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FULL PAPER

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Photometric Analysis of Propolis from the Island of Samothraki, Greece. The Discovery of Red Propolis

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Propolis presents notable and variable antioxidant activity depending on the territory and the local flora. As a result, propolis collected from areas presenting botanical diversity can become an intriguing research field. In the present study, we examined propolis from different areas of Samothraki, a small Greek island in the north-eastern Aegean Sea, considered a hot-spot of plant biodiversity. The analysis of propolis samples presented huge variability in the antioxidant activity, the total polyphenol content and the total flavonoids content. Propolis from two areas presented high antioxidant activity with a maximum at 1741.48 μmol of Trolox equivalents per gram of dry propolis weight, very high polyphenol content, 378.73 mg of gallic acid equivalents per gram of dry propolis weight, and high flavonoid content with a maximum concentration of 70.31 mg of quercetin equivalents per gram of dry propolis weight. The samples that presented the best qualitative characteristics were all red propolis which is a type that has never been reported in any part of Europe.

Keywords: Propolis, Samothraki Island, antioxidant activity, polyphenolic content, flavonoid content, biological activity.

Introduction

Propolis is a fascinating natural bee product. For the production of propolis, bees harvest resins from various plant species, mix them with their secretions such as wax or saliva and carry them back to the colony where they use them to narrow the nest entrance in order to prevent invaders, to coat the inner walls of their hive, to cover holes and crevices, to attach new combs and to 'mummify' dead intruders too heavy to be removed from their nest.[1–7] Overall, propolis is considered a key product for the social immunity of the honeybee colony.[8]

Propolis has long been used in folk medicine, as documented in historical sources indicating its use by ancient Egyptians, Greeks and Romans.[6] It has been studied widely for many decades because of its valuable characteristics including its antioxidant,[9–11] bacteriostatic,[12–16] antifungal,[17–19] anti-inflammatory,[20–22] antiviral,[15,23,24] antitumor and anticancer,[25–29] properties. The pharmaceutical action of propolis is a result of the bioactive ingredients. These possess high chemical diversity with different categories of constituents, such as polyphenols, terpenoids, steroids, sugars and amino acids, having been identified.[30] Polyphenolic components, mainly

flavonoids, are gaining scientific and commercial interest. Studies have correlated flavonoid content of propolis with its antioxidant properties,[9,10,31,32] while many other medicinal properties of propolis have been also attributed to flavonoids.[33–35]

Generally, flavonoids, polyphenols and propolis components have been found to be quantitatively or qualitatively variable, depending on plant origin.[36,37] Furthermore, there is an important variation according to climatic zones or geographic location.[10,15] Gardana et al.[38] analyzed the propolis of various geographic areas and showed that European, Chinese and Argentinean propolis were characterized by the presence of phenolic acids and flavonoids, and that the most abundant were chrysin (2–4%), pinocembrin (2–4%), pinobanksin acetate (1.6–3%) and galangin (1–2%). Samples of Brazilian propolis contain mainly artepillin C, different caffeoyl quinic acids and some flavonoids. Popova et al.[39] described a new type of propolis, the Mediterranean type, characterized by a diterpene fingerprint profile. All of the above research was conducted with propolis samples collected in different continents, entire countries or extended geographical areas. Very few experimental results are available for limited areas with special territorial and climatic features such as those found on remote islands. In Greece, there are more than 225 inhabited islands (in a total of around 6000 islands and islets). In most of them, apiculture has been practiced since the prehistoric era.

One of Greece's most isolated islands is Samothraki, a small island in the north-eastern Aegean Sea, covering an area of 178 km^2 (0.13% of the total Greek territory). Despite its limited surface, Samothraki is a significant hotspot of biodiversity: from the 5800 plant species that have been reported in Greece, 1441 (24.84%) can be found in Samothraki, belonging to 559 genera and 123 families.[40] Samothraki hosts 18 taxa which have been published as local endemics restricted to the island.[40] The reasons for this floral richness are mainly because of Samothraki's geographic location (25 km from the closest shore), its proximity to three different floral zones,[41] and its rugged terrain. As described by Fischer–Kowalski et al.,[42] a large part of Samothraki's total surface area is mountainous, owing to the volcanic origin of the island (Mount Saos rises to 1624 m). Most of this mountain territory is currently protected as a Natura 2000 conservation area. A wet microclimate exists on the north side, with numerous springs, hundreds of waterfalls and scenic freshwater ponds. Lush vegetation shaded by century-old oriental plane trees reaches down to the beaches. The southern and western shores are typically Mediterranean in terms of climate and vegetation. Many of island's plant species have been reported as herbs, medicinal and apicultural plants. As a result, apiculture is well developed on the island and is focused mainly on honey production. Propolis is just a side product for beekeepers and little attention is paid to the production and commercialization of propolis-based products. No research has been undertaken to assess the characteristics and the quality of Samothraki's propolis.

Intrigued by the richness of the Samothracian flora (from which propolis is derived), we tried to evaluate some qualitative factors of the Samothracian propolis by analyzing samples from different areas and seasons. To analyze the different propolis samples, we used photometric methods to determine antioxidant activity (AA), total polyphenolic content (TPC) and total flavonoids content (TFC). Furthermore, the variation in the qualitative characteristics of samples and the relationship to the season of harvest and different pigmentation of collected propolis was also determined.

