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Comparative profiling of secondary metabolites and antioxidant properties of twelve *Morus* varieties: Insights into the diversity of *M. alba* and *M. nigra* grown in Sicily

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ABSTRACT

This work analyzed potential phytochemical differences in bioactive compounds, antioxidant activity and metabolic profiles of 12 different varieties of *Morus alba* and *Morus nigra* fruits grown in Sicily by Liquid chromatography coupled to High Resolution Mass Spectrometry. Through UV/Vis assays, variations in in bioactive compounds were observed, recording higher contents of polyphenols and anthocyanins in *M. nigra* species. Spectrophotometric assays also revealed the absence of ellagitannins and proanthocyanidins in the same samples, while the amount of anthocyanins varied not only among species but also among cultivars. From a functional point of view, both scavenging and radical-reducing abilities were evaluated by biochemical assays, while Cellular Antioxidant assay was used to monitor antioxidant properties on a human cell-based biological system. These assays confirmed that *M. nigra* varieties possessed higher antioxidant activity in terms of radical scavenging and reducing antioxidant power. On the other hand, the cellular model system provided insights into the potential health implications of *Morus spp*. with values comparable to other common berries. Finally, metabolomic analysis illuminated the chemical diversity within *Morus spp*. by highlighting distinct metabolic fingerprints and allowing discrimination between species and varieties. Anthocyanins emerged as key metabolites for this discrimination, with additional compounds contributing to genotype-specific profiles. Overall, these results underscore the importance of genetic diversity in assessing the health properties of *Morus spp*. with implications for improving their nutritional and medicinal value in Mediterranean areas, particularly Sicily.

1. Introduction

The Moraceae family, often named 'mulberry family', includes a large number of plant species, with 38 genera and more than 1000 species. Within this family, the genus *Morus* includes several significant species, such as *Morus alba* (White Mulberry) and *Morus nigra* (Black Mulberry) (Rodrigues et al., 2019). White and black mulberry cultivation globally involves a significant area, with a diverse selection of cultivars for both species. Mulberries are indeed cultivated on a large scale, with a large number of varieties adapted for the different climates and consumer preferences. As with many fruit crops, their cultivation continues to make progress, with international cultivars being introduced to regions outside their traditional habitats (Kadam et al., 2019; Kılınçer et al., 2024).

M. alba cultivation is successful in regions where the climate is suitable, growing mainly in temperate and subarctic regions. Its cultivation covers about 2.5 million hectares worldwide. *M. alba* has considerable diversity, with more than 1000 cultivars developed over the years (Pawlowska et al., 2008). In recent years, there has been an increasing interest in cultivating *M. alba* along the coastal regions of the

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Mediterranean area (Mena et al., 2016). This expansion is attributed to the mild climate, which is favorable to the effective growth of *M. alba*. As a result, the introduction of various international cultivars has diversified *M. alba* cultivation in these areas (Mena et al., 2016). Additionally, the fruits of *M. nigra* have gained significant importance, particularly in regions with temperate climates. It is planted, and often naturalized, in the west throughout Europe and in the east in China. Today it is also widespread in other geographic areas, including Afghanistan, Iraq, India, Pakistan, Iran, Syria, Lebanon, Jordan and Turkey, covering about 1.2 million hectares globally. (Ercisli & Orhan, 2007; Pawlowska et al., 2008). Over time, interest in expanding the cultivation of *M. nigra* has grown, with emphasis on introducing cultivars with greater adaptability and productivity. Its diversification has contributed to the popularity of *M. nigra* in various regions of the world, resulting in a wide range of cultivars with different distinctive traits (Ercisli & Orhan, 2007).

Fruit quality attributes, including color, aroma, flavor, taste, and texture, play a significant role in the commercial value of *M. alba* and *M. nigra*. Consequently, in response to the growing consumer interest for the health-promoting aspects of foods, the nutraceutical value of mulberry species is also becoming an effective factor to evaluate the real fruit quality. Both *M. alba* and *M. nigra* fruits are natural sources of antioxidant phytochemicals, recognized as contributors radical scavenging activity (Truzzi et al., 2024).

Despite higher production levels in non-European countries such as China and Japan, mulberry cultivation in Europe remains both sustainable and realistic. For instance, countries like Greece and Turkey rank as the main contributors to European mulberry production (Zanier, 2022). In this scenario, also Italy and Spain are developing pedoclimatic conditions for the successful cultivation of *M. alba* and *M. nigra* because current climate changes (Mena et al., 2016). These conditions are especially favorable in the Tyrrhenian and Mediterranean coastal areas of South Italy (Truzzi et al., 2024). In Sicily, in addition to well-established cultivars, can be actually found the cultivation of local genotype, albeit they suffered of a very limited distribution in the market.

Despite the extensive research devoted to exploring the nutraceutical properties of different Morus species, there is relatively little emphasis on the distinct varieties of *M. alba* and *M. nigra*, particularly when grown in regions other than their native environments. Based on our current knowledge, there is a small number of research examining the profiling and antioxidant capacities of fruits derived from Mulberry cultivation in South Italy, and especially in Sicily. Consequently, this study holds promise for advancing the valorization of a previously unexplored non-indigenous product within the Sicilian domain. Furthermore, while other fruits have been extensively studied, there is still much to be explored in terms of bioactive compounds and potential health benefits of mulberry species. Indeed, despite some previous studies have investigated the phytochemical characterization of some cultivars of *M. alba* or *M. nigra*, a significant gap persists in the scientific literature in terms of integrating variations of the metabolic profiles of the different cultivars with biochemical evaluation. Specifically, the existing literature suggests the presence of intriguing bioactive compounds in both *M. alba* and *M. nigra*, including various polyphenols such as anthocyanins, chlorogenic acids, and resveratrol, as well as alkaloids such as morusimine and sanguiinin H-6. However, it has not been evaluated whether the observed antioxidant properties are directly related to the intrinsic characteristics of these bioactive compounds or whether they involve other biochemical and cellular pathways.

In order to have a more complete understanding of the functional attributes and phytochemical profiles of *M. alba* and *M. nigra*, this study aimed to analyze the fruit extracts of six different cultivars of white mulberry and six cultivars of black mulberry grown in Sicily, trying to shed light on their distinctive qualities. The results of this investigation are potentially able to provide valuable insights into the health benefits and commercial applications of the different mulberry cultivars, thus contributing not only to their wider recognition and use in the field of horticulture and nutrition, but also may provide useful insights into the selection of the species or cultivar according to their phytochemical and functional profile.

