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Integrated Orbitrap-HRMS and Chemometric Profiling Reveals Metabolite Diversity and Antioxidant Signatures in Cerrado *Eugenia* Fruits

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ABSTRACT

Fruits from the Brazilian Cerrado, such as pitanga (*Eugenia uniflora*), jambolao (*E. jambolana*) and murta (*E. punicifolia*), are recognized for their nutritional and therapeutic potential, largely due to their phenolic-rich profiles. However, systematic studies integrating high-resolution mass spectrometry (HRMS) with chemometric tools to correlate metabolite diversity with antioxidant activity remain scarce. Hydroalcoholic fruit extracts were analysed using Orbitrap-HRMS with data-dependent MS/MS for metabolite annotation. Spectrophotometric and voltammetric assays were performed to assess antioxidant activity, while total phenolic content was quantified. Chemometric analyses, including Fold change analysis, PCA, HCA, PLS-DA and OPLS-DA, were applied to classify samples and identify discriminant metabolites. Visualization strategies such as Van Krevelen diagrams, heatmaps and chord plots were used to explore compositional similarities and metabolite classes. Jambolao extract showed high antioxidant activity (DPPH IC_{50}: 3.10 ± 0.10 μg/mL, ABTS: 189.0 ± 3.2 μg/mL) and total phenolic content (41.60 ± 1.05 mg GAE/100 g), confirming a strong correlation between phenolic content and antioxidant potential. HRMS profiling revealed both shared and species-specific metabolites, including anthocyanins, flavonoids, tannins and phenolic acids. Chemometric models allowed the identification of cyanidin and delphinidin derivatives as key discriminant markers. Fold change analysis revealed distinct metabolic signatures among the species, with jambolao enriched in low-mass aglycone polyphenols, while pitanga and murta were characterized by higher-mass glycosylated polyphenols. Multivariate clustering grouped jambolao and pitanga together due to their similar metabolic and antioxidant profiles, whereas murta formed a distinct cluster. The findings highlight jambolao and pitanga as rich sources of bioactive polyphenols, supporting their potential for nutraceutical and functional food applications.

1 | Introduction

The Cerrado, the second largest biome in South America and Brazil, is recognized as a biodiversity hotspot due to its unique species, many of which are endemic and biologically significant. Spanning over 2 million km^2, the Cerrado covers nearly a quarter of Brazil's territory, approximately 23% of the national land area [1]. Fruits from this biome, rich in bioactive compounds,

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macronutrients and micronutrients such as vitamins, folates, carotenoids and phenolic compounds, are widely used in various bioproducts like jams, ice creams, liqueurs, juices, pharmaceuticals and cosmetics, offering substantial nutritional and economic benefits [2].

The Myrtaceae family, with approximately 121 genera, is one of the significant families of commercial fruit trees globally [3]. The genus *Eugenia*, which includes around 350 species native to Brazil, is particularly notable, with *E. uniflora L.* (pitanga) being the most studied for its medicinal and nutritional properties [4, 5]. The fruit exhibits significant genetic diversity, especially in its colour, which evolves from green to purple as it ripens [5]. Pitanga is rich in phenolic compounds and anthocyanins, which have potential health benefits and can be natural alternatives to synthetic food colourants [6]. Other *Eugenia* species, such as *E. jambolana* (jambolao) and *E. punicifolia* (murta), are also gaining attention for their biotechnological and nutritional potential, mainly due to their anthocyanin pigments, which have pharmacological properties and are used in traditional medicine to treat conditions related to oxidative stress such as diabetes and inflammation [7, 8].

Despite the growing interest in *Eugenia* species' pharmacological and nutraceutical properties, only some studies have linked these properties to the metabolic profiles of their fruits. To accurately identify the bioactive constituents responsible for nutritional benefits, it is essential to conduct comprehensive metabolic assessments. Traditional methods for isolating and structurally elucidating chemical components from complex matrices are labour-intensive and time-consuming. In this context, metabolomic approaches employing advanced analytical technologies and bioinformatics tools have become indispensable for discovering new bioactive molecules. Mass spectrometry (MS) is notably recognized for its ability to analyse various chemical species with diverse physicochemical properties, even from minute amounts of complex mixtures. Its high selectivity, sensitivity and speed make it a valuable tool in food chemistry, enabling detailed analysis of food products [8, 9]. As a result, MS-based platforms have been increasingly applied in research laboratories to profile plant metabolomes with food relevance [10, 11]. However, the large volume of data generated by MS necessitates robust bioinformatic tools and chemometrics methods to explore and organize the metabolite information for targeted species.

Interpreting large spectral datasets and translating molecular composition into biological insights is challenging, leading to the development of advanced tools for spectral analysis. Chemometric methods, such as multivariate statistical techniques, are essential for enhancing metabolomic data interpretation. These methods uncover relationships between variables, such as mass-to-charge ratios in MS, enabling the detection of similarities, differences and mass spectra classification. Tools like Principal Component Analysis (PCA) and Partial Least Squares (PLS) are widely used in MS analysis, with PLS being particularly effective in handling datasets with high collinearity and noise [12]. Chemometrics plays a crucial role in food analysis, improving data interpretation and quality control and is increasingly applied in areas like food fraud and authentication [13], fruit and vegetable screening [14], flavours and aroma profiles [15].

This study aims to employ high-resolution mass spectrometry (HRMS) coupled with chemometric approaches to comprehensively investigate the molecular diversity of *Eugenia uniflora*, *E. punicifolia* and *E. jambolana* Lam., commonly known as pitanga, murta and jambolão, respectively, and to correlate their metabolite profiles with free radical scavenging potential. Previous studies have extensively characterized the polyphenolic composition of *Eugenia* species, particularly *E. uniflora* and *E. jambolana*, reporting high levels of anthocyanins, flavonoids, tannins and phenolic acids. The metabolite classes identified in the present study are in strong agreement with these reports, while the integration of high-resolution metabolomics with chemometric modelling provides additional insights into species-specific distribution patterns and their relationship with antioxidant activity.

2 | Materials and Methods

2.1 | Plant Material

Edible fruits from *E. uniflora*, *E. punicifolia* (Kunth) DC and *E. jambolana* Lam were collected from three locations in Maranhão, Brazil. The *E. uniflora* samples were obtained in Pirapemas (Latitude: −3.724741, Longitude: −44.224341), *E. punicifolia* in Paço do Lumiar (Latitude: −2.418712, Longitude: −44.15359) and *E. jambolana* in Pirapemas (Latitude: −3.7263, Longitude: −44.229698). All fruits were harvested during the same season (dry season, between August and September 2023) to minimize seasonal metabolic variability. Fruits were collected at full ripeness, determined based on uniform peel coloration characteristic of each species and ease of detachment from the plant. After harvesting, fruits were immediately transported to the laboratory, washed and processed within 24 h.

Fresh fruits (100 g) were subjected to extraction at room temperature using a hydroalcoholic solution (70% ethanol in water) at a sample-to-solvent ratio of 1:1 (w/v), performed in three successive extraction cycles (100 mL per cycle) under continuous agitation. The extraction was carried out at room temperature under continuous agitation for 24 h in each extraction cycle. Three successive extraction cycles were performed to ensure exhaustive recovery of polar and semi-polar metabolites. The hydroalcoholic extracts were concentrated using a rotary evaporator and subsequently freeze-dried.

2.2 | MS Analysis and Putative Compound Annotation

MS/MS analyses were conducted using a Q-Exactive hybrid Quadrupole-Orbitrap high-resolution mass spectrometer (Thermo Scientific) with an electrospray ionization (ESI) source operating in positive mode. A sample injection volume of 30 µL was used. The ESI source parameters were configured as follows: spray voltage at 3.5 kV, capillary temperature set to 250°C, S-lens RF level at 60 V, sheath gas flow rates of 47 L min^{-1}, and auxiliary gas flow rates of 11 L min^{-1}. High-resolution mass spectra were acquired using Full MS/data-dependent MS2 (dd-MS2) mode, covering a mass range of m/z 100–1200. The top five most abundant precursor ions (TopN = 5) were sequentially

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selected for fragmentation via collision-induced dissociation with energies of 20, 30 and 35 eV. The resolving power was 140,000 for Full MS scans and 70,000 for dd-MS2 acquisitions.

