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Bioprospecting Italian biodiversity for identifying potential neuroprotective agents

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Neurodegenerative diseases represent a growing global health burden. With current treatments limited to symptom management, natural biodiversity offers a promising source of novel, multi-target compounds. Bioprospecting thus emerges as a sustainable strategy for discovering innovative neuroprotective therapies, underscoring the importance of preventing biodiversity for future drug discovery. An initial metabolomic screening of Italian plants was performed to support a bioprospective approach, in which 27 plant extracts were evaluated for their ability to inhibit acetylcholinesterase and tyrosinase, targeting mechanisms involved in neurodegenerative diseases. *Adenophora liliifolia* and *Carissa macrocarpa* emerged as the most promising species through multi-parameter screening. Metabolomic analysis showed that most identified metabolites exhibit activity against acetylcholinesterase and/or tyrosinase, supporting observed bioactivities. These extracts represent potential candidates for developing therapies against neurodegenerative diseases. These findings highlight systematic bioprospecting as a powerful strategy for identifying novel candidates with neuroprotective activity. By integrating traditional knowledge with modern metabolomic and biochemical approaches, this study demonstrates how underexplored plant biodiversity, such as *Adenophora liliifolia* and *Carissa macrocarpa*, can yield promising therapeutic candidates for targeting the complex multi-target mechanisms underlying neurodegenerative disorders.

Keywords Biodiversity, *Carissa macrocarpa*, *Adenophora liliifolia*, Tyrosinase inhibitors, Acethylcolinesterase inhibitors, Secondary metabolites, Taxonomic approach

Abbreviations

AChE	Acetylcholinesterase
AUC	Area under the curve
CID	Collision-induced dissociation
DDA	Data-dependent acquisition
DMSO	Dimethyl sulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
ESI	Electrospray ionization
FRS	Free radical scavenging
GAE	Gallic acid equivalent
GBD	Global burden disease
HLB	Hydrophilic–lipophilic balance
ID	Identificative
MeOH	Methanol
NBFC	National biodiversity future center
ORAC	Oxygen radical absorbance capacity
PCA	Principal component analysis
qTOF	Quadrupole time-of-flight
ROS	Reactive oxygen species

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SPE	Solid phase extraction
TE	Trolox equivalent
TPC	Total phenolic compounds
Tyr	Tyrosinase
UPLC-ESI-HRMS	Ultra high performance liquid chromatography–electrospray ionization–high resolution mass spectrometry

Neurodegenerative diseases are marked by the gradual loss and dysfunction of neurons, resulting in significant deficits in both cognition and movement. These conditions represent some of the most prevalent neurological disorders affecting middle-aged and elderly populations, with an escalating global impact [1]. According to the World Health Organization, approximately 50 million people were affected by dementia in 2019, while by 2021, around 3.4 billion individuals (43.1% of the global population) were living with some form of neurological conditions, resulting in over 11 million deaths annually [2]. Although these disorders affect all demographics, aging remains the most critical risk factor. In Europe, for example, the prevalence of dementia in individuals aged 65–69 is approximately 1.6%, rising steeply to 11% among those aged 85–89 [3]. These statistics underscore the urgent need for intensified research, early intervention, and preventive strategies, especially as neurological disorders have now become the leading global cause of disease burden, according to the Global Burden of Disease Study (GBD 2021) [2]. Despite advances in understanding the pathophysiology of neurodegenerative disorders, available treatments remain limited, primarily focusing on symptom management rather than halting or reversing the progression of the disease, thus highlighting the pressing necessity for innovative therapeutic approaches.

Given the absence of effective disease-modifying therapies, the chemical diversity offered by nature can be a valuable source of novel compounds with potentially innovative and multi-target mechanisms of action. Nature has long served as a reservoir of bioactive compounds suitable for drug discovery and development. Despite the use of approximately 70,000 plant species in traditional medicine, only about 15% have been scientifically investigated for their medicinal properties [4]. Nevertheless, nearly one-third of the drugs currently on the market are either derived directly from plants or synthesized from plant-based molecules [5]. This largely untapped reservoir of bioactive substances may be especially relevant in the context of neurodegenerative diseases. Bioprospecting, or biodiversity prospecting, involves the systematic search within biodiversity for organisms (plants, animals, fungi, bacteria, etc.) with potential application in research or industry, and has emerged as a powerful approach to uncover new therapeutic agents that may hold promise for the prevention or treatment of neurodegenerative diseases [6]. Integrating traditional knowledge with modern scientific methodologies may pave the way toward novel, effective solutions to one of the most pressing health challenges of our time. Biodiversity, or biological diversity, refers to the vast array of life forms that exist in nature, shaped over millions of years by evolutionary processes, forming the foundation of ecological balance and directly supporting human well-being [7]. Preserving biodiversity is not only a moral and ecological imperative, but also essential for sustainable development and the responsible use of natural resources.

Recognizing the crucial link between biodiversity and human health, especially in the discovery of natural compounds with therapeutic potential, this study focuses on the exploration and valorization of Italian biodiversity. The research was carried out in collaboration with the National Biodiversity Future Center (NBFC) (Home [online] - NBFC. https://www.nbfc.it/), which leads a nationwide Italian initiative to promote the value of Italian biodiversity [8]. A bioprospective approach has been applied to systematically explore natural resources to identify compounds with potential applications in the field of neurodegenerative disorders. Specifically, this research focuses on identifying plant extracts able to inhibit acetylcholinesterase (AChE) [9] and tyrosinase (Tyr) [10,11]. AChE is a serine hydrolase essential for the breakdown of acetylcholine, a neurotransmitter vital for memory, attention, learning, and sensory processing. In neurodegenerative conditions, the degeneration of cholinergic neurons leads to a decrease in acetylcholine availability [12], impairing synaptic transmission, and causing cognitive decline. AChE inhibitors slow down the breakdown of acetylcholine, helping maintain cholinergic function and alleviating cognitive symptoms. Consequently, AChE inhibitors have been extensively studied and clinically associated with neurodegenerative conditions such as Alzheimer's disease. Conversely, Tyr is the key enzyme responsible for melanin production in skin cells and for neuromelanin synthesis in *substantia nigra* dopaminergic neurons. Progressive accumulation of neuromelanin in dopaminergic neurons contributes to their degeneration. Additionally, tyrosinase activity in dopamine metabolism can generate dopamine metabolites and reactive oxygen species (ROS), contributing to oxidative stress and dopaminergic neuron loss, both hallmarks of Parkinson's disease [10]. Therefore, Tyr inhibitors have been recently proposed as a therapeutic target to counteract neurodegeneration by reducing neuromelanin and ROS production, thus preserving dopaminergic function and mitigating cognitive symptoms. Through this dual-target screening approach, this study aims to advance the discovery of natural compounds with therapeutic relevance for neurodegenerative diseases. This research ultimately seeks not only to identify potential neuroprotective candidates against neurodegeneration but also to highlight the value of systematic bioprospecting as a strategic and sustainable approach for discovering novel therapeutic leads from Italy's rich and underexplored botanical heritage. The pursued approach couples the rational chemotaxonomic approach for plant selection and a systematic bioactivity evaluation to increase the possibility to achieve our goal.

Materials and methods

Plant material

The NBFC plant species investigated in this work were retrieved from three main origins: botanical gardens (Padua and Pavia, Italy), the institutional nursery of Veneto Agricoltura (Centre for biodiversity and outside forests activities; Montecchio Precalcino, Vicenza, Italy), and sampling in the open field. All samplings were

carried out in accordance with relevant guidelines and regulations. Fresh plant material (leaves and eventually herbaceous stems) was collected in triplicate, according to the number of individuals available for each species. When more than one individual was available for each replicate, pooling of the collected organs was performed. For species sampled in the open field, plants were gathered from three separate areas representing distinct plant populations to serve as biological replicates, minimizing the risk of collecting genetically similar individuals. Sampling details of the investigated species are reported in Table 1.

Extraction protocol

Leaves and young stems of fresh plant material were harvested and immediately frozen in liquid nitrogen, ground to powder using an A11 basic analytical mill (IKA-Werke, Staufen, Germany), and stored at -80 °C. The resulting frozen powder was extracted with 10 volumes (w/v) of 100% LC-MS grade methanol (Honeywell, Seelze, Germany), vortexed vigorously for 30 s, and sonicated on ice for 10 min at 40 kHz in an ultrasonic bath Sonica® Ultrasonic Cleaner (SOLTEC, Milan, Italy). Later, the extracts were centrifuged at 14,000x *g* for 10 min at 4 °C. Supernatants were divided into 1 mL aliquots, each corresponding to 100 mg of fresh plant tissue, then dried using a Speed-Vac system (Heto-Holten, Frederiksborg, Denmark) and stored at -20 °C.

Extract analysis

UPLC-ESI-HRMS analysis of plant extracts and metabolite annotation

Extracts were diluted (1:10 to 1:200) with LC-MS grade water (Honeywell, Seelze, Germany) and filtered with 0.22 µm Minisart RC4 filters (Sartorius-Stedim Biotech, Göttigen, Germany). Analyses were performed using an Acquity I Class UPLC system (Waters, Singapore) equipped with a BEH C18 column (Waters), coupled online to a photodiode array (PDA) detector (UV-Vis range 180–800 nm) and a Xevo G2-XS qTOF mass spectrometer (Waters) featuring an electrospray ionization (ESI) source. Sample injections were carried out via a temperature-controlled autosampler maintained at 8 °C, with a constant flow rate of 0.350 mL/min. The mobile phase consisted of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). The gradient elution program was as follows: 0–1 min, 1% B; 1–10 min, 1–40% B; 10–13.5 min, 40–70% B; 13.5–15 min, 70–90% B; 15–16.5 min, 90–100% B; 16.5–20 min, 100% B; 20–20.1 min, 100–1% B; and 20.1–25 min, 1% B to re-establish initial conditions.

Species	Family	Place of sampling or supplier	*n**	Organs
Acanthus mollis L.	Acanthaceae	Padua Botanical Garden	3(1)	Leaves
Adenophora liliifolia (L.) Ledeb. ex A.DC.	Campanulaceae	Veneto Agricoltura Nursery	6(2)	Leaves and stems
Allium angulosum L.	Amaryllidaceae	Veneto Agricoltura Nursery	6(2)	Leaves
Allium lusitanicum Lam.	Amaryllidaceae	Veneto Agricoltura Nursery	6(2)	Leaves
Aloysia citrodora Paláu	Verbenaceae	Padua Botanical Garden	1	Leaves
Althaea officinalis L.	Malvaceae	Veneto Agricoltura Nursery	6(2)	Leaves
Aquilegia atrata (L.) W.D.J.Koch	Ranunculaceae	Veneto Agricoltura Nursery	6(2)	Leaves
Beta vulgaris L.	Amaranthaceae	University of Verona	9(3)	Leaves
Carissa macrocarpa (Eckl.) A.DC.	Apocynaceae	Padua Botanical Garden	3(1)	Leaves
Castanea sativa Mill.	Fagaceae	Padua Botanical Garden	1	Leaves
Dianthus superbus L. subsp. *superbus*	Caryophyllaceae	Veneto Agricoltura Nursery	6(2)	Leaves and stems
Echium vulgare L.	Boraginaceae	Padua Botanical Garden	12(4)	Leaves
Eryngium maritimum L.	Apiaceae	Veneto Agricoltura Nursery	6(2)	Leaves
Euonymus europaeus L.	Celastraceae	Padua Botanical Garden	1	Leaves
Gratiola officinalis L.	Plantaginaceae	Veneto Agricoltura Nursery	6(2)	Leaves and stems
Inula salicina L.	Asteraceae	Veneto Agricoltura Nursery	6(2)	Leaves and stems
Lythrum salicaria L.	Lythraceae	Veneto Agricoltura Nursery	6(2)	Leaves and stems
Malva alcea L.	Malvaceae	Veneto Agricoltura Nursery	6(2)	Leaves
Oxalis dillenii Jacq.	Oxalidaceae	Pavia Botanical Garden	3(1)	Leaves and stems
Petasites paradoxus (Retz.) Baumg.	Asteraceae	Veneto Agricoltura Nursery	6(2)	Lleaves
Philadelphus coronarius L.	Hydrangeaceae	Padua Botanical Garden	1	Leaves
Russelia equisetiformis Schltdl. & Cham.	Plantaginaceae	Padua Botanical Garden	3(1)	Leaves
Salvia pratensis L.	Lamiaceae	Veneto Agricoltura Nursery	6(2)	Leaves
Sicyos angulatus L.	Cucurbitaceae	Banks of the Po River (Portalbera, Pavia)**	15(5)	Leaves and stems
Succisa pratensis Moench	Dipsacaceae	Veneto Agricoltura Nursery	6(2)	Leaves
Typha laxmannii Lepech.	Typhaceae	Veneto Agricoltura Nursery	6(2)	Leaves
Wisteria sinensis (Sims) DC.	Fabaceae	Padua Botanical Garden	1	Leaves

Table 1. Sampling details of the NBFC plant species investigated. * *n* = number of plants from which the plant material was collected (in brackets, the number of individuals pooled per each biological replicate). ** The collection of *Sicyos angulatus* does not fall under IUCN restrictions.