Results and Discussion

Propolis was collected from seven areas of Samothraki Island (*Figure 1*) with different vegetation types. Selected areas were Potamia, Alonia, Chlabaria, Chora, Makrilies, Baxedes and Therma.

Color Variation of Propolis Samples

Propolis samples collected from different areas of Samothraki presented remarkable color variation (*Figure 2*). Colors ranged from light yellow (*Figure 2E*), all shades of brown (*Figure 2B*, *2C*), mixtures of brown and red (*Figure 2D*) up to dark brown/black (*Figure 2F*). However, the most interesting propolis that was collected during trials was the red propolis from the areas of Potamia and Baxedes (*Figure 2A*). Red propolis is produced mainly in Brazil, Cuba, Mexico, China and Nigeria.[43] No red propolis has ever been reported in a Mediterranean or European area. *Dalbergia ecastophyllum* has been determined as the main source of red propolis originating in Brazil and Cuba, though the contribution of other plant species cannot be assumed.[44] Such plant species are distributed in tropical and subtropical areas and are not present in Greece or on the island of Samothraki. As a result, the determination of the botanical origin of red propolis

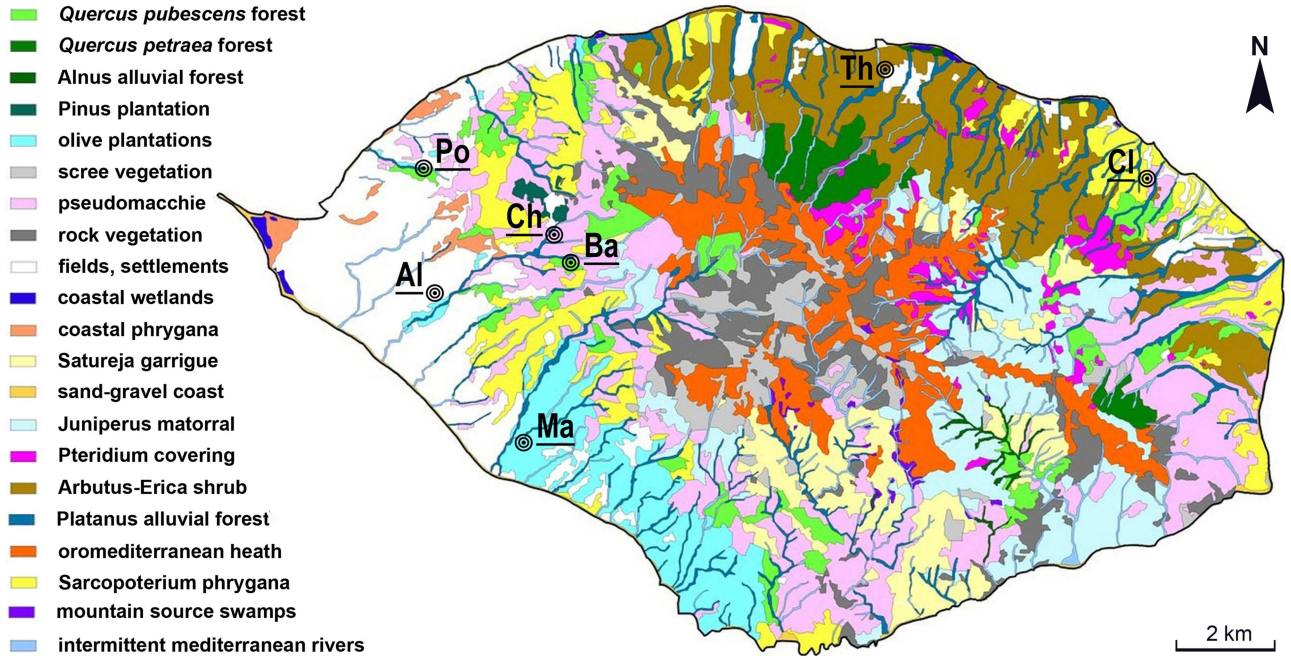

Figure 1. Vegetation units of Samothraki and sampling areas. Po: Potamia, Al: Alonia, Cl: Chlabaria, Ch: Chora, Ma: Makrilies, Ba: Baxedes, Th: Therma. Figure by Biel and Tan,[40] with permission.

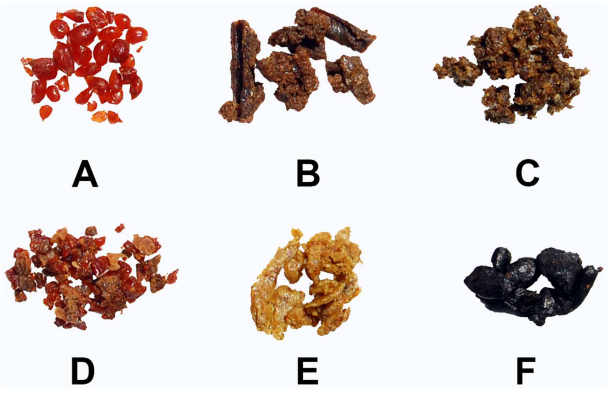

Figure 2. Propolis of different color from five apiaries of Samothraki. A) Red propolis from Potamia (demonstrated the highest antioxidant activity, total polyphenol and flavonoids content), B) Alonia, C) Therma, D) Baxedes, E) Potamia (autumn), F) Makrilies.

from Samothraki is an interesting field for future research.

