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Comparative metabolomic and bioactivity profiling of wild and greenhouse–Transplanted *Plantago coronopus* L

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ABSTRACT

This study investigates the influence of growing conditions on the metabolomic composition and associated bioactivities of *Plantago coronopus* L. Methanol extracts were prepared from freeze-dried biomass of both wild and greenhouse-transplanted plants and evaluated for *in vitro* antioxidant capacity and inhibitory effects on enzymes relevant to Alzheimer's disease (acetylcholinesterase, butyrylcholinesterase), type 2 diabetes (α-glucosidase, α-amylase), and obesity-related disorders (lipase). An untargeted metabolomic analysis was performed using mass spectral library matching and *in silico* annotation tools to characterise the chemical composition of the extracts. Extracts from wild specimens exhibited stronger antioxidant activity and cholinesterase inhibition, coinciding with higher levels of total phenolics, flavonoids, and carbohydrates. These differences were associated with increased levels of bioactive metabolites, including caffeic acid derivatives, terpenoids, and lipid-like compounds, possibly linked to environmental stress responses. Despite lower bioactivity, greenhouse-grown plants still produced valuable compounds such as acteoside, echinacoside, and plantamajoside. These findings highlight the potential of *P. coronopus* as a source of bioactive metabolites and suggest that manipulating environmental conditions in controlled cultivation could enhance phytochemical yields.

1. Introduction

The halophyte *Plantago coronopus* L. (Plantaginaceae) is a small rosette-forming herb characterized by deeply pinnatifid linear to lanceolate leaves, often arranged in a basal rosette. The erect inflorescences bear slender, leafless spikes with small, inconspicuous flowers tightly clustered along the axis (Koyro, 2006; Ceccanti et al., 2022a). It has a global distribution, thriving in both saline and human–impacted coastal areas often characterised by fluctuating environmental conditions, such as irregular water availability, extreme temperatures and nutrient limitations (Koyro, 2006; Pereira et al., 2016). The species is native to Europe, North Africa, and Western Asia, but has expanded to other temperate regions, including parts of Southern Africa. In South Africa, *P. coronopus* is considered a naturalised species, well established in coastal habitats, disturbed soils and ruderal areas (African Plant Database, 2025; iSpot Nature, 2025). Traditionally, it is used in Balkan and Mediterranean cuisine, as a cooked vegetable and as a salad ingredient, and ethnomedicine for its analgesic, anti–inflammatory, antipyretic, anticancer, and emollient properties (Ceccanti et al., 2022a; Pereira et al., 2016). Research into *P. coronopus* as a source of bioactive compounds has identified phenolics, especially caffeic acid derivatives, which exhibit significant antioxidant, anti–inflammatory and anticancer activities with potential pharmaceutical and nutraceutical applications (Gálvez et al., 2005, 2003; Pereira et al., 2016; Rodrigues et al., 2014; Tegin et al., 2018). Moreover, *P. coronopus* boasts a rich nutritional profile, including minerals such as magnesium, calcium, potassium, sodium, and essential amino acids (Pereira et al., 2016; Rodrigues et al., 2014) making it a valuable candidate as a functional food ingredient (Ceccanti et al., 2022a).

Given its emerging importance and wide range of potential uses, the domestication of *P. coronopus* is essential for the sustainable production of cost–effective biomass and, consequently, valuable bioactive molecules (Ceccanti et al., 2022b; López-Laredo et al., 2012). However, the environmental conditions in which plants grow significantly impact

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their productivity of bioactive compounds (Ceccanti et al., 2022b; López-Laredo et al., 2012). Factors such as soil composition, water availability, temperature, light, and nutrient levels play crucial roles in the production of these metabolites (Bachheti et al., 2021; Haruna and Yahaya, 2021; Ramawat and Arora, 2021). One of the major risks of plant domestication is that a deficiency in any of these parameters in greenhouse conditions might result in a lower production of bioactive compounds, such as phenolics and flavonoids, which are typically produced in response to environmental stresses (Ceccanti et al., 2022b; López-Laredo et al., 2012; Ramawat and Arora, 2021). Therefore, understanding how growing conditions affect the plant metabolome, and especially the production of bioactive compounds, is crucial for advancing plant biology and optimizing their domestication, cultivation, and applications (Bachheti et al., 2021; Ramawat and Arora, 2021). On one hand, metabolomics has been used in plant sciences to reveal the complex effects of environmental factors on plant metabolism (Sampaio et al., 2016). On the other hand, bioactivity studies describe the biological effects of plant compounds on human health, significantly influencing their medicinal and nutritional value (Ramawat and Arora, 2021). Thus, bridging the gap between plant–environment interactions and the production of valuable bioactive compounds is fundamental for the future cultivation and exploitation of medicinal plants (Bachheti et al., 2021; Sampaio et al., 2016).

While *P. coronopus* has been previously characterized for its antioxidant and phytochemical composition in wild populations from Portugal and other Mediterranean regions (Pereira et al., 2016; Ceccanti et al., 2022a), no studies to date have explored how environmental conditions influence its metabolomic profile and bioactivity under controlled cultivation. Understanding whether domestication impacts the accumulation of key secondary metabolites is essential to ensure both the efficacy and reproducibility of plant-based applications. Given the increasing interest in halophytes for sustainable agriculture and natural product discovery, this study aims to fill this gap by conducting an integrative comparison between wild-collected and greenhouse-grown *P. coronopus*, using untargeted metabolomics combined with *in vitro* antioxidant and enzymatic inhibition assays.