The ESI ion source operated with the following settings: capillary voltage at 0.8 kV, sampling cone voltage at 40 V, source offset at 80 V, source temperature at 120 °C, and desolvation temperature at 500 °C. The cone gas and desolvation gas flow rates were 50 L/h and 1000 L/h, respectively. Nitrogen was used as both the nebulizing and desolvation gas, while argon served as the collision gas for collision-induced dissociation (CID). Mass spectrometry data were collected in continuum mode across a mass range of 50–2000 m/z, in both positive and negative ionization modes, using a fixed collision energy of 35 V. MS/MS data were collected with Fast-DDA analysis of 1:10 diluted samples, setting a collision energy ramp of 10–40 eV (20–80 eV for high masses). Instrument stability over time was monitored by periodically analyzing, throughout the sample sequence, a mixture of six authentic standards prepared at different concentrations (1 ng/µL chlorogenic acid, daidzin, naringenin; 2 ng/µL phenylalanine; 3 ng/µL gallic acid, quercetin, dihydroartemisinic acid). The accuracy of the qTOF mass spectrometer was checked constantly during each analysis by infusing a solution of 100 pg/µL leucine-enkephalin at a flow rate of 10 µL/min and generating a signal of 556.2771 m/z in positive mode and 554.2615 m/z in negative mode. Data acquisition and processing were performed using MassLynx v4.2 software and Progenesis QI software (both by Waters).

Metabolite annotation was performed by comparing accurate mass (deduced from the m/z ratio and isotopic pattern), retention time, and fragmentation pattern (from the Fast-DDA analysis) with a proprietary library of authentic standard compounds, with an in silico proprietary library of plant compounds, and with scientific literature and public databases (e.g., Pubchem, MassBank; MassBank of North America). A mass error threshold of 5 ppm was considered reliable for annotation in negative ionization conditions. Characteristic neutral losses and diagnostic fragments commonly observed for plant metabolites supported the putative annotations when no other information was available. All annotations were assigned confidence levels according to MSI guidelines (Online Resource 1).

SPE chlorophyll removal method

A Solid Phase Extraction (SPE) chlorophyll removal method was applied, conducted using a manifold and a vacuum pump [13]. The cartridges Waters OASIS HLB 1 cc were mounted on the manifold and conditioned with methanol. 500 µL of extract at 5 mg/mL in MeOH were loaded into the cartridge, the vacuum was applied, and 1 mL of fresh MeOH was used for elution. The derived extract was evaporated under reduced pressure. The procedure has been applied to multiple extracts at a time.

Total phenolic compounds (TPC)

Total Phenolic Content[14] was assessed spectrophotometrically. 2 N Folin-Ciocâlteu reagent was diluted 1:10 in distilled water, along with a stock solution of gallic acid at six different concentrations in methanol (from 0.125 mM to 4.5 mM). For the assay, 100 µL of the diluted Folin-Ciocâlteu reagent was mixed with 80 µL of sodium carbonate solution and 10 µL of the extract (1 mg/mL in MeOH) or calibration standard. The plate was then incubated at room temperature for 30 min in the dark. After the incubation period, the absorbance of the wells was measured at 765 nm. The total phenolic content was quantified using the gallic acid standard calibration curve. The measured concentration of total phenolics was expressed as milligrams of gallic acid equivalents per mL of extract (mg GAE/mL).

All tests were performed in triplicate.

Preliminary bioactivity screening

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

The antioxidant activity of the extracts was assessed spectrophotometrically [15] using a multi-well reader. Specifically, 10 µL of the extract (1 mg/mL in methanol) was mixed with 190 µL of a 0.15 mM DPPH methanolic solution. For the blank solution, 190 µL of methanol was mixed with 10 µL of the extract, while for the control solution, 190 µL of a 0.15 mM DPPH solution was mixed with 10 µL of methanol. The plate was incubated in the dark at room temperature for 30 min, after which the absorbance was measured at 515 nm using a microplate reader. The percentage of radical scavenging activity was calculated as follows:

$$\% \text{ scavenging activity } = [(\text{Abs control} - (\text{Abs sample} - \text{Abs blank})) \ / \ \text{Abs control}] \times 100$$

All tests were performed in triplicate.

Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay [16,17] was conducted using a multi-well plate reader. Each well received 25 µL of extract (5 mg/mL in methanol) or 25 µL of Trolox at decreasing concentrations, ranging from 800 µM to 25 µM (eight dilutions in 10 mM phosphate buffer, pH 7.4). After this, 150 µL of fluorescein (10 µM in phosphate buffer) was added to each well. The plate was then incubated at 37 °C for 30 min in a microplate reader without shaking. Following the incubation, 25 µL of AAPH (240 nM) was added, and a 120-minute kinetic analysis began, with fluorescence readings taken every 20 s (Excitation: 485 nm, Emission: 520 nm).

Calculate AUC and Net AUC following the equations herein reported:

$$\text{AUC} = (R_1/R_1) + (R_2/R_1) + (R_3/R_1) + \ldots + (R_n/R_1)$$

Where R_1 is the fluorescence reading at the initiation of the reaction, and R_n is the last measurement.

The antioxidant activity was determined by calculating the net AUC:

$$\text{Net AUC} = \text{AUC sample} - \text{AUC blank}$$

Trolox equivalent values were obtained by interpolating the net AUC values of the extract with the standard calibration curve of Trolox.

All tests were performed in triplicate.

Tyrosinase inhibition assay

In brief, the activity of mushroom tyrosinase [18](in phosphate buffer 50mM, pH 6.8 solution at 1000 U/mL) was monitored colorimetrically using L-tyrosine as a substrate (at 0.5 mg/mL in distilled water) and a sample solution as an inhibitor (5 mg/mL in MeOH) for dopachrome formation. The reaction mix of the test well was composed of 60 µL of L-tyrosine solution, 15 µL of enzyme solution, 30 µL of extract, and 195 µL of buffer to reach a total of 300 µL per well. The plate was left to incubate in the dark at room temperature for 30 min; later, the absorbance was measured at 480 nm on a microplate reader. The inhibition activity of the extracts is calculated by applying the following equation:

$$\%\ \text{of inhibition} = [1 - ((\text{Abs}_{\text{sample}} - \text{Absblank})/\text{Abs}_{\text{control}})] \times 100$$

All tests were performed in triplicate.

Acetylcholinesterase inhibition assay

The anti-acetylcholinesterase activity[19] was assessed colorimetrically using an enzyme solution (0.22 U/mL in TRIS-HCl 20 mM pH 7.5), acetylcholine iodide (0.075 M in distilled water) as substrate, DTNB (19.8 mg and 7.5 mg of $NaHCO_3$ in 5mL of phosphate buffer 0.1 M pH 7) and a sample solution as an inhibitor (5 mg/mL in DMSO). The reaction mix of the test well was composed of 206 µL of phosphate buffer 0.1 M pH 8, 3 µL of extract to test, 30 µL of DTNB solution, and 5 µL of enzyme solution, and left to incubate at room temperature in the dark for 5 min. After incubation, the reaction mix was divided into two aliquots of 98 µL; one was added with 2 µL of phosphate buffer and considered as a blank, and the other was added with 2 µL of acetylthiocholine solution and considered as a test well. The absorbance of the seeded wells was measured at 412 nm at different timepoints (0, 2, 5, and 8 min), and the inhibition activity is calculated by applying the following equation:

$$\%\ \text{of inhibition} = [(\text{Slope}_{\text{Control}} - \text{Slope}_{\text{Inhibitor}})/\text{Slope}_{\text{Control}}] \times 100$$

All tests were performed in triplicate.

Statistical analysis

Biological activity data are reported as mean ± standard deviation (SD) from three independent experiments. Statistical analysis was performed using GraphPad Prism 3 software (Boston, MA). PCA analysis was carried out in Python 3.12 using the RDKit, scikit-learn, pandas, and NumPy modules, and visualized using Matplotlib.

Results

Plant species analysis and selection

The initial phase of this study focused on selecting representative plant material through a two-step approach. First, Italian plant biodiversity was surveyed to identify the most represented botanical families. Subsequently, metabolomic studies were conducted to prioritize species known to produce a wide range of secondary metabolites (Fig. 1). This strategy aimed to select a manageable number of plants that would both represent the studied flora and provide extracts covering broad chemical space.

This preliminary survey based on chemotaxonomic data led to the selection of 27 species for comprehensive investigation (Table 1). Leaves (eventually with herbaceous stems) were harvested and subjected to ultrasound-

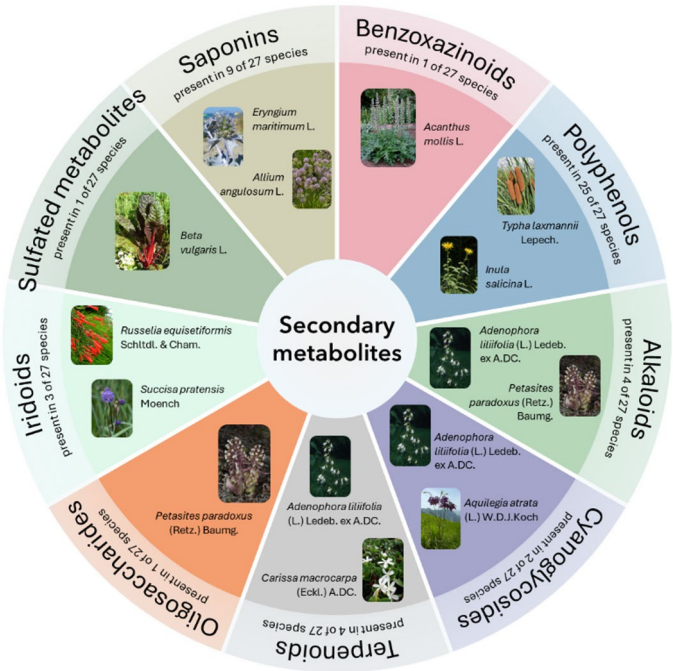


Fig. 1. Class of secondary metabolites identified in the considered extracts and examples of producing plants.

assisted extraction using methanol as solvent. This method was selected for its compatibility with high-throughput screening protocols [20–22]. Preliminary metabolomic profiling was obtained using UPLC-ESI-HRMS. Preliminary metabolomic profiling via UPLC-ESI-HRMS revealed substantial phytochemical diversity among the selected species. As anticipated from sampling across phylogenetically distinct plant families with varying biosynthetic capacities, the extracts exhibited markedly different profiles of accumulated specialized metabolites. The main findings obtained thanks to this phytochemical characterization are summarized in Table 2. It should be underlined that several selected species had received limited or no prior phytochemical investigation. While polyphenols and terpenoids emerged as the most prevalent metabolite classes, numerous compounds remained unidentified due to the insufficient supporting literature.

