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Article

# Chemical Markers in Italian Propolis: Chrysin, Galangin and CAPE as Indicators of Geographic Origin

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**Abstract:** The knowledge in chemical composition of propolis is crucial for understanding the characteristics of products of different origin, but also for quality control and regulatory purposes. To date, official monographs or official analyses that allow to evaluate propolis in a proper way have not yet been released. This study focuses on the characterization of twenty-seven Italian propolis and the identification of chemical markers that define its geographical provenance. Total polyphenol (TP) and total flavonoid (TF) content, alongside the quantification of pinocembrin, chrysin, galangin, and caffeic acid phenethyl ester (CAPE), were identified as potential markers. Additionally, DPPH assays were conducted to evaluate antiradical activity of propolis samples. Our findings demonstrated that TP, TF and pinocembrin differed in propolis of different origin, especially in samples from the islands. However, the quantification of the sum of chrysin and galangin and CAP provided a clear distinction of the geographical origin of the propolis samples. In contrast, the DPPH assay did not prove useful for this purpose, as most results were similar and, therefore, not significant. This study lays the groundwork for future research on propolis. These findings could contribute to the development of more refined methods for distinguishing propolis origins, enhancing the understanding, valuation and quality control of this natural product in various applications.

**Keywords:** Italian propolis; flavonoids; pinocembrin; chrysin; CAPE; geographical diversity

## 1. Introduction

In relation to bee propolis, ethnopharmacology has reported and described similar properties for a variety of products. These products have been identified as having distinct botanical and geographic origins, but they share antioxidant, immunomodulatory, and antimicrobial (or more accurately, "anti-pathogen" activities) properties. [1]. On one hand, this recognizes the univocal role of propolis in worldwide bee-hives: a sanitizing putty, mainly made of leaf buds, but also of other plant secretions [2], consisting in volatile and non-volatile terpenes, wax and phenolic compounds [3]. On the other hand, these general concepts are not enough to move towards a rational application of bee propolis in the modern medicine. In-depth study of propolis pharmacology and chemistry is mandatory to investigate the mechanism of action, targets, and signaling involved in the activity of the specific constituents and particular phytocomplexes that characterize bee propolis of different origins. Given an example, artemillin C, pinocembrin and caffeic acid phenethyl ester (CAPE) are all phenolic compounds with immunomodulatory effect, but artemillin C is a prenylated cinnamic acid found in green Brazilian propolis, known as an activator of TRPA1 channels [4], pinocembrin, an inhibitor of MAPK p38, is a flavanone common in Euro-Asian poplar propolis, while [5] CAPE, known as inhibitor of NF- $\kappa$ B, is a caffeic acid ester found in several propolis of different origin [6].

A higher knowledge in chemical composition of propolis is crucial for understanding and maximizing the potential of different propolis, but also for quality control purposes, a challenging concern not yet fixed. To date, there is no official Pharmacopoeia monograph available for bee propolis, nor any other official texts that include modern chemical assays [3,6]; currently, the best official reference is represented by the Chinese Pharmacopoeia, where a minimum of 2% of chrysin and 1% of galangin are required for raw propolis (poplar-type, mainly from *Populus* spp.) [7].

Moreover, the analysis and characterization of propolis and propolis-based formations are also critical to regulatory issues. Indeed, the marketing and use of specific preparations of propolis, such as liquid ethanolic extracts, alcohol-free extracts or dry extracts, are allowed only depending on regulatory framework of different countries; to cite the example of European Union (EU), bee propolis is included only as mother tincture in some homeopathic Pharmacopoeias (such as in France and Germany) for the preparation of homeopathic medicines, and allowed as ingredient for food supplements only if traditionally extracted with permitted solvents, without refining and purification, to not cross the frontier of novel foods [8]. Thus, the chemical characterization of a propolis-based product still remains the best way for legitimating or not the use of this bee product in various areas of human health.

These concerns are seriously taken into account by the scientific community in Italy, being the country with the fastest growing market within the EU for human health products [9] and, based on this ground, the aim of our work was to focus on Italian propolis to better investigate and to clarify the chemical composition from different geographical areas, providing an update and an insight of the current literature.

Italian propolis could be generically categorized as poplar-type propolis, although Gardini et al., [10], on the basis of chemical characteristics, proposed a distinction between propolis from the Mediterranean area and that from temperate region, which includes the river Po plain area; To date, this study [10] could be considered the most important reference for Italian propolis for the number of samples tested (43 collected in 2013), and for statistical analyses performed. In detail, the authors reported the quantification of total balsam content and more than 20 phenolic acids and flavonoids, showing that flavones, flavonols, flavanones and dihydroflavonols were higher in propolis from Po plain area. In addition, the study showed that the chrysin content, the most enriched single flavonoid (as mean content) in samples divided by ecoregional provenance, was higher in propolis from Mediterranean and in Po plain area compared to the its content in propolis from other temperate areas located in Northern and Central Regions or in island Southern locations.

Finally, the study showed that interestingly some samples from Sicily and Sardinia resulted poor in flavonoids and that, among sample has not been reported a substantial difference in antioxidant activity.

In a previous study conducted by Papotti et al., [11], 20 samples collected in 2007 in the area of Bologna (Emilia Romagna) were analyzed to compare different harvesting methods and solvents of extraction: as a result, in all samples galangin, pinocembrin and pinobanksin 3-acetate resulted the most enriched constituents, and the use of wooden wedge and acetone provided the highest yields in phenolics and the most impactful antioxidant activity.

A third paper that considered 5 samples of Italian propolis [12] collected in 2007 in different location of Central Italy, focused mainly on allergenic esters benzyl cinnamate and benzyl salicylate and reported that chrysin and galangin were found to be the most important single constituents.

Considering these premises, in this work, we analyzed 25 samples of propolis collected in 2020 in different Italian geographical areas, according to the conventional division made by the Italian Ministry of Tourism (Northern, Central, Southern regions) and two insular samples, specifically one from Sicily and one from a Tuscan island (Isola di Capraia), were added.