Antioxidant Activity, Total Polyphenol Content and Total Flavonoid Content

The analysis of samples collected from the seven locations of Samothraki (*Table 1*) presented differing values for all examined factors. The antioxidant activity (AA) was very high in some samples from Potamia, Baxedes, Chlabaria and Alonia which in many cases exceeded 1100 μmole of Trolox equivalents per gram of dry propolis weight (TRE/g).

The higher values of over 1600 TRE/g were recorded in six samples collected in late summer in the apiary at Baxedes and six in early summer from the area of Potamia, where the maximum value reached 1813.2 TRE/g. However, there were areas and samples with very low AA, such of those recorded at Chora, Makrilies and Alonia, demonstrating values between 5–20 TRE/g, with a minimum of just 1.75 TRE/g recorded at Alonia.

Samples which presented the highest AA exhibited also the highest polyphenolic content. Values between 349.5 and 380.2 mg of gallic acid equivalents per g of dry propolis weight (GAE/g) were recorded for samples from Baxedes and Potamia, respectively. To our knowledge, these TPC values recorded in Samothraki are the highest ever reported in Greece or in Europe.[10,16,45,46] Even higher polyphenolic content has been reported only in samples from Brazil,[47] China,[48] Japan,[49] and Canada,[50] with the first two, referring to red propolis. Low TPC values, ranging from 12.85 to 15.45 GAE/g, were also evaluated in propolis samples from Samothraki at Alonia, Chora and Makrilies.

Analysis of propolis samples revealed that the TFC was around 26% of the TPC. The highest values were

Table 1. Antioxidant activity, total polyphenolic content and total flavonoid content of propolis methanolic extracts from Samothraki. Data presented are number of samples: *n*, Mean ± SEM: Standard Error (Minimum – Maximum).[a]

Area	*n*	Antioxidant Activity DPPH· Scavenging Capacity (Trolox equivalents μmole/g propolis)	Total Polyphenolic Content (mg Gallic acid equivalents/ g propolis)	Total Flavonoid Content (mg Quercetin equivalents/ g propolis)
Po	36	927.30[a] ± 83.24 (264.28 – 1813.20)	133.72[a] ± 16.39 (27.00 – 380.20)	36.55[a] ± 3.25 (11.18 – 70.80)
Al	18	240.85[c] ± 91.64 (1.75 – 1066.80)	39.07[c] ± 10.05 (12.85 – 134.93)	11.80[c] ± 2.26 (5.55 – 33.40)
Cl	3	1094.88[a,b] ± 43.95 (1007.30 – 1144.90)	107.66[a] ± 3.502 (100.66 – 111.26)	36.29[a,b] ± 2.93 (32.26 – 42.01)
Ch	18	149.94[c] ± 44.32 (5.89 – 564.79)	46.03[c] ± 10.39 (15.07 – 141.93)	16.88[c] ± 3.51 (4.50 – 52.51)
Ma	15	126.65[c] ± 19.41 (14.17 – 228.17)	49.86[b,c] ± 5.52 (15.45 – 81.91)	11.59[c] ± 1.28 (5.43 – 19.65)
Ba	15	561.01[a,b] ± 129.50 (84.73 – 1346.60)	144.73[a,b] ± 33.87 (30.03 – 349.51)	26.49[a,b] ± 6.34 (5.27 – 70.57)
Th	15	264.71[b] ± 21.40 (142.74 – 381.03)	65.45[b] ± 4.72 (34.57 – 94.03)	16.84[c] ± 2.10 (8.18 – 32.94)
K–W	*p*	< 0.001	< 0.001	< 0.001

[a] Po: Potamia, Al: Alonia, Cl: Chlabaria, Ch: Chora, Ma: Makrilies, Ba: Baxedes, Th: Therma. Mean values in the same column followed by different superscript letters are statistically significant (significance level $p < 0.05$) according to the results of a series of Mann–Whitney tests. K–W: Significance according to Kruskal–Wallis test

from samples of Potamia and Baxedes with maximum values of 70.8 and 70.5 mg of quercetin equivalents per g of dry propolis weight (QE/g) respectively. Low values, between 4.5 and 5.53 QE/g, were found in samples from Chora, Baxedes, Makrilies and Alonia. In contrast to the high TPC, the TFC was within normal levels for Greek propolis. Similar values have been reported by other researchers who analyzed propolis samples from the Greek mainland and from Greek islands.[16,45,46]