Following the characterization, all extracts were subjected to chemical and enzymatic bioactivity screenings. Specifically, we evaluated the total phenolic content (TPC) and antioxidant activity [28], together with inhibitory activity on acetylcholinesterase (AChE) and tyrosinase (Thyr), enzymes critically implicated in neurodegenerative processes [10,29–31].

Total phenolic compounds (TPC) and free radical scavenging activity

TPC determination and antioxidant activity evaluation have been performed both before and after the application of the purification protocol designed to remove chlorophylls from plant extracts[13]. Chlorophyll interference can compromise assay reliability. In accordance with both our previously published findings and current results, chlorophyll removal generally increases both TPC amounts and antioxidant activity in treated extracts (Fig. 2). Based on this evidence, subsequent analyses focused only on the extracts without chlorophylls to ensure data reliability.

Euonymus europaeus (EuEu) and *Philadelphus coronarius* (PhCo) displayed the highest antioxidant activities at 93% ± 3.2% and at 94% ± 19.6%, respectively, as measured by DPPH free radical scavenging (%FRS). While 11 extracts showed antioxidant activity below 70%, only five extracts exhibited %FRS activity higher than 90% (PhCo, EuEu, AcMo, SuPr, and InSa) (Fig. 2) designating them as the most promising candidates based on this parameter.

Regarding phenolic content, *Lythrum salicaria* (LySa) and *Petasites paradoxus* (PePa) displayed the highest TPC values at 39.8 ± 0.5 and 35 ± 1.7 gallic acid equivalents (mg GAE/mL), respectively. Conversely, *Allium angulosum* (AlAn) and *Dianthus superbus* (DiSu) registered the lowest values (close to 0 mg GAE/mL). Notably, while metabolomic analyses identified glycosylated flavones in *Dianthus superbus* (DiSu), this extract showed minimal phenolic content by Folin-Ciocâlteu assay. This discrepancy likely reflects steric hindrance imposed by glycosylation, which can prevent flavonoid aglycones from reacting with Folin-Ciocâlteu reagent, as already reported in the literature[32]. TPC distribution proved to be more variable than %FRS activity. Six extracts with GAE values higher than 25 mg GAE/mL (LySa, PePa, SaPr, TyLa, and AcMo) were identified as the most promising based on phenolic content (Fig. 3).

Acetylcholinesterase and tyrosinase inhibition activity

Anti-AChE and anti-Tyr activities were assessed to evaluate the neuroprotective potential. All chlorophyll-free extracts were tested at a concentration of 500 µg/mL.

Anti-AChE activity ranged from 5% ± 0.2% (AcMo) to 40% ± 0.4% (ErMa). The five most potent AChE inhibitors, all exceeding 30% inhibition, were *Eryngium maritimum* (ErMa, 40% ± 0.4%), *Carissa macrocarpa* (CaMa, 38% ± 1.9%), *Sicyos angulatus* (SiAn, 32% ± 0.3%), *Aloysia citrodora* (AlCi, 32% ± 0.6%), and *Adenophora liliifolia* (AdLi, 31% ± 0.6%). Except for ErMa, which exhibited low antioxidant activity and phenolic content, the top-performing extracts generally demonstrated robust antioxidant capacity and moderate to high GAE values. These findings corroborate the established correlation between polyphenolic content and anti-AChE activity in plant extracts [33] (Fig. 4).

Anti-Tyr activity spanned a broader range, from 12% ± 0.7% (RuEq) to 87% ± 3.7% (CaMa). The most potent tyrosinase inhibitors were *Carissa macrocarpa* (CaMa, 87% ± 3.7%), *Inula salicina* (InSa, 80% ± 4.4%), and *Adenophora liliifolia* (AdLi, 73% ± 6.2%). 14 of 27 extracts demonstrated > 40% tyrosinase inhibition. Notably, the three most active extracts (CaMa, AdLi, and InSa) also displayed strong antioxidant activity and elevated GAE values, further supporting the hypothesis that polyphenolic compounds contribute significantly to both antioxidant and neuroprotective activities.

The comparative screening of bioactivities (Fig. 5A) revealed clear interspecific variability in antioxidants, anti-AChE, and anti-Tyrosinase activities, with *Adenophora liliifolia* and *Carissa macrocarpa* displaying high values of inhibitory activity. Figure 5B shows a Principal Component Analysis (PCA), which reduces multidimensional bioactivity data into principal components, clustering extracts according to shared bioactive profiles. Extracts with higher anti-AChE and anti-Tyr activities are clustered in the upper-right quadrant, indicating a positive correlation between these enzymatic activities and phenolic composition. Following preliminary bioactivity screening and based on the PCA-guided selection, seven extracts (CaMa, AdLi, InSa, SaPr, SiAn, ErMa, and AlCi), based on anti-Tyr or anti-AChE activity, demonstrated notable neuroprotective potential and were identified for further investigations. Among these, *Adenophora liliifolia* and *Carissa macrocarpa* emerged as the most promising candidates, being the only extracts exhibiting substantial activity against both targets (31% and 38% AChE inhibition; 73% and 87% Tyr inhibition, respectively). Although *A. liliifolia* and *C. macrocarpa* exhibited slightly below 90% antioxidant activity by DPPH assay (86% and 68%, respectively), these values remained satisfactory.

ID	Species	Main metabolites identified in the plant methanolic extracts	Reference*
AcMo	*Acanthus mollis* L.	Benzoxazinoids (BOA, DIBOA and HBOA glucosides); vicenin-2; apigenin and methoxyflavones O-glycosides (mainly glucuronides)	
AdLi	*Adenophora liliifolia* (L.) Ledeb. ex A.DC.	Pyrrolidine alkaloids (radicamine A and isomers); aromatic aminoacids; triglochinin and other cyanogenic glycosides; neodiosmin; vicenin-2; triterpenoids acids (oleanolic/ursolic acid isomers)	
AlAn	*Allium angulosum* L.	Steroidal saponins	
AlLu	*Allium lusitanicum* Lam.	Steroidal saponins	23
AlCi	*Aloysia citrodora* Paláu	Verbascoside (and isomers) and other phenylethanoid glycosides; iridoids glucosides	24
AlOf	*Althaea officinalis* L.	C_{13} norisoprenoids sulfo-glycosides; hyploaetin O-glycosides	25
AqAt	*Aquilegia atrata* (L.) W.D.J.Koch	Swertisin and derivatives; cycloartane-type triterpenoids (aquilegiosides); cyanogenic glycosides; unidentified metabolites	25
BeVu	*Beta vulgaris* L.	Apigenin C-glycosides and acylated derivatives; sulfated metabolites; oleanolic acid saponins	
CaMa	*Carissa macrocarpa* (Eckl.) A.DC.	Epicatechin and condensed tannins (olygomeric procyanidins from medium to high degree of polymerization, including procyanidin C1); O-glycosylated flavonols; saponins; triterpenoids acylated with coumaroyl and feruloyl moieties	
CaSa	*Castanea sativa* Mill.	Chestanin, cretanin, and other gallotannins/ellagitannins; unidentified glycosilated C_{13} metabolites; rutin and other quercetin O-glycosides	
DiSu	*Dianthus superbus* L. subsp. *superbus*	C-glycosylated flavones; oleanane-type saponins (gypsogenic and polygalacic acid glycosides); unidentified metabolites	26
EcVu	*Echium vulgare* L.	Pyrrolizidine alkaloids (echimidine N-oxide); rosmarinic acid, lithospermic acid	
ErMa	*Eryngium maritimum* L.	Unidentified metabolites; kaempferol and quercetin O-glycosides; oleanane-type saponins	25
EuEu	*Euonymus europaeus* L.	Unidentified C_{13}-ionone metabolites; quercetin and kaempferol O-glycosides and acylated derivatives	
GrOf	*Gratiola officinalis* L.	Arenarioside/forsythoside B, verbascoside and other phenylpropanoid/phenylethnaoid glycosidic esters; apigenin glycosides; elaterinide (gratiotoxin) and other cucurbitane-type saponins	27
InSa	*Inula salicina* L.	Caffeic acid derivatives (chlorogenic acid, dicaffeoylquinic acids, tricaffeoylglucaric acids, coumaroylcaffeoylquinic acids); quercetin and kaempferol glycosides	
LySa	*Lythrum salicaria* L.	Gallotannins/ellagitannins (salicarinins isomers, pedunculagin); luteolin and apigenin C-glycosides	
MaAl	*Malva alcea* L.	Unidentified metabolites (some exhibiting sulfate groups); myricetin and quercetin O-glycosides; apigenin C-glycosides	
OxDi	*Oxalis dillenii* Jacq.	Apigenin and luteolin C-glycosides	
PePa	*Petasites paradoxus* (Retz.) Baumg.	Oligosaccharides, caffeic acid derivatives (chlorogenic acid and dicaffeoylquinic acids); quercetin O-glycosides and acylated derivatives; sesquiterpenes; otonecine-type pyrrolizidine alkaloids (petasitenine, senkirkine)	25
PhCo	*Philadelphus coronarius* L.	Quercetin and kaempferol O-glycosides; oleanane-type saponins (medicagenic and zanhic acid glycosides)	
RuEq	*Russelia equisetiformis* Schltdl. & Cham.	Catalpol and other iridoids, verbascoside and other phenylpropanoid/phenylethanoid glycosidic esters	
SaPr	*Salvia pratensis* L.	Rosmarinic acid and derivatives; phenylpropanoid/phenylethnaoid glycosidic esters; luteolin O-glycosides; oct-3-en-1-ol O-glycoside	25
SiAn	*Sicyos angulatus* L.	Unidentified N-containing metabolite; kaempferol glycosides	
Continued			

ID	Species	Main metabolites identified in the plant methanolic extracts	Reference*
SuPr	*Succisa pratensis* Moench	Caffeic acid derivatives; apigenin, and luteolin C-glycosides; secoiridoids glucosides (gentiopicroside, oleoside, sweroside, swertiamarin); oleanane-type saponins (Akebia saponin D)	26
TyLa	*Typha laxmannii* Lepech.	Flavonols 3-O-rutinosides (rutin, nictoflorin, narcissoside); flavan-3-ols (catechin, epicatechin, gallocatechin), and proanthocyanidins (dimeric and trimeric procyanidins and prodelphinidins); caffeic acid derivatives	25
WiSi	*Wisteria sinensis* (Sims) DC.	Caffeoylmalic acid, luteolin, and apigenin O-glycosides; supinidine derivative	

Table 2. Main phytochemicals characterizing the methanolic extracts of the plant species investigated from the NBFC collection; *, reference is provided if the phytochemical characterization of the same plant extract has been previously published.