## 2. Results and Discussion

### 2.1. Chemical Analyses of Propolis Samples

#### 2.1.1. Total Polyphenols and Total Flavonoids Quantification

As reported in the reference of the Chinese Pharmacopoeia [7], but also in the cited literature [6,10,11] and again by Popova et al., 2007 [13], poplar propolis is mainly characterized by a high content of polyphenols and in particular of flavonoids; for this reason, the analysis of total polyphenols (TP) and total flavonoids (TF) represented the first analytical step of this work.

The analysis of TP and TF in propolis of different geographical Italian regions (North, Center, South, and Islands) revealed significant variations in the content of these bioactive compounds in different samples (**Table 1**).

**Table 1.** Content of total polyphenols, expressed as gallic acid equivalent, and total flavonoids expressed as galangin equivalent. Sample are grouped according to different geographical Italian areas, North (samples N1-N5), Central (C1-C13), South (S1-S7) and Islands, namely Capraia Island (Tuscany), I1 and Sicily, I2. Values are expressed as a percentage w/w (mean  $\pm$  SD).

Sample code	TP (% w/w)	TF (% w/w)	Sample code	TP (% w/w)	TF (% w/w)
N1	5.34 $\pm$ 0.64	3.38 $\pm$ 0.17	C10	23.84 $\pm$ 1.08	21.78 $\pm$ 1.78
N2	21.86 $\pm$ 3.07	18.13 $\pm$ 0.06	C11	22.70 $\pm$ 2.06	19.63 $\pm$ 2.36
N3	10.45 $\pm$ 1.88	9.26 $\pm$ 0.18	C12	27.36 $\pm$ 3.35	21.42 $\pm$ 0.98
N4	18.20 $\pm$ 2.09	11.04 $\pm$ 0.37	C13	16.58 $\pm$ 1.61	11.60 $\pm$ 0.14
N5	14.78 $\pm$ 3.28	12.28 $\pm$ 0.63	S1	27.88 $\pm$ 4.24	24.28 $\pm$ 3.27
C1	28.31 $\pm$ 4.61	26.64 $\pm$ 2.01	S2	21.41 $\pm$ 2.58	16.65 $\pm$ 0.05
C2	23.24 $\pm$ 4.74	21.29 $\pm$ 0.02	S3	22.53 $\pm$ 2.06	19.90 $\pm$ 0.65
C3	22.67 $\pm$ 4.28	18.15 $\pm$ 0.60	S4	8.26 $\pm$ 2.19	5.33 $\pm$ 0.01
C4	27.85 $\pm$ 6.44	20.70 $\pm$ 2.16	S5	12.10 $\pm$ 1.04	11.39 $\pm$ 0.01
C5	36.41 $\pm$ 0.30	18.33 $\pm$ 2.29	S6	20.14 $\pm$ 2.30	16.56 $\pm$ 0.90
C6	31.45 $\pm$ 4.82	25.65 $\pm$ 3.74	S7	19.64 $\pm$ 0.22	17.67 $\pm$ 0.04
C7	31.90 $\pm$ 5.78	20.50 $\pm$ 1.45	I1	3.68 $\pm$ 0.37	3.57 $\pm$ 0.12
C8	21.67 $\pm$ 2.30	18.94 $\pm$ 0.24	I2	3.04 $\pm$ 0.15	0.90 $\pm$ 0.10
C9	20.82 $\pm$ 2.01	20.43 $\pm$ 0.06			

Within propolis from Northern regions, the sample collected in Piedmont N1 (Biella province) markedly differed from other samples having low TP and TF content. Within the central regions, samples C13 from Latium showed marked differences from the other samples, revealing low TP and TF content, whereas C5 from the Florence countryside showed the highest TP content but medium TF content. In general, propolis from Tuscany, C1-C10 in this work showed a very similar TP and TF content respect to samples from Umbria, C11 and C12, probably due to the similar climatic conditions of these two regions. In propolis samples from Southern regions, sample S4 and S5 from Apulia, were those with lower content of TP and TF. In agreement with [10], in propolis from Islands we found the overall lowest content of TP and in the case of I2 from Tuscany, also the lowest content of TF.

Afterwards, in order to provide a more visual and comprehensive overview of the chemical similarities and differences across regions; TP and TF content of the 27 samples, grouped by their geographical provenance, were summed. This approach allowed for a clearer and more understandable comparison of the results based on regional divisions as reported in **Table 2**

In propolis from Northern regions, the mean TP content was 14.13  $\pm$  6.47% w/w. This value was lower compared to that recorded for propolis from Central and Southern regions but higher than that observed in samples from Islands (as graphically depicted in **Figure 1, panel A**). Propolis from

Central regions exhibited the highest mean TP content,  $25.75 \pm 5.41\%$  w/w, same results for TF, as depicted in **Figure 1**, panel A and B. Statistical analysis was performed to highlight different of TP and TF within the four Italian regions. Results showed a significant difference of samples from Central regions compared to samples from Northern regions ( $p < 0.05$ ), but not compared to samples from Southern Regions. In propolis from Southern regions the mean TP content was  $18.85 \pm 6.60\%$  w/w, not statistically different from the mean value of propolis from Northern regions.