2. Materials and methods

2.1. Chemical reactives

Absolute ethanol, Folin-Ciocalteau's reagent, Gallic acid (GA), 4-Dimethylaminocinnamaldehyde (DMAC), Potassium chloride (KCl), Sodium acetate, Cyanidin-3-glucoside, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), Potassium persulfate ($K_2S_2O_8$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-Tri (2-pyridyl)-s-triazine (TPTZ), Ferric chloride $FeCl_3$, RPMI medium, Fetal Bovine Serum (FBS), L-glutamine, Penicillin-Streptomycin, Trypsin-EDTA, Formic acid, Acetonitrile were purchased from Sigma-Aldrich spa (Milan, Italy). Proanthocyanidin (PAC) A2-type standard from Extrasynthese®. Chromatohrapic column (Luna C18 column) from Phenomenex (Castel Maggiore, Bologna, Italy). HepG2 cell line from ATCC (Manassas, Virginia, USA).

2.2. Plant material

The six varieties of *Morus alba* (Restrelli, Shimanouchi, White Kokuso, Tagowase, Kokka, and Datekagi) and *Morus nigra* (Korinne, Miura, Red Kokuso, Miuki, Kayriou, and Limoncina) were grown in Sicily (38°06 N, 13°07 E, 103 m) (Tinebra et al., 2021). Harvesting of the fruits took place when fully ripened, which aligns with the characteristic ripening periods of each species. Fruit color, as outlined by Sánchez (Sánchez-Salcedo et al., 2015), served as a reliable indicator of ripeness. After harvesting, a portion of the fresh fruits was dried at 50 °C for 48 h until the weight remained constant for two consecutive h. The remaining fruits were promptly frozen at −20 °C, preserving them until the extracts were prepared.

2.3. Preparation of the extracts

Since the water content was not statistically different among the examined varieties, the fresh fruits were used to prepare the hydro-alcoholic extracts. For the extraction process, the fruits were carefully thawed, brought to room temperature (23 ± 2 °C), and then homogenized. Extraction was performed using absolute ethanol as the solvent, maintaining a ratio of 1:5 (w/v) for the extraction. To each homogenate from every fruit, weighing around 2 g, an appropriate volume of the extraction solvent was added. The samples were thoroughly mixed using a vortex for 5 min, followed by a 20-min sonication at 22 °C. Subsequently, they were left under continuous agitation at room temperature (23 ± 2 °C) for a duration of 24 h. Consequently, the samples underwent centrifugation at 2000 rpm for 10 min at 20 °C and were filtered using Whatman 0.45 μm filter paper. The resulting supernatant was carefully collected. The extraction procedure was iterated until the insoluble residue became colorless, and the supernatants from various extraction cycles for each sample were combined. The obtained ethanolic extracts were then aliquoted and stored at −20 °C. Three different replicates of extraction process were performed for each variety.

2.4. Total polyphenol content (TPC)

TPC was determined by the reduction of phosphotungstic-phosphomolybdic acid using Folin-Ciocalteau's reagent, monitoring the formation of blue pigments in an alkaline solution, as previously described (Giuseppe Mannino et al., 2023). Briefly, 6 μL of the phosphotungstic-phosphomolybdic acid mixture was combined with 10 μL of 20% (w/v) sodium carbonate solution and 4 μL of the appropriately diluted sample. The volume was then brought up to 200 μL with

distilled water. After 90 min of stirring on an orbital shaker at room temperature (23 ± 2 °C), the absorbance was measured at 734 nm using a microplate reader, with a blank serving as the reference. GA calibration curves were employed for quantification, and the results were expressed as mg of GA equivalents (GAE) per 100 g of fresh weight (FW). All measurements were carried out in triplicate to ensure accuracy and reliability (Singleton, 1966).

2.5. Total proanthocyanidin content (TPAC)

TPAC was conducted through the DMAC assay, following the methodology outlined by Prior and co-authors (Prior et al., 2010). In brief, 170 μL of the reaction mixture, which included 1 mg/mL of DMAC reagent dissolved in 75% (v/v) ethanol acidified with 12.5% (v/v) hydrochloric acid, was incubated with 60 μL of fruit extract appropriately diluted in 75% (v/v) acetone acidified with 0.5% (v/v) acetic acid. The mixture was stirred on an orbital shaker at room temperature (23 ± 2 °C) for 10 min, followed by an additional 10-min incubation at room temperature (23 ± 2 °C). The absorbance of each well was then measured at 640 nm using a microplate reader, with a blank as the reference. The quantification of tPAC involved the use of an external calibration curve with pure PAC-A2 type as the standard (Extrasynthese®, France). Results were expressed as mg of PAC per 100 g of FW. All measurements were conducted in three independent replicates.

2.6. Total anthocyanin content (TAC)

TAC in *M. alba* and *M. nigra* fruit extracts was conducted using the pH-differential method (Palmieri et al., 2017). Briefly, the ethanolic extract were mixed with 0.025 M KCl (pH 1.0) or 0.4 M sodium acetate (pH 4.5) buffers using a 1:50 (v/v) ratio. Absorbance readings at 535 nm and 700 nm were taken, with 70% (v/v) EtOH serving as a blank. The TAC was calculated using the following formula:

$$TAC = \frac{\Delta Abs\ x\ MW\ x\ DF\ x\ EV\ x\ 1000\ x\ l}{\varepsilon\ x\ FW}$$

Where, ΔAbs represents the difference between absorbance at 515 nm and 700 nm at both pH 1.0 and pH 4.5; MW and ε refer to the molecular weight (449.2 g/mol) and molar extinction coefficient (26,900 mM^{-1} cm^{-1}) of cyanidin-3-glucoside (utilized as the standard); DF is the sample dilution factor, and l is the path length (1 cm); EV is the amount of extraction solvent used for each sample; FW is the weight of each sample before the extraction process. TAC were expressed as mg of cyanidin-3-glucoside equivalents per 100 g of fresh weight (FW).

2.7. Antioxidant activity

2.7.1. ABTS assay

A solution of 7 mM ABTS was prepared in water and allowed to react with 2.45 mM potassium persulfate. Following a 16-h incubation in darkness, the $ABTS^{+}$ solution was diluted with ethanol until it reached an absorbance of 0.70 at 734 nm at room temperature (23 ± 2 °C). Samples and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), employed as a reference standard, underwent incubation at various and suitable dilutions. After a 5-min incubation, the decolorization of the mixture was measured at 734 nm. Radical scavenging activity was measured using the following equation:

$$AA\% = \frac{Abs\ blank - Abs\ sample}{Abs\ blank} x\ 100$$

where: AA% is the percentage of color reduction of the reagent; Abs blank is the absorbance of blank and Abs sample the absorbance of the sample at 734 nm (Pisani et al., 2021). Results were expressed as mmol of Trolox equivalent (TE) per 100 g of FW weight perfoming the same experimental procedures on the pure standard. All measurements were performed in three different replicates.

