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RESEARCH ARTICLE

Quantifying the Impact of High-Pressure Processing on the Phenolic Profile, Antioxidant Activity, and Pollen Morphology in Honey

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ABSTRACT

Honey can benefit from non-thermal processing techniques such as high-pressure processing (HPP) to improve its quality and bioactivity. This study investigated the impact of HPP (600 MPa for 5, 10, and 15 min) on honey's quality, including the levels of hydroxymethylfurfural (HMF), antioxidant activity, total phenolic content (TPC), and phenolic profile. HPP treatment did not significantly affect HMF or TPC levels but led to selective changes in the phenolic profile. Despite a reduction in certain phenolic compound content, HPP for 5 and 15 min caused a significant increase in the antioxidant activity (2,2-diphenyl-1-picrylhydrazyl [DPPH]) of honey from the mean value of 41.8% to values of 45.4% and 49.6%, respectively. On the other hand, HPP for 10 min did not change the antioxidant activity of tested honey. A 27.5% reduction in the equatorial diameter of pollen grains was observed after HPP combined with temperature at 75°C, suggesting an improved release of bioactive compounds. The content of specific phenolic compounds, including caffeic acid, *p*-coumaric acid, sinapic acid, naringin, kaempferol, and the TPC, significantly affected the DPPH activity. The increment in the antioxidant activity of HPP honey may be attributed to selective changes in the content of certain phenolic compounds and improved their extraction from pollen grains.

1 | Introduction

Honey, a natural product with a long history of medicinal use, is increasingly sought after for its potential therapeutic benefits, which are linked to its bioactive compounds, primarily phenolic compounds [1]. These compounds contribute to honey's antioxidant and antibacterial properties [2, 3], offering potential health benefits such as cardiovascular protection and cancer prevention [4, 5].

The composition of honey is highly diverse, primarily due to differences in floral or geographical origin. However, other factors, such as environmental, seasonal, and processing conditions, can also influence its composition. Honey processing, such as conventional thermal processing, microwave, or ultrasound used for liquefaction and pasteurization of honey negatively impacts honey's antioxidant phenolics [6, 7]. These treatments can reduce honey's antioxidant and antibacterial activities, which are mediated by heat-sensitive compounds such as polyphenols. To preserve honey's health-related properties, minimal or no thermal processing is recommended [7]. This has led to a growing interest in non-thermal processing techniques as a safer and more effective alternative for preserving honey's quality and bioactivity. High-pressure processing (HPP) is a modern technology commercially applied at pressures up to 600 MPa for 5–6 min, at or below 25°C, to extend shelf life and improve food safety. This non-thermal process aims to destroy vegetative pathogenic microorganisms while preserving nutritional and sensory attributes compared to thermally processed products [8–10].

Recently, HPP has shown promise as an alternative approach for preserving [11] and/or enhancing the nutritive value of honey without affecting its overall quality characteristics (e.g., 5-hydroxymethylfurfural [HMF] content, diastase, viscosity, and color) [12, 13]. Akhmazillah et al. [14] have reported a substantial increase in total phenolic content (TPC), with an increment of 47.16%, of manuka honey subjected to HPP at 600 MPa at ambient temperature (~30°C) for 10 min, highlighting its potential to produce a more nutritive honey. Similarly, Chaikham and Prangthip [15] studied the effect of pressure at a range of 300–500 MPa at ambient temperature (25°C) for 5–20 min on TPC, total flavonoids, and antioxidant capacity (2,2-diphenyl-1-picrylhydrazyl [DPPH] and FRAP assay) in longan flower-honey. The level of TPC and antioxidant capacity of the honey increased with higher pressure (500 MPa) and time (20 min). Similarly, Leyva-Daniel et al. [16] reported an increase in the TPC of honey at 600 MPa for 15 min. The mechanisms behind the increase of TPC and antioxidant activity of honey after HPP are currently unknown, and it might be associated with increased extractability of some active antioxidant components. Phenolic compounds, as well-described honey antioxidants, are likely incorporated into honey through nectar and/or pollen collected from plants visited by honeybees [5]. It is hypothesized that the high antioxidant activity of honey after HPP is attributed to the extraction of the phenolic compounds from specific organelles within pollen cells in honey [17]. The phenolic compounds found in bee pollen include flavonoids, and their content in bee pollen can be as high as 2.5%, and they occur mainly as glycosides [18]. HPP treatment improved the extractability of phenolic compounds contained inside the bee pollen grain and increased the antioxidant potential of treated bee pollen [19].

Therefore, this study aims to investigate the impact of HPP at 600 MPa (the level of pressure used for commercial application of HPP) on the antioxidant properties of honey, as measured by changes in its phenolic profile, and to explore whether HPP and pressure combined with temperature at 75°C (pressure-assisted thermal processing, PATP) cause morphological changes in pollen cell structure, which could facilitate mass transfer between cellular content and honey, potentially improving extraction efficiency and increasing the content of phenolic compounds.

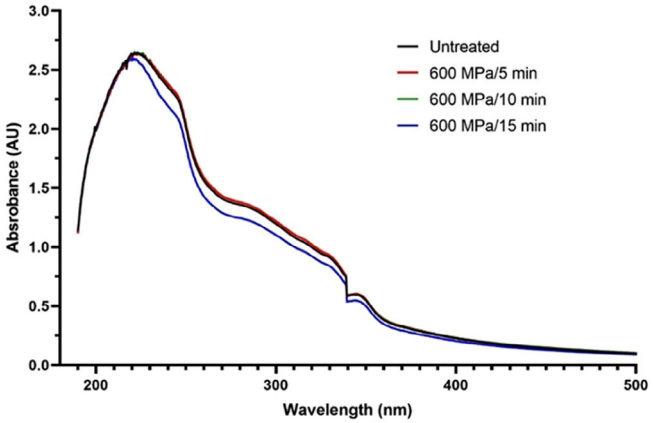

FIGURE 1 | Comparison of average UV–Vis spectra of untreated and HPP-treated honey samples in the wavelength interval of 190–500 nm.

2 | Results and Discussion

2.1 | Botanical Origin of Honey

The various pollen types were identified, and their frequencies of occurrence were categorized into the following classes: dominant pollen (>45% of the pollen spectrum); accompanying pollen (15%–45%); important pollen (3%–15%); minor pollen (1%–3%); and other pollen (≤1%). The dominant type of pollen was *Castanea sativa*; the accompanying pollen was *Quercus* sp. and *Myosotis* sp. Important and minor pollen types were *Acer* sp., *Apiaceae*, *Asteraceae* sp., *Campanula* sp., *Centaurea* sp., *Citrus* sp., *Dianthus* sp., *Echium* sp., *Erica* sp., *Genista* sp., *Lavandula* sp., *Pyrus* sp., *Prunus* sp., *Ranunculus* sp., *Robinia pseudoacacia*, *Rubus* sp., *Salvia* sp., *Taraxacum* sp., *Thymus* sp., *Tilia* sp., *Trifolium* sp., and *Vicia* sp. The honey used in this study contained a dominant pollen type *C. sativa*; however, this percentage was insufficient (<90%) to meet the minimum requirement for classification as a monofloral chestnut honey (at least 90% of *Castanea* pollens) [20]. Consequently, the honey was classified as multifloral.