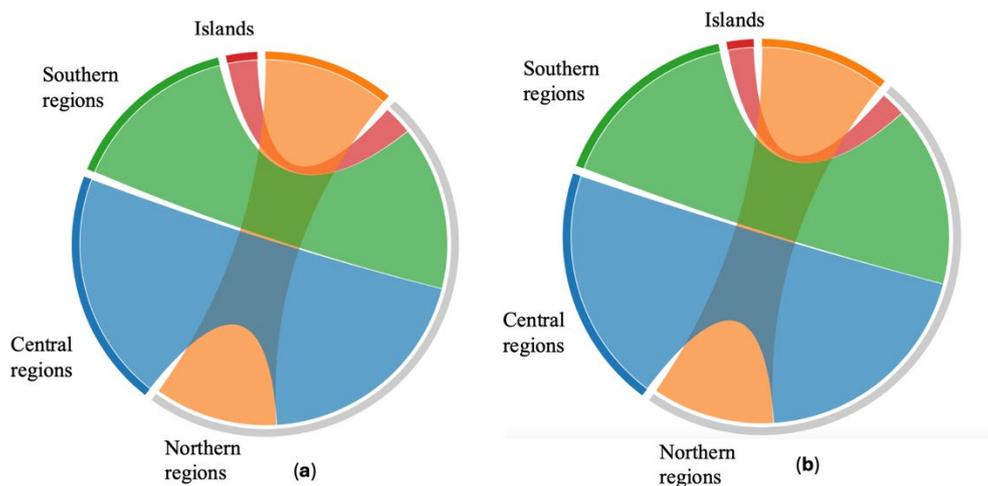
Propolis samples from Tuscan and Sicilian Islands displayed the lowest polyphenol content, with an average of  $3.36 \pm 0.45\%$  w/w. Statistically significant differences were observed compared to samples from all other regions. Interestingly, despite clear distinct climatic conditions that characterize the Tuscan archipelago and Sicily, the two insular propolis samples maintained a similar poor content of TP, **Figure 1** panel A, as already reported in [10] for propolis from Sicily and Sardinia.

As regards TF (**Table 2**), these secondary metabolites composed the main phenolic subclasses in almost all samples, with the exception of I2; in many cases, TF represented more than 90% of TP. The analysis of the ratio between TF and TP in samples divided by geographical area, gave similar values, ranging from  $0.63 \pm 0.48$  (Islands) to  $0.83 \pm 0.10$  w/w% (Southern Regions) (**Table 2**).

Propolis samples collected in Northern regions displayed a content of TF of  $10.82 \pm 5.32\%$  w/w. Similar to TP, TF content in propolis from the Northern regions (N) was lower than in propolis from the Center and South but higher than in the Islands (**Figure 1**, panel A and B). Propolis samples from the Central Regions (C) also showed the highest mean TF content at  $20.39 \pm 3.65\%$  w/w; the difference compared to the North was statistically significant ( $p = 0.007$ ), but the differences between propolis from Central and Southern Regions were not significant. The mean TF content in propolis from Southern Regions, in fact, was intermediate,  $15.97 \pm 6.09\%$  w/w, with no significant difference found when compared also to propolis from the North. The two propolis samples collected in Italian Islands deeply differed for TF content, even if both values were very low,  $3.57 \pm 0.12\%$  w/w in propolis from Tuscan Capraia Island and only  $0.90 \pm 0.10\%$  w/w in the Sicilian propolis. As in the case of TP, differences were found compared to all other regions: North ( $p = 0.02$ ), Center ( $p = 0.004$ ), and South ( $p = 0.002$ ).

**Table 2.** Total polyphenols content expressed as gallic acid equivalent, and total flavonoids, expressed as galangin equivalent. Samples are grouped according to geographical areas: North (N)), Central regions (C), South (S) and Islands (I), Values are expressed as a percentage w/w  $\pm$  SD (mean  $\pm$  standard deviation). Ratio between TF and TP in propolis divided by geographical area, N, C, S and I is also reported.

Geographical region	TP (% w/w)	TF (% w/w)	Flavonoids/Polyphenols ratio
N	$14.13 \pm 6.47^a$	$10.82 \pm 5.32^a$	$0.76 \pm 0.13^a$
C	$25.75 \pm 5.41^b$	$20.39 \pm 3.65^b$	$0.81 \pm 0.13^a$
S	$18.85 \pm 6.60^{ab}$	$15.97 \pm 6.09^{ab}$	$0.83 \pm 0.10^a$
I	$3.36 \pm 0.45^c$	$2.24 \pm 1.89^c$	$0.63 \pm 0.48^a$



**Figure 1.** Chord diagrams representing the sum of (a) total polyphenols (TP) and (b) total flavonoids (TF) values divided into the four Italian geographical areas.

In line with the aim of this work, results obtained by analyzing propolis samples collected in different geographical Italian areas, allowed us to deepen previous works and showed that results related to TP and TF content were in agreement with Gardini et al. (2018) [10] and Popova et al. (2007) [13]. Nevertheless, we found that a distinction could be made in order to distinct propolis from Northern, Central, Southern regions and Islands because of marked differences in TP content, but also in TF as in the case of the comparison North/Central and Islands with other areas.

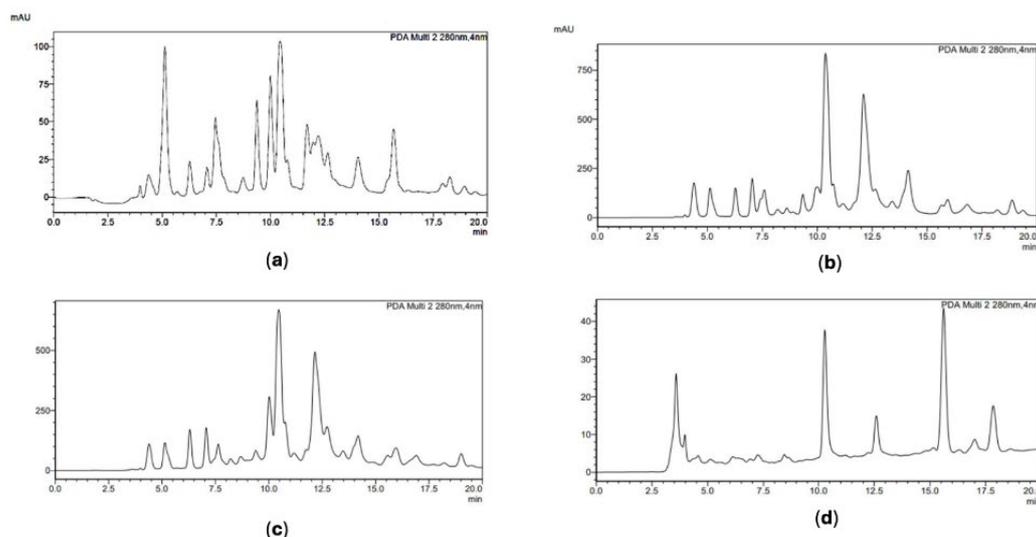
Temperature and soil composition could be responsible for the variations in propolis composition, as well as the differing presence of *Populus* species and other plants in the Salicaceae family across various locations. However, regarding this latter point, it's important to note that in Italy, *Populus* species are primarily found in the regions of Piedmont, Lombardy, and Emilia-Romagna [14], where collected propolis samples resulted poor both in TP and TF content (with the exception of N2). The question of the harvesting method, as claimed by Papotti et al. (2012) [11], newly emerges as a supplementary factor that could affect propolis quality.