In general, the values of the three parameters examined for the propolis of Samothraki, revealed huge variability, not only between areas but also between colonies of the same apiary. There was even variability within the same sample of a propolis trap from a single colony when it was analyzed in triplicates (i.e., *Figure 3*, samples 1, 2 and 3 or 40, 74 and 78). Results have therefore been analyzed and presented as separate units and not as a plotted sample of a triplicate. This variability can be explained by the diversity of resins that honeybees can find and harvest throughout the island. Though foragers are able to choose and harvest profitable food sources,[51,52] (i.e., nectar or pollen), there is no evidence that resin quality affects what is collected.[8] In fact, it has been shown that instead of resins, honeybees might collect even petroleum derivatives from vicinal asphalt.[53] Propolis is not consumed by honeybees, so foragers will harvest any easily reached source of resins. The sources of resins in Samothraki appear to be numerous and deposition of propolis on traps represented different types of propolis according to color. In many cases, the same trap was covered by multicolored propolis within a period of 2–3 days (*Figure 2D*). The distance between apiaries cannot explain the differences of the qualitative characteristics of propolis because most apiaries were within flying distance of honeybee foragers.[54] In some cases, there were apiaries only 600 m apart (Baxedes–Chora), but the differences in all tested parameters were very high, with Baxedes presenting very high values of AA, TPC and TFC, while samples from Chora presented very low values. An observation, regarding the vegetation units of each experimental area showed that the samples which presented the higher values (Potamia and Baxedes) derived from *Quercus pubescens* forests (*Figure 1*), so a possible relation between pubescent oak trees and the quality of propolis could be examined in the future. Other dominant plant species found on both areas are *Prunus dulcis* and *Prunus amygdaliformis* trees as well as *Paliurus spina-christi* bushes. Furthermore, all samples of red propolis were collected from these two areas. The 12 samples of red propolis presented the higher values of AA, TPC and TFC, indicating that the color of propolis has a significant role on the classification of the samples. Both color of propolis and the period of harvesting were further investigated through the statistical analysis of the results.

The variability of the qualitative characteristics of propolis samples, in relation to area, season and color, is presented in *Figures 3* and *4*. According to the dendrogram of *Figure 3* (Hierarchical Cluster Analysis), the samples are distributed in two main clusters (samples 1–84 and 85–120). The first cluster, with 70% of the samples (*Figure 3A*), contains all the

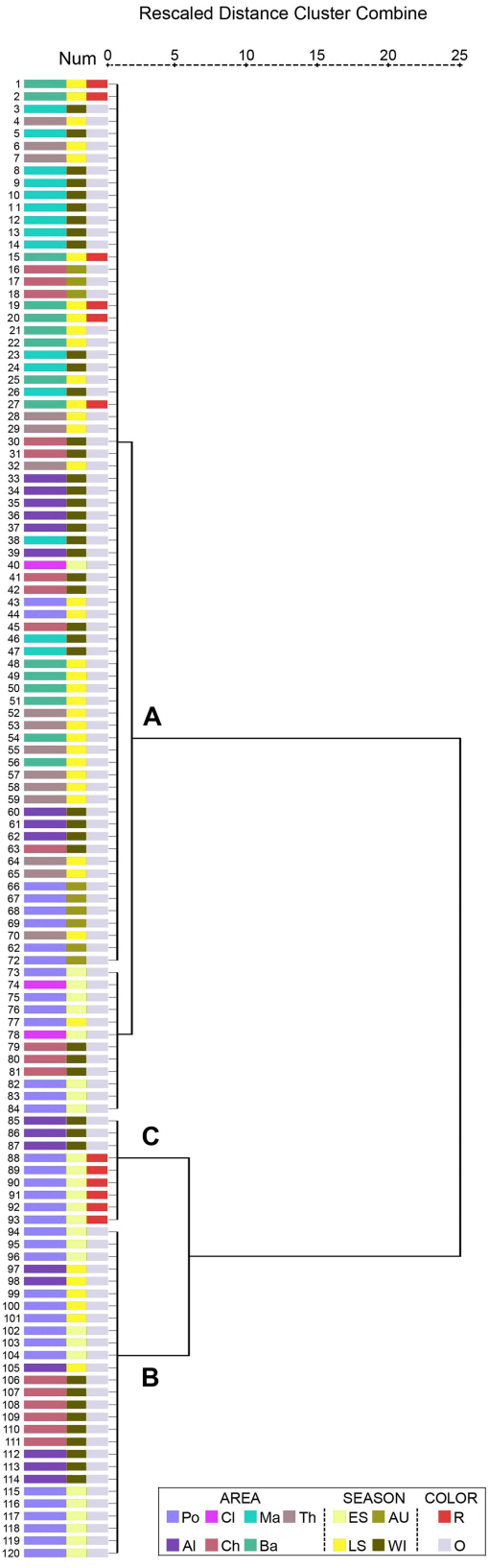

Figure 3. Dendrogram from Hierarchical Cluster Analysis of the propolis samples from Samothraki. Po: Potamia, Al: Alonia, Cl: Chlabaria, Ch: Chora, Ma: Makrilies, Ba: Baxedes, Th: Therma. ES: Early summer, LS: Late summer, AU: autumn, WI, winter. R: red, O: other colors.

propolis from Baxedes, Makrilies, Chlabaria and Therma as well as samples from the other three sampling areas. All autumn propolis is included in this group as well as the majority of late-summer samples. Red propolis from Baxedes is grouped in the large branch. The second cluster (30% of the samples) is subdivided into two branches. The larger one (*Figure 3B*) contains 27 samples (22.5%) from Potamia, Alonia (both summer harvest) and Chora (winter) while the smaller group (*Figure 3C*) contains the 6 (5%) red propolis samples from Potamia (early summer) and 3 (2.5%) winter samples from Alonia. The comparisons of the centroids of the three clusters relative to the three qualitative characteristics of the propolis samples (AA, TPC and TFC) are presented in *Table 2*. The values of the η^2 coefficients indicate that all three characteristics contribute almost equally to the construction of the three main branches of the dendrogram ($p < 0.001$).