Raw data files generated by the Q-Exactive mass spectrometer were converted to the (.mzML) format using MSConvert software (ProteoWizard, Palo Alto, CA, USA) before analysis with MZmine version 2.53. MS1 and MS2 mass detection was performed in centroid mode with noise levels set to 1.0×10^6 and 1.0×10^4, respectively. MS1 chromatograms were constructed using the ADAP chromatogram builder with a minimum group size of five scans, group intensity threshold of 3.0×10^6, minimum highest intensity of 3.0×10^6 and an m/z tolerance of 10 ppm. Chromatogram deconvolution was carried out using the baseline cut-off algorithm with a minimum peak height of 3.0×10^6, peak duration range of 0.02–2.0 min and baseline level of 1.0×10^6. Isotopic peak grouping was performed with an m/z tolerance of 10 ppm, retention time tolerance of 0.05 min and maximum charge of 2. Feature alignment was conducted using the Join Aligner with m/z and retention time tolerances of 10 ppm and 0.05 min, respectively. Gap filling was applied using the peak finder algorithm and only features with associated MS/MS spectra were retained. The processed feature table was exported and subsequently used for chemometric analyses in MetaboAnalyst.

Metabolite identification was performed by comparing experimental spectra to the GNPS spectral library, utilizing the Classical Molecular Networking (MN) tool as described by Wang et al. [16].

2.3 | Chemometric Data Analysis

Fifteen samples were analysed, including five from Pitanga, five from Jambolao and five from Murta. Chemometric analyses were performed using MetaboAnalyst 6.0 version and Origin Pro 2024b software. The dataset was pre-processed through normalization, autoscaling and square root transformation. Exploratory data analysis was conducted using PCA to investigate data structure and visualize clustering trends among the *Eugenia* species [17].

Hierarchical clustering analysis (HCA), another unsupervised multivariate method, was employed to identify inherent patterns and relationships within the data. Selecting a suitable distance function to calculate inter-cluster and inter-observation distances was critical, as it significantly impacts the clustering results [17]. In addition, Partial Least Squares Discriminant Analysis (PLS-DA), a classification method based on PLS regression, was also applied for sample classification. The variable importance in projection (VIP) scores derived from the PLS-DA model was used to identify key m/z variables contributing to species discrimination. Variables with VIP scores more outstanding than 1 were considered significant contributors to species differentiation.

In addition, heatmaps were employed as visual tools to represent metabolite abundance across samples, highlighting patterns, relationships and variations through a colour gradient. Venn diagrams and Van Krevelen plots were constructed to identify metabolites shared or unique among different groups, providing further insights into the samples' metabolic profiles.

2.4 | Free Radical Scavenging Activity and Total Phenolic Content Determination

The DPPH radical scavenging activity was assessed following the protocol outlined by Wang et al. [18]. Similarly, the ABTS radical scavenging activity was evaluated based on the procedure described by Yu et al. [15]. Voltammetric analyses were conducted using a μAutolab III potentiostat/galvanostat, integrated with NOVA 2.1 software (Metrohm), using the analytical method detailed by Moreno et al. [19]. The total phenolic content was determined in triplicate using the method described by Shahidi and Naczk [20].

The antioxidant activity of the fruit extracts was evaluated using the DPPH radical scavenging assay. A DPPH• solution was prepared by dissolving 4.0 mg of DPPH in 100 mL of analytical-grade ethanol. A rutin standard stock solution (4 mg mL^{-1}) was obtained by dissolving 16 mg of rutin in 4 mL of ethanol, followed by serial dilutions to yield concentrations of 2.0, 1.0, 0.5 and 0.25 mg mL^{-1}. The same dilution procedure was applied to fruit extracts. For the reaction, 2.0 mL of the DPPH• solution was mixed with 0.4 mL of either standard or extract solution, and the mixtures were incubated for 30 min at room temperature in the dark. All assays were performed in triplicate. Absorbance was measured using a UV–Vis spectrophotometer, and the percentage of radical scavenging activity was calculated based on the decrease in absorbance relative to the control DPPH• solution.

The $ABTS^{\bullet+}$ radical scavenging activity was determined according to the method described by Yu et al. [15], with minor modifications. An aqueous ABTS solution (7.0 mmol L^{-1}) was prepared by dissolving 3.84 mg of ABTS salt in 1 mL of distilled water, while an aqueous potassium persulfate solution (7.0 mmol L^{-1}) was prepared by dissolving 37.84 mg of the salt in 1 mL of distilled water. The $ABTS^{\bullet+}$ radical was generated by mixing 17.6 μL of the potassium persulfate solution with 1 mL of the ABTS solution and incubating the mixture for 16 h at room temperature in the dark. On the day of analysis, the $ABTS^{\bullet+}$ solution was diluted with methanol until an absorbance of 0.70 ± 0.02 at 734 nm was reached. Trolox was used as the reference antioxidant, with a stock solution (2000 μM) prepared by dissolving 25 mg of Trolox in 50 mL of analytical-grade methanol and subsequently diluted to obtain concentrations ranging from 100 to 2000 μM. Fruit extracts were prepared by dissolving 5 mg of each extract in 10 mL of ethanol and further diluted to final concentrations between 5 and 40 μg mL^{-1}. Under light-protected conditions, aliquots of 100 μL of Trolox solutions, extract solutions or blank were mixed with 1.9 mL of the $ABTS^{\bullet+}$ solution in quartz cuvettes. After 6 min of reaction, absorbance was measured at 734 nm using a digital visible spectrophotometer. All measurements were carried out in triplicate, and antioxidant capacity was expressed as Trolox Equivalent Antioxidant Capacity (TEAC) based on the Trolox calibration curve.

The total phenolic content of the extracts was determined using the Folin–Ciocalteu colorimetric method. Gallic acid (GA) was used as the reference standard to construct calibration curves over the concentration range of 10–160 μg mL^{-1}. For the reaction, 2.0 mL of distilled water was mixed with 250 μL of Folin–

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Ciocalteu reagent and 250 μL of the extract solution. After an incubation period of 8 min in the dark, 100 μL of sodium carbonate solution (10% w/v) was added. The mixtures were homogenized and allowed to react for 1 h at room temperature in the absence of light. Absorbance was measured at 760 nm using a UV–Vis spectrophotometer, and the total phenolic content was calculated from the GA calibration curve and expressed as mg of gallic acid equivalents (GAE) per g of dry extract. GA (≥ 98%, UV, HPLC) was used as the standard, and the results are expressed as GAEs (mg GAE g^{-1}).

Statistical analysis of total phenolic content and antioxidant assays was performed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Differences were considered statistically significant at $p < 0.05$.

Voltammetric analyses were performed using a μAutolab III potentiostat/galvanostat coupled with NOVA 2.1 software. Measurements were conducted in a single-compartment electrochemical cell with a total volume of 2 mL, employing a three-electrode system consisting of a carbon paste working electrode, an Ag/AgCl (3.0 M KCl) reference electrode, and a platinum wire auxiliary electrode. Differential pulse voltammetry measurements were carried out using a pulse amplitude of 50 mV, a pulse width of 0.5 s and a scan rate of 10 mV s^{-1}. Square wave voltammetry was performed with a pulse amplitude of 50 mV, a frequency of 50 Hz and a potential increment of 2 mV, corresponding to a scan rate of 100 mV s^{-1}. Cyclic voltammetry experiments were recorded over a potential range from 0.0 to 1.0 V at a scan rate of 100 mV s^{-1}. All voltammograms were baseline-corrected, and data processing was performed using Origin 9.0 software. All electrochemical experiments were conducted in triplicate at room temperature (21 ± 1°C) using 0.1 M phosphate buffer solution (PBS, pH 7.0) as the supporting electrolyte.