2.7.2. DPPH assay

A solution of 0.1 mM of $DPPH^{\bullet}$ radical was prepared in ethanol, and 1 mL of this solution was introduced to various concentrations of the sample. The mixture underwent vigorous shaking and was allowed to stand for 30 min in darkness, with absorbance measured at 517 nm. Samples and Trolox, employed as a reference standard, were subjected to incubation at different and suitable dilutions. After a 20-min incubation period at room temperature (23 ± 2 °C), the decolorization of the mixture was assessed at 517 nm (Platzer et al., 2022). Radical scavenging activity was then measured using the previous equation.

2.7.3. Ferric reducing antioxidant power (FRAP) assay

A composite solution comprising 0.3 M acetate buffer (pH 3.6), 10 mM TPTZ, and 20 mM $FeCl_3$ was formulated in an 8:1:1 (v/v/v) ratio. The mixture underwent a 10-min incubation at 37 °C with a suitably diluted sample. Subsequent absorbance readings were taken at 595 nm (Rumpf et al., 2023). Quantification was conducted utilizing an external calibration curve of Trolox, and the outcomes were expressed as millimoles of Trolox Equivalent (TE) per 100 g of FW. To ensure reliability, all measurements were carried out in triplicate.

2.7.4. Cellular antioxidant activity (CAA) assay

The ethanolic extracts underwent examination using the Cellular Antioxidant Activity (CAA) assay, employing the HepG2 cell line (hepatocarcinoma cells) obtained from the American Type Culture Collection (ATCC) in Rockville, MD, USA. Cultured in RPMI supplemented with 5% (v/v) FBS, 2 mM l-glutamine, 50 IU/mL penicillin, and 50 μg/mL streptomycin, the cells were maintained in a humidified atmosphere with 5% CO_2 at 37 °C, routinely cultured in 75 cm^2 culture flasks and trypsinized with trypsin-EDTA upon reaching approximately 80% confluence.

In accordance with our previous protocol (Kellett et al., 2018), the EtOH concentration in the wells did not exceed 0.25% (v/v) in each experiment, with culture medium containing 0.25% (v/v) EtOH serving as the control. The calculation of the CAA value for each fruit extract concentration involved integrating the area under the curve of fluorescence versus time using the formula:

$$CAA = \frac{\int SA}{\int CA}$$

Where, ∫SA represents the integrated area from the sample curve, and ∫CA is the integrated area from the control curve. The concentration required for 50% inhibition of dichlorofluorescein formation (CAA_{50}) for each extract was determined from concentration–response (CAA) curves using linear regression analysis. Each reported result represents the mean value derived from three separate experiments conducted in triplicate.

2.8. HRMS-based phytochemical analysis

A Data Dependent Acquisition Method was employed for the analysis of the diverse *Morus* species samples, by an Orbitrap Fusion Tribrid (Thermo Fisher Scientific, Waltham, Massachusetts, USA) MS analyser, in a mass range of 100–1000 *m/z*. Fragmentation was performed in Higher-energy collisional dissociation (HCD) mode, the collision energy was set at a value of 28. Chromatographic separation involved a Luna C18 column (150 × 2 mm, 3 μm, Phenomenex, Castel Maggiore, Bologna, Italy) kept at 40 °C, with a chromatographic gradient of 0.1% (v/v) formic acid in H_2O (Solvent A) and Acetonitrile acidified with 0.1% formic acid (v/v) (Solvent B). The chromatographic run started injecting 10 μL of each sample solution. The elution proceeded maintaining 5% (v/v) Solvent B from 0 to 4 min, followed by an increase to 100% (v/v) over the next 30 min. To ensure effective elution, 100% (v/

v) Solvent B was maintained for additional 3 min before returning to the initial conditions for the next analysis. Solvent flow was set at 200 μL min $^{-1}$.

Mass spectrometry analyses were conducted in both positive and negative ion modes to obtain comprehensive molecular information from the samples. The ionization source coupled with Orbitrap was electrospray ionization (ESI) set at 4000V for positive ion mode and 3200 V for negative ion mode. The ion transfer tube temperature was set at 300 °C, and the vaporization temperature at 275 °C. Raw files generated by the mass spectrometer were analyzed using Xcalibur software (Abu-Izneid et al., 2023). Peak picking, feature detection and alignment were performed through MZmine 3 software (Schmid et al., 2023), on centroided MS spectra. The features annotation was lead using public libraries downloaded from MONA (Mass bank Of North America) for both positive and negative modes. A final matrix, including the accurate mass of the molecular ion, retention time, peak area, hypothetical identity and presence/absence in individual samples was generated. For each annotated molecule, its presence and likelihood were verified both in the chromatographic peaks of individual samples and in fragmentation (Schmid et al., 2023).

2.9. Statistical analysis

Statistical analyses were executed using SPSS package version 25. To assess differences among various species, spectrophotometric, analytical, and biological data were subjected to the one-way analysis of variance (ANOVA; general linear model), followed by Tukey's multiple range test with a significance threshold of $p \leq 0.05$. Furthermore, the phytochemical profiles obtained through HPLC-DAD-MS/MS were normalized by median, transformed in Log10 and scaled using Pareto method. Data normalized are reported in Supplementary Fig. S1. Metabolomic analysis were performed using a Web-based multi-omics software (MetaboAnalyst 4.0) and differences were plotted via principal component analysis (PCA) (Ewald et al., 2024; Pang et al., 2022).

3. Results and discussion

3.1. UV/Vis determination of bioactive compounds

The potential health benefits associated with the consumption of plant-based foods stem from a range of secondary metabolites. Conversely, the intricate interplay between the content of specific phytochemical substances and factors such as ripening stage and storage conditions also extends to the distinct genetic makeup. Understanding how these variables shape the qualitative parameters of fruits becomes particularly pertinent given the strong variability of secondary metabolites among varieties of the same species (Kassa et al., 2023). In this context, our preliminary assessment focused on discerning the levels of polyphenols in 12 distinct samples of *M. alba* and *M. nigra*, each distinguished by its genotype (Fig. 1).