In this context, the primary goal of this study is to compare the metabolomic profiles and bioactive properties of wild–grown and greenhouse–transplanted *P. coronopus* plants. By conducting comprehensive metabolomic analyses and bioactivity assays, we aim to identify key metabolites influenced by growing conditions and correlate them with observed bioactivities. Methanol extracts from both groups were prepared and evaluated for *in vitro* antioxidant activity by radical and metal–based assays, as well as for inhibition of enzymes commonly used as *in vitro* models to screen compounds with potential relevance to neurodegenerative (AChE, BuChE), metabolic (α–glucosidase, α–amylase, lipase), and dermatological (tyrosinase) processes.. Differences in the total contents of phenolics (TPC), flavonoids (TFC), and carbohydrates (TCC) were established using colorimetric methods, followed by an untargeted metabolomics approach, including a feature annotation through traditional MS/MS library match and *in silico* tools

2. Material and methods

2.1. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), gallic acid, acetylcholinesterase (EC 3.1.1.7), butyrylcholinesterase (EC 3.1.1.8), acetylthiocholine iodide, butyrylthiocholine iodide, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), α-amylase from porcine pancreas (EC 3.2.1.1), soluble starch, α-glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20), p-nitrophenyl-α-D-glucopyranoside (pNPG), lipase (EC 3.1.1.3) from porcine pancreas type II, 4-nitrophenyl dodecanoate (PNP), tyrosinase (EC 1.14.18.1) from mushroom, L-tyrosine, galantamine, acarbose, orlistat, arbutin, and pyrocatechol violet were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and ethanol (HPLC grade), and other solvents and reagents were obtained from VWR International (Leuven, Belgium).

2.2. Plant material and growing conditions

Wild specimens of *Plantago coronopus* were collected in April 2021 from saline areas surrounding aquaculture ponds at the Fish Farming Pilot Station in Olhão (EPPO), in the Portuguese Institute for the Sea and Atmosphere (IPMA) in Algarve, Portugal. The plants were carefully uprooted, ensuring minimal disturbance to their root systems, and transported to the greenhouse facilities located at EPPO. Upon arrival at the greenhouse, the wild specimens were transplanted into pots filled with a soil mixture composed of peat and perlite (3:1, v/v). These plants were irrigated with brackish water from a local well, with a conductivity of 20.7 us/cm, and fertilization was carried out bi–weekly using a commercial nutrient solution (NPK 7–5–6) to support growth. The plants were grown under these conditions for 3 months to allow for full acclimatization and growth stabilization. Both wild–grown and greenhouse–transplanted plants were harvested on the same day and belonged to the same population and collection site, to ensure comparability and reduce genetic variability between groups (Table 1, Fig. 1). For all biochemical and metabolomic analyses, three biological replicates were used per condition, each consisting of pooled aerial parts from five individual plants, resulting in a total of 15 plants per group. A voucher specimen has been deposited at the XtremeBio herbarium, under the reference codes XBH02–01 (wild) and XBH02–02 (greenhouse). The harvested plant material was thoroughly washed with deionized water to remove any soil or debris. The cleaned plant material was then freeze–dried and stored at –20 °C until further analysis.

A schematic diagram of the experimental workflow, from plant collection to extract analysis, is provided in Fig. 2.

2.3. Extracts preparation

Freeze–dried plant material was ground into a fine powder using a coffee grinder. The extracts were prepared by macerating 1 g of powdered plant material in 40 mL of methanol for 24 h at room temperature with continuous stirring. Methanol was selected due to its polarity and proven efficiency in extracting phenolic compounds and other polar secondary metabolites from *P. coronopus* (Pereira et al., 2016; Rodrigues et al., 2014). The extracts were filtered through the Whatman No 4 filter paper, and the filtrates were concentrated under reduced pressure using a rotary evaporator. The concentrated extracts were reconstituted in 70 % ethanol to a final concentration of 10 mg/mL and stored at –20 °C until analysis.

Table 1
Meteorological parameters from April to June 2021 (Source: Weather Spark, 2021)). Na: Not applicable.

Parameter	Wild			Greenhouse		
	April 2021	May 2021	June 2021	April 2021	May 2021	June 2021
Mean air temperature (°C)	16.2	18.7	21.8	23.9	23.1	24.8
Mean maximum temperature (°C)	18.6	21.3	24.4	44.6	45.0	45.5
Mean minimum temperature (°C)	13.7	15.9	18.7	11.3	7.8	12.8
Total precipitation (mm)	44	25	5	Na	Na	Na
Mean relative humidity (%)	71	67	64	58	55	59

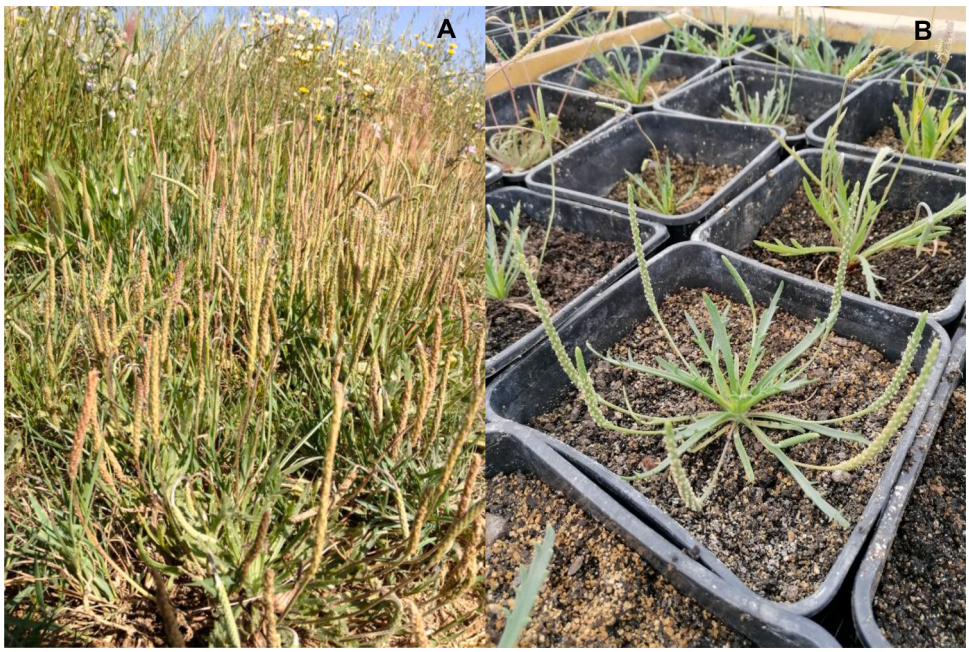

Fig. 1. Representative photographs of *P. coronopus* plants collected in the wild (A) and cultivated under greenhouse conditions (B).