3 | Results and Discussion

3.1 | Free Radical Scavenging Activity and Total Phenolic Content

Comprehensive analyses using appropriate methodologies are necessary to assess the redox profile of the fruits from pitanga (*E. uniflora*), jambolao (*E. jambolana*) and murta (*E. punicifolia*). Electrochemical experiments were conducted in triplicate. Anodic peaks observed at approximately $E_{pa1} = 0.2$ V or lower potentials are characteristic of polyphenolic compounds, as Rebelo et al. reported [21]. These low overpotential values in the oxidation process suggest that these compounds possess significant free radical scavenging activity.

The samples exhibited similar voltammetric profiles, particularly closely resembling pitanga and jambolao. According to the Cottrell equation [22], the intensity of the anodic peak is directly related to the concentration of electroactive species in the sample. Based on this correlation, pitanga likely contains the highest concentration of antioxidant compounds, whereas murta appears to have the lowest.

Research indicates that phenolic acids, flavonoids and tannins can be involved in redox reactions and contribute significantly to the antioxidant properties of plant extracts. These compounds are known for neutralizing free radicals through various mechanisms, including electron donation and radical scavenging [23, 24]. This information aligns with the observed voltammetric data, suggesting that the phenolic content in pitanga and jambolao is a key factor in their higher antioxidant potential than murta.

Figure 1 illustrates the voltammetric profiles of pitanga, jambolao and murta extracts.

Regarding the inhibition assay of the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ free radicals (Table 1), the lowest concentrations required for 50% inhibition of $DPPH^{\bullet}$ radicals were observed in the jambolao extract (3.1 ± 0.10 μg/mL), followed closely by pitanga (3.3 ± 0.12 μg/mL), with murta showing the highest IC_{50} value (3.7 ± 0.15 μg/mL) among the fruit samples. The reference standard rutin (flavonoid) exhibited the most potent DPPH scavenging activity with an IC_{50} of 2.9 ± 0.08 μg/mL, demonstrating its superior antioxidant potential. For the ABTS assay, Trolox, the reference antioxidant, showed the highest radical scavenging activity with an IC_{50} value of 147.0 ± 2.5 μg/mL. Among the fruit extracts, jambolão showed the highest ABTS radical scavenging activity (189.0 ± 3.2 μg/mL), followed by pitanga (190.0 ± 3.5 μg/mL) and murta (191.0 ± 3.8 μg/mL). However, despite these numerical differences, no statistically significant differences were observed among the samples ($p > 0.05$), indicating comparable antioxidant capacity in the ABTS assay. The lack of significant differences observed in the ABTS assay among the three fruits may be attributed to the broader reactivity spectrum of the $ABTS^{\bullet+}$ radical, which is sensitive to both hydrophilic and lipophilic antioxidants. Unlike DPPH, ABTS does not selectively respond to phenolic compounds alone, and its response may be influenced by non-flavonoid antioxidants such as ascorbic acid, tocopherols, carotenoids and volatile compounds, including essential oils [29].

Therefore, similar ABTS values may reflect the combined contribution of diverse antioxidant classes rather than differences in polyphenolic composition. This highlights the complementarity of antioxidant assays and reinforces the importance of integrating multiple analytical approaches when evaluating antioxidant capacity in complex plant matrices.

The total phenolic content (Table 1) varied slightly among the fruit extracts. Jambolao exhibited the highest phenolic content (41.6 ± 1.05 mg GAE/100 g), followed by pitanga (40.93 ± 1.10 mg GAE/100 g), while murta had the lowest content (39.54 ± 1.20 mg GAE/100 g). Although the total phenolic contents partially overlapped among the fruits and did not differ significantly ($p > 0.05$), a consistent trend was observed, with jambolao and pitanga exhibiting slightly higher mean values than murta. This trend was reflected more clearly in the DPPH assay, suggesting that qualitative differences in phenolic composition, rather than total content alone, play a key role in antioxidant performance.

Among the three fruits, jambolao is the most effective antioxidant source, followed closely by pitanga, while murta showed comparatively weaker activity. Jambolao is recognized for its high antioxidant capacity due to its rich content of phenolic compounds such as anthocyanins, ellagic acid, quercetin and rutin, which contribute significantly to its ability to neutralize free radicals and inhibit oxidative stress [5]. In the case of

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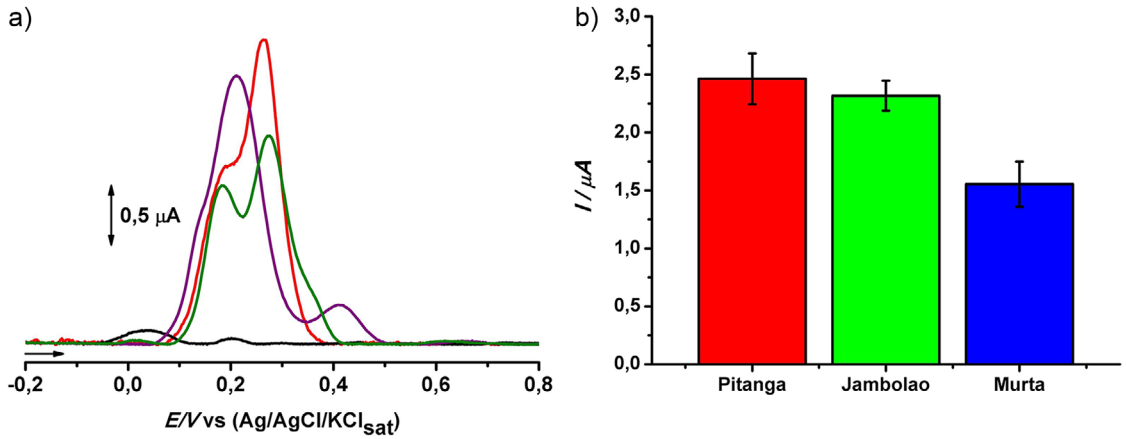

FIGURE 1 | Voltammetry profiles of fruit extracts of pitanga (*Eugenia uniflora*), jambolao (*E. jambolana*) and murta (*E. punicifolia*). (A) Voltammograms showing anodic peaks (0.1–0.4 E/V vs. Ag/AgCl/KCl_sat) related to phenolic oxidation. (B) Peak current intensities (I, µA) indicate higher antioxidant capacity. Error bars represent standard deviations ($n = 3$).

TABLE 1 | The total phenolic content (mg GAE/100 g) and the IC_{50} values for the inhibition of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ free radicals were evaluated in fruit extracts of pitanga (*Eugenia uniflora*), jambolão (*E. jambolana*) and murta (*E. punicifolia*). The results are presented as the mean ± standard deviation, based on analyses performed in triplicate.

Sample	DPPH inhibition assay (µg/mL)	ABTS inhibition assay (µg/mL)	Total phenolic content (EAG mg/100 g)
Pitanga	3.3 ± 0.12	190.0 ± 3.5	40.93 ± 1.10
Jambolao	3.1 ± 0.10	189.0 ± 3.2	41.6 ± 1.05
Murta	3.7 ± 0.15	191.0 ± 3.8	39.54 ± 1.20
Rutin	2.9 ± 0.08	—	—
Trolox	—	147.0 ± 2.5	—

pitanga, particularly the purple variety, its antioxidant power is attributed mainly to its high anthocyanin content. Studies have shown that purple-fleshed pitanga has the highest total phenolic and anthocyanin contents among different varieties, which correlates with its excellent antioxidant potential. Purple pitanga's in vitro antioxidant capacity has been evaluated, demonstrating significant potential to protect against oxidative stress [25]. These findings highlight the importance of phenolic compounds in determining the antioxidant capacities of these fruits, with jambolao and pitanga being particularly rich sources.

The correlation analysis (Figure 2) revealed a strong negative association between total phenolic content and DPPH radical scavenging values ($r \approx -0.9$), indicating that samples richer in phenolic compounds exhibited greater antioxidant capacity. This inverse relationship reinforces the pivotal role of phenolics as major contributors to the overall antioxidant potential of the analysed fruit extracts. In contrast, weak or negligible correlations were observed between total phenolics and the ABTS assay, as well as between DPPH and ABTS values, suggesting that these assays may respond differently depending on the structural diversity and reactivity of the antioxidant molecules present. Despite the limited sample size, these findings are consistent with the widely reported capacity of phenolic compounds to effectively quench free radicals, highlighting their relevance in the antioxidant defence mechanisms of tropical fruits.