In general, notable differences were observed among the twelve distinct varieties of *Morus* spp. TPC ranged from 97.44 ± 1.61–453.01 mg g^{-1} FW. It is worth highlighting that the TPC of *M. alba* fruits consistently exhibited lower values compared to the *M. nigra* varieties, with measurements of 115.74 ± 16.56 and 254.29 ± 126.88 mg g^{-1} FW, respectively (Fig. 1). Among white mulberries, the Restrelli variety showed the highest TPC value, closely followed by Shimanouchi and Tagowase. On the other hand, Korinne, Miura, and Red Kokuso were the *M. nigra* varieties with the highest TPC content. In contrast, the Dateakagi variety, a type of white mulberry, displayed the lowest TPC value. Comparable TPC values have been previously reported by Ercisli and Orhan (2007) in other cultivated varieties of *Morus* spp. in Turkey (Ercisli & Orhan, 2007).

Typically, the quantification of TPC in berries, assessed through the Folin-Ciocalteau assay, exhibits a range from 30 to 2000 mg 100 g^{-1}. The highest concentrations are commonly observed in dark-colored

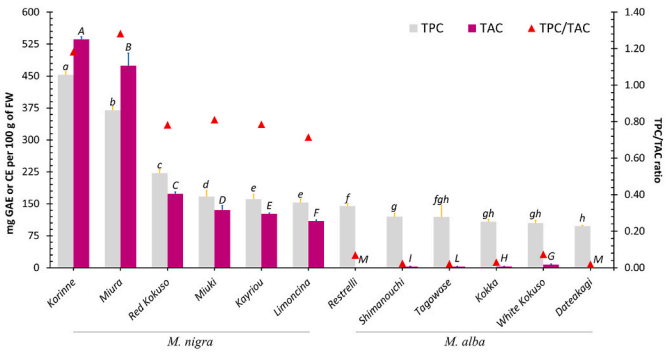

Fig. 1. Total polyphenol content (TPC) and anthocyanin content (TAC) in the different varieties of *M. alba* and *M. nigra*. The gray bars represent TPC, expressed as mg of gallic acid equivalents (GAE) per 100 g of fresh weight (FW). Similarly, the pink bars represent TAC, expressed as mg of cyanidin equivalents (CE) per 100 g of FW. For each bar, the vertical lines indicate the standard deviation, while lowercase letters denote significant differences in TPC and uppercase letters denote significant differences in TAC among the analyzed varieties, as measured by one-way ANOVA followed by Tukey's test. The TPC/TAC ratio is shown in the figure as a red triangle, providing insights into the contribution of anthocyanins to the TPC value.

berries, such as black elderberry (1950 mg 100 g^{-1}), black chokeberry (1752 mg 100 g^{-1}), black raspberry (980 mg 100 g^{-1}), and blackcurrant (821 mg 100 g^{-1}) (Hill et al., 2021). Berries showcase diverse phenolic compounds, with distinct polyphenol profiles characterizing each species. The predominant polyphenols of these fruits include anthocyanins (especially in colored berries), ellagitannins, and proanthocyanidins (Hill et al., 2021). Despite the established methodology for detecting and quantifying anthocyanins, the intricate and polymeric molecular structures of ellagitannins and proanthocyanidins present challenges for their accurate quantification using chromatographic techniques. This complexity may indeed lead to potential underestimation. Therefore, spectrophotometric assays are preferred for their obvious utility in detecting and quantifying these compounds (Rauf et al., 2019). Notably, our analysis using the DMAC assay revealed the absence of this class of polyphenols in the fruits of both *M. alba* and *M. nigra* varieties. Previous reports, including that by Truzzi et al. (2024), observed the presence of PACs in some varieties of *Morus* cultivated across different regions of Italy, as suggested by Vanillin assay (Truzzi et al., 2024). However, the levels detected by the authors were remarkably low, raising concerns about potential false positives attributed to the complexometric mechanism characteristic of the assay employed, which produces a spectrophotoemtrically detectable product at 510 nm, a typical wavelength for absorbing even anthocyanins. Consequently, the observed interference of anthocyanins may have contributed to the increased results (Giuseppe Mannino et al., 2021). Although the mechanism of action of DMAC is not currently known, the formation of the colorimetric product detectable at 640 nm highlights the importance of preferring the DMAC assay as the most reliable method for the quantification of proanthocyanidins, particularly in plant matrices prone to the presence of anthocyanins (Prior et al., 2010).

Concerning anthocyanins, their amount varies widely between berries. The highest levels were found in black elderberry (1317 mg 100 g^{-1}), black chokeberry (878 mg 100 g^{-1}), and blackcurrant (592 mg 100 g^{-1}). Intermediate levels of anthocyanins are found in lowbush blueberry (187 mg 100 g^{-1}), highbush blueberry (134 mg 100 g^{-1}), and blackberry (173 mg 100 g^{-1}). The lowest levels are found in strawberry (73 mg 100 g^{-1}), red raspberry (71 mg 100 g^{-1}), lingonberry (60 mg 100 g^{-1}), American Cranberry 50 mg 100 g^{-1}), gooseberry (6.6 mg 100 g^{-1}), and cloudberry (3.4 mg 100 g^{-1}) (Hill et al., 2021). In our *Morus* fruit samples, a significant variability in TAC was observed among the twelve mulberry varieties under study. As expected, the highest TAC values were recorded in *M. nigra* fruits, demonstrating an average

content of 254.29 ± 126.88 mg CE g^{-1} of FW, while white mulberries exhibited a median content of 2.78 ± 2.84 mg CE g^{-1}. The highest TAC was found in the Korinne and Miura varieties, with values approximately 5 times lower in the remaining *M. nigra* fruits (Fig. 1). It is noteworthy that, despite white mulberries not displaying typical anthocyanin pigmentation, they were detected in the fruits of some varieties, such as White Kokuso (Fig. 1). Our results are consistent with previously reported literature findings (Xu et al., 2021). Moreover, by calculating the TAC/TPC ratio (Fig. 1), we identified a strong positive correlation between TAC and TPC (ρ = 0.86), suggesting a substantial contribution of TAC to the overall TPC value. However, although TPC via Folin-Ciocalteu is commonly used to express total polyphenol content as GAE, it tends to underestimate the presence of anthocyanins (Bajčan et al., 2021). Additionally, the pH differential method, despite being an officially recognized analytical method by AOAC (J. Lee et al., 2005), is strongly influenced by the indirect measurement of anthocyanin content. Indeed, by this UV/Vis assay, despite anthocyanins have different ε and MW, are still approximated to cyanidin-glucoside equivalents (S. G. Lee et al., 2016). Consequently, TAC, TPC, and TAC/TPC ratio can provide useful information more as a general trend and for comparisons with other species or varieties, rather than as reliable analytical measurement (Tonutare et al., 2014).