#### 2.1.2. Quantification of Pinocembrin, Chrysin, Galangin and CAPE through HPLC-DAD

The UV methods used to quantify TP and TF have some important strengths because they are reliable, unexpensive and rapid, therefore very useful for a preliminary chemical screening of multiple samples. Nevertheless, these methods are not sufficient to provide a high-quality characterization of natural products and, in the case of propolis, they do not allow to investigate more subtle quali-quantitative differences in the flavonoid profile of samples from different Italian areas.

HPLC-DAD analysis was therefore performed to characterize the flavonoid fraction of propolis under analysis and allowed the identification of the main constituents. In accordance with what previously reported by Biagi et al. (2016) [9], we identified pinocembrin (PIN), with a retention time (RT) of 10.4 min. While for chrysin (CHR) and galangin (GAL) we were not able to perfectly separate these two peaks that partially overlapped with RT of 12.1 and 12.5 min., respectively. Therefore, we chose to quantify CHR and GAL together to avoid errors depending on different overlays recorded in samples. Caffeic acid phenethyl ester (CAPE) was identified and quantified in all samples at RT = 11.2 min. **Figure 2A-D** show the chromatogram of N1, C11, S6 and I2, representative of samples from different origins.

PIN was found the flavonoid at highest content in several samples, as reported in **Table 3**: In particular, in five samples from Central Regions its content was > 10% w/w. In propolis from Northern Regions, 2 (N2, N5) out of 5 samples PIN ranged from 5.5% m/m to 8.2% w/w. With the exception of S4, in propolis from Southern Regions. PIN ranged from 5.1% w/w to 9.9% w/w. Finally, in samples from islands, PIN was in high amount compared to TF.



**Figure 2.** Chromatograms recorded at 280 nm. (a) sample N1 from Piedmont (Biella); (b) sample C11 from Umbria, Perugia province; (c): sample S6, Calabrian propolis from Cosenza province; (d) sample I2 from Sicily (Messina). PIN retention time (RT) = 10.4 min., CAPE at RT = 11.2 min, CHR at RT = 12.1 min, GAL at RT = 12.5 min.

**Table 3.** Content of pinocembrin (PIN), the sum of chrysin and galangin (CHR + GAL) and caffeic acid phenethyl ester (CAPE) in different propolis samples expressed as a percentage w/w (mean  $\pm$  SD).

Sample code	PIN (% w/w)	CHR and GAL (% w/w)	CAPE (% w/w)	Samples code	PIN (% w/w)	CHR and GAL (% w/w)	CAPE (% w/w)
N1	1.04 $\pm$ 0.14	0.60 $\pm$ 0.01	0.86 $\pm$ 0.41	C10	10.16 $\pm$ 0.05	8.67 $\pm$ 0.14	1.65 $\pm$ 0.05
N2	8.20 $\pm$ 0.16	2.79 $\pm$ 0.17	1.30 $\pm$ 0.04	C11	9.30 $\pm$ 0.08	10.03 $\pm$ 0.34	1.70 $\pm$ 0.04
N3	2.28 $\pm$ 0.10	2.52 $\pm$ 0.07	0.96 $\pm$ 0.03	C12	11.27 $\pm$ 0.05	7.13 $\pm$ 0.13	1.62 $\pm$ 0.07
N4	*coelution	<0.05	0.81 $\pm$ 0.03	C13	4.91 $\pm$ 0.05	3.15 $\pm$ 0.07	1.01 $\pm$ 0.05
N5	5.53 $\pm$ 0.03	4.87 $\pm$ 0.11	1.37 $\pm$ 0.01	S1	9.87 $\pm$ 0.32	12.22 $\pm$ 0.13	1.93 $\pm$ 0.05
C1	10.07 $\pm$ 0.12	9.38 $\pm$ 0.15	1.52 $\pm$ 0.02	S2	8.14 $\pm$ 0.15	4.55 $\pm$ 0.01	1.96 $\pm$ 0.05
C2	8.16 $\pm$ 0.15	5.68 $\pm$ 0.10	1.80 $\pm$ 0.02	S3	8.33 $\pm$ 0.03	6.24 $\pm$ 0.10	1.43 $\pm$ 0.01
C3	6.88 $\pm$ 0.09	5.38 $\pm$ 0.01	1.16 $\pm$ 0.01	S4	3.08 $\pm$ 0.01	1.20 $\pm$ 0.03	1.59 $\pm$ 0.03
C4	8.05 $\pm$ 0.09	6.11 $\pm$ 0.18	1.53 $\pm$ 0.01	S5	5.13 $\pm$ 0.22	2.63 $\pm$ 0.12	1.54 $\pm$ 0.05
C5	8.01 $\pm$ 0.24	6.58 $\pm$ 0.42	1.37 $\pm$ 0.01	S6	8.01 $\pm$ 0.84	7.53 $\pm$ 0.42	1.74 $\pm$ 0.21
C6	11.53 $\pm$ 0.12	10.02 $\pm$ 0.40	1.57 $\pm$ 0.01	S7	7.13 $\pm$ 0.13	7.70 $\pm$ 0.14	1.17 $\pm$ 0.07
C7	5.14 $\pm$ 0.17	4.31 $\pm$ 0.18	1.19 $\pm$ 0.01	I1	1.46 $\pm$ 0.10	0.28 $\pm$ 0.01	0.14 $\pm$ 0.03
C8	9.97 $\pm$ 0.03	4.57 $\pm$ 0.04	1.54 $\pm$ 0.43	I2	0.24 $\pm$ 0.11	0.07 $\pm$ 0.01	0.10 $\pm$ 0.01
C9	10.95 $\pm$ 0.13	6.47 $\pm$ 0.27	1.96 $\pm$ 0.41				

\*Quantification vitiated by a marked overlay: approximate value ranging from 4.5% to 6.0% .