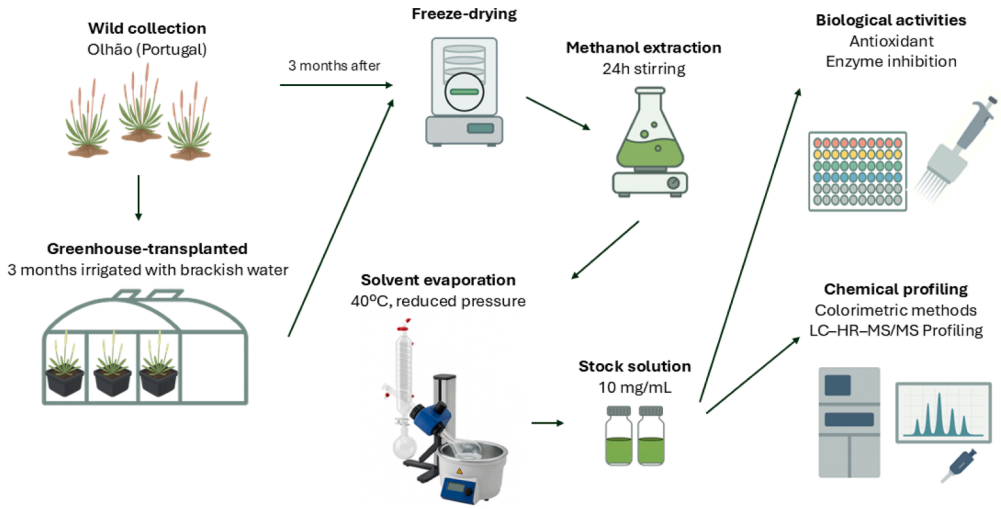

Fig. 2. Overview of the experimental workflow used in this study. Wild *P. coronopus* plants were collected in Olhão (Portugal), and a subset was transplanted to greenhouse conditions for three months under brackish irrigation. At the end of this period, both wild and greenhouse-grown plants were harvested simultaneously from the same site and population. Aerial parts were freeze-dried, extracted with methanol, and analysed for antioxidant activity, enzyme inhibition, and metabolite composition using colorimetric assays and untargeted LC–HR–MS/MS.

2.4. Antioxidant activity

2.4.1. DPPH scavenging assay

The radical scavenging activity (RSA) against 1,1–diphenyl–2–picrylhydrazyl (DPPH•) was evaluated by the method of Brand Williams et al. 1995 (Brand-Williams et al., 1995) adapted to 96–well microplates. Briefly, 22 μL of extracts were mixed with 200 μL of DPPH solution (120 μM in ethanol) and incubated for 30 min at RT in the darkness. The absorbance was measured at 515 nm. BHT (1 mg/mL) was used as a positive control.

2.4.2. ABTS scavenging assay

The RSA on 2,2′–azinobis(3–ethylbenzothiazoline–6–sulfonic acid), (ABTS•+) radical cation was evaluated as previously described by *Re* et al. 1999 (*Re* et al., 1999), adapted to 96–well microplates. First, a stock ethanol solution containing ABTS•+ (7.4 mM) and potassium persulfate (2.6 mM) was prepared and incubated overnight in darkness at 4 °C. A working solution was then prepared by diluting the stock solution to an absorbance of 0.7 at 734 nm. For the assay, 10 μL of the extracts were mixed with 190 μL of ABTS•+ working solution and incubated in darkness at RT for 6 min. The absorbance was measured at 734 nm. BHT (1 mg/mL) was used as a positive control.

2.4.3. Ferric reducing antioxidant power (FRAP)

Ferric reducing antioxidant power (FRAP) was evaluated using the method described by Megías et al. 2009 (Megías et al., 2009). Plant extracts (50 μL) were mixed with 50 μL of 1 % potassium ferricyanide and 50 μL of distilled water. After an incubation at 50 °C for 20 min, 50 μL of 10 % trichloroacetic acid and 10 μL of 0.1 % ferric chloride were added. Microplates were further incubated for 10 min at 50 °C and the

final absorbance was measured at 700 nm. BHT (1 mg/mL) was used as standard.

2.4.4. Copper (CCA) and iron (ICA) chelating activities

Copper (CCA) and iron (ICA) chelating activities followed the protocols described by Megías et al. (Megías et al., 2009). To assess the capacity of *P. coronopus* to chelate copper, 30 μL of extracts were mixed with 200 μL of 50 mM sodium acetate buffer (pH 6), 6 μL of pyrocatechol violet (PV, 4 mM in the acetate buffer), 100 μL of copper sulphate (50 μg/mL in water) and the final absorbance was measured at 632 nm. Similarly, ICA was determined by mixing 30 μL of the extracts with 200 μL of distilled water and 30 μL of an iron (II) chloride solution (0.1 mg/mL in water) and incubated for 30 min at RT. Afterwards, 12.5 μL of ferrozine solution (40 mM in water) was added and the absorbance was measured at 562 nm. Ethylenediamine tetraacetic acid (EDTA 1 mg/mL) was used as a positive control for both CCA and ICA.

2.5. Enzymatic inhibition

2.5.1. Inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE)

The inhibition of the extracts on acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) was evaluated following Ellman at al. 1961 (Ellman et al., 1961). Briefly, 20 μL of the extracts were mixed with 140 μL of sodium phosphate buffer (0.1 mM, pH 8.0) and 20 μL of AChE or BChE solution (0.28 U/mL in sodium phosphate buffer 0.1 mM, pH 7.0) and incubated at RT for 15 min. The reaction was started with the addiction of 10 μL of the substrates (acetylthiocholine or butyrylthiocholine iodide, 4 mg/mL diluted in sodium phosphate buffer 0.1 mM, pH 8.0) and 20 μL of 5,50–dithio–bis (2–nitrobenzoic acid) at a concentration of 1.2 mg/mL in ethanol. After 10 min of incubation at RT, the absorbance at 412 nm was measured and galanthamine (1 mg/mL) was used as the positive control.

2.5.2. Inhibition of α–amylase

The α–amylase inhibitory activity was determined following Kwon et al. 2008 (Kwon et al., 2008). Extracts (40 μL) were mixed with 40 μL of 0.1 % starch solution (diluted in the previous buffer and 40 μL of amylase solution (100 U/mL in 0.1 M sodium phosphate buffer, pH 7.0) and incubated for 10 min at 37 °C. After that, 20 μL of 1 M hydrochloric acid (HCl) and 100 μL of iodide solution (5 mM iodine (I_2), 5 mM potassium iodide (SSKI oral solution) (KI), in distilled water) were added. The absorbance was measured at 580 nm and acarbose (10 mg/mL) was used as positive control.