3.2. Radical scavenging, reducing and cellular antioxidant activity

Considering both the diverse array of molecules with redox-active properties and the significant implications of oxidative stress in various chronic diseases, there is a notable focus on researching plant-derived matrices due to their distinct antioxidant characteristics. These matrices encompass not only the traditional antioxidant vitamins but also include bioactive phenolic compounds capable of acting as antioxidants. The mechanisms by which these molecules confer antioxidant functionality to food are multifaceted, involving direct detoxification of free radicals through scavenging or reducing mechanisms, as well as indirect modulation of enzymatic antioxidant systems. Additionally, these molecules can exert their biological effects independently or through synergistic/antagonistic interactions with other compounds, contributing exclusive antioxidant properties to each specific food item (Giuseppe Mannino et al., 2022; Rumpf et al., 2023).

Several and different assays, principally conducted in solution, have been developed to measure the total antioxidant capability of plant extracts. Despite criticism regarding the inherent limitations of *in vitro* chemical methods, these assays remain highly favored for technological and nutritional purposes. They indeed provide valuable insights into the intrinsic reducing properties of the intricate blend of redox-active molecules present in foods and contribute to unraveling the mechanisms behind the observed activities (Towanou et al., 2023). Recognizing the limitations of a single assay in predicting the genuine antioxidant potential of plant extracts, this study examines the redox-active properties of extracts from *Morus* spp. fruits using the ABTS, DPPH, and FRAP assays (Fig. 2).

Despite ABTS and DPPH share a very similar mechanism of action, involving the stabilization of chemical radical species through electron scavenging, the general trend in the results highlights a significantly higher value for ABTS compared to DPPH. Variations in pH levels and hydrophilicity within the assay's reaction mixture, coupled with differences in the stability of reactive species produced across distinct assays, collectively contribute to explaining the variances observed in the reducing activity of the studied samples in various experimental conditions (Rumpf et al., 2023). Despite its drawbacks, like not involving a natural physiological radical, large size, and varying speeds of antioxidant reactions, the ABTS method remains valuable. It is fast, requires minimal processing, and is highly versatile across different pH levels. It allows wavelength adjustments to minimize spectrophotometric interference, accommodates both hydrophilic and lipophilic compounds, and is easily adaptable for high-throughput applications like microplate

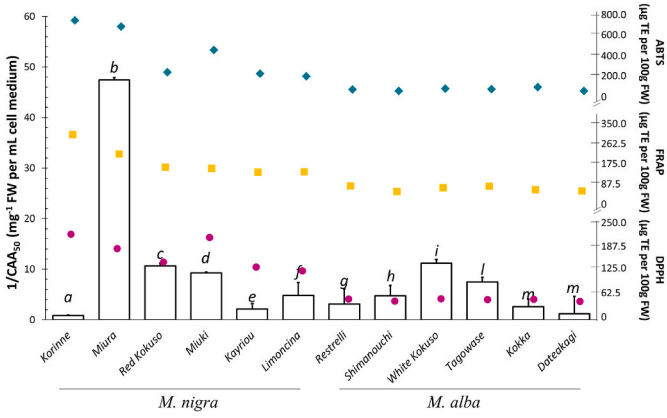

Fig. 2. Antioxidant properties of different genotypes of *M. alba* and *M. nigra*. The white bars represent the values obtained from the Cellular Antioxidant Assay (CAA), shown as $1/CAA_{50}$ and expressed as mg per milliliter of cell medium per gram of fresh weight (FW). The vertical lines represent the standard deviation, while lower case letters denotes statistical differences among the different samples, as measured by one-way ANOVA followed by Tukey post hoc. The different curves represent the values obtained from assays evaluating radical scavenging activity (DPPH: purple; ABTS: green) or metal-reducing activity (FRAP: yellow). The antioxidant activity values are expressed as μg of Trolox Equivalents (TE) per 100 g of FW.

assays and HPLC, making it almost ideal for comprehensive antioxidant testing (Cano et al., 2023).

Specifically, a median value of 215.06 mmol TE 100 g^{-1} FW was measured for samples using the ABTS assay, while it was 87.45 mmol TE 100 g^{-1} FW with the DPPH assay. Concerning ABTS, the variety with the highest antioxidant value was Korinne (714.96 ± 44.09 mmol TE 100 g^{-1} FW), followed by Miura (656.12 ± 20.54 mmol TE 100 g^{-1} FW) and Miuki (424.12 ± 2.49 mmol TE 100 g^{-1} FW). These three varieties were characterized by being red-colored berries, belonging to the *M. nigra* species. Regarding the other *M. nigra* species (Kayriou, Limoncina, and Red Kokuso), values approximately 3-times lower were recorded, averaging 187.23 mmol TE 100 g^{-1} FW. In any case, these values were still markedly higher than those measured in *M. alba* varieties, ranging between 21.75 ± 1.78 and 57.64 ± 4.16 mmol TE 100 g^{-1}. A similar trend was also observed for the DPPH assay. However, in this case, the red varieties exhibited very similar values, ranging between 131.71 ± 1.58 and 217.50 ± 9.22 mmol TE 100 g^{-1}. Also in this case, Kayriou, Limoncina, and Red Kokuso recorded the lowest DPPH values. Similarly to the ABTS assay, the trend shown for DPPH indicates *M. alba* varieties as fruits with the lowest antioxidant value, recording a content ranging between 12.29 ± 0.98 and 19.42 ± 0.44 mmol TE 100 g^{-1}.