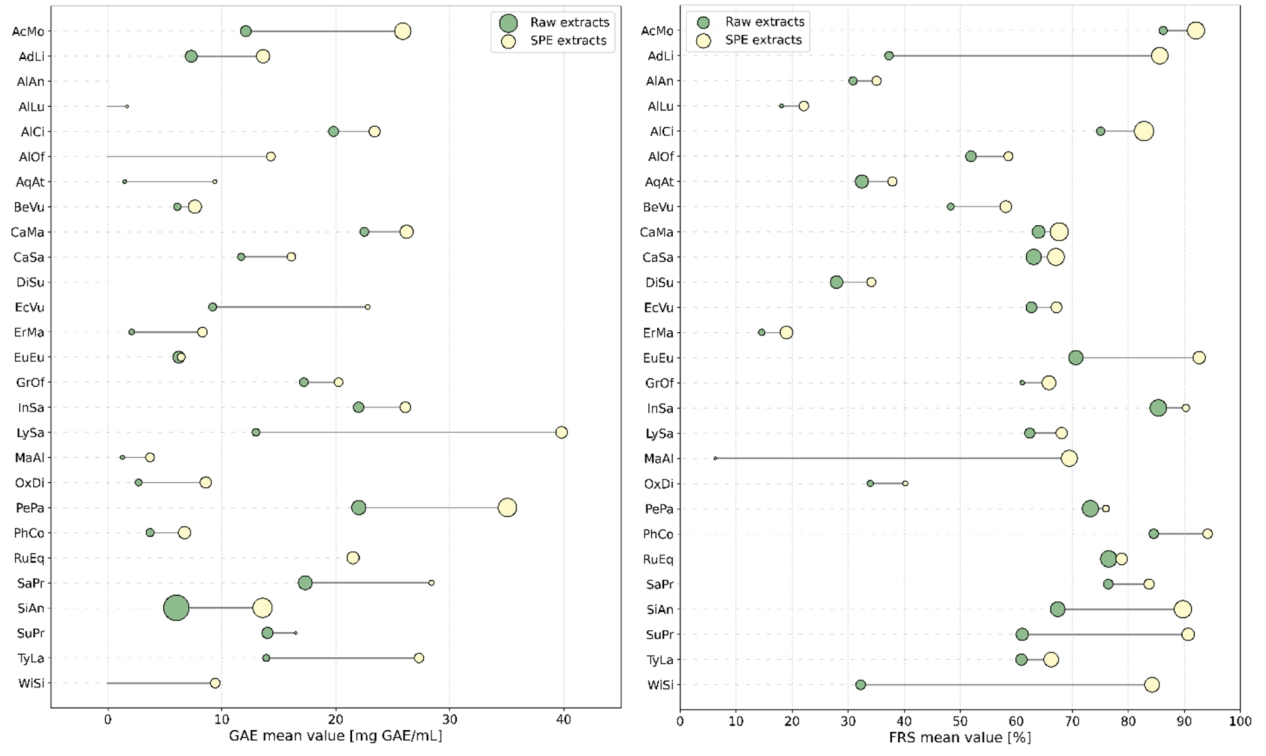


Fig. 2. Total phenolic characterization and Free Radical Scavenging Activity evaluation of the 27 considered extracts before (Raw extract) and after (SPE extract) chlorophyll removal. Ball dimensions represent standard deviation. Assays were performed in triplicates.

ORAC assay

To confirm antioxidant capacity, we performed the Oxygen Radical Absorbance Capacity (ORAC) assay, a superior method for evaluating antioxidant activity under physiological-like conditions [34]. Due to the assay's technical demands and time requirements, ORAC analysis was limited to these two most active extracts (Fig. 6).

Adenophora liliifolia (AdLi) demonstrated good antioxidant capacity with an ORAC value of 18.2 ± 0.2 µM Trolox Equivalents [µM TE], consistent with its strong DPPH activity (86%). *Carissa macrocarpa* (CaMa) showed moderate to good antioxidant activity with the ORAC assay (10.6 ± 0.1 µM TE), aligning with medium antioxidant DPPH activity (68%).

The ORAC results thus confirmed the DPPH findings, while providing more biologically relevant data, as ORAC better replicates in vivo conditions through hydrogen atom transfer in aqueous environments [34]. Taken together, these results identified *Adenophora liliifolia* (AdLi) and *Carissa macrocarpa* (CaMa) as the most interesting extracts.

Carissa macrocarpa and *Adenophora liliifolia* UPLC/MS metabolomic profiling

The phytochemical profile of the methanolic extract of *Adenophora liliifolia* leaves and herbaceous stems is shown in Fig. 7. The extract is characterized by the massive accumulation of polar metabolites, including

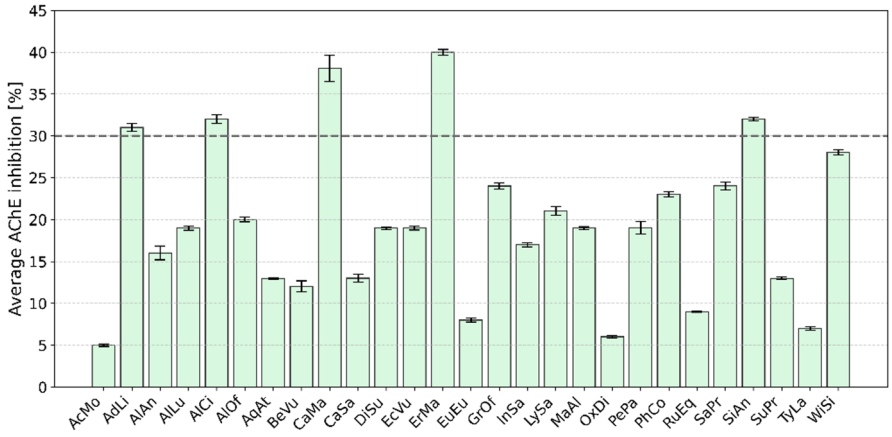


Fig. 3. Anti-AChE activity of the extracts considered. Assay was performed in triplicate.

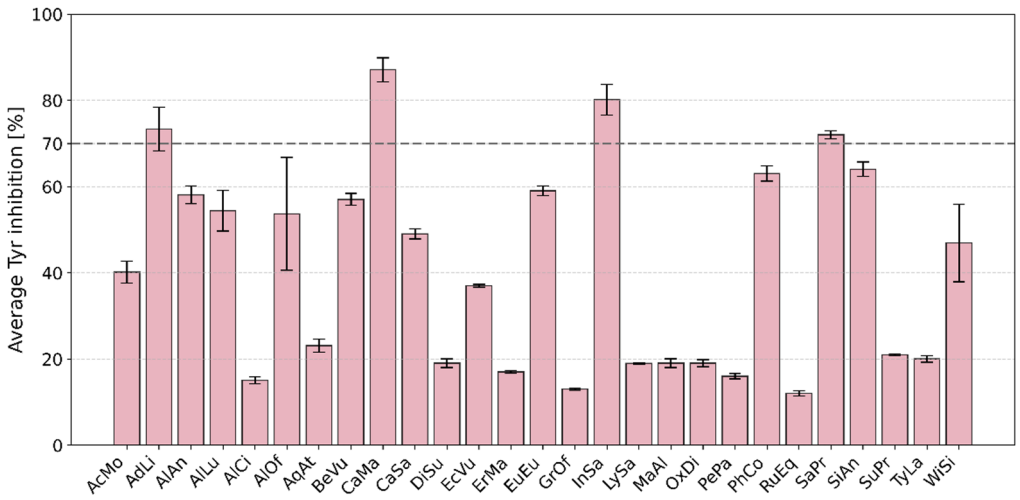


Fig. 4. Anti-Tyr activity of the extracts considered. Assay was performed in triplicate.

glutamine (**1**), quinic acid (**2**), along with an unidentified derivative (**5**), and a di-hexose sugar (**3**); in the low-polar region, two main metabolites (**75**, **76**) were identified as structural isomers of oleanolic/ursolic acids. In the medium-polar region of the chromatogram, a wide and diversified array of phytochemicals was detected, with four metabolites accumulating at high levels: the pyrrolidine alkaloid radicamine A (**10**), the tyrosine-derived cyanogenic glycoside triglochinin (**26**), and the flavones vicenin 2 (apigenin-6,8-di-C-glucoside, **48**) and neodiosmin (diosmetin-7-O-neohesperidoside, **67**). Other metabolites included lignans glycosides (pinoresinol diglucoside, **52**; lariciresinol dihexoside, **55**; coniferyl alcohol dihexoside, **57**; urolignoside, **61**), coumaric acid derivatives including esters with quinic (**37**, **49**) and aldaric (**29**, **31**, **35**, **38**) acids, benzyl alcohol glycosides (**39**, **46**, **47**), di-C-glycosides (**41**, **45**, **62**) and O-glycosides (**60**, **65**, **66**) of other flavonoid aglycons, aromatic amino acids (tyrosine, **15**; phenylalanine, **24**; tryptophan, **30**), and other nitrogen-containing compounds (**12**, **16**) including radicamine A isomers (**9**, **14**). Other metabolites present in the extract are reported in the Online Resource 3.

In contrast to *Adenophora liliifolia*, the *Carissa macrocarp*a methanolic leaf extract exhibits a phytocomplex more specialized toward the production of specific classes of metabolites (Fig. 8). The medium-polar region of the chromatogram is dominated by peaks attributed to the flavan-3-ol epicatechin (**28**) along with a wide array of condensed tannins (proanthocyanidins) consisting of B-type oligomers of (epi)catechin units (procyanidins) at various degree of polymerization; these include dimers (**12**, **22**, **44**), trimers (**21**, including procyanidin C1, **30**), a tetramer (**33**), a pentamer (**34**), an hexamer (**37**), an heptamer (**38**), a nonamer (**42**) and a decamer (**41**). At lower levels, other unidentified proanthocyanidin oligomers were detected. The other group of most abundant metabolites falls in the low-polar region of the analysis, comprising two unidentified triterpenoid saponins (**53**, **54**) with a core structure of $C_{30}H_{48}O_3$, putatively representing ursolic/oleanolic acid. Interestingly, in this region, uncommon acylated triterpenoids (**58**–**68**) were also detected at lower levels and characterized by the presence of $C_{30}H_{46}O_3$ (putatively 3β-hydroxyolean-11-en-28,13β-olide previously isolated from *C. macrocarpa*[35] and $C_{30}H_{44}O_2$ cores, harboring coumaroyl and feruloyl moieties. Specifically, two of these metabolites (**59**, **61**)