Similarly to what previously done with regards to the TP and TF in **Table 2**, in order to provide a general overview of chemical differences within the four Italian geographical area of interest, quantification of the identified flavonoids and CAPE as well as the ratio of PIN and TF, CHR and GAL and TF, CAPE and TP were calculated and reported in **Table 4**.

**Table 4.** Content of pinocembrin (PIN), the sum of chrysin and galangin (CHR + GAL) and CAPE and relative total flavonoids (TF) and total polyphenols (TP) ratio found in samples from North (N)), Central regions (C), South (S) and Islands (I). Values are expressed as a percentage w/w (mean  $\pm$  SD). Different letters indicate values significantly different ( $p < 0.05$ ), according to the Tukey's post hoc test.

Geographical region	PIN (%w/w)	PIN/TF ratio	CHR and GAL (%w/w)	CHR and GAL /TF ratio	CAPE (%w/w)	CAPE/TP ratio
N	4.26 $\pm$ 3.24 <sup>a</sup>	0.29 $\pm$ 0.19 <sup>a</sup>	2.16 $\pm$ 1.93 <sup>a</sup>	0.20 $\pm$ 0.15 <sup>a</sup>	1.06 $\pm$ 0.26 <sup>a</sup>	0.09 $\pm$ 0.04 <sup>a</sup>
C	8.80 $\pm$ 2.18 <sup>b</sup>	0.43 $\pm$ 0.08 <sup>a</sup>	6.73 $\pm$ 2.22 <sup>bc</sup>	0.33 $\pm$ 0.08 <sup>ab</sup>	1.51 $\pm$ 0.27 <sup>b</sup>	0.06 $\pm$ 0.02 <sup>ab</sup>
S	7.10 $\pm$ 2.28 <sup>ab</sup>	0.46 $\pm$ 0.06 <sup>a</sup>	6.01 $\pm$ 3.66 <sup>c</sup>	0.35 $\pm$ 0.11 <sup>b</sup>	1.62 $\pm$ 0.28 <sup>b</sup>	0.10 $\pm$ 0.05 <sup>a</sup>
I	0.85 $\pm$ 0.86 <sup>c</sup>	0.34 $\pm$ 0.10 <sup>a</sup>	0.18 $\pm$ 0.15 <sup>a</sup>	0.08 $\pm$ 0.01 <sup>ca</sup>	0.12 $\pm$ 0.03 <sup>c</sup>	0.04 0.01 <sup>ab</sup>

The mean content of PIN in propolis from Northern regions was 4.26  $\pm$  3.24% w/w, from Central regions was 8.80  $\pm$  2.18% w/w, from Southern regions was 7.10  $\pm$  2.28% w/w and in insular regions was 0.85  $\pm$  0.86% w/w. The statistical analysis revealed a significant difference in PIN content by comparing Northern and Central Regions ( $p = 0.003$ ) and in insular propolis compared to all other samples. PIN content resulted correlated to TF and the ratio between PIN and TF resulted in similar values for propolis from all geographical areas. In conclusion, with regards to PIN content, as already suggested by Gardini et al. (2018) [10] but also by Cui-Ping et al. (2015) [15] for Chinese propolis, in this work we reinforced the opinion that PIN could be considered a general marker of poplar-type propolis, but we also noticed that its high content distinguished propolis from Central and Southern provenance from Insular and Northern regions.

Regarding CHR and GAL a larger variation was recorded. In propolis from the Northern Regions CHR and GAL content ranged from  $< 0.05\%$  (N4) to 4.87% w/w (N5). In propolis from the Central Regions the range was 3.15 % (C13) up to 10.03% w/w (C11), in propolis from the Southern Regions values ranged from 1.20% (S4) up to 12.22% w/w (S1). Islands (I) presented the lowest CHR and GAL content (0.28% and 0.07% in I1 and I2, respectively), reinforcing the distinctiveness of insular propolis compared to mainland samples.

The very low CHR and GAL content obtained with the sample from the Alps, in Trentino, the one collected at the highest altitude ( $> 500$  m), was considered a point worthy of further investigation, which is currently difficult to discuss without other references from the same source.

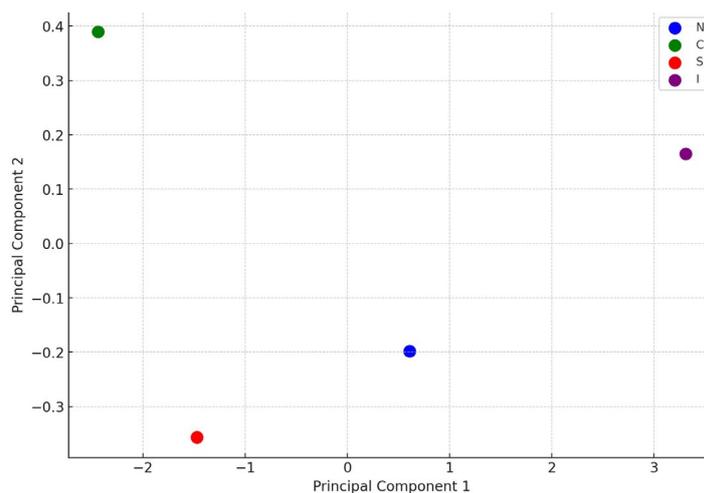
The mean content of CHR + GAL in samples collected in Northern Regions was 2.16  $\pm$  1.93% w/w, a value statistically different compared to the content of CHR + GAL in samples from Central Regions (6.73  $\pm$  2.22% w/w,  $p = 0.002$ ), but also compared to values recorded in samples from Southern Regions (6.01  $\pm$  3.66% w/w,  $p = 0.04$ ). On the other hand, mean content of CHR + GAL in samples from Central and Southern Regions did not differ in a significant manner. Propolis from Tuscan archipelago and Sicily once again deeply differed from the others from Central Regions and South (and North, even not in statistically significant manner). A less marked difference was noted in the ratio between CHR + GAL and TF content among samples from North, Center and South but, similar to PIN, the statistical analysis showed a significant difference between propolis from North and South ( $p = 0.04$ ). Again, a clear difference was observed by comparing propolis from Islands and those from Central and Southern Regions ( $p < 0.001$ ) (**Table 4**). Results partly confirm what reported in [10] that stated a lower CHR content in propolis from Italian temperate areas, namely areas far from the sea and Po river.