Principal component analysis showed that the first component explains almost the entire variability of the three qualitative characteristics (AA, TPC and TFC) of the samples (92.7% of total variance). The dense cloud of samples depicted in *Figure 4A* revealed that some areas are more homogenous than others. In areas such as BA and Po, the variability was greater than the others. The impact of the period of harvesting is demonstrated in *Figure 4B*. The summer samples are distinguished clearly with the early summer propolis presenting the highest values, followed by the autumn samples. Winter samples presented the lowest values for all the parameters examined. In addition, early summer samples showed greater variability than the other periods. Propolis presented a clear differentiation according to color. All red samples were differentiated from the rest (*Figure 4C*).

The AA of early summer samples was significantly higher than the rest periods ($p < 0.001$). The TPC and TFC were also significantly higher from late summer ($p < 0.001$) but did not vary from autumn samples ($p = 0.392$ and $p = 0.967$ for TPC and TFC, respectively). There were no differences between late-summer and autumn samples regarding AA and TPC ($p = 0.089$ and $p = 0.428$, respectively), while TFC presented higher values in autumn samples ($p = 0.018$). Our results are in agreement with previous research showing that the polyphenolic and the flavonoid content of propolis as well as the antioxidant activity increase during the warmest periods of the year.[11,55,56] Comparison of red and non-red samples showed significant differences for all examined factors ($p < 0.001$ for AA, TPC and TFC). Overall, red propolis samples presented the most distinct profile in the classification of Samothracian

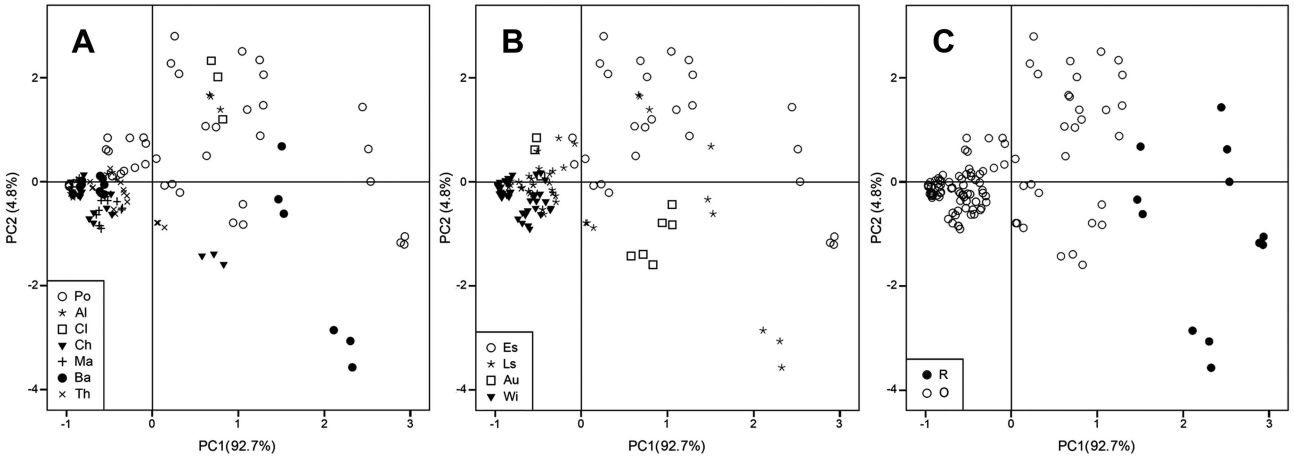

Figure 4. Principal Component Analyses (PCA) of the propolis samples from Samothraki. A) PCA of different areas of sampling, B) PCA of different periods of sampling, C) PCA according to the color of samples. Po: Potamia, Al: Alonia, Cl: Chlabaria, Ch: Chora, Ma: Makrilies, Ba: Baxedes, Th: Therma. ES: Early summer, LS: Late summer, AU: autumn, WI, winter. R: red, O: other colors.