2.2 | Ultraviolet–Visible (UV–Vis) Spectral Analysis

The mean spectra obtained for the unprocessed honey and HPP-honey samples are presented in Figure 1, showing similar spectra within the samples. The similarity in the spectrum can be attributed mainly to sugar composition because fructose and glucose are the most abundant compounds. So, similar ratios and types of sugars in different honey samples can result in similar spectral profiles, as sugars have characteristic absorption patterns in the UV and visible ranges.

Honey contains various minor components, such as phenolic compounds, which are related to the nectar's botanical origin. Although these components are present in smaller quantities, they can still affect the spectral profile. The presence of similar types and concentrations of these minor components in different

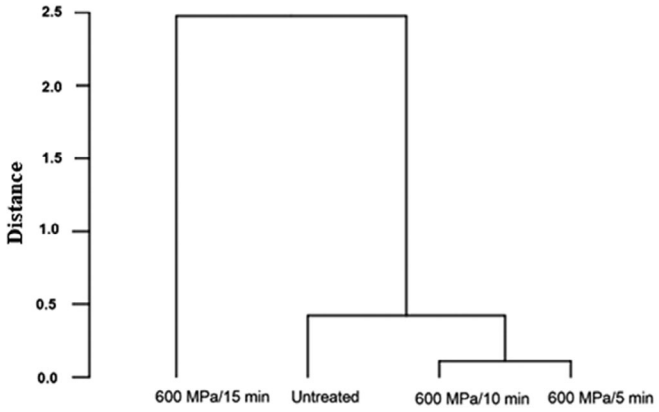

FIGURE 2 | Dendrogram of hierarchical clustering of UV spectra of untreated and HPP-treated honey samples.

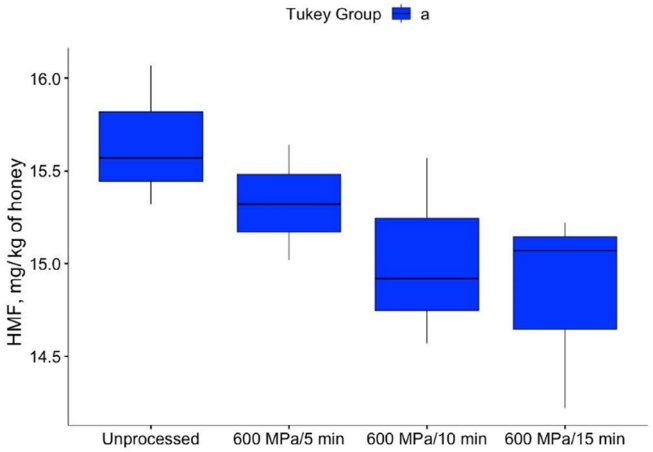

FIGURE 3 | Boxplot illustrating the results for the 5-hydroxymethylfurfural (HMF) content in honey before and after HPP at 600 MPa for 5, 10, and 15 min. The horizontal lines in the box denote the 25th, 50th, and 75th percentile values. The error bars denote the 5th and 95th percentile values. The height of the box is the measure for tolerance. Data were generated from three independent experiments with triplicated measurement.

honey samples can lead to spectral similarities. To verify the differences among spectra, hierarchical clustering was performed, and the obtained dendrogram is presented in Figure 2.

The hierarchical clustering solution obtained from the spectral data showed 99.12% similarity. This suggests that the clustering method has almost perfectly preserved the original distances between the spectra, providing a highly accurate representation of the data structure. Samples HPP-5 and HPP-10 are highly similar, followed by the untreated honey, with sample HPP-15 min being the most distinct.

A similar analysis was performed using a correlation matrix between the spectra and expressed as a percentage, with 100.00% indicating identical spectra and lower percentages indicating increasing dissimilarity. The results indicated that the spectra HPP-5 and HPP-10 are identical (100% similarity), the spectrum of untreated honey is nearly identical to HPP-5 and HPP-10 (99.99% similarity), and the spectrum of HPP-15 is slightly less similar but still highly correlated (99.85%–99.90% similarity). This suggests that all four spectra (untreated, HPP-5, HPP-10, and HPP-15) are very similar to each other, with minor variations.

The similarity in the UV spectra of untreated and HPP-processed honey samples is mainly due to the predominance of monosaccharides, which are major components of honey [21]. However, the differences, although minor, can be attributed to variations in the chemical composition of the honey samples, such as the presence of minor components, such as the phenolic compounds (phenolic and flavonoid compounds with absorbance at 280–300 nm) [22].

2.3 | HMF Content

The content of HMF is an important legislative parameter (with an upper limit of 40 mg/kg) as it relates to honey freshness and possible overheating during processing. The content of HMF in untreated honey was 15.7 ± 0.4 mg/kg and did not change significantly after HPP treatment (Figure 3). Previous studies have shown that HMF content of HPP-treated honey neither changed immediately after HPP nor changed over the entire storage period compared to untreated honey [11]. Similarly, no significant changes in the HMF content were found between different untreated honeys (e.g., manuka honey and stingless bee honey) and HPP-treated honey samples [13, 23].

2.4 | Total Phenolic Content

In the present study, the TPC value for the untreated honey was 1063.5 ± 24.2 mg gallic acid equivalents (GAE)/kg, which was lower than that in monofloral Spanish chestnut honey (1143.6 mg GAE/kg) [24] and Portuguese chestnut honey (1418 mg GAE/kg, containing less than 90% *C. sativa* pollen) [25]. The quantity of TPC in honey and, consequently, the antioxidant capacity vary widely depending upon the geographical and floral origin of honey. Honey dominated by pollen from *C. sativa* is rich in polyphenols and, consequently, exhibits higher antioxidant activity and potential benefits for human health [26]. Statistical analysis showed that HPP did not result in significant variations in the TPC between untreated (1063.5 ± 24.2 mg GAE/kg) and HPP-treated honey samples (Figure 4). Although there were some differences in the mean TPC values (the highest being in HPP-15, 1063.8 ± 23.3 mg GAE/kg, and the lowest being in HPP-10, 1044.4 ± 22.8 mg GAE/kg), they were not sufficient to justify separate classifications.