Concerning the FRAP assay, it goes beyond simply assessing the reducing potential of samples, providing insights into their underlying antioxidant mechanisms. Indeed, during the assay's redox reaction, antioxidant molecules with iron-chelating abilities facilitate the reduction of Fe(III) to Fe(II). TPTZ then chelates the Fe(II), producing the characteristic violet coloration. However, not all antioxidants can chelate Fe(III), leading to potential discrepancies between FRAP and ABTS or DPPH results (Rumpf et al., 2023). Under our experimental conditions, the antioxidant profiles of *M. alba* and *M. nigra* berries exhibited strong correlations with ABTS (ρ = 0.912) and DPPH (ρ = 0.940), indicating that a significant portion of their bioactive compounds can interact with metal ions (Fig. 2). Among the *M. nigra* varieties, Korinne displayed the highest reducing activity (303.87 ± 5.95 mmol TE 100 g^{-1} FW), followed closely by Miura (219.59 ± 5.67 mmol TE 100 g^{-1} FW). The remaining *M. nigra* varieties exhibited comparable activities, ranging from 142.44 ± 3.16 mmol TE 100 g^{-1} FW (Kayriou) to 163.57 ± 4.87 mmol TE 100 g^{-1} FW. Despite confirming the lower reducing power of all *M. alba* varieties, the FRAP assay recorded only a 1.5-fold difference compared to their red counterparts (Fig. 2). In

contrast to the 10-fold higher ABTS and DPPH values observed for *M. nigra* compared to *M. alba* fruits, the narrower gap between the two species in FRAP assays indicates that *M. alba* possesses a greater abundance of bioactive compounds containing ortho/meta-oriented hydroxyl groups relative to *M. nigra* berries.

Given the potential of antioxidants in mitigating the risk of cancer and other chronic diseases, precise and biologically relevant evaluation of antioxidant potential is crucial. This assay represents a significant advancement over traditional chemical assays due to its ability to mimic cellular processes that normally occur *in vivo*. By incorporating aspects of cellular uptake, metabolism, and distribution of bioactive compounds, which are key modulators of bioactivity, the CAA assay offers enhanced predictive power in anticipating the antioxidant behavior of pure compounds, foods, dietary supplements, or other formulations, also providing a more comprehensive understanding of the potential antioxidant activity within biological systems (Kellett et al., 2018; Wolfe & Liu, 2007). Under our experimental conditions, the average $1/CAA_{50}$ of the *Morus* spp. fruit extracts was 330 μg FW mL^{-1} of cell medium. In comparison with the values determined by Wolfe and Liu for several common fruits, the antioxidant capacity measured in our extracts was comparable to that measured for other berries fruit extracts, including blueberry, cranberry, blackberry, and raspberry (Wolfe & Liu, 2007). In contrast to other chemical antioxidant assays, in our investigation no clear distinction was observed between *M. alba* and *M. nigra* fruit extracts (Fig. 2). Additionally, despite exhibiting high TPC, TAC and AOA values, not all *M. nigra* varieties consistently demonstrated the highest $1/CAA_{50}$ values, with the exception of Miura, which displayed the highest $1/CAA_{50}$ value among all assessed mulberries (Fig. 2). This observation, coupled with the low correlation coefficient between CAA_{50} and TAC ($\rho = 0.505$) suggests that compounds other than anthocyanins contribute 1/significantly to the actual antioxidant activity of the extracts in HepG2 cells. Apart from Miura, Red Kokuso and White Kokuso also exhibited notable $1/CAA_{50}$ values (Fig. 2). $1/CAA_{50}$ and higher total antioxidant capacity measured by ABTS and DPPH assays suggests that antioxidant compounds significantly enhance radical scavenging activity and intracellular mechanisms, including transcriptional and enzymatic modulation, leading to comprehensive oxidative protection. As a result, some samples with elevated ABTS and DPPH values (indicating higher antioxidant concentrations) also recorded high $1/CAA_{50}$ values, suggesting these compounds are highly effective. Since $1/CAA_{50}$ is the reciprocal of the concentration required for 50% inhibition of oxidative stress, a higher value indicates greater potency. Bioavailability and synergistic interactions among antioxidants further influence this correlation (Dai & Mumper, 2010; G. Mannino et al., 2020; Wolfe & Liu, 2007).

3.3. Metabolomic analysis

Metabolomic analyses play a primary role in the characterization and discrimination of plants extracts, especially when it comes to understanding the chemical diversity of their secondary metabolites. These compounds, often subjected to a wide range of biochemical modifications during biosynthetic processes, are responsible for multiple biological and physiological functions, and their variation among species or varieties can be a significant indicator of their identity and properties (Zou et al., 2024).

In this study, an untargeted high-throughput metabolomic method based on HPLC-ESI-HRMS/MS using an Orbitrap was developed to obtain information regarding the complete metabolome of M*orus* spp. and to explore the effect of genotype. In particular, the application of untargeted global metabolomic analysis protocols represent a comprehensive approach to explore the complexity of secondary metabolites in these plants, not only discriminating between different taxa but also between varieties within the same species. In a broader context, the ability to effectively discriminate among varieties based on their metabolomic profiles not only enriches our understanding of plant biodiversity, but also has significant implications for a variety of applications, including the development of new cultivars with desired traits, the selection of plants for pharmaceutical or food purposes, and the conservation of genetic diversity (Sánchez-Salcedo et al., 2015). Furthermore, the identification of specific metabolites can provide valuable insights into the biochemical mechanisms involved in plant response to environmental factors and biotic stress, offering opportunities to improve the resilience of agricultural crops under adverse conditions (Song et al., 2023).

An initial analysis was undertaken to identify metabolites that could be reliably discriminated between the two species using a volcano plot. The volcano plot is a visualization tool commonly used in metabolomic analysis to identify metabolites that show significant differences between two groups of samples, combining the p-value and the overall fold change. Consequently, based on the distance between the origin point, it is possible to understand not only how large the average difference between the two groups of samples is (fold change) but also how statistically significant the difference is (p-value) (Elessawy et al., 2023). By setting a Log2(FC) cutoff of $> \pm 1$, it was possible to reduce the significant features to 358, differently distributed between the two genera, as indicated by the different positive or negative values of Log2 (FC) (Fig. 3, Panel A). In particular, metabolites with a positive Log2(FC) are depicted in red on the right side of the volcano plot, representing the major contributors to *M. alba* species. Conversely, those with negative values are shown in blue on the left side, indicating the major contributors to *M. nigra* species. The plot also includes a region of non-significance (gray dots between the two lines), labelling metabolites equally represented between the two species. Among them, 237 were more present in *M. alba*, 194 in *M. nigra*, while the remaining 739 equally distributed between the two genera. To further reduce system noise and demonstrate the discriminant potential provided by the above data, supervised OPLS-DA models were built for the classification of *Morus* genera. As shown in Fig. 3, Panel B, the different cultivars of *M. nigra* were well discriminated from *M. alba* varieties, proving clear differences in the metabolites of the two genera. The panel C of Fig. 3 shows the 20 compounds that mostly determined this clustering of the two species. The predominant compounds in *M. nigra* were found to be anthocyanins, and in particular, glycosylated forms. Among these, kuromanin, idein, primulin, and oenin were present, however, also the respective aglyconic forms of delphinidin, malvidin, and cyanidin were observed. This result align with previous reports, in which anthocyanin were detected in *M. nigra* species (Truzzi et al., 2024). The expectation that anthocyanins would emerge as primary discriminatory metabolites between the two species of *Morus* was based on the conspicuous phenotypic dichotomy, with one species displaying a reddish hue and the other maintaining a pale coloring. However, as illustrated in panel C of Fig. 3, other secondary metabolites play an active role in genetic differentiation. In particular, chlorogenic acids and astragalin showed a fair prevalence in the red varieties, while vicenin and vitexin, along with other flavonoids, showed a characteristic presence instead of the *M. alba* profile.