2.5.3. Inhibition of lipase

The inhibitory activity on porcine lipase was evaluated according to method of McDougall et al. 2009 (McDougall et al., 2009). Plant extracts (20 μL) were mixed with 200 μL of Tris–HCl buffer (100 mM, pH 8.2), 20 μL of the enzyme solution (1 mg/mL in Tris–HCl buffer), 20 μL of the substrate (4–nitrophenyl dodecanoate, 5.1 mM in ethanol) and incubated for 10 min of incubation at 37 °C. The final absorbance was read at 410 nm and orlistat (1 mg/mL) was used as the positive control.

2.5.4. Inhibition of tyrosinase

The inhibitory activity against tyrosinase was determined in 96–well microplates using the method reported by Zengin 2016 (Zengin, 2016). Briefly, the extracts (70 μL) were mixed with 30 mL of the enzyme (333 units/mL in phosphate buffer, pH 6.5) and incubated for 5 min at RT. Afterwards, 110 μL of the substrate (L–tyrosine, 2 mM in water) were added and incubated for 30 min at RT. The final absorbance was measured at 492 nm and arbutin (1 mg/mL) was used as positive control.

2.6. Chemical profiling

2.6.1. Colorimetric methods

Total phenolics content (TPC) was determined by the Folin–Ciocalteau assay and results were expressed as gallic acid equivalents in milligrams per gram of extract (mg GAE/g dry weight, DW) using a calibration curve of gallic acid standard solutions (from 500 to 10 mg/mL) (Velioglu -et al., 1998). Total flavonoid content (TFC) was estimated by the aluminium chloride ($AlCl_3$) method (Zou et al., 2011). Results were expressed as milligrams of quercetin equivalents per gram of dried sample (mg QE/g, DW) using a calibration curve of rutin (from 500 to 10 mg/ml). Total carbohydrate content (TCC) was estimated by the phenol–sulfuric acid method, using D–glucose as standard (DuBois et al., 1956). Results were expressed as milligrams of glucose equivalents per gram of dried sample (mg GE/g DW) using a calibration curve of glucose (from 500 to 10 mg/mL).

2.6.2. LC–HR–MS/MS profiling of extracts

The extracts were analysed in triplicates using a Thermo Scientific™ UltiMate™ 3000 LC equipped with an Orbitrap Elite (Thermo Fisher Scientific, Waltham, MA, USA) mass spectrometer and a Heated Electro–Spray Ionization source (HESI–II; Thermo Scientific). Chromatographic profile followed the protocol reported in Silva et al. 2022 (Silva et al., 2022). Xcalibur v4.1 Qual Browser (Thermo Scientific, Waltham, MA, USA) was used for LC–MS data acquisition. Thermo -".raw" files acquired in positive ion mode were converted to ".mzML" format in centroid mode using Proteowizard (Chambers et al., 2012) an imported in Mzmine version 3.2.3 (Schmid et al., 2023) for feature finding, alignment and extraction. Final features were exported as ".mgf" and ".csv" files and analysed in Global Natural Products Social Molecular Networking (Wang et al., 2016) (GNPS; http://gnps.ucsd.edu, accessed on 24 June 2024) platform for feature−based molecular networking (Nothias et al., 2019). The data was filtered by removing all MS/MS fragment ions within +/− 17 Da of the precursor *m/z*. MS/MS spectra were window filtered by choosing only the top 6 fragment ions in the +/− 50 Da window throughout the spectrum. The precursor ion mass tolerance was set to 2.0 Da and the MS/MS fragment ion tolerance to 0.02 Da. A molecular network was then created where edges were filtered to have a cosine score above 0.6 and >6 matched peaks. Further, edges between two nodes were kept in the network if and only if each node appeared in each other's top 10 most similar nodes. Finally, the maximum size of a molecular family was set to 100, and the lowest–scoring edges were removed from molecular families until the molecular family size was below this threshold. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.8 and at least 4 matched peaks. SIRIUS version 5.8.3 (Dührkop et al., 2019) was used to assign features molecular formulas and predict their structures and chemical classes based on the fragmentation patterns in MS/MS spectra and the MS1 (parent ion) isotope patterns (Böcker et al., 2009). Within the workflow, ZODIAC (Ludwig et al., 2020), CSI:FingerID (Hoffmann et al., 2021), and CANOPUS based on ClassyFire ChemOnt ontology (Djoumbou Feunang et al., 2016; Dührkop et al., 2020) tools were employed. Blank was used for background feature removal. Features annotation was based on four distinct levels (Sumner et al., 2007). The spectra in the network were then searched against GNPS spectral libraries (Horai et al., 2010; Wang et al., 2016). The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.7 and at least 3 matched peaks. SIRIUS version 5.8.3 (Dührkop et al., 2019) was used to assign features molecular formulas and predict their structures and chemical classes based on the fragmentation patterns in MS/MS spectra and the MS1 (parent ion) isotope patterns (Böcker et al., 2009). Within the workflow, ZODIAC (Ludwig et al., 2020), CSI:FingerID (Hoffmann et al., 2021), and CANOPUS based on ClassyFire ChemOnt ontology (Djoumbou

Feunang et al., 2016; Dührkop et al., 2020) tools were employed. A solvent blank was used for background feature removal. Features annotation was based on four distinct levels (Sumner et al., 2007) with a strict consensus (Trentin et al., 2024).

2.7. Statistical analyses

Statistical analyses were performed in R–Statistics 3.2.3 version. When the biological activities of the extracts (at 10 mg/mL) were above 50 %, maximal effective concentrations (EC_{50} mg/mL) and the half–maximal concentration values (IC_{50} mg/mL) were calculated. Data resulting from antioxidant, enzymatic, and colorimetric assays were tested for normal distribution (Shapiro–Wilk's Normality Test) and homogeneity of variance (Levene's test). The data were analysed using one–way ANOVA or its non–parametric equivalent (Kruskal–Wallis's test), followed by Tukey's post–hoc or non–parametric post–hoc test (Dunn test). Data obtained using the untargeted metabolic approach were analysed through different steps (multivariate and univariate). After blank removal and data normalization, the "pheatmap" package was employed for heatmap visualization. Four additional R packages ("ggsci", "matrixStats", "ggrepel", and "tidyverse") were used to compare treatment and control samples by one–way ANOVA followed by Tukey's post hoc test.