In this study we also analyzed the content of CAPE in all samples, being one of the most peculiar caffeic acid derivatives of propolis [16] but rarely taken into account in large comparative analysis. We identify and quantified CAPE in all samples.

As showed in **Table 3**, the range of CAPE content was narrower than in the other parameters previously considered, and in 22 of the 27 samples CAPE ranged between 1.0% and 2.0% w/w. Nevertheless, in line with previous findings here obtained, in 3 out of 5 samples from Northern Regions (N1, N3, N4) CAPE was below 1% w/w and also a very low amount of this compound was found in insular samples. As summarized in **Table 4**, samples from Northern Regions had a moderate CAPE content ( $1.06 \pm 0.26\%$  w/w), propolis from Central Regions a mean content of  $1.51 \pm 0.27\%$  w/w, whereas samples from Southern Regions had the highest mean content of CAPE:  $1.62 \pm 0.28\%$  w/w; in line with our previously findings, insular regions presented the lowest CAPE content ( $0.12 \pm 0.03\%$  w/w). The statistical analysis revealed that CAPE content in propolis from Northern Regions was significantly different from that of Central and Southern Regions ( $p = 0.01$  and  $p = 0.006$ , respectively) and, as observed for other parameters, a great difference was recorded comparing CAPE content in propolis from Islands and that of all other samples ( $p < 0.001$  for all comparisons).

A differential study of CAPE content respect to TP was finally performed (**Table 4**) and it was observed low differences in samples from Northern, Central and Southern Regions, but a lower ratio in propolis from Islands, with differences statistically significant with North and South ( $p = 0.03$  and  $p = 0.006$ , respectively).

The Principal Component Analysis (PCA) plot (**Figure 3**) visually represents the differences in the chemical composition of propolis samples from various geographical regions. Each region is depicted using different colors, with the Northern area shown in blue, the Central area in green, the Southern area in red, and the Insular area in purple. The separation of these regions on the plot indicated that the chemical markers used in the analysis (such as PIN, CHR, GAL, and CAPE, TP and TF) effectively distinguished the samples based on their geographical origin. The Insular samples were notably distinct from the others, appearing clearly separated on the plot, which suggested a different chemical profile for these samples. This distinctiveness could be attributed to the specific environmental conditions or plant sources found on the islands. The Northern and Central samples, while closer to each other, still showed noticeable differences, implying some level of similarity between them, yet with distinct characteristics. The Southern samples also formed a separate group, highlighting their unique chemical profile compared to the Northern and Central regions. Overall, the PCA analysis provided a clear and effective visualization of how geographical factors influence the chemical composition of propolis. The distinct clustering of samples from different regions supported the conclusion that these regions have unique propolis profiles, particularly emphasizing the uniqueness of the Insular samples. This visualization strengthened the understanding of regional variations in propolis composition. In this work we could update and simplify the division of geographical clusters of Italian propolis underlining a marked difference in propolis from Northern Regions respect to those from Central and Southern Regions, more enriched in TP and TF, but also and more specifically in PIN, CHR + GAL and CAPE. However, the most evident difference in propolis samples was observed for those collected in Sicily and in Capraia, chemically very poor. Partly not in accordance with [10], we couldn't distinct in a clear way propolis collected near the seacoast and far from the sea and, more in general, we observed strong similarities in propolis from Central and Southern Regions, regardless latitude and altitude. Differently from [10], unfortunately we weren't able to collect and analyze propolis from Po plain areas and we consider this one limit of this work that did not allow to provide a supplementary comparison. Moreover, as mentioned before, the very low content of CHR + GAL in the sample collected above 500 m in the Alps opened the interest in focusing on mountain propolis in the future to understand if the different flavonoid profile may be a peculiar characteristic.



**Figure 3.** Principal Component Analysis (PCA) plot built (Phyton) with %w/w of pinocembrin, chrysin and galangin, CAPE, total polyphenols and total flavonoids grouped by geographical areas.

## 2.2. Antiradical Activity of Propolis Samples

In line with other studies [17,18], we also performed a simple, but validated antiradical assay through the DPPH (2,2-diphenyl-1-picrylhydrazyl) test.

Of note, we measured a strong activity for most part of samples  $\leq 100 \mu\text{g/mL}$  for all samples with the exception of Insular propolis. Samples from Central Regions showed the narrowest range of  $\text{IC}_{50}$  values, whereas samples from Southern Regions showed the largest range of activity (**Table 5**).

**Table 5.**  $\text{IC}_{50}$  of samples in DPPH assay, expressed as  $\mu\text{g/mL}$ . The standard deviation for all measures is  $< 20\%$  of the mean calculated value,.

