic and terpenoid constituents of the extract.^{91,94} These compounds not only scavenge ROS directly but also modulate copper-induced redox cycling, a key driver of sustained ROS production.⁹⁵ The overall lipophilic profile of MOA—characterized by high β -sitosterol content and low unsaturated fatty acids (Table 3)—suggests potential membrane-stabilizing properties,^{96,97} which could complement the antioxidant effects of the abundant hydroxycinnamic acids detected in the hydroalcoholic extract. Phytosterols like β -sitosterol are known to incorporate into neuronal membranes and enhance the bioavailability of polyphenols in brain tissue.^{96–98} In our previous study on the dandelion *Taraxacum officinale* spontaneous mountain ecotype (MSE),²⁰ identical experimental protocols were applied for the preparation of the hydroalcoholic extract and the analysis of *ex vivo* gene expression (TNF- α , NOS-2, BDNF) in the prefrontal cortex and hypothalamus of LPS-stimulated mice. Using the same standardized methodology, MOA hydroalcoholic extracts ($100\text{--}200 \mu\text{g mL}^{-1}$) mirrored the modulation of LPS-induced neuroinflammation observed with MSE, reducing TNF- α and NOS-2 expression to baseline or below in both brain regions, while upregulating BDNF to restore neurotrophic balance. These effects could be attributed to their shared high content of hydroxycinnamic acids (caftaric $454 \text{ mg } 100 \text{ g}^{-1} \text{ DW}$ in MOA; elevated levels in MSE). Distinctively, MOA also extended neuroprotection to cortical tissues stimulated with β -amyloid (0.05 mg mL^{-1}), significantly decreasing the expression of IL-6 and AChE at $50\text{--}200 \mu\text{g mL}^{-1}$ ($p < 0.01$ compared to β -amyloid alone), suggesting enhanced efficacy in counteracting neuroinflammatory and neurodegenerative mechanisms beyond the broader anti-inflammatory profile shared with dandelion MSE.

Throughout this study, biological interpretations are strictly limited to the results obtained with the hydroalcoholic extract, whereas the analysis of the non-polar fraction is intended solely to provide a comprehensive chemical and comparative characterisation of the species.

4. Conclusion

This study provides the first comprehensive metabolomic and biological characterization of *Melissa officinalis* subsp. *altissima* (MOA), integrating high-resolution NMR, GC-MS, and a range of *in vitro* and *ex vivo* assays. MOA exhibited a distinctive phytochemical profile highlighted by exceptionally high levels of rosmarinic acid along with abundant chlorogenic and caftaric acids, compounds well recognized for their potent antioxidant and neuroprotective properties. Remarkably, the lipophilic fraction was notable for a high content of β -sitosterol, coupled with a marked depletion of total and unsaturated fatty acids, pointing to a unique membrane-stabilizing phytosterol profile rarely reported in lemon balm species. The volatile fraction was dominated by germacrene and (*E*)- β -ocimene, molecules linked to antimicrobial and anti-inflammatory activities. Biological assays confirmed that the hydroalcoholic extract, characterised by a complex phytochemical composition, trans-



lates into functional antioxidant and neuroprotective effects. Hydroalcoholic extracts demonstrated a strong antioxidant capacity by protecting hypothalamic cells from hydrogen-peroxide-induced cytotoxicity, significantly down-regulating the pro-inflammatory mediators TNF- α and NOS-2, and increasing BDNF levels beyond control values. In cortical tissue challenged with β -amyloid, the extracts suppressed IL-6 and AChE expression, thus indicating a neuroprotective effect that may be related, at least in part, to the content of hydroxycinnamic acids and other bioactive constituents found in MOA acting within a complex phytochemical matrix, likely through synergistic mechanisms rather than the action of single compounds. Taken together, these findings highlight MOA as a promising source of bioactive compounds with antioxidant, anti-inflammatory, and neuroprotective properties. Its unique metabolic signature, particularly the enrichment in phytosterols and the distinctive volatile profile, supports its potential use as a functional ingredient for nutraceutical, pharmaceutical, and food applications, while underscoring the value of wild aromatic plants as reservoirs of health-promoting metabolites.

However, this study has some limitations that should be acknowledged. Notably, the findings are based on *in vitro* and *ex vivo* assays, and no *in vivo* validation or bioavailability assessment has been performed. Therefore, while the data support potential functional benefits, causal relationships between individual compounds and the observed biological effects cannot be definitively established within the scope of the present study and should be further investigated in future studies with a dedicated bioactivity-guided fractionation and *in vivo* validation to confirm the physiological relevance and therapeutic potential of MOA.

Author contributions

Conceptualisation, D. A., and C. I.; data curation, D. A., F. M., and C. I.; formal analysis, D. A. and M. E. A.; funding acquisition, L. M., A. M. and C. I.; investigation, D. A., F. M., M. E. A., A. C., and C. D. S.; methodology, D. A. and C. I.; project administration, C. I., L. M., and A. M.; resources, L. M., C. F., and C. I.; supervision, C. I., and A. M.; validation, C. I., A. C., L. M., C. F.; visualisation, D. A., F. M., and C. I.; writing – original draft, D. A. and F. M.; writing – review and editing, D. A., and C. I. All authors have read and agreed to the published version of the manuscript.

Conflicts of 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.

Data availability

The data that support the findings of the study are available from the corresponding author upon reasonable request. For further information or to request access to the datasets, please contact cinzia.ingallina@uniroma1.it.

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