3. Results

3.1. Antioxidant activity and enzymatic inhibition

The crude extracts of greenhouse–transplanted and wild–collected specimens of *P. coronopus* were tested for radical scavenging, metal chelating and enzymatic inhibitory potential using a combination of several *in vitro* assays (Fig. 3). Both extracts from wild–collected and greenhouse–transplanted plants showed RSA values (Fig. 3a) above 50 % towards DPPH• (EC_{50} = 0.14 and 0.42 mg/mL, respectively) and ABTS•+ (EC_{50} = 0.30 and 1.71 mg/mL, respectively) radicals (Table 1). Similarly, both the FRAP (Fig. 3a) and the CCA (Fig. 3b) assays showed higher values (Table 1) in wild–collected plants (EC_{50} = 1.15 mg/mL for FRAP and 4.17 mg/mL for CCA) than in greenhouse–transplanted ones (EC_{50} = 2.75 mg/ml for FRAP and 8.83 mg/mL for CCA). Conversely, the ICA was significantly lower in the extracts from wild–collected plants compared to the greenhouse–transplanted ones (Fig. 3b). The inhibitory effects of *P. coronopus* extracts were also tested against AChE, BChE, α–amylase, α–glucosidase, lipase and tyrosinase (Fig. 3c). Statistically significant differences in inhibitory activity were observed in the AChE, BChE and α–amylase assays, with wild–collected specimens of *P. coronopus* showing markedly higher inhibitory potential towards both AChE and BChE and lower inhibitory activity against α–amylase. In particular, AChE inhibition was above 50 %, with average IC_{50} values of 0.89 mg/ml for wild–collected plants and 2.12 mg/ml for greenhouse–transplanted ones (Table 2).

3.2. Chemical profiling

The extracts from both wild–collected and greenhouse–transplanted specimens of *P. coronopus* were evaluated for total phenolics (TPC), flavonoids (TFC) and carbohydrates (TCC) content (Table 3). Generally, extracts from wild–collected plants exhibited significantly higher values of TPC (99.33 vs. 48.99 mg GAE/g DW), TFC (20.59 vs. 13.23 mg QE/g DW), and TCC (174.23 vs. 142.71 mg GAE/g DW) than those from greenhouse–transplanted ones.

A total of 67 features were extracted through the bioinformatic analysis of *P. coronopus* extracts (Table S1). Among these, 55 features showed significant differences between the wild–collected and greenhouse–transplanted *P. coronopus* (ANOVA, Benjamini–Hochberg adjusted p–value < 0.05, Table S2. Figure S1). Library spectral matches enabled the annotation of 14 features (level 2), while an *in silico* approach allowed for the chemical class prediction of 16 features (level 3). The level 2 and 3 annotated features of *P. coronopus* extracts were visualized in a hierarchical clustering heatmap (Fig. 4). Most of these features belonged to phenylpropanoids, polyketides, lipids and lipid–like molecules.

Among the level 2 annotated compounds (Table 4), several phenylethanoid glycosides stood out due to their high relative abundance, particularly in wild plants. Acteoside was the most intense feature, representing 35.72 % of wild extracts compared to 23.92 % in greenhouse samples. Echinacoside and plantamajoside also showed higher intensities in wild specimens (14.26 % and 2.29 %, respectively), while

Table 2
Half maximal effective concentrations (EC_{50}, mg/mL) and inhibitory concentrations (IC_{50}, mg/mL). Values represent the mean ± standard deviation (SD) performed six times (n = 6); nt: not tested; *positive control. For the same column, different letters indicate significantly different (Multiple Comparisons of Means: Tukey Contrast, 95 % family–wise confidence level).

	EC_{50}				IC_{50}	
	DPPH	ABTS	FRAP	CCA	AChE	BChE
Greenhouse	0.42 ± 0.08 ^{b}	1.71 ± 0.57 ^{b}	2.75 ± 0.28 ^{b}	8.83 ± 0.97 ^{c}	2.12 ± 0.19 ^{b}	nt
Wild	0.14 ± 0.06 ^{a}	0.30 ± 0.06 ^{a}	1.15 ± 0.26 ^{a}	4.17 ± 0.27 ^{b}	0.89 ± 0.20 ^{a}	3.70 ± 0.30 ^{b}
BHT*	0.05 ± 0.01 ^{a}	0.16 ± 0.03 ^{a}				
EDTA*				0.14 ± 0.04 ^{a}		
Galanthamine*					0.62 ± 0.20 ^{a}	1.47 ± 0.50 ^{a}

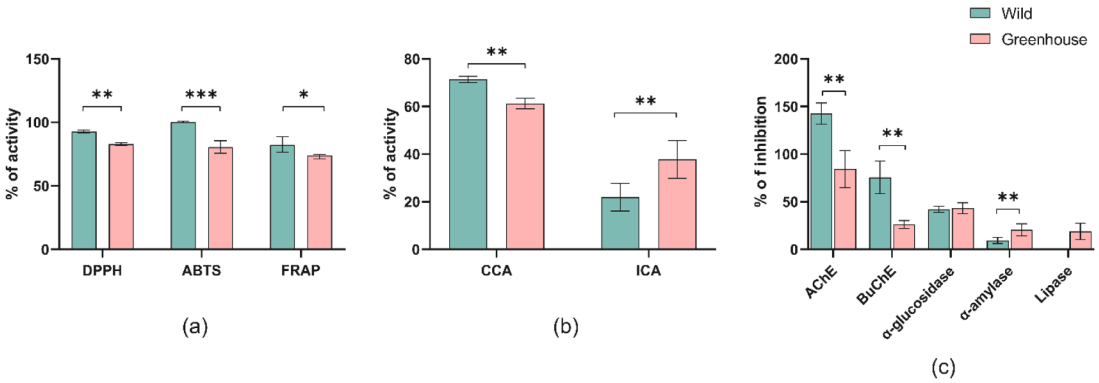

Fig. 3. Radical–scavenging activities (a), metal–chelating activities (b) and enzymatic inhibitory properties (c) of the extracts from *P. coronopus* at the concentration of 10 mg/mL. Asterisks indicate statistically significant differences ('***' = p–value < 0.001; '**' = p–value < 0.01 and '*' = p–value < 0.05).