Table 2. Centroids (mean values) of the three clusters (A, B, C) of propolis samples from Samothraki.[a]

Cluster	*n*	Antioxidant Activity DPPH• Scavenging Capacity (Trolox equivalents μmole/g propolis)	Total Polyphenolic Content (mg Gallic acid equivalents/ g propolis)	Total Flavonoid Content (mg Quercetin equivalents/ g propolis)
A	84	194.22[a]	45.20[a]	12.92[a]
B	27	1021.01[b]	140.42[b]	39.27[b]
C	9	1509.68[c]	331.26[c]	68.77[c]
K–W	*p*	<0.001	<0.001	<0.001
η^2		0.84	0.86	0.83

[a] Mean values in the same column followed by different superscript letters are statistically significant different (significance level $p<0.05$, according a series of Mann–Whitney tests). η^2: percentage of variance between clusters. K–W: Significance according to Kruskal–Wallis test

propolis, regardless of the analysis performed. Results of AA, TPC and TFC according to period of sampling and color of propolis are presented in *Table 3*.

Correlations between Antioxidant Activity, Total Polyphenol Content and Total Flavonoid Content

The correlation between AA and the TPC and TFC samples was examined (*Figure 5A* and *5B*). Furthermore, the correlation between TFC with TPC was evaluated (*Figure 5C*). In all of the tests, there was a strong correlation between all combinations. Spearman's rho was 0.912 for AA and TPC and 0.900 between AA and TFC. Spearman's rho was 0.894 for the correlation between TPC and TFC. In all cases, *p* was <0.001. The correlation between propolis-scavenging activity of DPPH• and its polyphenolic content has been studied extensively. Though most studies demonstrated a strong correlation between AA and TPC or TFC,[9,10,32,56–58] controversial results, mainly from recent research, showed variable, weak or no correlation at all.[59–62] Though the results of the present study appear to agree with studies supporting strong correlation between AA, TPC and TFC, analysis separating red propolis samples from the rest provided some interesting findings suggesting that all hypotheses are partly correct. AA and TPC appeared to be strongly related in all but the red samples (*Figure 5D*). However, while AA increases in the 12 samples of red propolis, there is no proportional increase of the TPC. On the contrary, in the first six samples presenting values of AA over 1000 TRE/g from the area of Baxedes, a decrease in the polyphenolic content of more than 100 GAE/g does not affect the increase of AA, while the highest content of polyphenols of around 380 GAE/g (3 samples) from the area of

Table 3. Antioxidant activity, total polyphenolic content and total flavonoid content of propolis methanolic extracts from Samothraki, according to sampling period and color. Data presented are Number of samples: *n*, Mean, ± SEM: Standard Error (Minimum – Maximum).[a]

	n	Antioxidant Activity DPPH· Scavenging Capacity (Trolox equivalents μmole/ g propolis)	Total Polyphenolic Content (mg Gallic acid equivalents/ g propolis)	Total Flavonoid Content (mg Quercetin equivalents/ g propolis)
ES	27	1126.23[a] ± 81.58 (475.21 – 1813.17)	159.51[a] ± 18.95 (64.30 – 380.20)	41.15[a] ± 3.39 (19.04 – 70.80)
LS	39	461.76[b] ± 60.84 (84.73 – 1345.62)	98.71[b] ± 14.47 (30.03 – 349.51)	21.80[c] ± 2.66 (5.27 – 70.57)
AU	9	546.94[b] ± 69.47 (264.28 – 852.52)	104.11[ab] ± 17.75 (27.00 – 146.10)	38.69[ab] ± 6.40 (11.18 – 57.88)
WI	45	91.78[c] ± 12.48 (1.75 – 304.94)	32.80[c] ± 2.92 (12.85 – 81.91)	10.12[d] ± 0.74 (4.29 – 19.65)
K–W	*p*	< 0.001	< 0.001	< 0.001
Red	12	1438.91[a] ± 90.89 (1038.06 – 1813.17)	311.21[a] ± 15.79 (241.58 – 380.20)	61.69[a] ± 3.75 (38.16 – 70.80)
Other	108	372.34[b] ± 36.70 (1.75 – 1411.99)	63.29[b] ± 4.02 (12.85 – 159.73)	18.75[b] ± 1.30 (4.29 – 57.88)
Mann–Witney	*p*	< 0.001	< 0.001	< 0.001

[a] ES: Early Summer, LS: Late Summer, AU: Autumn, WI: Winter. Mean values in the same column followed by different superscript letters are statistically significant (significance level $p < 0.05$) according to the results of a series of Mann–Whitney tests. K–W: Significance according to Kruskal–Wallis test