Using the data obtained from Fig. 3, panel A, a clustering analysis coupled with heatmap visualization was also performed (Fig. 3, Panel D). The statistical analysis allowed the formation of two large clusters, one mainly from the Red Kokuso, Korinne, Miura, Karyriou, Muki, and Lemoncina varieties, and the other instead composed of Tagowase, Kokka, Restrelli, Datekagi, and Shimanouchi. Interestingly, the variety of *M. alba* called White Kokuso found a better clustering together with the *M. nigra* varieties, despite the low content of anthocyanins respect to the varieties belonging this group.

In addition to highlighting the existing differences between the *M. alba* and *M. nigra* genera, metabolomic analyses also proved to be capable of discriminating different varieties within the same specie. In particular, by PCA analysis on the data obtained exclusively on the varieties of *M. nigra*, it was possible to notice how the Korinne, Muki, and Miura varieties were separated by positive PCA2 scores from the

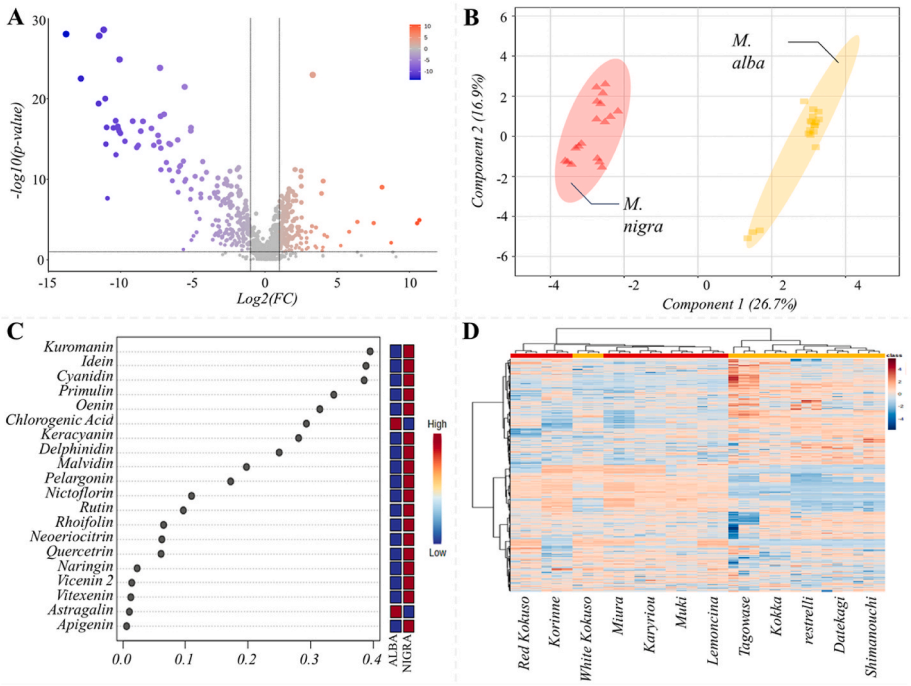

Fig. 3. Metabolomic analysis showing the differences between the metabolome of the fruit extracts from *M. alba* and *M. nigra*. In Panel A, a volcano plot is depicted, derived from data obtained in both negative and positive modes. This plot combines Fold Change (FC) Analysis and T-tests, offering users a comprehensive view to discern significant features based on biological or statistical significance. FC was calculated as ratio of *M alba/M. nigra* specie. Panel B showcases Partial Least Squares Discriminant Analysis (PLS-DA), emphasizing the covariance between X (data) and Y (group). The variance exhibited in the plot explains the variance for X. In Panel C, the variable importance in projection (VIP) and the weighted sum of absolute regression coefficients (coef.) are illustrated. The colored boxes on the right indicate the relative concentrations of corresponding metabolites across the studied groups. Panel D offers an intuitive visualization of the data, combining Hierarchical Clustering Dendrogram with Heatmap. Each cell's color on the map corresponds to a concentration value, with samples arranged in rows and features/compounds in columns.

Karyriou and Lemoncina varieties, while Red Kokuso was placed in the middle. PCA1 scores instead allowed to discriminate Korinne and Karyriou from Red Kokosu and Miura, while Lemoncina instead was placed at scores very close to zero (Fig. 4, Panel A). The main metabolites determining the separation of genotypes were morin, paeoniflorin, dipaclone, hesperidin, viscutin, and guaiaverin. In particular, Korinne is in almost all cases the variety that had the highest contents of the selected metabolites, followed by Karyriou and Miura. On the other hand, Red Kokuso, Muki, and Lemoncina were instead the varieties that showed the lowest contents (Fig. 4, Panel B).

Regarding the varieties belonging to the *M. alba* species, PCA analysis showed how all the varieties, except Restrelli and Shimanouchi, were metabolically different. In particular, positive PCA2 scores separated Tagowase, Restrelli, and Shimanouchi from the other varieties which instead were separated by negative PCA2 scores. Negative PCA1 scores allowed to separate Datekagi, Kokka, Restrelli, and Shimanouchi from White Kokuso and Tagowase (Fig. 4, Panel C). In this case, phlorizin, mulberrin, and morusin were the metabolites that mostly allowed this clustering. The genotype with the highest contents of the selected metabolites was White Kokuso, followed by Tagowase. Restrelli and Shimanouchi instead showed intermediate contents, often not very different from each other, and for this reason, it was not possible to correctly discriminate them through PCA. The cultivars with the lowest content were Kokka, immediately followed by Datekagi (Fig. 4, Panel D).