Similarly, our recent study did not reveal any significant changes in the TPC of multifloral pasture honey after HPP at 600 MPa for 5, 15, and 30 min [11]. Nevertheless, HPP-induced changes in the TPC of different honey types have been described in several studies [14, 15, 27, 28]. Previous studies focusing on single honey types, such as those by Razali (kelulut honey) [29] and Akhmazillah (manuka honey) [14], reported significant increases in TPC (~47%) after processing at 600 MPa for 10 min. However, Faraz reported that although TPC increased in one honey type (Jarrah honey) by 4.7% at 600 MPa for 15 min, it either decreased (maximum decrease of 8%) or remained unchanged

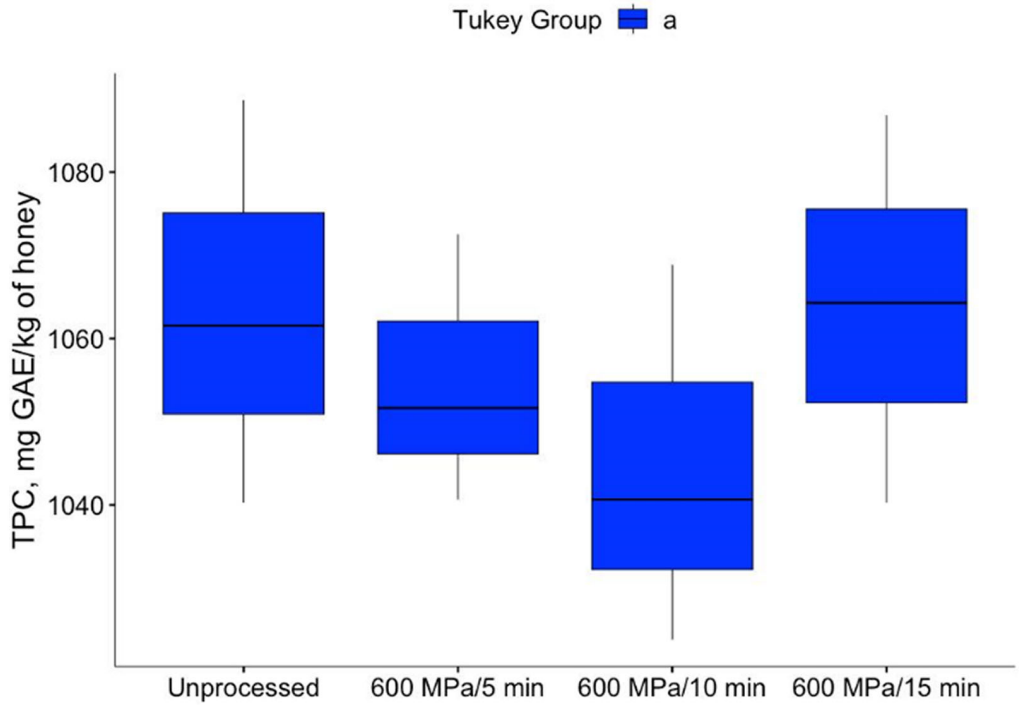

FIGURE 4 | Boxplot illustrating the results for the total phenolic content (TPC) in honey before and after HPP at 600 MPa for 5, 10, and 15 min. The horizontal lines in the box denote the 25th, 50th, and 75th percentile values. The error bars denote the 5th and 95th percentile values. The height of the box is the measure for tolerance. Data were generated from three independent experiments with triplicated measurement.

in other honey types (multifloral and Jarrah honey, manoongar, leatherwood, sugar gum, and paperbark honey) [28]. On the basis of these findings, they concluded that changes in the TPC of HPP honey samples depend on the botanical origin of the honey. Honey derives its phenolic compounds (flavonoids and phenolic acids) from specific organelles within pollen cells naturally present in the honey. The increase in phenolic content observed in plant and fruit cells after HPP has been attributed to cell membrane disruption and release of phenolics from specific organelles, resulting in higher extractability. HPP increases mass transfer by damaging cell membranes, enhancing permeability, and facilitating the diffusion of secondary metabolites during solvent extraction. Additionally, the disruption of weak interactions between phenolics and the cell wall promotes their release [30]. Taken together, these observations suggest that the increase of TPC in HPP honey depends on pollen disintegration and the release of phenolic compounds.

2.5 | Morphological Changes in Pollen Cells

The impact of HPP on plant tissues, organelles, cell walls, and membranes varies with pressure levels. For instance, in a study on prickly pears [31], higher pressures (350–600 MPa) led to cell wall collapse, enhancing phenolic extractability. Increasing pressure and treatment time further facilitated phenolic extraction by compromising cell wall integrity.

Therefore, this study investigated whether HPP at room temperature (HPP-15) and PATP (600 MPa, 15 min, 75°C) affect the total number of pollen grains and the morphological changes in pollen cells naturally present in honey. Additionally, PATP at 75°C for 15 min was included for comparison.

No statistically significant differences in the total number of pollen grains were observed among the unprocessed, HPP-15 honey, and PATP honey samples ($p > 0.05$).

The pollen grains of *C. sativa* show changes in exine and in the size of pollen grains (Figure 5).

Changes in the morphology and size of pollen grains at different carbohydrate concentrations were observed [32] and were also determined in our study according to the different honey treatments. The unprocessed and HPP samples were morphologically similar (Figures 5A and 8B), whereas the PATP honey sample showed a less visible exine and shorter but wider pollen grains in equatorial view (Figure 5C), indicating that combining high pressure and heat modifies honey's structural attributes more significantly than HPP alone.

In the morphometric characteristics, there were statistically significant differences in the equatorial plane of the pollen grains in each group, whereas the polar axis did not show any significant differences (Table 1).

Wang et al. described differences in morphometric characteristics and partial destruction of pollen between dry and fresh samples after pulsed vacuum treatment. By contrast, pollen grain destruction was not observed in our study [33]. We attribute the differences in the equatorial plane to pollen grain adaptability to environmental stress, a phenomenon known as harmomegathy [34].

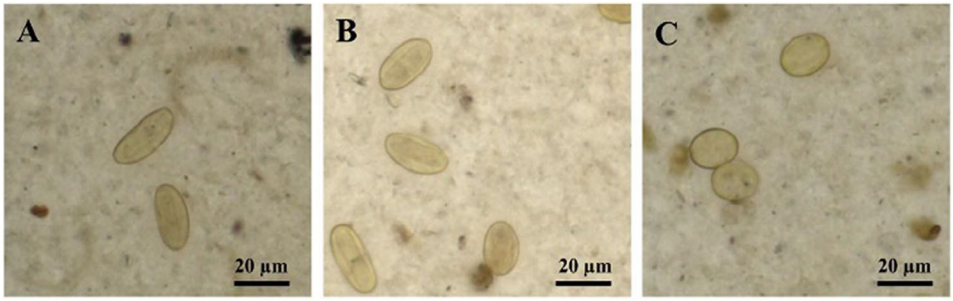

FIGURE 5 | Microscopic structure of *Castanea sativa* pollen grain cells before and after processing: (A) unprocessed honey; (B) HPP honey (600 MPa for 15 min at room temperature); (C) PATP (600 MPa for 15 min at 75°C).