Table 3
Total contents in phenolics (TPC; mg GAE/g DW, GAE: gallic acid equivalents), flavonoids (TFC; mg QE/g DW, QE: quercetin equivalents) and carbohydrates (TCC; mg GE/g DW, GE: glucose equivalents) of extracts from wild and greenhouse samples of *P. coronopus*. For the same column, different letters indicate significantly different (Multiple Comparisons of Means: Tukey Contrast, 95 % family–wise confidence level).

	TPC (mg/GAE g DW)	TFC (mg QE/g, DW)	TCC (mg/GAE g DW)
Greenhouse	48.99 ± 4.78 [a]	13.23 ± 0.42 [a]	142.71 ± 27.05 [a]
Wild	99.33 ± 6.04 [b]	20.59 ± 0.76 [b]	174.23 ± 8.82 [b]

verbasoside was almost exclusively detected in wild plants (0.84 % *vs.* 0.03 %). On the other hand, trehalose and lauryldiethanolamine were slightly more abundant in greenhouse extracts. Lipid-related metabolites, such as phytosphingosine, LysoPC(16:0), glycyrrhetinic acid, and oleanolate, were present in both extracts at relatively low but comparable intensities.

In addition, Principal component analysis (PCA) revealed a clear separation between wild and greenhouse-grown samples based on their metabolomic profiles (Fig. 5), with PC1 and PC2 explaining 72.6 % and 8.3 % of the total variance, respectively. This differentiation further supports the role of environmental factors in shaping the accumulation of secondary metabolites with potential antioxidant and neuroprotective properties in *P. coronopus*.

4. Discussion

Methanol extracts of *P. coronopus* showed significant *in vitro* antioxidant activity, metal chelation and enzyme inhibition, with wild–collected plants consistently outperforming greenhouse-grown specimens. These findings are in line with previous reports on wild *P. coronopus,* which showed high RSA towards DPPH• and ABTS•+, using methanol, ethyl acetate and water extracts from aerial parts collected from the wild in Spain, Portugal and Turkey (Gálvez et al., 2005; Pereira et al., 2016; Rodrigues et al., 2014; Tegin et al., 2018). Similarly, methanol/water (4:1) extracts from the closely related species *P. altissima* and *P. lanceolata* collected from Serbian mountains showed also strong iron reducing power (Beara et al., 2012; Rodrigues et al., 2014). Additionally, the capacity of *P. coronopus* extracts to chelate Cu^{2+} and Fe^{2+} ions was tested and IC_{50} values were similar to those obtained by Pereira et al. (2016) (Pereira et al., 2016), confirming the wide

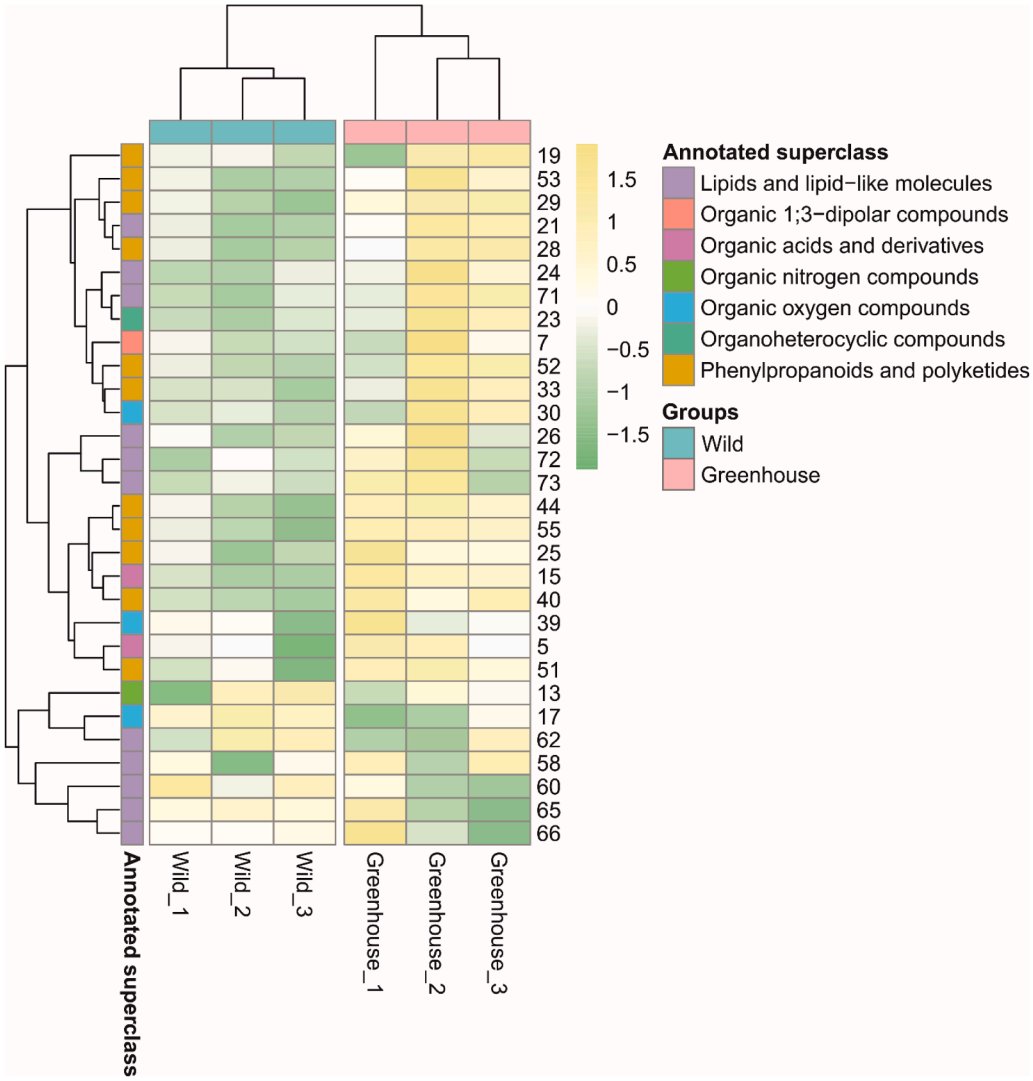

Fig. 4. Hierarchical clustering analysis of *P. coronopus* features (level 2–3 annotation). Features ID are indicated at the side of each raw. Colours from yellow to green indicate the normalized relative abundance values of metabolites from low to high according to the scale bar. Treatments are displayed in columns, with replicates coded based on their origin: "wild" for wild–collected and "greenhouse" for greenhouse–transplanted plants. Each replicate is followed by a suffix number (1, 2, or 3) to indicate the specific replicate.