3.2 | Chemometric Approaches for the Discrimination of *Eugenia* Species

Using HRMS, an initial approach was performed to assess the spectral complexity of the data and detect critical differences among the fruit samples of pitanga (*E. uniflora*), jambolao (*E. jambolana*) and murta (*E. punicifolia*). For clarity, the common names of the fruits were used to facilitate sample identification. This preliminary screening focused on the number of shared peaks among the species to evaluate the extent of metabolomic overlap. Beeswarm plots (Figure 3a) and Venn diagrams (Figure 3b) were constructed, revealing peak distributions within the 150–950 Da range, with the highest abundance observed between *m/z* 200 and 600, a range typically associated with polyphenols commonly found in *Eugenia* species [26].

Pitanga samples exhibited 1.149 unique ions, representing distinctive metabolic traits exclusive to this species. Among these, 435 ions were shared with murta, indicating some metabolic overlap. Murta samples, in turn, presented 800 unique ions, highlighting species-specific metabolites. A significant overlap of 1.518 ions was identified across all three species, demonstrating considerable similarity in their metabolic profiles. Overall, the Venn diagram analysis underscored both the unique and shared metabolic profiles among the studied species, providing critical insights into the chemical diversity of *E. jambolana*, *E. punicifolia*

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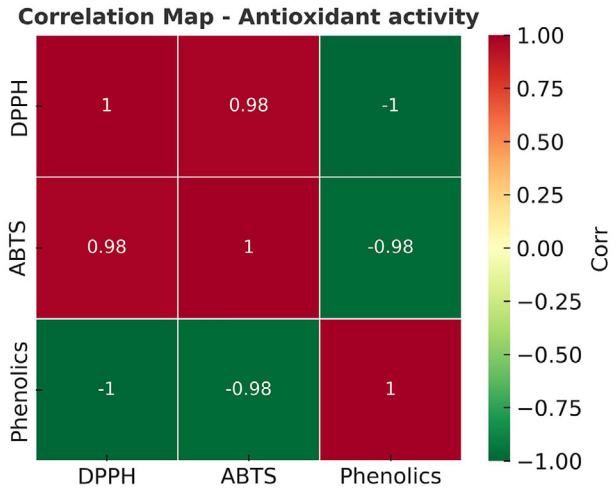

FIGURE 2 | Correlation map showing the relationships among DPPH and ABTS radical scavenging activities and total phenolic content in selected fruit extracts. A strong negative correlation between total phenolics and DPPH values indicates that phenolic-rich samples possess higher antioxidant capacity. Colour intensity represents the strength and direction of the Pearson correlation coefficients (*r*), ranging from –1 (strong negative correlation) to +1 (strong positive correlation).

and *E. uniflora* (Figure 3). These findings establish a foundation for understanding these fruits' metabolomic composition and potential functional roles.

Univariate statistical analysis revealed pronounced and robust metabolic distinctions among the three species, underscoring unique chemical signatures with potential as discriminatory biomarkers. Application of t-tests and volcano plot analyses highlighted numerous statistically significant ions contributing to the differentiation of these fruits, while fold change analysis further delineated the magnitude and direction of metabolic shifts, pinpointing species-specific enrichment patterns. For instance, comparisons between jambolao and pitanga identified ions at *m/z* 815.6 and 426.8 as predominantly elevated in jambolao, whereas *m/z* 368.2 and 351.7 were more abundant in pitanga, confirming the presence of differential metabolic markers. Contrasts between jambolao and murta revealed enriched metabolites for each group, emphasizing chemical specificity (Figure 4). The murta–pitanga comparison revealed 31 upregulated and 16 downregulated ions, many exceeding *m/z* 400, indicative of glycosylated polyphenols such as anthocyanins and other flavonoids, compounds widely recognized for their antioxidant and bioactive properties. Notably, in jambolao, most significantly upregulated ions fell within *m/z* 300–400, a range characteristic of aglycone polyphenols, contrasting with murta and pitanga, where higher-mass glycosylated polyphenols predominated. This structural divergence may have functional implications, as aglycone polyphenols are generally reported to exhibit higher intestinal absorption and bioavailability compared to their glycosylated counterparts, in addition to distinct biological activities [30, 31]. Collectively, these results demonstrate that jambolao, pitanga and murta possess discrete chemical profiles defined by significant variations in polar metabolites and high-molecular-weight polyphenols.

To better elucidate the relationship between chemical composition and biological activity, two independent PCA models were constructed using different data matrices, as described below. The first PCA model was constructed using the MS-derived chemical composition data in order to evaluate differences in the metabolomic profiles among the fruit samples (Figure 5). The second PCA model was built using the antioxidant activity data to discriminate the samples according to their biological performance, allowing their classification into high and medium antioxidant potential groups (Figure 6).

A detailed compositional similarity analysis among the samples was performed using unsupervised multivariate chemometric methods, PCA and HCA (Figure 5). These complementary tools enable data dimensionality reduction and visualization of variance patterns (PCA) while identifying natural groupings and hierarchical relationships among samples (HCA). The PCA analysis revealed that PC1 and PC2 accounted for 37.87% and 33.60% of the total variance, respectively. A clear separation between the groups was observed, with a slight overlap between pitanga (*E. uniflora*) and jambolao (*E. jambolana*). In the HCA dendrogram, all replicates from each fruit are grouped into distinct clusters, confirming consistency within each species. Notably, pitanga and jambolao clustered together, sharing approximately 40 % similarity, while murta (*E. punicifolia*) formed a separate cluster, reflecting its distinct metabolic profile compared to the other two species. This combined multivariate approach highlights shared and unique metabolic characteristics among the *Eugenia* fruits.

To more precisely assess the compositional differences among the three species and correlate these variations with their radical scavenging activity, as well as their potential nutritional value, unsupervised multivariate chemometric methods were employed (Figure 6a,b), focusing on the antioxidant properties of each fruit. The results indicated that both the PCA loading plot (Figure 6a) and the HCA dendrogram (Figure 6b), derived from the antioxidant profile, effectively clustered the samples according to their antioxidant capacity. The PCA model accounted for 82.6% of the total variance (PC1: 67.2%, PC2: 15.4%), representing a more robust explanation than the previous model based solely on MS data. The PCA analysis segregated the samples into two distinct groups based on antioxidant potential: one with high and the other with medium antioxidant activity. Pitanga and jambolao were grouped in the high-antioxidant cluster, while murta was classified separately in the medium-antioxidant cluster. The HCA dendrogram further substantiated these findings, with samples exhibiting high antioxidant potential clustering together. At the same time, murta was distinctly positioned in a separate cluster, consistent with its classification as having medium antioxidant activity.

This study applied supervised chemometric models such as PLS-DA and OPLS-DA (Orthogonal Partial Least Squares Discriminant Analysis) (Figure 7a,b). PLS-DA was used to identify differential metabolites and develop classification models, while OPLS-DA is a supervised statistical method focused on the predictive component. Both models could accurately classify the samples, explaining 81% of the variance in the first two components.