TABLE 1 | Morphometric characteristics of *Castanea sativa* pollen grains in equatorial view.

Sample	Equatorial plane (µm ± SD)	Polar axis (µm ± SD)
Untreated	18.03 ± 1.69[a]	10.22 ± 0.99[a]
HPP, 600 MPa, 15 min	16.48 ± 1.66[b]	9.93 ± 2.36[a]
PATP, 600 MPa, 15 min, 75°C	13.08 ± 1.14[c]	10.60 ± 0.84[a]

Note: Data are expressed as a mean value, with standard deviation (SD), of three independent experiments with triplicated measurement. Different letters (a–c) in columns indicate significant differences among groups ($p < 0.05$).

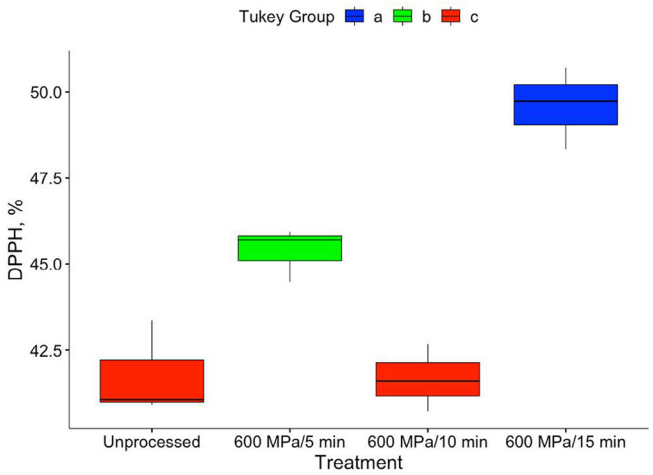

FIGURE 6 | Boxplot illustrating the results for the DPPH radical scavenging activity of honey before and after HPP at 600 MPa for 5, 10, and 15 min. The horizontal lines in the box denote the 25th, 50th, and 75th percentile values. The error bars denote the 5th and 95th percentile values. The height of the box is the measure for tolerance. Data were generated from three independent experiments with triplicated measurement. DPPH, 2,2-diphenyl-1-picrylhydrazyl.

2.6 | Antioxidant Activity

Furthermore, DPPH is widely used to evaluate the free radical-scavenging activity of several types of honey [35]. As a result of the DPPH assay, HPP-15 showed the highest antioxidant capacity (49.6% ± 1.2%), followed by HPP-5 with an intermediate value (45.4% ± 0.8%) (Figure 6). HPP-10 (41.7% ± 1.0%) did not significantly change the antioxidant capacity compared to untreated honey (41.8% ± 1.4%), which contradicts the findings of Fauzi, who reported a 30% increase in DPPH activity in Manuka honey after HPP at 600 MPa for 10 min [27]. Indeed, owing to the complex nature of the matrix and the involvement of multiple reaction characteristics and mechanisms, the antioxidant capacity of honey should be evaluated by a combination of assays to provide more comprehensive information on the antioxidant properties of HPP-treated honey.

Nevertheless, in agreement with our findings, Faraz reported a significant increase (11.48%) in DPPH activity in paperbark honey after treatment at 600 MPa for 15 min, with no significant changes in TPC [28]. However, they observed an increase in the total flavonoid content of the honey, from 173.33 to 206.67 mg quercetin equivalent/kg. The differences in antioxidant activity among honey samples may be attributed to variations in the type and quantity of individual polyphenolic compounds [7], as HPP can selectively increase the levels of certain phenolic compounds [17, 30].

2.7 | HPLC Analysis of Phenolics

Phytochemical screening of multifloral honey (chestnut as the dominant pollen) revealed the presence of biologically active constituents such as phenolic compounds, including phenolic acids, flavonoids, and hydroxybenzaldehyde. The content of identified analytes in untreated honey, HPP-5, HPP-10, and HPP-15 honey samples is presented in Table 2. A representative chromatogram is presented in Figure S1.

Naringin, 4-hydroxyphenylacetic acid, and protocatechuic acid were found in the highest quantity (21.15 ± 1.11, 11.01 ± 0.49, and 9.45 ± 0.52 mg/kg, respectively). 4-Hydroxyphenylacetic acid and protocatechuic acid have been regularly identified in chestnut honey samples [36, 37]. Moreover, the high content of naringin was also found in other types of honey, such as acacia,

TABLE 2 | Content of individual phenolic compounds (mg/kg) in untreated and HPP-treated honey.

Phenolic compound	Untreated honey	HPP-5	HPP-10	HPP-15
Phenolic acids				
Gallic acid	4.06 ± 0.09^{b}	4.30 ± 0.13^{b}	4.87 ± 0.23^{a}	4.23 ± 0.09^{b}
Protocatechuic acid	9.45 ± 0.52^{a}	9.50 ± 0.43^{a}	9.77 ± 0.35^{a}	9.26 ± 0.23^{a}
4-Hydroxyphenylacetic acid	11.01 ± 0.49^{a}	10.59 ± 0.59^{ab}	10.94 ± 0.48^{a}	9.32 ± 0.55^{b}
4-Hydroxybenzoic acid	6.84 ± 0.49^{a}	6.96 ± 0.34^{a}	7.21 ± 0.19^{a}	6.87 ± 1.52^{a}
Vanillic acid	5.62 ± 0.16^{a}	5.56 ± 0.18^{a}	5.38 ± 0.19^{a}	4.56 ± 0.07^{b}
Chlorogenic acid	7.25 ± 0.55^{a}	7.38 ± 0.20^{a}	7.50 ± 0.17^{a}	5.96 ± 0.05^{b}
Caffeic acid	3.21 ± 0.21^{a}	3.01 ± 0.55^{a}	2.93 ± 0.14^{a}	2.86 ± 0.06^{a}
Syringic acid	2.10 ± 0.10^{a}	2.11 ± 0.02^{a}	2.10 ± 0.08^{a}	1.38 ± 0.06^{b}
p-Coumaric acid	5.90 ± 0.51^{a}	6.41 ± 0.18^{a}	6.19 ± 0.38^{a}	4.44 ± 0.12^{b}
Ferulic acid	3.70 ± 0.30^{a}	3.92 ± 0.02^{a}	3.67 ± 0.12^{a}	3.64 ± 0.22^{a}
Sinapic acid	1.84 ± 0.10^{a}	1.84 ± 0.13^{a}	1.77 ± 0.08^{a}	0.95 ± 0.01^{b}
Flavonoids				
Naringin	21.15 ± 1.11^{ab}	22.70 ± 0.82^{a}	22.05 ± 1.15^{a}	18.84 ± 0.59^{b}
Kaempferol-3-*O*-glucoside	1.21 ± 0.07^{a}	1.22 ± 0.03^{a}	1.22 ± 0.04^{a}	1.23 ± 0.07^{a}
Naringenin	0.21 ± 0.08^{a}	0.25 ± 0.14^{a}	0.16 ± 0^{a}	0.17 ± 0.02^{a}
Quercetin	1.59 ± 0.47^{a}	1.25 ± 0.26^{a}	1.29 ± 0.28^{a}	1.51 ± 0.47^{a}
Kaempferol	1.0 ± 0.06^{a}	0.95 ± 0.02^{a}	0.98 ± 0.03^{a}	0.98 ± 0.05^{a}
Pinocembrin	0.96 ± 0.13^{b}	1.19 ± 0.03^{a}	1.16 ± 0.08^{ab}	1.04 ± 0.07^{ab}
Hydroxybenzaldehyde				
4-Hydroxybenzaldehyde	1.64 ± 0.07^{a}	1.63 ± 0.05^{a}	1.63 ± 0.01^{a}	1.30 ± 0.04^{b}
Σ of total phenolic acids	60.97 ± 3.16^{a}	61.59 ± 2.02^{a}	62.31 ± 1.34^{a}	53.49 ± 2.40^{b}
Σ of total flavonoids	26.11 ± 1.36^{ab}	27.56 ± 1.23^{a}	26.86 ± 1.15^{a}	23.77 ± 0.55^{b}
Σ other	1.64 ± 0.07^{a}	1.63 ± 0.05^{a}	1.63 ± 0.01^{a}	1.30 ± 0.04^{b}