Table 4
Annotated compounds (level 2) in methanol extracts of wild and greenhouse–cultivated plants of *P. coronopus*.

Feature ID	*m/z* value	RT [min]	Adduct	Annotated Molecular Formula	Annotated compound	Relative Intensities (%)	
						Wild	Greenhouse
15	118.0855	0.9705	$[M+H]+$	$C_5H_{11}NO_2$	Valine	1.23	0.96
17	365.1033	0.9763	$[M+Na]+$	$C_{12}H_{22}O_{11}$	Trehalose	0.56	1.03
19	485.1604	9.0555	$[M+Na]+$	$C_{20}H_{30}O_{12}$	Verbasoside	0.84	0.03
25	658.2299	13.2776	$[M+H_3N+H]+$	$C_{29}H_{36}O_{16}$	Plantamajoside	2.29	1.36
39	804.2877	14.1185	$[M+H_3N+H]+$	$C_{35}H_{46}O_{20}$	Echinacoside	14.26	10.07
44	642.2363	14.398	$[M+H_3N+H]+$	$C_{29}H_{36}O_{15}$	Acteoside	35.72	23.92
58	274.2729	23.8209	$[M+H]+$	$C_{16}H_{35}NO_2$	Lauryldiethanolamine	1.68	2.40
60	318.2987	24.0337	$[M+H]+$	$C_{18}H_{39}NO_3$	Phytosphingosine	0.31	0.52
62	290.2676	24.1973	$[M+H]+$	$C_{16}H_{35}NO_3$	Hexadecaphytosphingosine	0.34	0.55
65	520.3372	24.4993	$[M+H]+$	$C_{26}H_{50}NO_7P$	LysoPC(18:2)	0.22	0.03
66	496.3371	25.3276	$[M+H]+$	$C_{24}H_{50}NO_7P$	LysoPC(16:0)	0.34	0.25
71	471.3441	25.9314	$[M+H]+$	$C_{30}H_{46}O_4$	Glycyrrhetinic acid	0.55	0.39
72	439.3543	30.1358	$[M-H_2O+H]+$	$C_{30}H_{48}O_3$	Oleanolate	0.93	0.95

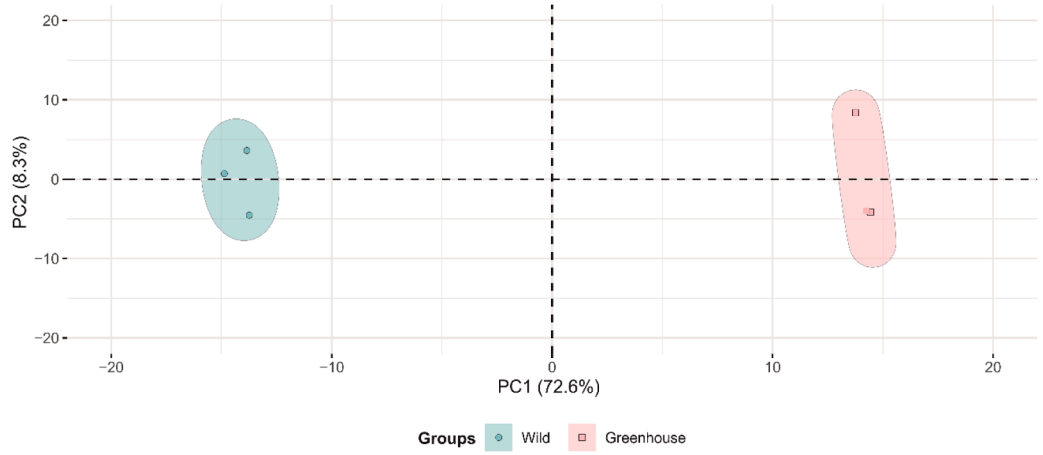

Fig. 5. Principal component analysis (PCA) of metabolomic profiles of wild–collected (light blue) and greenhouse–transplanted (pink) *P. coronopus*. The x– and y–axes represent principal coordinates 1 and 2, respectively, in brackets the percentages of the overall variance explained by each principal coordinate.

antioxidant potential of this species. *P. coronopus* extracts were further explored as enzymatic inhibitors. Notably, extracts from wild-collected plants inhibited cholinesterase enzymes (AChE and BChE) by >50 %, suggesting this species may be a promising source of bioactive compounds with *in vitro* inhibitory activity against enzymes implicated in neurodegenerative processes (Özaslan et al., 2022). Similar inhibitory activity has been described for methanol, hexane, ethanol, and water extracts of *P. subulata* collected in Turkey (Kurt et al., 2018; Özaslan et al., 2022). To the best of our knowledge, this is the first report on the AChE and BChE inhibotry activity of *P. coronopus* extracts.

The antioxidant and enzyme-inhibitory activities are likely linked to the presence of phenolics, flavonoids and carbohydrates (Adom et al., 2017; Ceccanti et al., 2022b; Gálvez et al., 2005, 2003; Pereira et al., 2016; Rodrigues et al., 2014; Tegin et al., 2018). Colorimetric assays confirmed significantly higher TPC, TFC and TCC values in extracts from wild-collected plants. The TPC and TFC values observed were comparable to those previously reported for methanol extracts of *P. coronopus* (Pereira et al., 2016; Rodrigues et al., 2014), while the TCC values were similar to those found in *P. major* (Alizadeh Behbahani et al., 2017).