A recent study conducted by Truzzi et al. (2024) delved into the phytochemical and functional composition of 13 varieties of *M. alba* cultivated in Italy. Among the evaluated cultivars, Datekagi, Restrelli, and White Kokuso were present as well. (Truzzi et al., 2024). Although direct comparison of the data was hindered by the diverse growth regions of the species, the findings revealed intriguing disparities. Specifically, Datekagi exhibited a phytochemical profile remarkably similar to that of White Kokosu, whereas Restrelli stood out as distinct from the aforementioned varieties. This robustly underscores that factors beyond genotype play a significant role in shaping the production of secondary metabolites, including environmental influences (Palmieri et al., 2017). Analyzing the data obtained using heatmap visualization coupled with clustering analysis, it was also possible to highlight any genotype similarities. Regarding *M. nigra*, Miura and Korinne were the varieties that mostly differed from the others, which instead were clustered within a single group. However, the metabolic differences allowed to highlight differences in genotype, resulting in a different branching for each of the varieties under study (Supplementary Fig. S2, Panel A). Regarding the genotypes of *M. alba*, the variety with the greatest differences in terms of secondary metabolites was White Kokuso, which mostly differed from the other varieties. Also in this case, although the different genotypes were clustered within the same branch, the different quantities of bioactive compounds allowed clustering in different branches, confirming the previously obtained data (Supplementary Fig. S2, Panel B).

4. Conclusion

The comprehensive analysis conducted on the extracts of *Morus alba* and *Morus nigra* fruits sheds light on the intricate interplay between genotype, phenolic composition, and antioxidant activity. Through UV/Vis determination, significant variations in polyphenol content were observed among the distinct varieties, with *M. nigra* generally exhibiting higher levels compared to *M. alba*. Further exploration via spectrophotometric assays revealed the absence of ellagitannins and proanthocyanidins in the fruits of both species, while TAC varied widely, particularly between *M. nigra* and *M. alba* varieties.

In assessing radical scavenging and reducing capabilities, significant disparities emerged among the varieties, with *M. nigra* generally displayed higher antioxidant activity compared to *M. alba*, as evidenced by

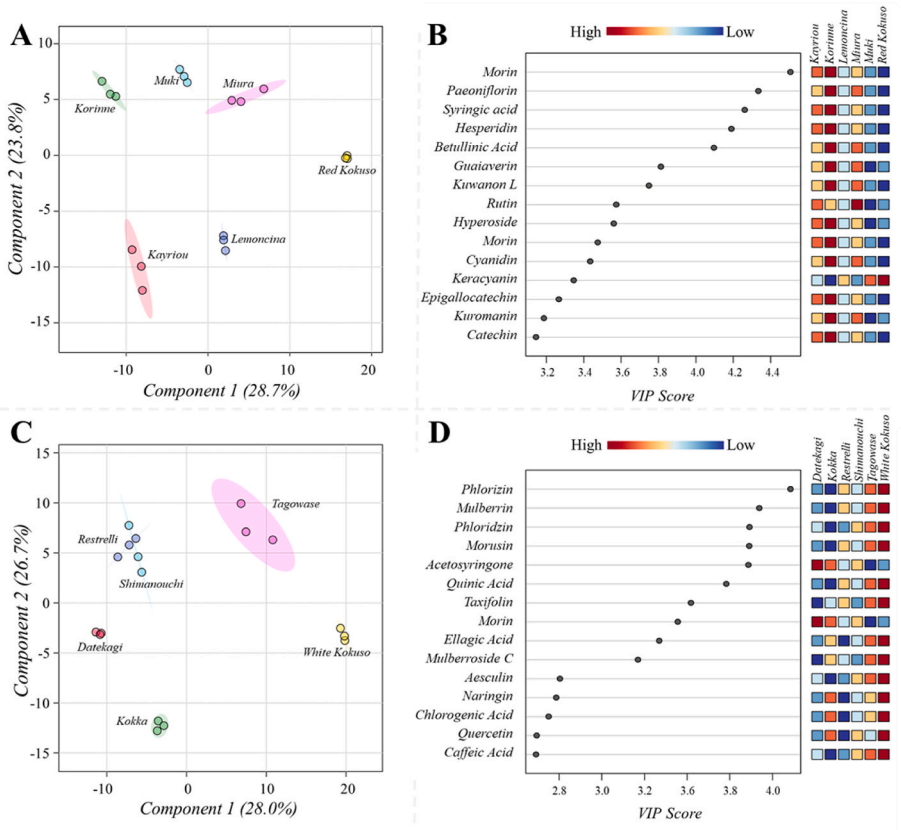

Fig. 4. Metabolomic analysis showing different among the different varieties of *M. nigra* (Panel A and B) and *M. alba* (Panel C and D) metabolites. In Panel A and C, showcases Partial Least Squares Discriminant Analysis (PLS-DA), emphasizing the covariance between X (data) and Y (group). The variance exhibited in the plot elucidates the explained variance for X. In Panel B and D, the variable importance in projection (VIP) and the weighted sum of absolute regression coefficients (coef.) are illustrated. The colored boxes on the right indicate the relative concentrations of corresponding metabolites across the studied groups.

ABTS, DPPH, and FRAP assays. Notably, the cellular antioxidant activity assay provided insights into the potential health implications of Morus spp. fruit extracts, with values comparable to other commonly consumed berries.

Metabolomic analysis further elucidated the chemical diversity within Morus spp., allowing discrimination not only between species but also among varieties within each species. Anthocyanins emerged as key differentiating metabolites, particularly in *M. nigra*, with additional compounds contributing to genotype-specific profiles. Clustering analysis highlighted distinct metabolic signatures, underscoring the potential for targeted breeding and selection of varieties with desired traits.

Overall, these findings underscore the importance of considering both genetic diversity and phenolic composition in evaluating the health-promoting properties of Morus spp. fruits. Future research exploring the underlying biochemical mechanisms driving these differences may offer valuable insights for enhancing the nutritional and medicinal value of these fruits.

CRediT authorship contribution statement

Graziella Serio: Writing – original draft, Formal analysis, Data curation. **Alberto Asteggiano:** Formal analysis, Data curation. **Noemi Gatti:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Lorenza La Rosa:** Formal analysis, Data curation. **Cinzia Margherita Bertea:** Writing – review & editing, Resources, Project administration, Funding acquisition, Data curation, Conceptualization. **Vittorio Farina:** Resources. **Claudio Medana:** Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Giuseppe Mannino:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Carla Gentile:** Writing – review & editing, Visualization, Validation, Supervision, 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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2024.104782.

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