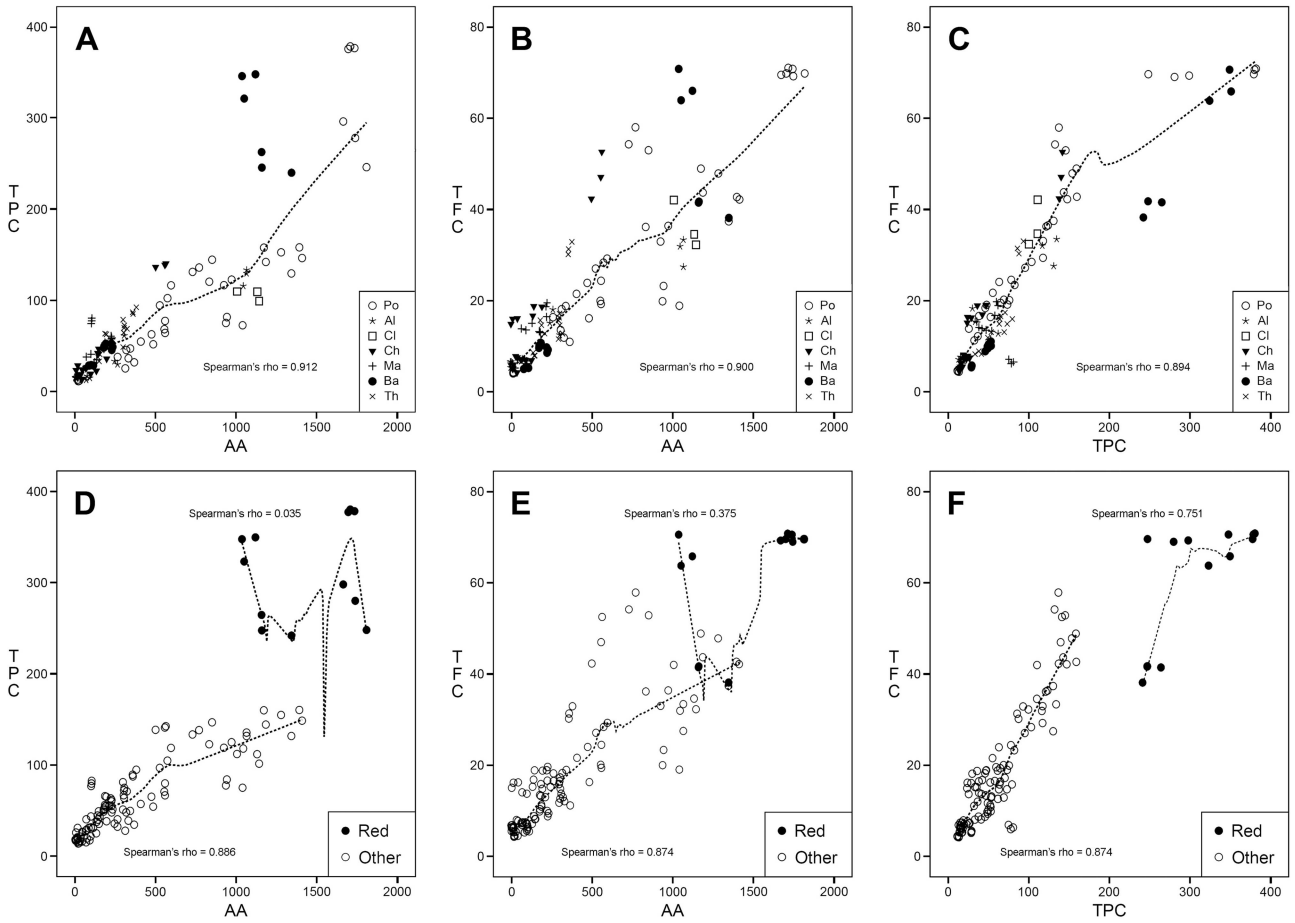

Figure 5. Correlation between antioxidant activity (AA), total polyphenolic content (TPC) and total flavonoid content (TFC) of the propolis samples from Samothraki. A) Correlation between AA and TPC according to sampling areas. B) Correlation between AA and TFC according to sampling areas. C) Correlation between TPC and TFC according to sampling areas. D) Correlation between AA and TPC according to color. E) Correlation between AA and TFC according to color. F) Correlation between TPC and TFC according to color. Po: Potamia, Al: Alonia, Cl: Chlabaria, Ch: Chora, Ma: Makrilies, Ba: Baxedes, Th: Therma. R: red, O: other colors. Best fitted line (s) has been plotted using the Lowess method.

Potamia have similar AA values when compared with the other three samples from the same area. These findings suggest that substances other than polyphenols presented in red propolis from Samothraki contribute to the high AA. Such substances could belong to terpenes since previous research has shown that Greek and Mediterranean propolis in general is not very rich in polyphenols and is considered rather poor in flavonoids, but it very rich in diterpenes which provide high antioxidant properties in the samples analyzed.[16,39,63–64]

Similar to TPC, red propolis samples from Baxedes presented a drop in TFC values from 70.57 QE/g to 38.15 QE/g, while AA increased from 1038 TRE/g to 1346 TRE/g (*Figure 5E*). The analysis of non-red propolis samples (*Figure 5F*) showed that the TPC and TFC correlation is almost linear. Red propolis samples presented a different pattern with the TFC remaining constant in 9 of the 12 samples while TPC increased. These results indicate that polyphenols other than flavonoids are present in red propolis of Samothraki and they may contribute to its qualitative characteristics. We can assume that other polyphenols except for flavonoids, such as phenolic acids and phenolic acid esters, might play a key role to the increased AA of Samothracian red propolis. Red propolis presenting high AA, originating from Brazil Cuba and China, has been found to contain caffeic acid, ferulic acid and coumaric acid.[47,48,65] Since the determination of chemical profile of propolis has not been the objective of the present study, all the above assumptions offer an intriguing field of future research.