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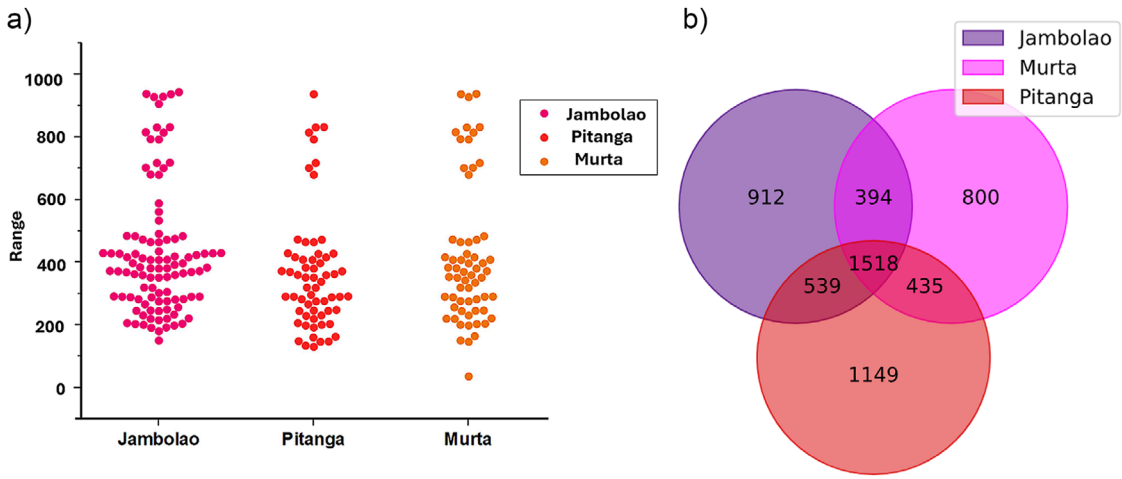

FIGURE 3 | Beeswarm plot (a) and Venn diagram (b) illustrating the number of ions detected in the ethanolic extracts of jambolão, pitanga and murta fruits, as well as the metabolite complexity identified in each species.

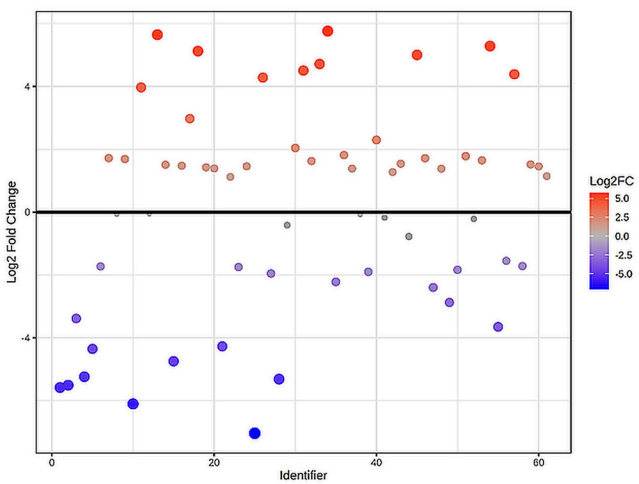

FIGURE 4 | Fold change analysis of metabolites in jambolao versus murta. The plot illustrates significantly upregulated (up, red) and downregulated (down, blue) ions between the two species.

The VIP score was evaluated to assess the importance of individual variables in the PLS-DA model (Figure 7c), providing insight into the contribution of each variable to the model's predictive power. The VIP score is determined by calculating a weighted sum of the squared correlations between the original variables and the components of the PLS-DA model. The weights reflect the proportion of variance explained by each component in the model. In this study, the anthocyanins with the highest VIP scores were Cyanidin-3-*O*-sophoroside, Delphinidin-3-*O*-laminaribioside, Delphinidin-3-*O*-galactoside, Cyanidin-3-*O*-caffeoyl-diglucoside, Cyanidin-3-*O*-pentoside, Pelargonidin-3-*O*-glucoside and Delphinidin-3-*O*-coumaroyl-glucoside. Variables with VIP scores above 1, such as Cyanidin-3-*O*-pentoside, Pelargonidin-3-*O*-glucoside and Delphinidin-3-*O*-galactoside, were identified as highly influential, with Cyanidin-3-*O*-pentoside contributing to the high antioxidant potential group and Pelargonidin-3-*O*-glucoside and Delphinidin-3-*O*-galactoside being significant in both high and medium antioxidant potential groups, respectively.

Anthocyanins are the main pigments found in fruits of the genus *Eugenia* and represent the class of metabolites that most strongly contributes to the free radical scavenging activities evaluated in this study. Their identification in the fruit extracts was based on high-resolution MS/MS data, combining accurate mass measurements and diagnostic fragmentation patterns. MS/MS fragmentation analysis revealed characteristic ions consistent with anthocyanin aglycones and their corresponding glycosylated forms, allowing confident putative annotation at MSI Level 2. Glycosylated phenolic compounds such as anthocyanins present at high abundance in the extracts were readily recognized by the neutral loss of sugar moieties, with typical losses of 162 Da for hexosides, 146 Da for deoxyhexosides and 132 Da for pentosides in *O*-glycosylated flavonoids, in agreement with previously reported fragmentation behaviour [27, 28]. For instance, malvidin-3,5-*O*-diglucoside ($[M+H]^+$ *m/z* 655) yielded fragment ions at *m/z* 493 and 331, corresponding to successive losses of glucose units and formation of the aglycone ion. Similarly, petunidin-3,5-*O*-diglucoside ($[M+H]^+$ *m/z* 641) and delphinidin-3,5-*O*-diglucoside ($[M+H]^+$ *m/z* 627) generated diagnostic product ions at *m/z* 479/317 and 465/303, respectively. Monoglycosylated anthocyanins such as petunidin-3-*O*-glucoside ($[M+H]^+$ *m/z* 479) and delphinidin-3-*O*-glucoside ($[M+H]^+$ *m/z* 465) were identified by the characteristic neutral loss of 162 Da, yielding the corresponding aglycone ions at *m/z* 317 and 303. These fragmentation features, together with accurate mass measurements and spectral library comparisons, confirm the presence of anthocyanins as the major contributors to the antioxidant profiles of the analysed *Eugenia* fruit extracts.

Considering that anthocyanins are an essential class of compounds in the *Eugenia* genus and their contribution as a measure of a variable's importance in the PLS-DA model, heatmap plots (Figure 8a) were constructed to assess the abundance of these compounds in the fruit extracts of *E. uniflora* (pitanga), *E. jambolana* (jambolao) and *E. punicifolia* (murta) and correlate these findings with the results of antioxidant assays. The most abundant anthocyanins identified were A3 (delphinidin-3-*O*-galactoside) and A15 (cyanidin-3-*O*-pentoside), which exhibited higher concentrations in jambolao and pitanga. These compounds likely contribute to the higher antioxidant potential

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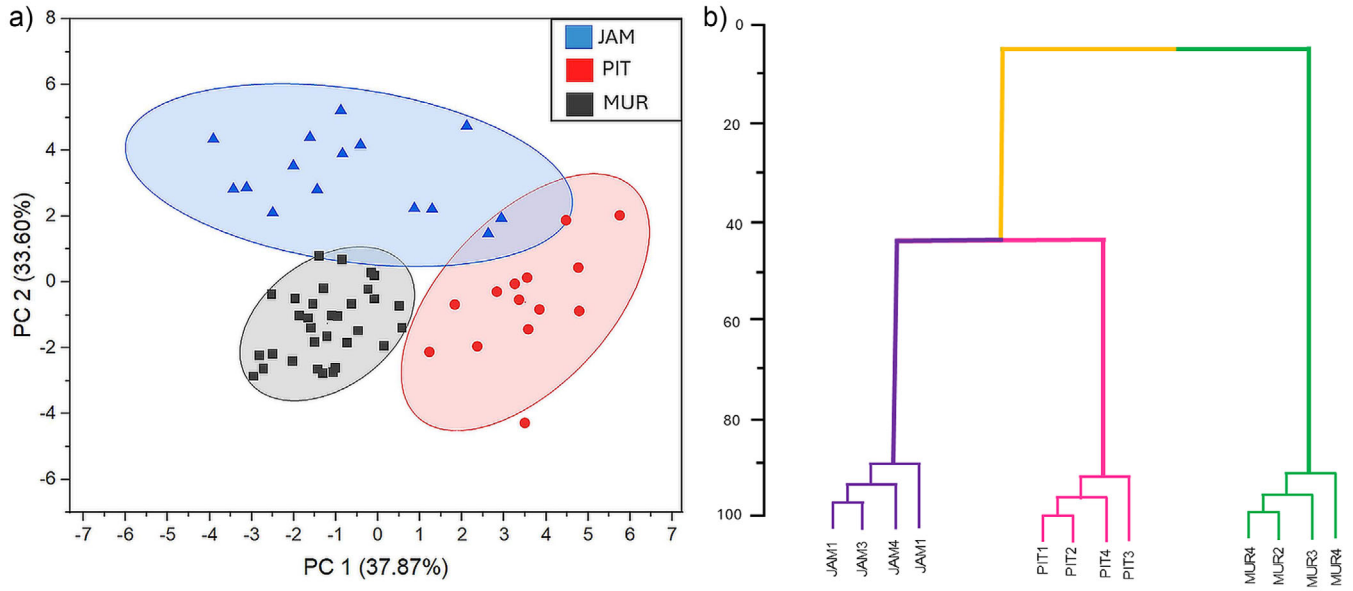

FIGURE 5 | Visualization of samples in 2D space using PCA scores (a) and HCA dendrograms corresponding to fruit extract samples from pitanga (*Eugenia uniflora*), jambolao (*E. jambolana*) and murta (*E. punicifolia*).