To better understand which compounds underlie the observed activities and the differences between wild and greenhouse–cultivated plants, an untargeted metabolomics approach was employed. Among the annotated features, the most intense were the acteoside, echinacoside and plantamajoside. These compounds are caffeic acid derivatives, also known as phenylethanoid glycosides (Agar and Cankaya, 2020), widely distributed across *Plantago* species. They play important protective roles in plants against high light, UV radiation, herbivores and pathogens, and have been associated with potential health benefits in humans (Agar and Cankaya, 2020; Gálvez et al., 2005, 2003; Pereira et al., 2016; Rodrigues et al., 2014). Acteoside, also known as verbascoside, was the main phenolic annotated in both wild and greenhouse extracts. It is considered as the most abundant compound in *P. coronopus*, with putative allelopathic activity against phytopathogenic fungi and well-documented antioxidant, anti–inflammatory, and cytotoxic activities (Gálvez et al., 2005, 2003; Pereira et al., 2016). Echinacoside, the second most abundant feature, is known for its antioxidant, neuroprotective and anti–inflammatory properties (Wang et al., 2023). Pure acteoside and echinacoside isolated from *P. subulata* have demonstrated effective inhibition of both AChE and BChE (Özaslan et al., 2022), supporting their likely contribution to the strong inhibitory activities observed in this study. Plantamajoside, the third most abundant phenylethanoid glycoside in *P. coronopus* extracts, also exhibits broad bioactivity, including anti–tumour, anti–inflammatory, diuretic, wound–healing, anti–asthmatic, hepatoprotective, anti–aging, and neuroprotective activities (Goyal et al., 2022; Ravn et al., 2015). In addition, verbasoside, another phenolic compound, was tentatively identified for the first time in this species.

Moreover, lipid and lipid−like molecules annotated in *P. coronopus* extracts included terpenoids, such as glycyrrhetinic acid and oleanolate, previously reported in *P. major* leaves (Adom et al., 2017). These compounds are known to selectively inhibit cyclooxygenase–2-mediated prostaglandin biosynthesis and are considered promising natural anti–inflammatory agents with potentially fewer side effects than conventional drugs (Ringbom et al., 1998). Additional lipid-related metabolites included lysophosphatidylcholines [LysoPC(18:2), LysoPC(16:0)] and sphingolipids (phytosphingosine, hexadecaphytosphingosine), which

participate in diverse cellular processes, although their roles and bioactivity in *Plantago* spp. remain largely unexplored (Drissner et al., 2007; Michaelson et al., 2016). Other annotated features included osmolytes such as trehalose and valine, commonly synthesized in response to osmotic stress (Vicente et al., 2004).

The observed differences in RSA, metal chelation and enzymatic inhibition between wild and greenhouse-grown *P. coronopus* extracts are likely attributed to their distinct chemical composition. Notably, wild specimens exhibited significantly higher TPC, TFC and TCC values, which correlated with enhanced biological activities. These trends were consistent with metabolomic data, which revealed a greater abundance of acteoside, echinacoside and plantamajoside in wild extracts. Furthermore, the clear separation between sample groups observed in the PCA underscores the strong influence of environmental conditions on the metabolomic profile of *P. coronopus*, reflecting shifts in secondary metabolism triggered by stress exposure. The accumulation of osmolytes (*e.g.*, polysaccharides, amino acids, polyols) and antioxidant compounds (phenolics, flavonoids) is a typical response of halophytes to environmental stress (Bueno and Cordovilla, 2021; Ltaeif et al., 2021). Indeed, *P. coronopus* and related species have shown increased TPC and TFC under saline conditions (Ltaeif et al., 2021). Understanding how such stress-induced metabolic adjustments affect bioactivity is essential for defining optimal cultivation strategies that preserve or enhance the phytochemical potential of this species (López-Laredo et al., 2012).

Meteorological data recorded during the collection period supports the hypothesis that the enhanced biological activity observed in wild *P. coronopus* is linked to environmental stress. Wild plants were exposed to moderate temperatures, higher humidity and a gradual reduction in precipitation, while greenhouse specimens faced extreme maximum temperatures (>44 °C), lower humidity and likely water stress. Such abiotic stressors are known to induce oxidative stress and stimulate the production of antioxidant compounds, particularly phenolics and flavonoids (Chaves et al., 2009; Khan et al., 2015). In wild habitats, additional factors such as natural UV-B radiation and fluctuating photoperiods further activate phenylpropanoid pathways and secondary metabolism (Ramakrishna and Ravishankar, 2011; Selmar and Kleinwächter, 2013). Although greenhouse conditions imposed thermal and hydric stress, the consistently higher TPC, TFC and TCC values in wild plants, along with greater levels of acteoside, echinacoside and plantamajoside, suggest that stress complexity and multifactorial cues in natural environments more effectively trigger metabolite accumulation (Barnes et al., 2023). These findings highlight the importance of mimicking key environmental factors when developing sustainable cultivation strategies for halophytes with pharmacological potential. Given that stress-related metabolite accumulation in *P. coronopus* appears highly responsive to environmental conditions, future work should focus on optimizing cultivation parameters, such as salinity, photoperiod, and light quality, to replicate or even enhance the production of bioactive compounds observed in wild specimens. In addition, investigating the influence of developmental stage and controlled stress exposure may provide further insight into the metabolic plasticity of this species.

Overall, this study confirms *P. coronopus* as a valuable source of antioxidants and cholinesterase inhibitors and underscores the need for further research to optimize greenhouse conditions that can reproduce the metabolite profiles and biological activity observed in wild-grown plants.

5. Conclusions

This study highlights significant differences in the metabolomic composition and bioactivity of *Plantago coronopus* grown in wild *versus* greenhouse conditions. Wild plants, exposed to moderate temperatures, natural UV radiation, and gradual water limitation, accumulated higher levels of phenolics, flavonoids, and key bioactive metabolites, resulting in superior antioxidant and cholinesterase inhibitory activities. These findings underscore the role of environmental stressors in enhancing phytochemical production and suggest that mimicking such conditions in controlled cultivation may improve metabolite yields. Given its stress tolerance and bioactive potential, *P. coronopus* holds promise for sustainable use in functional foods and as a source of bioactive compounds for further pharmacological screening, particularly in saline and arid regions such as those in South Africa.

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Supplementary Materials: Table S1. Annotated feature table. **Table S2.** ANOVA test comparing wild and greenhouse *P. coronopus* features. p: p–value for group–wise difference after taking multiple group–wise comparisons into account; p_Benjamini_Hochberg: adjusted p–value after taking multiple tests (N number of features) into account; Significance: Whether the corrected p–value is below 0.05; F: F–value. **Figure S1.** Normalized features boxplots.

CRediT authorship contribution statement

Riccardo Trentin: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Maria João Rodrigues:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Alexia Dos Santos:** Methodology. **Eliana Fernandes:** Methodology. **Luísa Custódio:** Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sajb.2025.07.038.

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