Note: Data were expressed as a mean value, with standard deviation (SD), of three independent experiments with triplicated measurement. Different letters indicate statistically significant differences, and conversely, the same letter indicates an insignificant difference among the values.
Abbreviation: HPP, high pressure processing.

wildflower, and Sundarban honey [38, 39]. Naringin, a flavanone, is metabolized in the liver to naringenin, and it has shown various pharmacological activities [40]. 4-Hydroxybenzoic acid found in a concentration (6.84 ± 0.49 mg/kg) in the analyzed honey is considered a helpful component for identifying chestnut honey [41].

The effect of HPP on the content of the monitored phenolic acids, flavonoids, and hydroxybenzaldehyde in honey was evident. Shorter processing durations (5–10 min) resulted in an increase in the concentration and/or stability of many phenolic compounds. However, an extended processing time (15 min) predominantly led to a decrease.

Further, the application of PCA (principal component analysis) to the HPLC results of the honey samples provided valuable insights into the chemical composition and variability of the samples, highlighting the most significant variations and patterns among the samples. The PCA results showed that three principal components represented 100% of the analytical data variability. The cumulative proportion of the variance represented by PC1 was 64.27%, which is high (standard deviation for PC1 is 3.7603), and PC2 increased the cumulative proportion to 85.25% (standard deviation of 2.1485).

Figure 7 presents the biplot of the first two principal components. The honey sample processed at 600 MPa for 10 min has the highest concentrations of gallic acid, 4-hydroxyphenylacetic acid, and naringin. The overall profile was characterized by higher levels of certain phenolic acids and flavonoids, suggesting a distinct chemical composition. The honey processed at 600 MPa for 5 min presents the highest concentrations of protocatechuic acid, 4-hydroxyphenylacetic acid, and naringin. This honey sample also shows high concentrations of phenolic acids and flavonoids, indicating a comparable chemical profile to that of the sample processed at 600 MPa for 10 min.

Conversely, the sample processed at 600 MPa for 15 min showed significantly lower concentrations of most compounds, particularly phenolic acids and flavonoids, suggesting a distinct chemical composition compared to samples processed for 5 and 10 min.

Overall, samples HPP-5 and HPP-10 were closely clustered, suggesting that they shared similar chemical compositions.

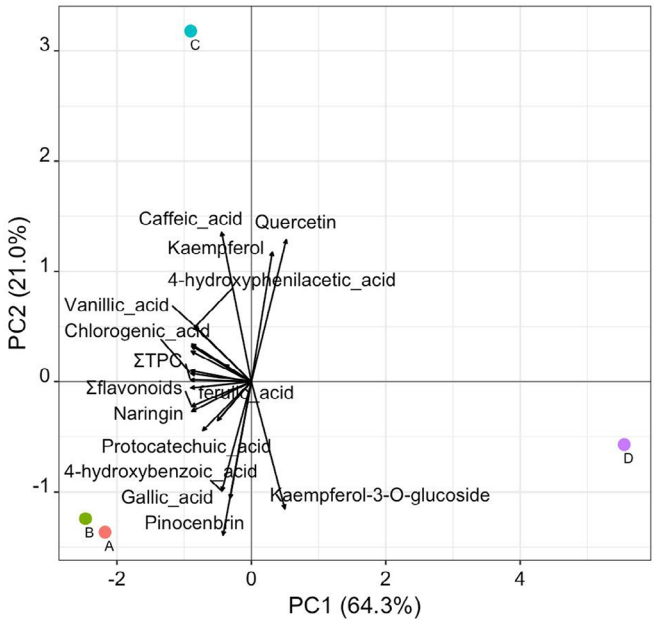

FIGURE 7 | Plot of PC1 and PC2 for HPLC analysis of honey before and after HPP at 600 MPa for 5, 10, and 15 min.

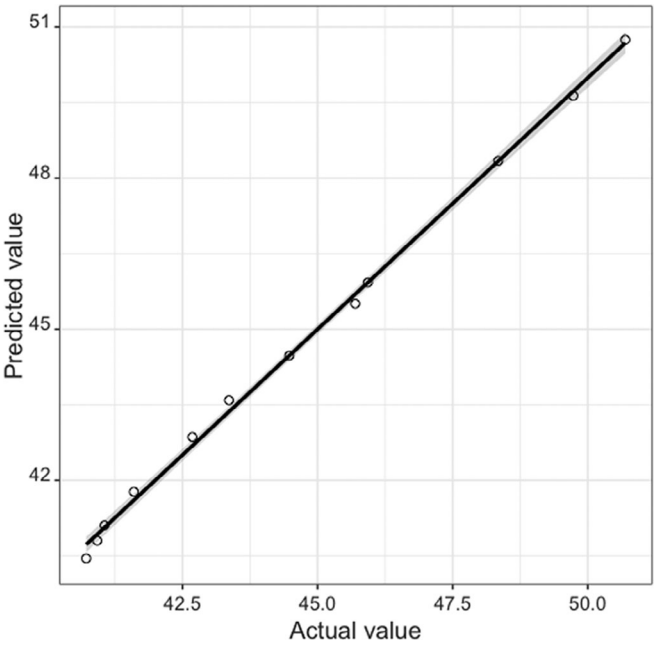

FIGURE 8 | Plot of the estimation linear model for DPPH variation using independent variables from the HPLC analytical data and TPC values.