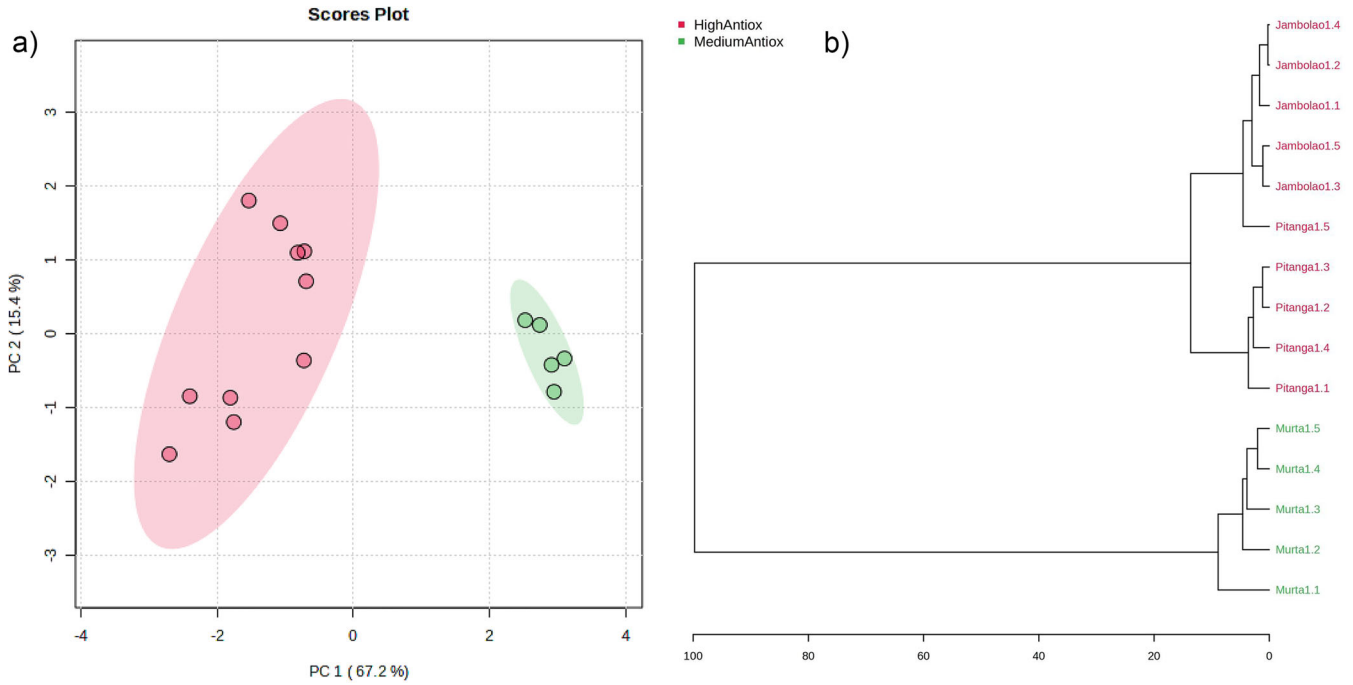

FIGURE 6 | PCA score plot (a) and HCA dendrogram based on the antioxidant potential of fruit extract samples from pitanga (*Eugenia uniflora*), jambolao (*E. jambolana*) and murta (*E. punicifolia*).

observed in these fruits. A3 showed significant levels across both jambolao and pitanga, correlating with the potent antioxidant activity of these fruits. A15, while abundant in jambolao and murta, further reinforces the relationship between anthocyanin abundance and antioxidant capacity.

In contrast, the compounds A2 (delphinidin-3-*O*-laminaribioside), A7 (cyanidin-3-*O*-sambubioside) and A11 (delphinidin-3-*O*-coumaroyl-glucoside) were found to be present in lower concentrations, especially in murta. The low abundance of these anthocyanins corresponds with the moderate antioxidant activity observed in these fruits. The data highlights the correlation between the levels of specific anthocyanins and the overall antioxidant potential of the fruit extracts, emphasizing the role of these compounds in the fruits' bioactive profiles.

To explore the contributions of various compound classes to the antioxidant potential of *Eugenia* species, we conducted a

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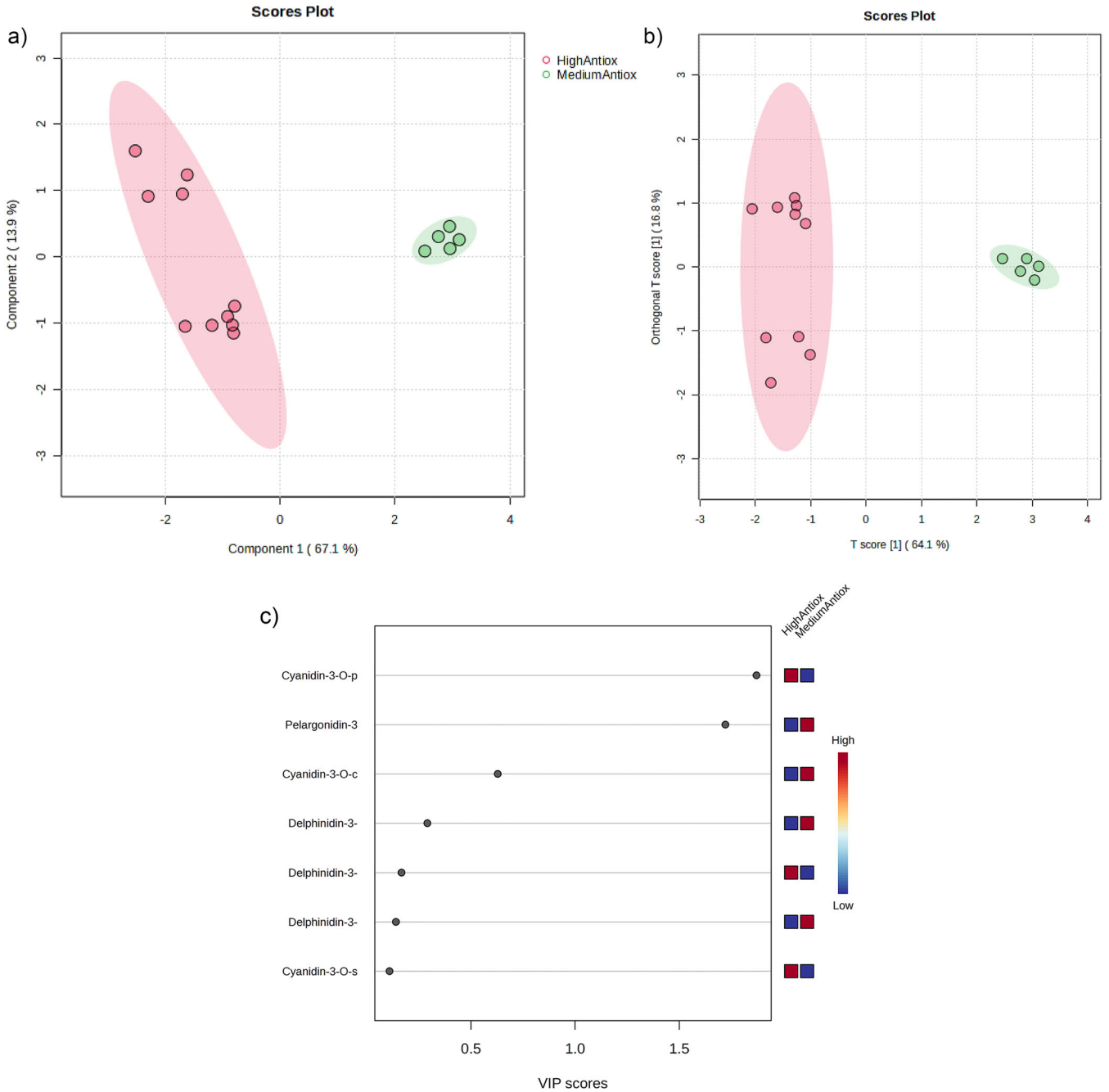