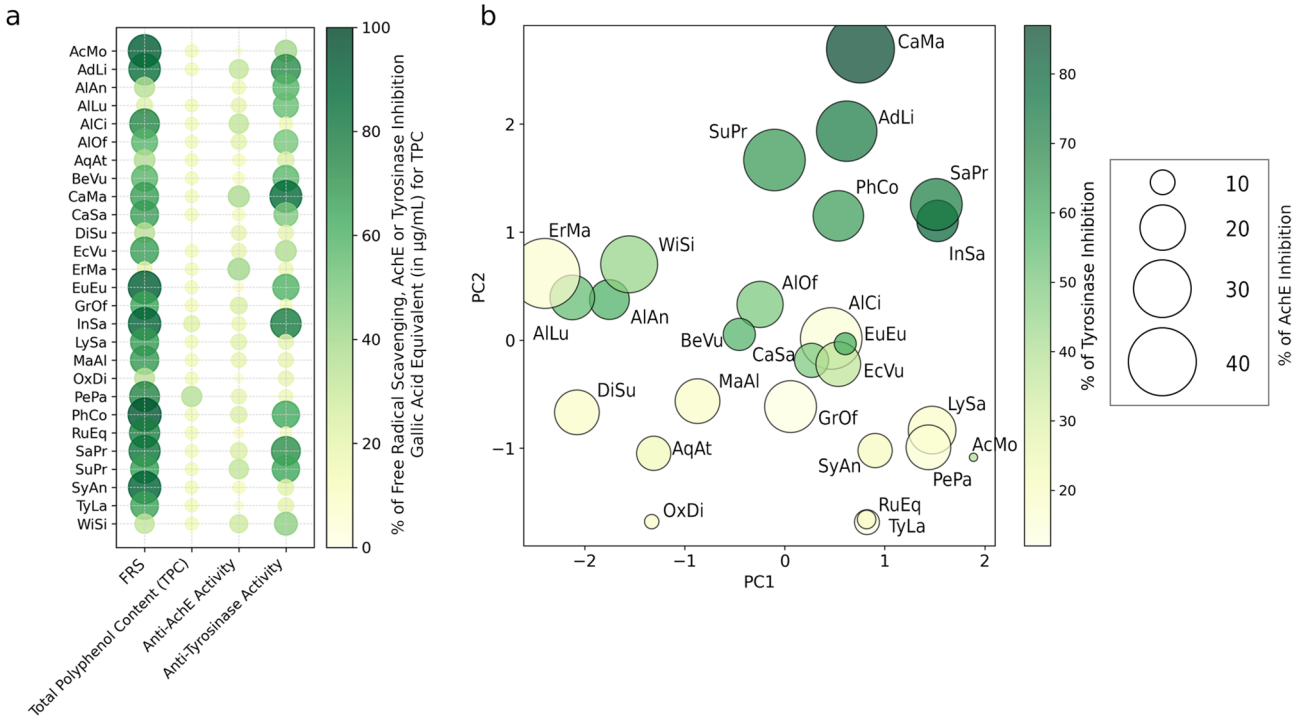


Fig. 5. Multivariate analysis of the bioactive profiles of plant extracts. (**a**) Comparative representation of Free Radical Scavenging activity (DPPH assay), total phenolic content (TPC), and inhibitory effects on acetylcholinesterase (AChE) and tyrosinase, expressed as percentage inhibition or gallic acid equivalents (mg/mL) for TPC. (**b**) Principal component analysis (PCA) based on bioactivity parameters. Bubble color indicates tyrosinase inhibition (%), while bubble size represents AChE inhibition (%). Extracts such as *Adenophora liliifolia* (AdLi) and *Carissa macrocarpa* (CaMa) show the highest combined inhibitory activities.

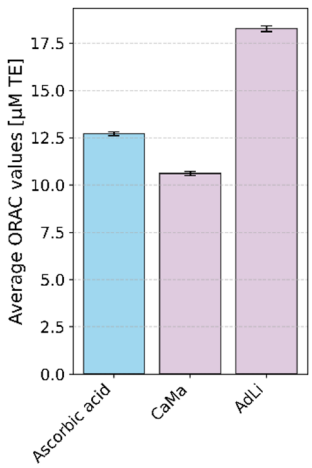


Fig. 6. ORAC results. Ascorbic acid is reported as a reference of antioxidant activity (12.7 ORAC values [µM TE]). Assay was performed in triplicate.

were putatively annotated as isomers of carissin, previously isolated from *C. carandas*[36]. Other metabolites characterizing the extract included caffeic (**11**, **17**, **19**, **27**, **35**) and coumaric (**12**, **18**) acid derivatives, kaempferol and quercetin O-glycosides (**32**, **39**, **43**, **46**), glycosides of aromatic (**24**) and cinnamyl (**26**) alcohols, a naringenin-di-C-hexoside (**20**), and one megastigmane glycoside (**47**). Other metabolites present in the extract are reported in the Online Resource 4.

Discussion

This work, conducted in collaboration with NBFC [37], represents a systematic exploration of Italian flora to identify plant species with potential therapeutic application against neurodegenerative pathologies. The focus on

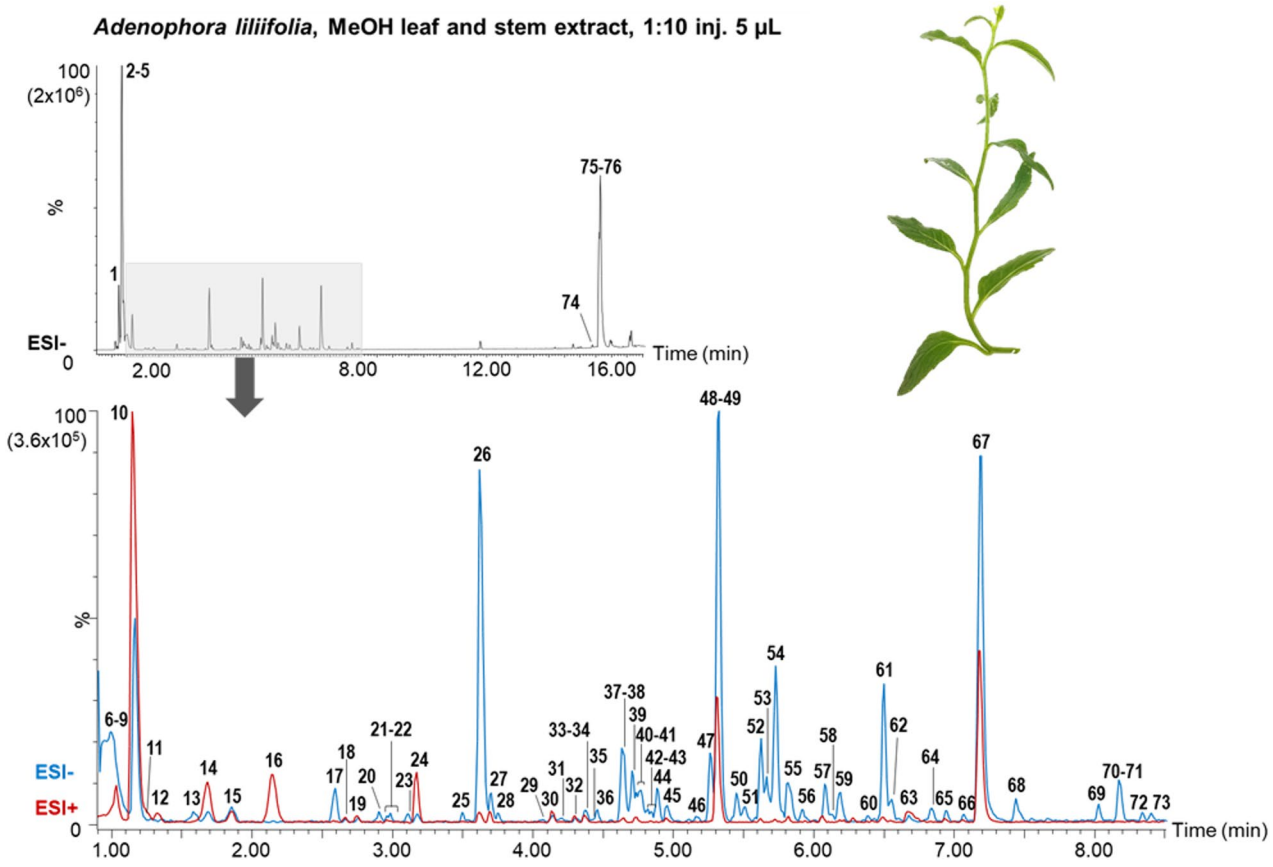


Fig. 7. Representative base peak chromatograms (ESI^-, negative ionization; ESI^+, positive ionization) obtained from the UPLC-ESI-HRMS analysis of the *Adenophora liliifolia* methanolic extract. Peak numbers refer to plant metabolites listed in the Online Resource 3.

whole phytocomplexes, rather than isolated compounds, is strategically significant. Phytocomplexes can exert their effects through multiple mechanisms of action simultaneously, offering a potentially more comprehensive therapeutic approach. This multi-target strategy is particularly relevant for neurodegenerative diseases, which involve complex, interconnected pathological pathways that single-target therapies often fail to address adequately. To achieve our objectives, we implemented a phylogenetically-informed bioprospecting approach. From approximately 12,000 plant species present in Italy, around 800 were selected based on taxonomic diversity criteria, ensuring broad chemical representation across different plant families. This strategy enabled the development of a comprehensive extract library that was subsequently analyzed using UPLC-HRMS in collaboration with the Universities of Verona and Salerno to obtain detailed metabolomic profiles. Based on the chemical diversity of secondary metabolites, 27 plant species exhibiting the most diverse metabolomic signatures were prioritized for biological evaluation.

Prior to biological evaluation, all extracts underwent solid-phase extraction (SPE) for chlorophyll removal. This preparatory step is critical for accurate bioactivity assessment, as chlorophylls present in leaf extracts interfere with spectrophotometric and spectrofluorometric assays through their strong absorbance and autofluorescence properties. We employed an innovative and validated method that enables simultaneous processing of multiple samples ($n = 27$, in this study) [13]. The de-greened extracts were then tested for total phenolic content and antioxidant capacity, as antioxidant properties are closely linked to neuroprotective effects. In neurodegenerative conditions, mitochondrial dysfunction triggers excessive reactive oxygen species (ROS) production, suggesting its key role in neurodegeneration [38].

In line with our previous findings [13] and with literature evidence, chlorophyll interference can significantly affect spectrophotometric measurements, leading to an underestimation of phenolic content and antioxidant capacity. Accordingly, an increase in both TPC and %FRS values was observed following chlorophyll removal. The antioxidant activity profile of the treated extracts revealed substantial variability among them, with only a limited number exhibiting very high %FRS values. Notably, the distribution of TPC values was more heterogeneous than that of antioxidant activity, indicating that high phenolic content does not necessarily translate into proportionally increased radical scavenging efficiency.

Following antioxidant characterization, all 27 extracts were screened for anti-tyrosinase and anti-acetylcholinesterase activity. These two assays were selected because both are indirectly related to neuroprotection, in particular, while AChE inhibition is a well-established strategy to preserve cholinergic transmission in neurodegenerative conditions, tyrosinase has emerged more recently as a target due to its role in Parkinson's

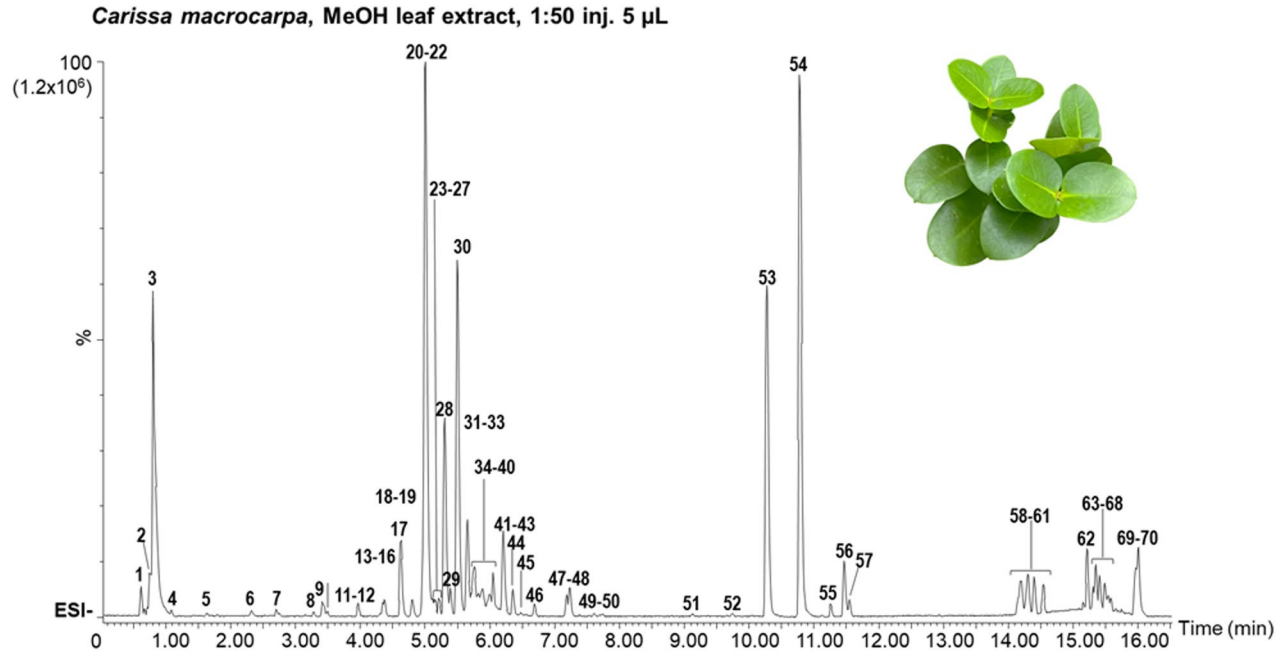


Fig. 8. Representative base peak chromatogram recorded in negative ionization (ESI⁻) obtained from the UPLC-ESI-HRMS analysis of the *Carissa macrocarpa* methanolic extract. The positive ionization chromatogram is not shown, as it does not provide any additional or complementary information to the ESI⁻ chromatogram. Peak numbers refer to plant metabolites listed in the Online Resource 4.

disease. Thus, tyrosinase is responsible for the production of neuromelanin in dopaminergic neurons, which accumulates, leading to neuroinflammation and neurodegeneration [39].