HPP-15 honey, however, was more distinct, likely due to its lower overall concentrations of phenolic acids (such as gallic acid, vanillic acid, chlorogenic acid, syringic acid, *p*-coumaric acid, and sinapic acid) and flavonoids (such as naringin). Interestingly, HPP-15 exhibited the highest DPPH activity.

However, increased antioxidant activity is not solely attributed to a high phenolic content. For example, in green tea extracts rich in phenolic compounds with antioxidant properties, 78% of the antioxidant activity was specifically due to individual phenolics such as catechins and catechin gallate esters [42].

2.8 | Estimation Model for DPPH Variation

The results of the model training demonstrated a high level of accuracy and predictive power. The selected model ($I = 6$) included six key variables: caffeic acid, *p*-coumaric acid, sinapic acid, naringin, kaempferol, and TPC. These variables contributed significantly to predicting the outcome variable (DPPH). Although including more variables improved the model's fit, it also increased complexity, which was avoided in favor of a simpler model for evaluation purposes.

Overall, the model selection and training process yielded a highly predictive model, capable of explaining the variance in the target variable with minimal error, as can be seen in Figure 8.

The performance metrics reveal a strong model fit. The trained *R*-squared (R^2) value of 0.9981 (adjusted R^2 of 0.9959) indicates that the model explains nearly all the variance in the training data. Furthermore, the RMSE (0.496) demonstrates low prediction errors. Cross-validation results were equally impressive, with *R*-squared values reaching 0.9999 at higher iterations, confirming the model's robustness across different data splits. The linear regression summary shows that most coefficients are highly significant; the low residual standard error (0.2248) and *F*-statistic (447.3) underscore the model's excellent fit.

Regarding the linear estimation model, the coefficients of the DPPH estimation model provide insights into how each variable influences the outcome. The intercept (−43.91) suggests that, in the absence of all other variables, the predicted DPPH value would be negative, though this is mainly theoretical as all predictors are present in the model.

A positive coefficient (coefficient = 1.14, $p = 0.0129$) indicates that an increase in caffeic acid is associated with an increase in DPPH activity.

The negative coefficient of *p*-coumaric acid (coefficient = −3.06, $p = 0.0017$) implies that higher concentrations of *p*-coumaric acid reduce DPPH activity.

Sinapic acid (coefficient = −7.39, $p < 0.0001$) has the strongest negative impact on DPPH activity, as shown by its large negative coefficient and extremely significant p-value.

Naringin (coefficient = 1.78, $p = 0.0003$) has a positive effect on DPPH, indicating that it strongly contributes to increasing antioxidant activity.

Kaempferol (coefficient = −70.23, $p < 0.0001$) has the largest negative coefficient, showing a substantial reduction in DPPH activity as its concentration increases.

TPC (coefficient = 0.14, $p < 0.0001$) positively affects DPPH activity, reflecting that increased phenolic content boosts antioxidant capacity.

Overall, the coefficients indicate that although increases in components, such as naringin, caffeic acid, and TPC, contribute positively to antioxidant activity, others, such as kaempferol, *p*-coumaric acid, and sinapic acid, have a negative impact.

The HPP-15 sample that exhibited significantly higher antioxidant activity (increased by 4.2% compared with untreated honey) had significantly lower concentrations of *p*-coumaric acid and sinapic acid (phenolics with negative coefficients), a significant decrease in naringin, and no significant changes in caffeic acid and TPC (variables with a positive coefficient). On the basis of this predicted model, the higher DPPH activity may be caused by the selective changes in individual phenolics.

When applying the model to new training data (Pred_train), the results remained consistent, with an *R*-squared of nearly 1 and a high *F*-statistic. This indicates that the model can effectively generalize and accurately predict the outcomes for unseen data.

3 | Conclusions

To the best of our knowledge, this study is the first to report on the individual phenolic composition of honey processed by HPP (at 600 MPa for 5, 10, and 15 min at room temperature) and its impact on the morphological changes in the structure of pollen grains naturally present in honey.

This study demonstrates that HPP at 600 MPa, especially at 15 min, effectively enhances the antioxidant activity (measured by DPPH) of honey without altering its overall quality as indicated by stable HMF levels and TPC.

This adaptation may support honey's enhanced antioxidant properties due to improved bioactive compound extraction from pollen, the size and width of which were changed after HPP treatment, especially when combined with high temperature.

In conclusion, HPP offers a viable method for increasing honey's antioxidant activity while preserving its quality, presenting a promising non-thermal alternative for honey processing to meet consumer demands for functional and nutritionally robust products.

Although HPP offers a promising non-thermal processing technique to improve honey's functional properties, further research is needed to explore the effects of different processing parameters and honey varieties. Additionally, a combination of antioxidant assays would provide a more comprehensive assessment of the overall antioxidant profile of HPP-treated honey.

4 | Experimental Section

4.1 | Preparation of Honey Samples

The honey, from *Apis mellifera*, was supplied by a beekeeper from an apiary located in the Trás-os-Montes region in Portugal. The honey was obtained in raw quality (no liquefied and no pasteurized) and processed by HPP and PATP 1 week after the harvesting. All honey samples were taken from a single batch.

The raw honey was manually homogenized inside a laminar flow chamber (Bio Safety Cabinet Telstar Bio II Advance, Terrassa, Spain) and aseptically placed into low-permeability polyamide–polyethylene bags (PA/PE-90, Albipack-Packaging Solutions, Portugal), which were then thermosealed using a vacuum packager (Packman, Albipack, Águeda, Portugal) to avoid, as much as possible, air bubbles inside the bags. All samples were prepared in triplicate. All honey samples were from the same honey batch. After processing, the samples were stored at −80°C until analysis.

4.2 | High-Pressure Processing

The honey samples were processed using pilot-scale HPP equipment (Hiperbaric 55 L, Hiperbaric S.A., Burgos, Spain) that consisted of a 55-L volume vessel with a 200 mm diameter and an automatic loading/unloading system. The pressure was generated by pumping water into the pressure chamber. The HPP pressure conditions were set at 600 MPa for 5, 10, and 15 min (Table 3) according to our recent study [11]. The input water temperature was 20°C ± 2°C, the compression rate was approximately 250 MPa/min, and the decompression time was less than 5 s. Immediately after processing, the honey samples were immersed in ice water (4°C) to rapidly cool. The processing conditions were selected based on previous studies that reported the maintenance of honey quality attributes while enhancing antioxidant activity and TPC.