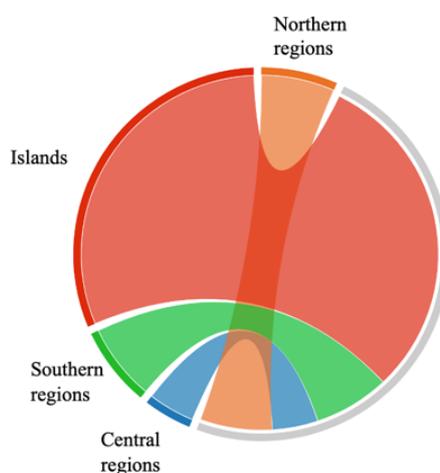
Samples	$\text{IC}_{50}$ ( $\mu\text{g/mL}$ )	Samples	$\text{IC}_{50}$ ( $\mu\text{g/mL}$ )
N1	67.27	C10	23.67
N2	27.34	C11	26.78
N3	40.76	C12	18.92
N4	37.78	C13	32.42
N5	33.48	S1	21.93
C1	25.82	S2	32.71
C2	30.04	S3	29.48
C3	26.80	S4	100.16
C4	27.09	S5	64.75
C5	24.12	S6	26.24
C6	27.70	S7	30.46
C7	26.18	I1	162.67

C8	36.13	I2	220.59
C9	24.25		

**Table 6.** IC<sub>50</sub> in DPPH test of propolis samples divided by geographical origin.

Geographical region	IC <sub>50</sub>
N	41.33 ± 15.36°
C	26.46 ± 4.09°
S	43.68 ± 28.60°
I	191.63 ± 40.96 <sup>b</sup>

The analysis of grouped samples, divided by geographical areas showed similar mean IC<sub>50</sub> values for samples from North, Center and South (41.33 ± 15.36, 26.46 ± 4.09 and 43.68 ± 28.60 µg/mL, respectively) but a very higher mean value for insular samples as depicted in **Figure 4**. ( $p < 0.001$  for all comparisons) (**Table 6 and Figure 4**).



**Figure 4.** IC<sub>50</sub> in DPPH test of propolis samples divided by geographical origin.

Delving into a possible correlation between antiradical activity and TP, PIN and CAPE content through IC<sub>50</sub> analysis of individual samples, an R<sup>2</sup> between 0.52 and 0.54 could be observed. However, the most valid correlation was found between the IC<sub>50</sub> values and TF content (R<sup>2</sup> = 0.59), and within the samples, those from the North, South and Islands the correlation was higher.

On the other hand, the correlation between the antiradical activity and CHR + GAL content was the worst (R<sup>2</sup>= 0.37), a reason claimed to explain the limited effect of geographical diversity of tested samples in DPPH test. Actually, we postulated that scarce differences observed in the assay mainly depended by the high content of red/ox active species present in all samples and we could say that in propolis the whole phytocomplex played a major role for antiradical and antioxidant activities. By the way, our findings about similar IC<sub>50</sub> values in DPPH test of propolis collected in different places are again in agreement with [10], that also reported similar scavenging/antioxidant activities of samples obtained through other essays. A future perspective about this point is to study fine red/ox activity differences in different propolis exploiting sensitivity and specificity of purely chemistry-based methods such as voltammetry, already demonstrated effective and reliable for this bee product [19]. Interestingly, after having discussed the scarce utility of the DPPH test, we confirmed that, about propolis, chemical insights as those conceived for this work are crucial to discriminate different samples, in this case from different Italian areas.

### 3. Conclusions

Chemical analyses of different propolis are pivotal for the rational and modern use of this fascinating bee product for human health purposes, as well as being the most important means of characterizing products from different geographical areas.

In this study, we aimed to update the knowledge of the chemical features of Italian poplar-type propolis by analyzing 27 samples collected in the same year from various regions covering Northern, Central, and Southern Italy, as well as Capraia Island in the Tuscan archipelago and Sicily.

Our findings revealed that it is possible to effectively distinguish propolis from different geographical regions using straightforward and rapid UV methods, such as total polyphenols and total flavonoids analyses. Additionally, the quantification of key markers like pinocembrin, chrysin, galangin, and CAPE via HPLC-DAD enabled a more precise determination of the geographical origin of propolis. While the DPPH assay provided valuable insights into antiradical activity, it proved suboptimal for differentiating geographical sources due to the similarity in results.

The main strength of this work lies in having established a solid foundation for developing more refined methods to assess and ensure the quality and origin of propolis.

We are fully aware that this study represents only a preliminary basis that needs further development. This includes expanding the comparison to include sub-areas (such as fluvial regions, Apennine and Alpine propolis, samples from inland beehives in Sicily and Sardinia) and significantly improving the analytical level beyond the analysis of the main constituents.

### 4. Materials and Methods

#### 4.1. Samples Collection

Twenty-seven propolis samples were obtained from beekeepers in different regions throughout the Italian peninsula and islands; All propolis were produced during spring 2020 and the samples were already supplied cleaned and de-waxed. In the laboratory, they were solubilised in EtOH 75% (v/v) at a concentration of 10 mg/mL. The solutions were all yellow-orange in color, tending to red-brown. Odor was balsamic.

Details of areas where samples were collected are shown in **Table 7**.

**Table 7.** List of analysed samples with their origin.

Sample s code	Area of origin	Gps coordinates	Region	Sample s code	Area of origin	Gps coordinates	Region
N1	Valdilana (BI)	45°39'25.66" N 8°09'01.85"E	Piedmont	C10	Arcidosso (GR)	42°52'20"N 11°32'15"E	Tuscany
N2	Arcisate (VA)	45°51'18.98" N 8°52'03.3"E	Lombardi a	C11	Castello delle Forme, Marsciano (PG)	42°58'47.06" N 12°21'22.21" E	Umbria
N3	Castellanz a (VA)	45°37'N 8°54'E	Lombardi a	C12	Deruta (PG)	42°59'N 12°25'E	Umbria
N4	Pergine Valsugan a (TN)	46°04'N 11°14'E	Trentino- Alto Adige	C13	Norma, Monti Lepini (LT)	41°35'N 12°58'E	Lazio