FIGURE 7 | PLS-DA score plot (a) and OPLS-DA score plot (b) based on the antioxidant potential of fruit extract samples from pitanga (*Eugenia uniflora*), jambolao (*E. jambolana*) and murta (*E. punicifolia*). (c) VIP scores of the key anthocyanins identified in the fruit extracts.

detailed analysis of secondary metabolites. The study evaluated the abundance of flavonoids, tannins and phenolic acids in the fruits of pitanga (*E. uniflora*), jambolao *(E. jambolana*) and murta *(E. punicifolia*). The findings are visualized in a Chord diagram (Figure 8b), illustrating the number of metabolites detected for each class.

The results show that flavonoids are most abundant in pitanga, followed by jambolao, with murta exhibiting the lowest levels. On the other hand, murta has a higher concentration of tannins than pitanga and jambolao, which display significantly lower levels. In addition, phenolic acids are more prevalent in murta, while pitanga and jambolao have similar but lower amounts.

It is important to note that the number of annotated metabolites within each chemical class does not necessarily reflect their relative contribution to antioxidant activity, which depends on both compound abundance and intrinsic redox properties.

Due to the limited number of polyphenolic metabolites detected in the dereplication analysis using spectral similarity and database matching, additional tools were employed to expand the metabolic coverage and identify other compounds contributing to the radical scavenging activity of the extracts. The Van Krevelen diagram (Figure 9a) showed promising results for detecting compounds not identified in the previous dereplication analysis. This diagram is a valuable tool in metabolomic data analysis,

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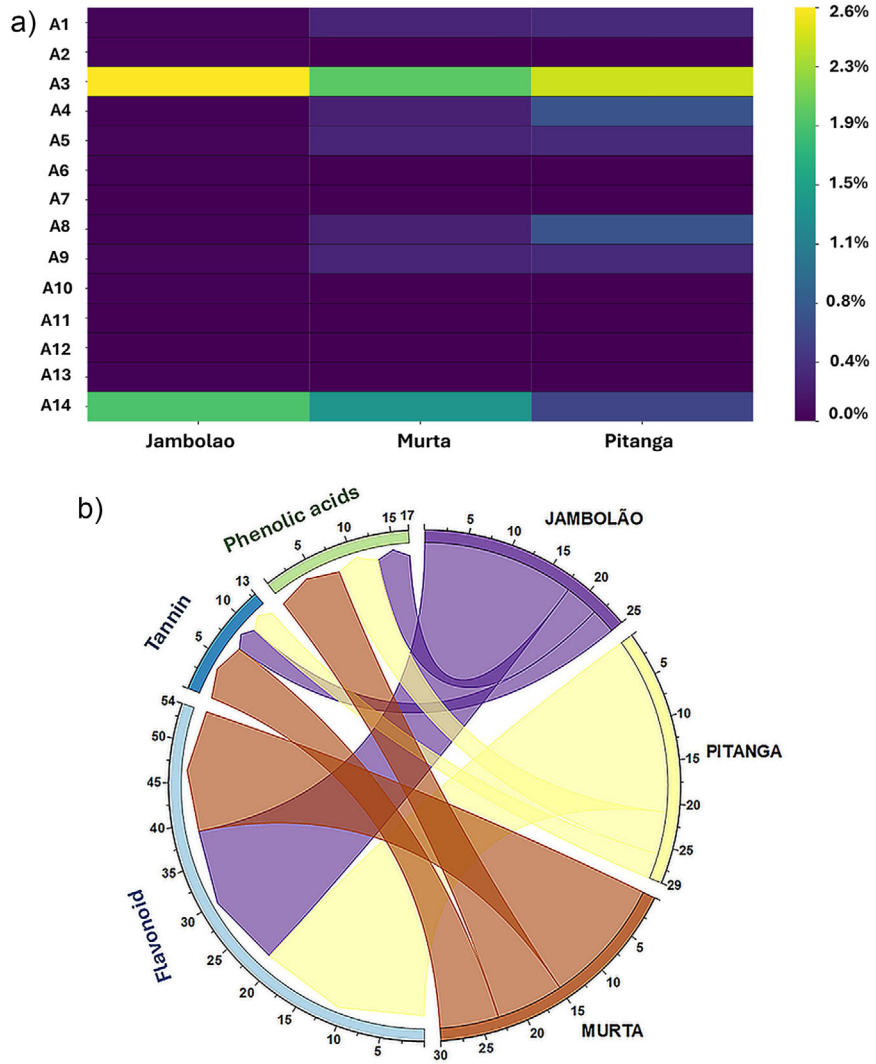

FIGURE 8 | (a) Heatmap graph representing the abundance of anthocyanins (A1 - Cyanidin-3-*O*-sophoroside, A2 - Delphinidin-3-*O*-laminaribioside, A3 - Delphinidin-3-*O*-galactoside, A4 - Cyanidin-3-*O*-galactoside, A5 - Cyanidin-3-*O*-laminaribioside, A6 - Cyanidin-3-*O*-sambubioside, A7 - Cyanidin-3-*O*-caffeoyl-diglucoside, A8 - Cyanidin-3-*O*-glucoside, A9 - Cyanidin-3-*O*-caffeoyl-glucoside, A10 - Delphinidin-3-*O*-coumaroyl-glucoside, A11 - Pelargonidin-3-*O*-glucoside, A12 - Cyanidin-3-*O*-malonyl-glucoside, A13 - Cyanidin-3-*O*-acetyl-glucoside, A14 - Cyanidin-3-*O*-pentoside) and, (b) chord diagram illustrating the abundance of flavonoids, tannins and phenolic acids in the fruit extracts of pitanga (*Eugenia uniflora*), jambolao (*E. jambolana*) and murta (*E. punicifolia*).

particularly for characterizing organic compounds based on their oxygen-to-carbon (O/C) and hydrogen-to-carbon (H/C) ratios, which can be derived from HRMS data. Polyphenols such as flavonoids and phenolic acids typically exhibit a low H/C ratio due to their aromatic skeleton and hydroxyl groups, positioning them in the diagram in regions with high O/C and low H/C compared to aliphatic compounds. Tannins, which have a more complex structure, were found in a region with low H/C but varied O/C values depending on the presence of phenolic groups and polymerization. Condensed tannins were located near flavonoids, while hydrolyzable tannins were found with very low H/C ratios. This approach detected many glycosylated flavonoids in pitanga, characterized by a high oxygen-to-carbon (O/C) ratio. Murta contained other hydrolyzable tannins that had not been previously identified. Jambolao was primarily composed of anthocyanins, and phenolic acids were predominantly found in murta. The Van Krevelen diagram also facilitated the identification of other oxygenated compounds, such as terpenes and saponins, which were not detected in the dereplication analysis. This method thus provided a broader identification of bioactive compounds in the fruit extracts.