4.3 | Pressure-Assisted Thermal Processing

The PATP was performed at 600 MPa for 15 min at 75°C in the pilot-scale HPP equipment (Hiperbaric 55 L, Hiperbaric, Burgos, Spain). The samples were preheated by immersing them in a temperature-controlled water bath before processing, which acted as the heating medium. The water temperature was set to 75°C before PATP to achieve the desired sample temperature. This temperature was chosen as it is commonly used for conventional thermal pasteurization of honey [7]. The preheated samples were then placed in an insulated polypropylene basket filled with water (the working fluid) at a temperature of 75°C. The basket was quickly transferred to the high-pressure equipment.

At an initial operating temperature of 70°C, the adiabatic temperature increases 3.7°C for every 100 MPa increase in pressure, up to 581.1 MPa [43]. On the basis of this finding, it is estimated that the average processing temperature in the present experiment (600 MPa/75°C) reached approximately 97.2°C due to adiabatic heating. Immediately after processing, the honey samples were immersed in ice water (4°C) to rapidly cool.

4.4 | Botanical Origin

The quantitative melissopalynology analysis was conducted according to Ohe et al. [44] with modifications by Pospiech et al. [45]. The samples were evaluated in duplicates, and the total pollen content and botanical taxa were evaluated. The frequency of the appearance of the different pollen types was divided into the following classes: dominant pollen (>45% of the pollen

TABLE 3 | Experimental conditions for honey processing.

Processing parameters	Honey samples				
	Untreated honey	HPP-5	HPP-10	HPP-15	PATP-75
Pressure (MPa)	0.1	600	600	600	600
Time (min)	—	5	10	15	15
Temperature (°C)	22	22	22	22	75[a]

Abbreviations: HPP, high-pressure processing; PATP, pressure-assisted thermal processing.
[a]Actual temperature was approximately about 97.2°C due to adiabatic heating.

spectrum); accompanying pollen (15%–45%); important pollen (3%–15%); minor pollen (1%–3%); and other pollens (≤1%).

Morphological evaluation of pollen grains was conducted using an Eclipse Ci-L microscope (Nikon, Tokyo, Japan), equipped with an automated microscopic view field scan via the ProScan III (Prior, Rockland, MA, USA) stage. Morphometric data were analyzed using the NIS-Elements AR 5.20 image analysis software (Laboratory Imaging, Prague, Czech Republic).

4.5 | 5-HMF Content

The determination of HMF content was based on the spectrophotometric method of the International Honey Commission (IHC) [46]. Briefly, honey (5 g) was dissolved in approximately 25 mL of distilled water and quantitatively transferred into a 50 mL volumetric flask. Then, 0.5 mL of Carrez solution I (15 g of potassium hexacyanoferrate(II) trihydrate $K_4Fe(CN)_6 \cdot 3H_2O$ diluted up to 100 mL with distilled water) and 0.5 mL of Carrez solution II (30 g zinc acetate dihydrate $Zn(CH_3COO)_2 \cdot 2H_2O$ diluted up to 100 mL with distilled water) (Sigma-Aldrich, Saint-Quentin Fallavier, France) were added to the honey solution, which was then made up to its final volume (50 mL) with distilled water. The solution was filtered through filter paper, and the first 10 mL of the filtrate was rejected. Then, 5 mL aliquots of the filtrate were put in two test tubes: (i) to one test tube were added 5 mL of distilled water (sample solution) and (ii) to the second tube were added 5 mL of 0.2% sodium hydrogen sulfite (Sigma-Aldrich, Saint-Quentin Fallavier, France) solution (0.20 g of $NaHSO_3$ diluted up to 100 mL with distilled water). The absorbance of the sample solution against the reference solution at 284 and 336 nm was determined in quartz cuvettes using a spectrophotometer (3100-Spectrophotometer, VWR International, Radnor, PA, USA). The content of HMF was calculated by the application of the following formula:

$$\text{HMF (mg/kg)} = [(A_{284\,\text{nm}} - A_{336\,\text{nm}}) \times F \times 5] / P \quad (1)$$

where $A_{284\,\text{nm}}$ and $A_{336\,\text{nm}}$ are the absorbance readings at 284 and 336 nm, F is a constant value of 149.7, and P is the weight of the sample in grams.

4.6 | Total Phenolic Content

The quantification of TPC in unprocessed and processed honey was determined using a 96-well microplate-based Folin–Ciocalteu assay according to the methodology described by Bobo-García et al. Folin–Ciocalteu reagent was diluted (1:4), and 100 µL was mixed with 20 µL of honey sample (0.1 g/mL) in flat-bottom 96-well microplate. After 4 min, 75 µL of sodium carbonate (Na_2CO_3) solution (100 g/L) were added and incubated for 2 h in dark at 20°C. The absorbance was recorded at 750 nm using the microplate reader (Multiskan GO Microplate spectrophotometer, Thermo Fisher Scientific Inc., USA). Gallic acid was used as a standard (0–200 mg/L) for calibration. The blank solution consisted of water instead of honey or standard and was subtracted from the absorbance of the reaction with honey sample. The results were expressed as mg GAE/kg of honey.

4.7 | Free Radical DPPH Scavenging Capacity

The reduction of DPPH was evaluated using the microplate antioxidant assay described by Bobo-García et al. [47]. Briefly, 20 µL of the honey sample (0.1 g/mL) was added to 180 µL of DPPH solution (150 µmol/L) in methanol–water (80:20, v/v) and shaken for 60 s in 96-well microplate. The absorbance was measured at 515 nm after 40 min incubation at 20°C in dark using the microplate reader (Multiskan GO Microplate spectrophotometer, Thermo Fisher Scientific Inc., USA). The radical DPPH scavenging activity was calculated by the following equation:

$$\text{DPPH \% inhibition} = \left[1 - \left(A_{\text{sample}} - A_{\text{blank}}\right) / \left(A_{\text{control}} - A_{\text{blank}}\right)\right] \times 100 \quad (2)$$

where A_{sample} is absorbance at 515 nm of 20 µL of honey sample with 180 µL DPPH solution after 40 min; A_{blank} is absorbance at 515 nm of 20 µL water with 180 µL of methanol–water (80:20, v/v) after 40 min; A_{control} is absorbance at 515 nm of 20 µL water with 180 µL of DPPH solution after 40 min. The results were expressed as the percentual (%) inhibition.

4.8 | Solid-Phase Extraction (SPE) Procedure for Phenolic Compounds

SPE of phenolic compounds from honey was performed using a Vac Elut 10 vacuum manifold system (Varian, Harbor City, CA, USA). The honey sample (10 g) was mixed with 25 mL of HCl 0.02 M (pH 2) until it was totally fluid, after which it was filtered through cotton and paper filters to remove any solid particles (e.g., pollen and crystals). Loading onto a 500 mg SPE Chromabond

TABLE 4 | Procedure of the solid-phase extraction (SPE) of phenolic compounds from honey.