Anti-AChE activity was generally moderate, with a limited subset of extracts exceeding 30% inhibition. Notably, most of the top AChE inhibitors also exhibited strong antioxidant activity and moderate to high phenolic content, supporting the well-established association between polyphenolic compounds and cholinesterase inhibition. In contrast, tyrosinase inhibition spanned a wider activity range and involved a larger number of active extracts. The strongest tyrosinase inhibitors also displayed elevated antioxidant activity and phenolic content, reinforcing the hypothesis that polyphenols play a central role in modulating both oxidative stress and enzyme-related neurodegenerative pathways.

The multivariate analysis of the enzymatic data further integrated the bioactivity data, revealing distinct clustering of extracts with shared inhibitory profiles and confirming positive correlations between phenolic composition, antioxidant capacity, and enzyme inhibition. PCA-guided prioritization enabled the identification of a subset of extracts with balanced and multi-target activity profiles. Among these, *Adenophora liliifolia* and *Carissa macrocarpa* emerged as the most promising candidates, as they were the only extracts showing substantial inhibitory activity against both acetylcholinesterase and tyrosinase, together with satisfactory antioxidant capacity. While these findings do not establish definitive neuroprotection, they support the selection of these extracts for further studies.

Given the central role of ROS overproduction and oxidative stress in neuronal loss and the neurodegenerative progression[40], we performed an additional antioxidant characterization of the two most promising extracts using the Oxygen Radical Absorbance Capacity (ORAC) assay. The ORAC assay relies on hydrogen atom transfer and uses peroxyl radicals, which better mimic in vivo oxidative processes if compared with DPPH assay. Furthermore, the ORAC assay is conducted in aqueous media and measures both the extent and duration of antioxidant activity, providing a more biologically relevant assessment[34]. The ORAC analysis confirmed the robust antioxidant activity of both *Carissa macrocarpa* and *Adenophora liliifolia*, supporting their neuroprotective potential.

Adenophora liliifolia (Campanulaceae) is a Eurasian perennial herb, classified as a threatened species, occurring in Italy along the southern Alpine arch [41]. *A. liliifolia* has received attention primarily from a conservation perspective [42] with no phytochemical information previously reported. The methanolic extract of leaves and herbaceous stems collected from young plants of *A. liliifolia* showed a rather diverse phytocomplex, with the occurrence of diverse nitrogen-containing compounds. Similar to observations in other Campanulaceae species, some metabolites that accumulated at higher levels belong to pyrrolidine alkaloids (such as radicamine A, previously reported in *A. triphylla* [43] and *Lobelia chinensis* [44] and to cyanogenic glycosides, such as triglochinin, which is biosynthetically derived from tyrosine; although cyanogenesis is considered relatively rare in Campanulaceae, it has already been reported in species of the genus *Campanula* [45]. On the polyphenolic side, neodiosmin (diosmetin-7-O-neohesperidoside) is among the metabolites with the highest accumulation. The occurrence of glycosides of luteolin and of its methylated derivative diosmetin (luteolin 4′-methyl ether) is

not novel within the Campanulaceae family (e.g., in *Lobelia* spp [46]. Numerous triterpenoids and triterpenoid saponins have been characterized in *A. triphylla*, whereas in the extract of *A. liliifolia*, the accumulation of non-glycosylated species was observed, consistent with isomers of oleanolic and ursolic acids. A visual summary of *A. liliifolia* metabolomic landscape is provided by the hierarchical clustering dendrogram (Fig. 9) to facilitate the understanding of biochemical relationships within the considered plant.

Most metabolites identified in *A. liliifolia* have documented activity against tyrosinase and/or acetylcholinesterase. Flavones, including neodiosmin and diosmetin, inhibit tyrosinase and melanin production compared to arbutin controls, while luteolin glycosides have demonstrated anti-tyrosinase activity with IC_{50} values of 2.95 ± 0.11 μM [47,48]. These flavones, along with cinnamic alcohols, esters, and their glycosides, also exhibit anti-acetylcholinesterase activity [49]. Triterpenoid acids, including oleanolic and ursolic acids, function as tyrosinase inhibitors (IC_{50} values of 207.79 and 221.51 μg/mL, respectively) and demonstrate anti-acetylcholinesterase properties [50–52]. Notably, certain nitrogen-containing compounds, specifically the pyrrolidine alkaloids (radicamine A and isomers) and the iridoid monoterpenoid daphylloside, have not been previously reported as tyrosinase or acetylcholinesterase inhibitors, warranting further investigation. Additionally, the presence of cyanogenic glycosides necessitates future safety evaluations before any therapeutic development.

Much more information is available in the current literature concerning the phytochemistry of *Carissa macrocarpa* (Apocynaceae) and, in general, of *Carissa* spp. Although not native to Italy, this species is naturalized in the southern regions and included in the Italian flora databases [41]. Our phytochemical profile aligns with previously published characterization of aerial organs [53–55], confirming the high abundance of condensed tannins (mainly B-type (epi)catechin oligomers, i.e., procyanidins). Interestingly, less-polar metabolites such as triterpenoids were just marginally investigated in previous works. We detected carissin (3β-hydroxy-27-E-feruloyloxyurs-12-en-28-oic acid), an acylated ursane-type triterpenoid first isolated from *Carissa carandas*[56], which was also detected in our *C. macrocarpa* leaf extract, together with several other triterpenoids featuring a C30 backbone and or feruloyl substituents. Further structural elucidation of the triterpenoid saponins present in the extract is still required.

The hierarchical clustering dendrogram (Fig. 10) visually represents the chemical similarity relationships between the various metabolites present in *Carissa macrocarpa*. This dendrogram effectively captures the complex biochemical landscape and provides insight into the metabolic composition and chemical diversity of the species.

Also in this case, most of the class of metabolites identified in the phytocomplex are reported in the literature as active on tyrosinase and acetylcholinesterase enzymes. Polar polyphenolic compounds are reported in literature for their known tyrosinase and acetylcholinesterase inhibition activity, as well as condensed tannins and procyanidins[49,57–59]. According to what has been said about *A. liliifolia*, triterpenoids are reported to be tyrosinase and acetylcholinesterase inhibitors. In particular, carissin has not been investigated yet, but extracts from *Carissa carandas* have shown anti-tyrosinase and anti-acetylcholinesterase activites [60–62]. Saponins and triterpenoid saponins have also shown anti-tyrosinase and anti-acetylcholinesterase activites [52,63]. Of note, megastigamane compounds and glycosides identified in the *C. macrocarpa* extract are the only metabolites and metabolite classes that did not show both considered activities. Some megastimane glycosides, not identified

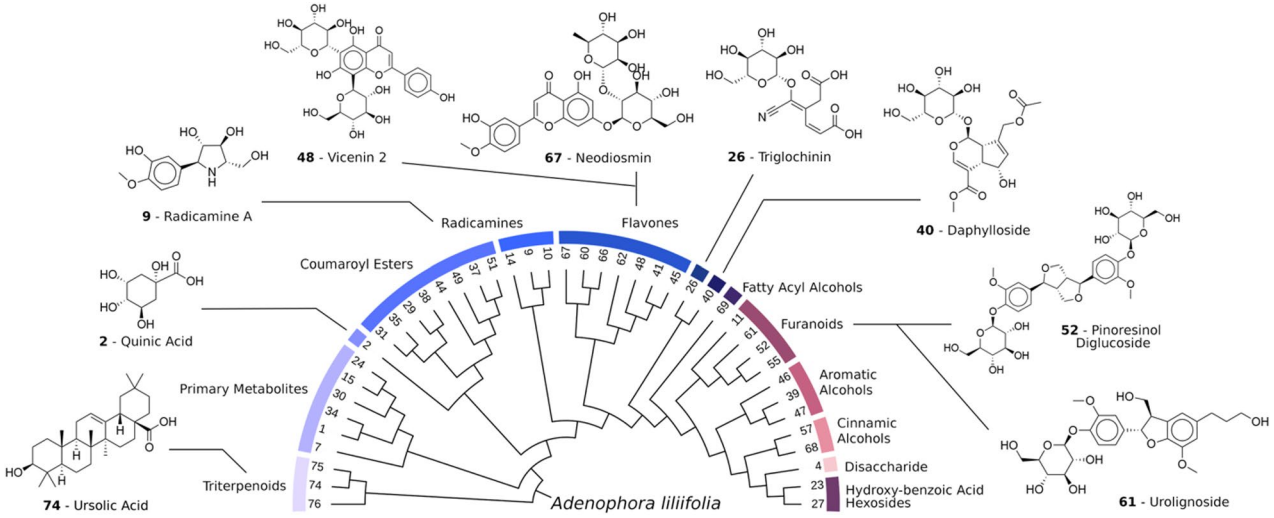


Fig. 9. Hierarchical clustering dendrogram (obtained with Python 3.0 RDKit module, based on ECFP4 fingerprint similarity) illustrating the chemical similarity relationships among metabolites identified in *Adenophora liliifolia* extract by mass spectrometry. Each number refers to the peak ID assigned in the chromatographic profile. Metabolites are grouped according to their chemical classes, and, when available, a representative structure for each class is displayed. Representative compounds were annotated based on accurate mass measurements and confirmed by comparison with analytical standards.