Additional approaches were applied using the molecular formulas derived from HRMS data, demonstrating that structural information of biological significance can be obtained from MS1 data alone. Polar heatmaps with dendrograms and stacked column graphs were constructed to investigate further the composition of nitrogen-containing compounds, such as amino acid derivatives, and the degree of glycosylation in the metabolites (Figure 9b). These visualizations were used to assess the presence of glycosylated flavonoids and aglycones, as they contribute differently to antioxidant potential. The degree of oxygenation, characterized by a high number of oxygen atoms, was also considered, as this may indicate the presence of sugar moieties. Pitanga

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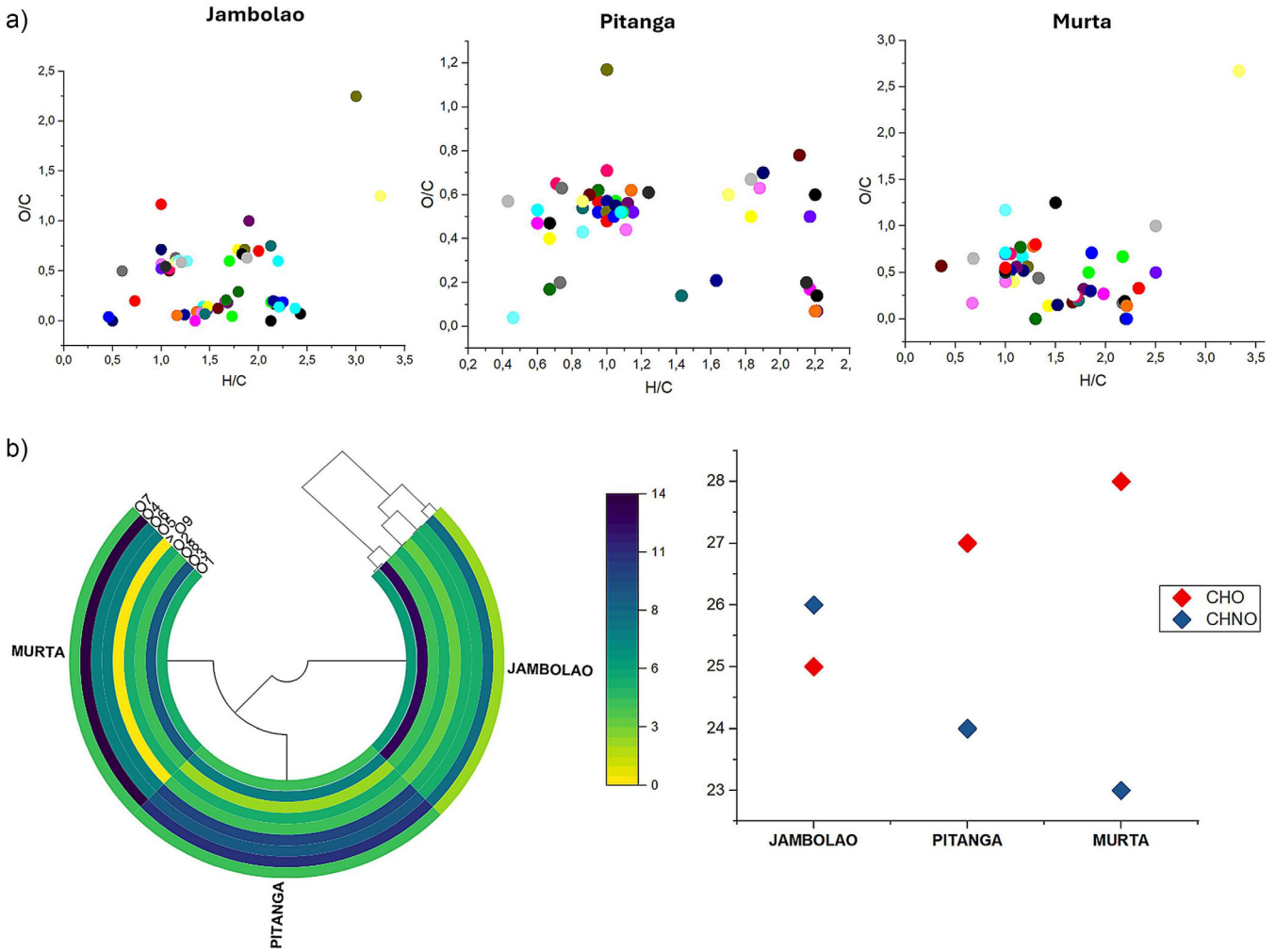

FIGURE 9 | (a) Van Krevelen diagram of the fruit extracts from pitanga (*Eugenia uniflora*), jambolao (*E. jambolana*) and murta (*E. punicifolia*). The diagram plots the oxygen-to-carbon (O/C) ratio against the hydrogen-to-carbon (H/C) ratio for the detected metabolites, with the O/C and H/C ratios calculated from molecular formulas derived from Orbitrap HRMS data. (b) Polar heatmap with dendrogram and scatter plot depicting the distribution of nitrogen-containing compounds and the degree of oxygenation of metabolites across the fruit extracts from pitanga (*E. uniflora*), jambolao (*E. jambolana*) and murta (*E. punicifolia*).

fruits showed the highest number of compounds with six oxygen atoms in their structures, followed by murta and jambolao, with the latter showing the lowest levels. Murta exhibited the highest number of compounds with four oxygen atoms and the lowest number containing more than nine oxygen atoms. Pitanga had the highest number of oxygenated compounds overall. Regarding nitrogen-containing compounds, jambolao presented the highest content, while murta had the lowest content.

4 | Conclusions

This study comprehensively assessed the potential of fruits from pitanga (*E. uniflora*), jambolao (*E. jambolana*) and murta (*E. punicifolia*) as antioxidants. These fruits' antioxidant potential and metabolic profiling were evaluated through a multidisciplinary approach, combining radical scavenging assays, electrochemical analysis, metabolomic profiling and HRMS-based chemometric approaches. Jambolao and pitanga exhibited higher antioxidant activity in the DPPH assay than murta samples. Electrochemical experiments further supported these findings, with anodic peaks characteristic of polyphenolic compounds indicating significant redox activity in jambolao and pitanga. Univariate statistical analysis robustly highlighted species-specific metabolic differences, revealing distinctive chemical signatures that can serve as reliable biomarkers for jambolao, pitanga and murta. Chemometric analysis of HRMS data, including HCA, PLS-DA and OPLS-DA, provided further insight into the distinct phenolic profiles of the fruits. The results demonstrated high classification accuracy based on the samples' antioxidant capacity in DPPH assay. Pitanga and jambolao cluster together, indicating their similar metabolic and antioxidant properties. These chemometric tools were critical in uncovering subtle differences in the fruit extracts and validating the findings from the other analytical techniques. Metabolomic profiling revealed distinct polyphenolic signatures among the species. While murta exhibited a higher number of annotated tannins and phenolic acids, pitanga and jambolao were characterized by a greater abundance of flavonoids and anthocyanins, which are more strongly associated with radical scavenging efficiency. These compositional differences

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help explain the superior antioxidant performance observed for pitanga and jambolao. Notably, anthocyanins such as cyanidin and delphinidin derivatives emerged as key contributors to their antioxidant properties.

These findings highlight the potential of jambolao and pitanga as valuable sources of bioactive compounds, emphasizing their suitability for functional food and nutraceutical applications. By integrating diverse analytical tools, this study underscores the critical role of polyphenols in the antioxidant activity of these fruits. It provides a foundation for further exploration of their health-promoting properties.

Author Contributions

Alexandre W. V. Nascimento: conceptualization, methodology, validation, formal analysis, investigation, data curation, writing – original draft. **Naará S. Balbino**: methodology, validation, formal analysis, investigation, data curation. **Salva Asghar**: methodology, formal analysis, investigation, data curation. **Erica A. Batista**: methodology, formal analysis, investigation. **Teresinha J. A. S. Andrade**: methodology, validation, formal analysis, investigation, data curation, project administration, supervision. **Eric S. Gil**: project administration, supervision. **Abeer M. Alawi Al-Sakkaf**: formal analysis, investigation, data curation, writing – original draft**. Boniek G. Vaz**: Project administration, Supervision. **Nerilson Marques Lima**: conceptualization, methodology, validation, formal analysis, investigation, data curation, writing – original draft, project administration, supervision.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The authors have nothing to report.

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