Phase	Conditions
Pre-conditioning of cartridge	Insert 10 mL of methanol (HPLC grade), then 10 mL of ultrapure water (at a flow rate of 5 mL/min) with use of compressor
Loading of sample	25 mL of honey solution at reduced flow rate (0.8 mL/min)
Washing of cartridge	10 mL of ultrapure water (at a flow rate of 5 mL/min)
Elution of particles	2 mL of methanol at reduced flow rate (0.8 mL/min)
Filtration	2 mL of the eluted mixture pass through a 0.2 μm syringe filter (previously washed with methanol) into dark flask used for automatic sampling of the HPLC equipment

cartridge (Macherey-Nagel, Dueren, Germany) and extraction was carried out according to the procedure shown in Table 4.

4.9 | HPLC Analysis

The phenolic composition of honey samples was identified by HPLC with photodiode array (PDA) detection according to our previous report [48]. Analyses were carried out in a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with an LC-20AD prominence pump, a DGU-20AS prominence degasser, a CTO-10AS VP column oven, a SIL-20A HT prominence autosampler, and an SPD-M20A PDA detector. A Gemini C_{18} column (250 × 4.6 mm^2, 5 μm) from *Phenomenex* (Torrance, California, USA) operated at 25°C was used. The mobile phase was composed by methanol (A) and water (B), both acidified with 0.1% formic acid, and the applied gradient was: 0–13 min: 20%–26.5% A; 13–18 min: 26.5% A; 18–25 min: 26.5%–30% A; 25–50 min: 30%–45% A; 50–60 min: 45%–50% A; 60–70 min: 50%–55% A; 70–90 min: 55%–70% A; 90–100 min: 70%–100% A. A 5-min post-run at initial conditions for equilibration of the column was performed before the next injection. The injection volume was 20 μL. Hydroxybenzoic acids (gallic, vanillic, protocatechuic, syringic, 4-hydroxyphenilacetic, and 4-hydroxybenzoic), 4-hydroxybenzaldehyde, naringin, naringenin, and pinocembrin were monitored at 280 nm, hydroxycinnamic acid derivatives (caffeic, chlorogenic, *p*-coumaric, ferulic, and sinapic) at 320 nm, and quercetin, kaempferol, and kaempferol-3-*O*-glucoside at 360 nm. For the identification of individual phenolic compounds, the retention time and their UV–Vis spectra were compared with the ones from commercial standards. Individual stock solutions (2000 mg/L) of the polyphenols were prepared in methanol, and their mixtures to plot calibration curves ranging from 1 to 50 mg/L were made in methanol–water (50:50, v/v). The results were expressed as mg of each phenolic compound per kg of honey.

4.10 | UV–Vis Spectrophotometry Analysis of Honey Solution

Honey solutions (25% w/v) from untreated and HPP samples were prepared and filtered through Whatman No. 1 filter paper. Then, serial dilutions were prepared by homogenizing 1 mL of each sample with 9 mL of ultrapure water. These dilutions were scanned separately in the UV–Vis spectrophotometer (3100-Spectrophotometer, VWR International, Radnor, PA, USA) over the wavelength range of 190–1100 nm. A quartz cuvette was used with a scan step of 1.0 nm, a recording speed of 35 nm/s, and an absorbance measurement reproducibility of 0.002.

4.11 | Statistical Analysis

To verify the similarity among the spectra from the untreated and HPP honey samples, a hierarchical clustering was performed, and the results were visualized through a dendrogram. The statistical analysis involved calculating the Euclidean distance matrix from spectral data, conducting hierarchical clustering using Ward's minimum variance method, and visualizing the results through a dendrogram to identify patterns and groupings in the data.

Moreover, the correlation matrix of the transposed spectra data was obtained to verify the similarity between spectral profiles. The correlation values were converted into a percentage-based similarity matrix for easier interpretation and comparison of similarities between individual spectra.

An analysis of variance (ANOVA) of one factor was performed for understanding the variability and the impact of different treatments on the honey samples. When the ANOVA results indicated that there were indeed significant differences in the means of certain parameters among the treatments, a Tukey post hoc test was performed to identify which specific treatments had different means.

PCA was applied to the HPLC results of honey samples to analyze their chemical composition. The data were scaled to ensure that each variable contributes equally to the PCA analysis, thereby enhancing the accuracy and reliability of the results. This technique allows a clear comparison of the samples based on their chemical profiles and filters out noise and minor variations, focusing on the principal components that explain the most variance, thus emphasizing the most noteworthy features of the data. This unsupervised technique approach allows the identification of the most relevant parameters by highlighting the principal components that capture most of the variance in the data.

To verify if the values of DPPH could be explained by the HPLC data and TPC value, a feature selection was done to obtain an estimation multilinear model of their dependence. The simulated annealing algorithm was applied that allowed to optimize the selection of variables by iterating through various feature combinations (between 2 and 10 independent variables) and minimizing a loss function (tau2). In each iteration, a linear

model is trained using the selected subset of features, with model performance assessed using cross-validation. Metrics, such as RMSE, *R*-squared, and predicted RMSE, are computed for each model, and the final model was selected considering RMSE <0.5 and *R*-squared >0.98 for the cross-validation.

Data processing was performed with R software (R version 3.3.2, released on October 31, 2016), a free software environment for statistical computing and graphics.

For morphometric characteristics of pollen cells, the statistical analysis was performed by XLSTAT 2024 (Addinsoft, USA), and the comparison of samples Kruskal-Wallis test with post hoc Dunn's procedure was used.

Author Contributions

Hana Scepankova and Juraj Majtan: conceptualization. **Hana Scepankova, Matej Pospiech, Manuela M. Moreira, and Luís G. Dias**: methodology. **Hana Scepankova and Matej Pospiech**: formal analysis. **Hana Scepankova, Matej Pospiech, and Juraj Majtan**: investigation. **Jorge A. Saraiva and Leticia M. Estevinho**: resources. **Hana Scepankova, Luís G. Dias, and Matej Pospiech**: data curation. **Hana Scepankova and Juraj Majtan**: writing–original draft preparation. **Hana Scepankova, Juraj Majtan, Carlos A. Pinto, and Jorge A. Saraiva**: writing–review and editing. **Hana Scepankova, Juraj Majtan, Luís G. Dias, Carlos A. Pinto, Jorge A. Saraiva, and Manuela M. Moreira**: visualization. **Jorge A. Saraiva, Leticia M. Estevinho, and Cristina Delerue-Matos**: supervision. **Jorge A. Saraiva**: project administration. **Jorge A. Saraiva, Leticia M. Estevinho, and Cristina Delerue-Matos**: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.