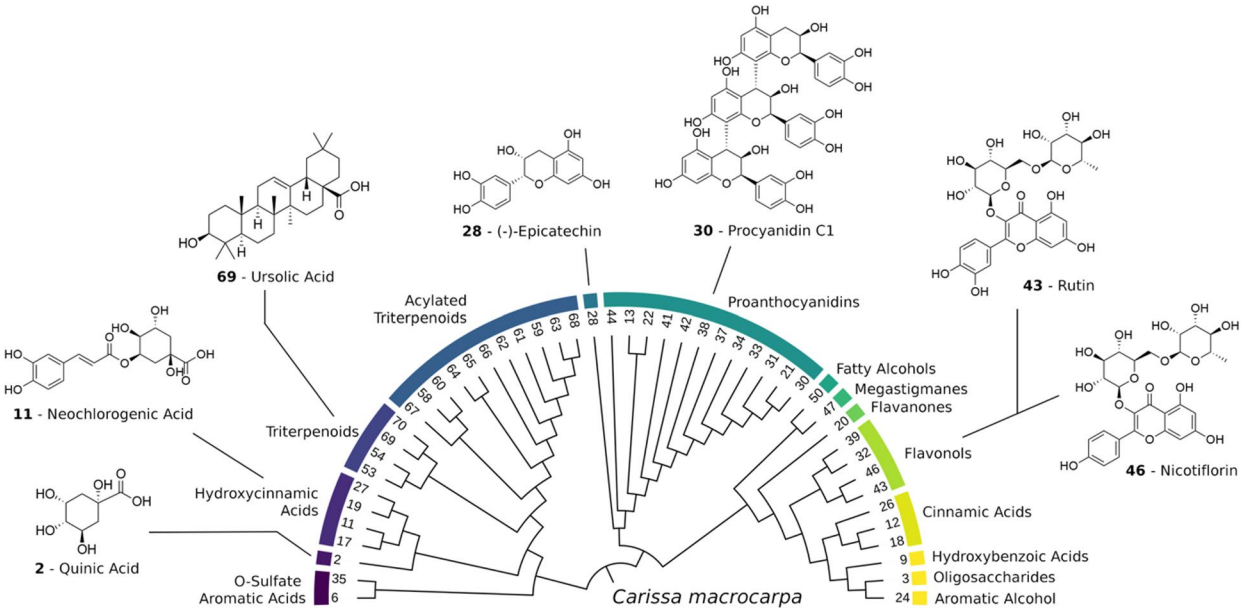


Fig. 10. Hierarchical clustering dendrogram (obtained with Python 3.0 RDKit module, based on ECFP4 fingerprint similarity) illustrating the chemical similarity relationships among metabolites in *Carissa macrocarpa* extract by mass spectrometry. Each number refers to the peak ID assigned in the chromatographic profile. Metabolites are grouped according to their chemical classes, and, when available, a representative structure for each class is displayed. Representative compounds were annotated based on accurate mass measurements and confirmed by comparison with analytical standards.

in *A. liliifolia* or *C. macrocarpa*, are reported in literature as promising anti-tyrosinase compounds, but no information was found related to their anti-acetylcholinesterase activity[64].

Figures 9 and 10 show that the phytochemical profile of the *Adenophora liliifolia* extract is primarily composed of polar to medium-polar metabolites, such as coumaroyl esters, radicamines, flavones, cyanogenic glycosides, iridoids, furanoids, and cinnamic acids. Only a few triterpenoids are found among the apolar metabolites, with the largest clusters in the profile being coumaroyl esters and flavones. By contrast, the phytochemical profile of the *Carissa macrocarpa* extract exhibits a more balanced distribution of polar and apolar metabolites, with acylated triterpenoids and proanthocyanidins forming the largest clusters. However, while *C. macrocarpa* presents a phytochemical profile primarily dominated by acylated triterpenoids, flavonoids, and phenolic acids; *A. liliifolia* fingerprint is more diversified in terms of metabolic composition, including quinic acids, coumaroyl esters, and furanoids.

Metabolomic analysis of *Adenophora liliifolia* and *Carissa macrocarpa* extracts revealed a complex and chemically heterogeneous profile, encompassing metabolites belonging to different phytochemical classes. The annotation of these compounds at different confidence levels supports the reliability of the metabolomic dataset and highlights the contribution of structurally diverse constituents to the observed bioactivities.

Notably, the identified metabolites, for which inhibitory activity against tyrosinase and/or acetylcholinesterase has been previously reported in the literature, include compounds active against either one or both enzymatic targets (Online Resources 4 and 5). This compositional profile is consistent with the moderate and balanced inhibitory activities observed in the anti-AChE and anti-Tyr assays and is further reflected in the PCA analysis, where these extracts cluster according to shared bioactivity patterns.

While the assignment of enzyme inhibitory properties is based on previously reported evidence and does not imply direct attribution of activity to individual metabolites within the extracts, the convergence of metabolomic annotations with enzymatic inhibition and antioxidant data offers a plausible explanation for the observed bioactivities. Thus, theese findings strongly support the measured enzymatic activity of both extracts and identify specific compounds of interest for potential isolation and further studies.

Conclusion

This study implemented a phylogenetic-informed bioprospecting approach to identify natural products from Italian biodiversity with potential against neurodegenerative conditions. In collaboration with NBFC and the University of Verona, 27 extracts have been selected based on metabolomic diversity and subjected to comprehensive bioactivity screening. Following chlorophyll removal, extracts were evaluated for antioxidant activity, total phenolic content, anti-tyrosinase and anti-acetylcholinesterase activites, parameters indirectly involved in neurodegeneration. This multi-parameter screening identified *Adenophora liliifolia* and *Carissa macrocarpa* as the most promising candidates, prompting detailed phytochemical characterization. *Adenophora liliifolia*, a threatened species with no prior phytochemical documentation, revealed several nitrogen-containing compounds, like pyrrolidine alkaloids (radicamine A) and cyanogenic glycosides (triglochinin), considered rare

in Campanulaceae, polyphenolic compounds (neodiosmin), triterpenoids, and triterpenoid saponins already characterized in other *Adenophora* genus. The presence of alkaloids and cyanogenic metabolites in this extract necessitates further safety assessment. *Carissa macrocarpa* exhibited a phytochemical profile rich in several already characterized polyphenolic compounds, triterpenoids, and triterpenoid saponins already identified in other *Carissa* genus.

Metabolomic analysis revealed that dozens of *A. liliifolia* and *C. macrocarpa* metabolites possess documented activity against tyrosinase and/or acetylcholinesterase, supporting their measured bioactivities. These secondary metabolites likely contribute to the neuroprotective effects observed and represent promising candidates for therapeutic development against neurodegenerative diseases. Future work should focus on bioassay-guided fractionation to identify the most active constituents, mechanistic studies to elucidate their modes of action, and toxicological assessments, particularly for *A. liliifolia*, to ensure safety profiles compatible with therapeutic application.

Data availability

The data underlying this study are available in the published article and its online supporting information.

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References

1. Raggi, A. et al. Incidence, prevalence and disability associated with neurological disorders in Italy between 1990 and 2019: An analysis based on the Global Burden of Disease Study 2019. *J. Neurol.* **269**, 2080–2098. https://doi.org/10.1007/s00415-021-10774-5 (2022).
2. Maher, C. & Ferreira, G. Time to reconsider what Global Burden of Disease studies really tell us about low back pain. *Ann. Rheum. Dis.* **81**, 306–308. https://doi.org/10.1136/annrheumdis-2021-221173 (2022).
3. Choi, B. C. K., Maza, R. A., Mujica, O. J. & Paho Strategic Plan Advisory Group, Paho Technical Team. The Pan American Health Organization-adapted Hanlon method for prioritization of health programs. *Rev. Panam. Salud Pública*. **43**, 1. https://doi.org/10.26633/RPSP.2019.61 (2019).
4. Süntar, I. Importance of ethnopharmacological studies in drug discovery: Role of medicinal plants. *Phytochem Rev.* **19**, 1199–1209. https://doi.org/10.1007/s11101-019-09629-9 (2020).
5. Newman, D. J. & Cragg, G. M. Natural products as sources of new drugs over the nearly four decades from 01/1981 to 09/2019. *J. Nat. Prod. Am. Chem. Soc.* **83**, 770–803. https://doi.org/10.1021/acs.jnatprod.9b01285 (2020).
6. Nambisan, P. Biodiversity and sharing of biological resources. *Introd. Ethic. Saf. Intellect. Prop. Rights Issues Biotechnol. [Internet]* (Elsevier) 189–209. https://doi.org/10.1016/B978-0-12-809231-6.00008-9 (2017). Accessed 7 Oct 2025.
7. Wilson, E. O. (Ed.) *The Diversity of Life. 1. Harvard University Press Paperb* (Belknap Press of Harvard University, 2010).
8. Cena, H. & Labra, M. Biodiversity and planetary health: A call for integrated action. *Lancet* **403**, 1985–1986. https://doi.org/10.1016/S0140-6736(24)00292-7 (2024).
9. Rees, T. M. & Brimijoin, S. The role of acetylcholinesterase in the pathogenesis of Alzheimer'sdisease. *Drugs Today*. **39**, 75. https://doi.org/10.1358/dot.2003.39.1.740206 (2003).
10. Jin, W., Stehbens, S. J., Barnard, R. T., Blaskovich, M. A. T. & Ziora, Z. M. Dysregulation of tyrosinase activity: A potential link between skin disorders and neurodegeneration. *J. Pharm. Pharmacol.* **76**, 13–22. https://doi.org/10.1093/jpp/rgad107 (2024).
11. Qi, S. et al. A new strategy for the treatment of Parkinson's disease: Discovery and bio-evaluation of the first central-targeting tyrosinase inhibitor. *Bioorg. Chem.* **150**, 107612. https://doi.org/10.1016/j.bioorg.2024.107612 (2024).
12. Moreira, N. C. D. S., Lima, J. E. B. D. F., Marchiori, M. F., Carvalho, I. & Sakamoto-Hojo, E. T. Neuroprotective effects of cholinesterase inhibitors: Current scenario in therapies for Alzheimer's disease and future perspectives. *J. Alzheimers Dis. Rep.* **6**, 177–193. https://doi.org/10.3233/ADR-210061 (2022).
13. Fossati, A., Cavalloro, V., Rossi, D., Collina, S. & Martino, E. A practical and easy-to-scale protocol for removing chlorophylls from leaf extracts. *Appl. Plant. Sci.* **13**, e70018. https://doi.org/10.1002/aps3.70018 (2025).
14. Attard, E. A rapid microtitre plate Folin-Ciocalteu method for the assessment of polyphenols. *Open. Life Sci.* **8**, 48–53. https://doi.org/10.2478/s11535-012-0107-3 (2013).
15. Brand-Williams, W., Cuvelier, M. E. & Berset, C. Use of a free radical method to evaluate antioxidant activity. *LWT - Food Sci. Technol.* **28**, 25–30. https://doi.org/10.1016/S0023-6438(95)80008-5 (1995).
16. *Determination of Antioxidant Potential Using an Oxygen Radical Absorbance Capacity (ORAC) Assay with Synergy H4*.
17. *Exploiting Kinetic Features of ORAC Assay for Evaluation of Radical Scavenging Capacity [Internet]*. https://www.mdpi.com/2076-3921/12/2/505. Accessed 7 Oct 2025.
18. Chlapanidas, T. et al. TNF-α blocker effect of naringenin-loaded sericin microparticles that are potentially useful in the treatment of psoriasis. *Int. J. Mol. Sci.* **15**, 13624–13636. https://doi.org/10.3390/ijms150813624 (2014).
19. Rui, M. et al. Identification of dual Sigma1 receptor modulators/acetylcholinesterase inhibitors with antioxidant and neurotrophic properties, as neuroprotective agents. *Eur. J. Med. Chem.* **158**, 353–370. https://doi.org/10.1016/j.ejmech.2018.09.010 (2018).
20. Li, S. et al. Ultrasound-assisted extraction optimization and cultivar screening for polyphenol recovery from thinned peach fruit: A comprehensive evaluation of 179 cultivars. *Foods* **14**, 1897. https://doi.org/10.3390/foods14111897 (2025).
21. Zhu, Y. et al. Determination of 12 halogenated organic pollutants in edible fish by ultra performance liquid chromatography-high resolution mass spectrometry combined with ultrasound-assisted extraction and gel permeation chromatography purification. *Chin. J. Chromatogr.* **43**, 68–77. https://doi.org/10.3724/SPJ.1123.2023.12028 (2025).
22. Hiranpradith, V., Therdthai, N., Soontrunnarudrungsri, A. & Rungsuriyawiboon, O. Optimisation of ultrasound-assisted extraction of total phenolics and flavonoids content from *Centella asiatica*. *Foods* **14**, 291. https://doi.org/10.3390/foods14020291 (2025).
23. De Soricellis, G. et al. Development of an analytical platform for the affinity screening of natural extracts by SEC-MS towards PPARα and PPARγ receptors. *Anal. Chim. Acta.* **1309**, 342666. https://doi.org/10.1016/j.aca.2024.342666 (2024).
24. Moretto, G. et al. *Aloysia citrodora* polyphenolic extract: From anti-glycative activity to in vitro bioaccessibility and in silico studies. *Nutrients [Internet]* **18**. https://doi.org/10.3390/nu18010115 (2025). Accessed 26 Jan 2026.
25. Argentino, G. et al. Screening of mediterranean plant-derived extracts for antioxidant effect in cell-free and human cell line models. *Antioxidants [Internet]* **14**. https://doi.org/10.3390/antiox14101217 (2025). Accessed 26 Jan 2026.
26. Giammona, A. et al. A novel strategy for glioblastoma treatment by natural bioactive molecules showed a highly effective anti-cancer potential. *Nutrients* **16**, 2389. https://doi.org/10.3390/nu16152389 (2024).
27. Bianchini, S. et al. Gratiola officinalis alcoholic extract targets Warburg effect, apoptosis and cell cycle progression in colorectal cancer cell lines. *Int. J. Mol. Sci.* **26**, 2220. https://doi.org/10.3390/ijms26052220 (2025).