<b>N5</b>	Castel San Pietro Terme (BO)	44°23'52"N 11°35'22"E	Emilia-Romagna	<b>S1</b>	Bellante (TE)	42°45'N 13°48'E	Abruzzo
<b>C1</b>	Quarrata (PT)	43°50'51"N 10°59'00"E	Tuscany	<b>S2</b>	Massiccio Del Matese	41°26'59.87" N 14°22'19.21" E	Molise/Campan ia
<b>C2</b>	Firenze Valdarno (FI)	43°39'24"N 11°26'58"E	Tuscany	<b>S3</b>	Campobasso (CB)	41°33'39.6" N 14°40'06.24" E	Molise
<b>C3</b>	Figline Valdarno (FI)	43°37'N 11°28'E	Tuscany	<b>S4</b>	Rodi Garganico (FG)	41°55'19.9" N 15°52'37.86" E	Puglia
<b>C4</b>	Grassano Ponte a Ema (FI)	43°44'22.42" N 11°17'51.73" E	Tuscany	<b>S5</b>	San Severo (FG)	41°41'42.4" N 15°22'45.4"E	Puglia
<b>C5</b>	Greve in Chianti (FI)	43°35'N 11°19'E	Tuscany	<b>S6</b>	San Basile (CS)	39°48'34.56" N 16°09'47.81" E	Calabria
<b>C6</b>	Reggello (FI)	43°41'N 11°32'E	Tuscany	<b>S7</b>	Cicala (CZ)	39°01'19.88" N 16°29'09.96" E	Calabria
<b>C7</b>	San Polo in Chianti (FI)	43°40'18.13" N 11°21'46.13" E	Tuscany	<b>I1</b>	Isola di Capraia (LI)	43°02'55.32" N 9°50'25.08"E	Tuscany
<b>C8</b>	Batignano (GR)	42°52'02.22" N 11°09'57.84" E	Tuscany	<b>I2</b>	Pianoconte (ME)	38°28'38.2" N 14°55'43.8"E	Sicily
<b>C9</b>	Montorsaiolo (GR)	42°53'26"N 11°12'13"E	Tuscany				

## 4.2. Phytochemical Analyses

### 4.2.1. Quantification of Total Polyphenols and Total Flavonoids

The total polyphenol content was quantified by the Folin-Ciocalteu (FC) colorimetric assay according to Finetti et al., 2020 [20] using ethanolic solution of propolis samples 10 mg/ml. Absorbance of each solution was measured with a spectrophotometer (UV/Vis spectrophotometer UV-1900i, Shimadzu, Kyoto, Japan) at 700 nm, using as a reference a blank consisting of ethanol reacted under the same conditions. The calibration line was made with gallic acid at concentrations between 0.06 and 5 mg/mL, with an  $R^2 > 0.99$ . The concentration of polyphenols in the samples was calculated as % w/w expressed as gallic acid equivalent (GAE).

Total flavonoids were quantified according to the method reported by Governa et al., 2020 [21] and Sberna et al., 2022 [22]. Samples (10 mg/ml in ethanol) were diluted 1:200 in ethanol 75% v/v and, using a VICTOR Nivo 3S multi-mode plate reader, (PerkinElmer, Waltham, Massachusetts, USA). Absorbance was read at 353 nm, and galangin was used as a standard (0.05-2 mg/mL,  $R^2 > 0.98$ ).

All tests were conducted in triplicate.

### 4.2.2. HPLC-DAD Analysis

HPLC analyses were conducted using a Shimadzu Prominence LC 2030 3D instrument equipped with a Bondapak® RP C18 column, 10  $\mu\text{m}$ , 125  $\text{\AA}$ , 3.9 mm x 300 mm (Waters Corporation, Milford, Massachusetts, USA). The solvents used were: A: water + 0.1% formic acid; B: methanol + 0.1% formic acid (Merck Sima-Aldrich, Darmstadt, Germany).

The chromatographic conditions were as follows: B from 60% at 0.01 min to 70% at 6.00 min and to 85% at 17.00 min and 3 minutes for returning to the initial conditions. Total run time was 20 minutes. Flux was set at 0.75 mL/min. Chromatograms were recorded at 280 nm.

Pinocembrin, chrysin, galangin and CAPE reference standard grade (Merck Sima-Aldrich) were used. The method guaranteed linearity and precision ( $R^2 > 0.99$  for all standards), repeatability (inter- and intra-day differences in replicates < 15%) and allowed to quantify all metabolites of interest in tested samples above the limit of quantification (LOQ), < 0.05  $\mu\text{g}$  in column.

Samples (10 mg/mL in ethanol) were diluted 10 folds in ethanol, filtered 0.44  $\mu\text{m}$  and injected (10  $\mu\text{l}$ ).

Compounds peaks were identified by comparing their retention times and UV spectra with those of the corresponding standards.

Analyses were conducted in triplicate.

## 4.3. Antiradical Activity of Propolis

The antiradical activity of propolis was determined using the DPPH (2,2-diphenyl-1-picrylhydrazyl) as described by Bonetti et al., 2021 [17]. The negative control was made with ethanol and DPPH (1:19). Pure ascorbic acid (Merck Sigma-Aldrich) was used as the reference substance. The percentage inhibition of DPPH was calculated according to the following formula: % inhibition =  $(\text{Absc} - \text{Absx}) / \text{Absc} \times 100$  and  $\text{IC}_{50}$  calculated.

## 4.4. Multivariate Modelling

The Principal Component Analysis (PCA) was performed to assess the variation in propolis composition across different geographical regions. The data used for this analysis included the concentrations of key compounds such as pinocembrin, chrysin + galangin, CAPE, total polyphenols and total flavonoids from propolis samples, specifically, the average of the four geographical regions: North (N), Central (C), South (S), and Islands (I). The data were centered and scaled to ensure comparability between variables. PCA was conducted using Python programming language, specifically with the scikit-learn library [23] for the PCA computation and the matplotlib library for visualization [24]. The analysis used a covariance matrix to identify the principal components that explain the maximum variance in the dataset. The first two principal components, which accounted

for the most variance, were plotted to visualize the clustering and distribution of the samples from each region. Each region is represented by a distinct color on the scatter plot.

#### 4.5. Statistical Analysis

Data were presented as mean  $\pm$  standard deviation (SD) of experimental triplicates. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by post-hoc Tukey test (with  $p < 0.05$  as significance level).

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