28. Jalouli, M. et al. Targeting natural antioxidant polyphenols to protect neuroinflammation and neurodegenerative diseases: A comprehensive review. *Front. Pharmacol.* **16**, 1492517. https://doi.org/10.3389/fphar.2025.1492517 (2025).
29. Carballo-Carbajal, I. et al. Brain tyrosinase overexpression implicates age-dependent neuromelanin production in Parkinson's disease pathogenesis. *Nat. Commun.* **10**, 973. https://doi.org/10.1038/s41467-019-08858-y (2019).
30. Walczak-Nowicka, Ł. J. & Herbet, M. Acetylcholinesterase inhibitors in the treatment of neurodegenerative diseases and the role of acetylcholinesterase in their pathogenesis. *Int. J. Mol. Sci.* **22**, 9290. https://doi.org/10.3390/ijms22179290 (2021).
31. Moss, D. E. & Perez, R. G. Anti-neurodegenerative benefits of acetylcholinesterase inhibitors in Alzheimer's disease: Nexus of cholinergic and nerve growth factor dysfunction. *Curr. Alzheimer Res.* **18**, 1010–1022. https://doi.org/10.2174/1567205018666211215150547 (2021).
32. Pérez, M., Dominguez-López, I. & Lamuela-Raventós, R. M. The chemistry behind the Folin–Ciocalteu method for the estimation of (poly)phenol content in food: Total phenolic intake in a Mediterranean dietary pattern. *J. Agric. Food Chem.* **71**, 17543–17553. https://doi.org/10.1021/acs.jafc.3c04022 (2023).
33. Wu, M. et al. The inhibition mechanism of polyphenols from Phyllanthus emblica Linn. fruit on acetylcholinesterase: A interaction, kinetic, spectroscopic, and molecular simulation study. *Food Res. Int.* **158**, 111497. https://doi.org/10.1016/j.foodres.2022.111497 (2022).
34. Schaich, K. M., Tian, X. & Xie, J. Reprint of hurdles and pitfalls in measuring antioxidant efficacy: A critical evaluation of ABTS, DPPH, and ORAC assays. *J. Funct. Foods.* **18**, 782–796. https://doi.org/10.1016/j.jff.2015.05.024 (2015).
35. Moodley, R., Chenia, H., Jonnalagadda, S. B. & Koorbanally, N. Antibacterial and anti-adhesion activity of the pentacyclic triterpenoids isolated from the leaves and edible fruits of Carissa macrocarpa. *J. Med. Plants Res. Acad. J.* **5**, 4851–4858. https://doi.org/10.5897/JMPR.9000125 (2011).
36. Siddiqui, B. S., Ghani, U., Ali, S. T., Usmani, S. B. & Begum, S. Triterpenoidal constituents of the leaves of *Carissa carandas*. *Nat. Prod. Res.* **17**, 153–158. https://doi.org/10.1080/1478641031000104109 (2003).
37. Home - NBFC [Internet]. https://www.nbfc.it/en. Accessed 7 Oct 2025.
38. Morén, C., deSouza, R. M., Giraldo, D. M. & Uff, C. Antioxidant therapeutic strategies in neurodegenerative diseases. *Int. J. Mol. Sci.* **23**, 9328. https://doi.org/10.3390/ijms23169328 (2022).
39. Qi, S. et al. A new strategy for the treatment of Parkinson's disease: Discovery and bio-evaluation of the first central-targeting tyrosinase inhibitor. *Bioorg. Chem.* **150**, 107612. https://doi.org/10.1016/j.bioorg.2024.107612 (2024).
40. Teleanu, R., Chircov, C., Grumezescu, A., Volceanov, A. & Teleanu, D. Antioxidant therapies for neuroprotection—A review. *J. Clin. Med.* **8**, 1659. https://doi.org/10.3390/jcm8101659 (2019).
41. Portale della Flora d'Italia - Portal to the Flora of Italy [Internet]. (2025). http://dryades.units.it/floritaly. Accessed 7 Oct 2025.
42. Mariotti, M. et al. Using selected Habitat European Directive species as garden plants: Challenges and opportunities. *Acta Hortic.* 315–326. https://doi.org/10.17660/ActaHortic.2023.1383.38 (2023).
43. Kim, Y. et al. Profiling of phytochemicals in *Adenophora triphylla* using LC-Q-TOF/MS-based untargeted metabolomics. *RSC Adv.* **15**, 18275–18282. https://doi.org/10.1039/D5RA00186B (2025).
44. Shibano, M., Tsukamoto, D., Masuda, A., Tanaka, Y. & Kusano, G. Two new pyrrolidine alkaloids, radicamines A and B, as inhibitors of.ALPHA.-glucosidase from Lobelia chinensis LOUR. *Chem. Pharm. Bull. (Tokyo).* **49**, 1362–1365. https://doi.org/10.1248/cpb.49.1362 (2001).
45. Rosatti, T. J. The genera of Sphenocleaceae and Campanulaceae in the Southeastern United States. *J. Arnold Arbor Arnold Arboretum Harv. Univ.* **67**, 1–64 (1986).
46. Zheng, Q., Fang, L., Huang, X., Wang, Y. & Zhang, S. Investigation of the mechanisms of neuroprotection mediated by Lobelia species via computational network pharmacology and molecular modeling [Internet]. *In Review* (2020). https://doi.org/10.21203/rs.3.rs-61537/v1. Accessed 7 Oct 2025.
47. Desmiaty, Y. et al. Two triterpenoids from Rubus fraxinifolius leaves and their tyrosinase and elastase inhibitory activities. *Sci. Rep.* **11**, 20452. https://doi.org/10.1038/s41598-021-99970-x (2021).
48. Wagleet al. et al. Discovery of a highly potent tyrosinase inhibitor, luteolin 5-O-β-d-glucopyranoside, isolated from Cirsium japonicum var. maackii (Maxim.) Matsum., Korean thistle: Kinetics and computational molecular docking simulation [Internet]. Vol. 3. https://doi.org/10.1021/acsomega.8b02694 (2018). Accessed 27 Oct 2025.
49. Khan, H., Marya, Amin, S., Kamal, M. A. & Patel, S. Flavonoids as acetylcholinesterase inhibitors: Current therapeutic standing and future prospects. *Biomed. Pharmacother.* **101**, 860–870. https://doi.org/10.1016/j.biopha.2018.03.007 (2018).
50. El-Nashar, H. A. S., El-Din, M. I. G., Hritcu, L. & Eldahshan, O. A. Insights on the inhibitory power of flavonoids on tyrosinase activity: A survey from 2016 to 2021. *Molecules* **26**, 7546. https://doi.org/10.3390/molecules26247546 (2021).
51. Loesche, A. et al. Ursolic and oleanolic acid derivatives with cholinesterase inhibiting potential. *Bioorg. Chem.* **85**, 23–32. https://doi.org/10.1016/j.bioorg.2018.12.013 (2019).
52. Wei, Y-M. et al. Triterpenoids as bivalent and dual inhibitors of acetylcholinesterase/butyrylcholinesterase from the fruiting bodies of *Inonotus obliquus*. *Phytochemistry* **200**, 113182. https://doi.org/10.1016/j.phytochem.2022.113182 (2022).
53. Souilem, F. et al. Phenolic profile and bioactive properties of *Carissa macrocarpa* (Eckl.) A.DC.: An in vitro comparative study between leaves, stems, and flowers. *Molecules* **24**, 1696. https://doi.org/10.3390/molecules24091696 (2019).
54. Ghanem, D. M. et al. Effect of *Carissa macrocarpa* (Eckl.) A. DC. aerial parts on some non-communicable diseases: In vitro study and HPLC-QTOF/MS-MS analysis. *Discov. Appl. Sci.* **6**, 238. https://doi.org/10.1007/s42452-024-05899-x (2024).
55. Orabi, M. A. A. et al. Human lung cancer (A549) cell line cytotoxicity and anti-Leishmania major activity of Carissa macrocarpa leaves: A study supported by UPLC-ESI-MS/MS metabolites profiling and molecular docking. *Pharmaceuticals* **15**, 1561. https://doi.org/10.3390/ph15121561 (2022).
56. Siddiqui, B. S., Ghani, U., Ali, S. T., Usmani, S. B. & Begum, S. Triterpenoidal constituents of the leaves of *Carissa carandas*. *Nat. Prod. Res.* **17**, 153–158. https://doi.org/10.1080/1478641031000104109 (2003).
57. Liu, J., Liu, Y., He, X., Teng, B. & McRae, J. M. Valonea tannin: Tyrosinase inhibition activity, structural elucidation and insights into the inhibition mechanism. *Molecules* **26**, 2747. https://doi.org/10.3390/molecules26092747 (2021).
58. Jeong, S. H. et al. Tyrosinase inhibitory polyphenols from roots of *Morus lhou*. *J. Agric. Food Chem.* **57**, 1195–1203. https://doi.org/10.1021/jf8033286 (2009).
59. Akbar Basha, S., Maheswaraiah, A. & Prasada Rao, U. J. S. Antioxidant profile, acetylcholinesterase inhibition, and platelet aggregation of polyphenols and proteins from germinating green gram (*Vigna radiata*). *Int. J. Food Prop.* **20**, S959–S971. https://doi.org/10.1080/10942912.2017.1325899 (2017).
60. Thitilertdecha, N. & Pintathong, P. The antioxidant and anti-tyrosinase activities of Carissa carandas Linn. fruit extracts and their applications in cosmetics. *Indian J. Pharm. Sci. [Internet]*. https://doi.org/10.36468/pharmaceutical-sciences.1017 (2022). [cited 2025 Oct 7];84.
61. Kareti, S. R. Molecular docking analysis of potential anti-Alzheimer's compounds present in chloroform extract of Carissa carandas leaf using gas chromatography MS/MS. *Curr. Ther. Res.* **93**, 100615. https://doi.org/10.1016/j.curtheres.2020.100615 (2020).
62. Singh, S., Sapra, A., Kakkar, S., Bhan, M. & Jangra, A. K. To evaluate in vitro acetylcholinesterase inhibitory activity of various Indian medicinal plants. *Asian Pac. J. Health Sci.* **9**, 205–212. https://doi.org/10.21276/apjhs.2022.9.2.41 (2022).
63. Zhang, H. & Zhou, Q. Tyrosinase inhibitory effects and antioxidative activities of Saponins from *Xanthoceras sorbifolia* nutshell (Hudson AO, ed.). *PLoS ONE* **8**, e70090. https://doi.org/10.1371/journal.pone.0070090 (2013).
64. Yu, L-L. et al. Gusanlungionosides A–D, potential tyrosinase inhibitors from *Arcangelisia gusanlung*. *J. Nat. Prod.* **74**, 1009–1014. https://doi.org/10.1021/np100900k (2011).

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Declarations

Competing interests

The authors declare no competing interests.

Statement and declarations

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