Conclusions

The propolis of Samothraki Island presented interesting and variable characteristics. Samples derived from the rich flora of a small island which is a hotspot of biodiversity varied in their antioxidant activity, total phenolic and total flavonoid content. Differences occurred between samples collected in neighboring areas or even within the same apiary. The impact of the propolis collecting season was also important as was the type of propolis, characterized by different pigment. The most important finding of the present study was the discovery of red propolis from two apiaries located in the north-west and center-west of the island. These samples showed very high antioxidant activity and were characterized by their high polyphenolic content. The correlation between antioxidant activity, polyphenol and flavonoid content indicated that substances other than polyphenols are present in Samothracian red propolis and contribute to its high antioxidant activity. Furthermore, the flavonoids must be accompanied by different polyphenolic compounds that also induce high antioxidant activity.

Experimental Section

Chemicals and Agents

The 2,2-diphenylpicrylhydrazyl (DPPH$^{\bullet}$) was obtained from Scientific Industries Inc. (N.Y., USA). Trolox and gallic acid were obtained from Sigma–Aldrich Chemie GmbH (Taufkirchen, Germany). Folin–Ciocalteu and monohydrated sodium phosphate were obtained by Merck (Darmstadt, Germany). $AlCl_3$ were from Fisher Scientific (Fair Lawn, NJ).

Sampling

Propolis samples were collected from seven different areas of Samothraki Island with differing vegetation. Selected areas were Alonia (AL, 40°27′29.60″ N, 25°29′46.30″ E): 18 samples in late summer and winter, Potamia (PO, 40°28′59.47″ N, 25°29′39.41″ E): 36 samples in all 4 periods, Makrilies (MA, 40°25′51.86″ N, 25°31′01.08″ E): 15 samples in winter, Chora (CH, 40°28′07.83″ N, 25°31′29.77″ E): 18 samples in autumn and winter, Baxedes (BA, 40°27′53.09″ N, 25°31′46.26″ E): 15 samples in late summer, Therma (TH, 40°29′55.69″ N, 25°36′13.70″ E): 15 samples in late summer, Chlabaria (CL, 40°28′43.80″ N, 25°40′00.72″ E): 3 samples in early summer. Colonies in the selected areas were in good health and were managed according to the regulations for bio-apiculture. They were all equipped with top-screened propolis traps, provided by ANEL (Athens, Greece) apicultural company. The specific propolis traps are made from low-density polyethylene (LDPE) and, during preliminary analyses, no residues were detected in the transfer from the trap to the sample to be analyzed (data not shown). When traps were filled with propolis by honeybees, they were removed and were placed in freezer at −20 °C for 24 h. Then, propolis was removed from the trap and was stored at the same temperature until extraction.

Extract Preparation

For the analysis, 5% (w/v) of propolis methanolic extract (PME) was prepared as described by Graikini

et al.[66] Crude propolis samples were ground in a chilled grinder and small amounts (0.5 g) of pulverized crude propolis were extracted after stirring with a 10 mL volume of absolute methanol for 5 min. Then, extracts were filtered with a 25 mm syringe filter (Nylon 66, 0.22 μm) and solutions were kept in tightly closed bottles stored at −20 °C. From each propolis load, three separate PME were prepared.

Determination of the Antiradical Activity (AA)

For AA determination, a previously described protocol was used.[67] Briefly, an aliquot of 0.025 mL of sample was added to 0.975 mL DPPH• solution (100 μM in MeOH) and the absorbance was read at $t=0$ and $t=$ 30 min. Trolox™ equivalents (mM TRE) were determined from linear regression, after plotting $\%\Delta A_{515}$ of known solutions of Trolox™ against concentration, where

$$\%\Delta A_{515} = \frac{A_{515}^{t=0} - A_{515}^{t=30}}{A_{515}^{t=0}} \times 100$$

($t=0$: the absorbance of the control reaction at time 0, $t=30$: the absorbance in the presence of the sample of the extracts after 30 min of reaction). The wavelength to measure DPPH• absorbance was 515 nm. Results were expressed as μmol TRE per g of dry propolis weight.

Determination of Total Polyphenol Content (TPC)

The total polyphenol content (TPC) from propolis extract was determined using the Folin–Ciocalteu method, as adapted in microscale by Arnous et al.,[68] with slight modifications. In a tube, 3.16 mL of distilled water, 0.04 mL of sample and 0.2 mL of FolinC-> Ciocalteu reagent were mixed. After shaking and resting for 1 min, 0.6 mL of sodium carbonate (20% w/v in distilled water) was added, the sample was mixed with vortex and stored in the dark for 120 min. Absorbance of the samples was measured at 750 nm using quartz cuvettes, at a UV/VIS spectrophotometer and the final results were expressed as mg gallic acid equivalents (GAE) per g of dry propolis weight.

Determination of the Total Flavonoid Content (TFC)

A previously published protocol was used,[69] with modifications. An aliquot of 0.5 mL of sample was mixed with 500 μL $AlCl_3$ reagent (2% [w/v] $AlCl_3$ in 5% [v/v] acetic acid in methanol) and 700 μl 5% [v/v] acetic acid in methanol and allowed to stand for 30 min at room temperature. The absorbance was obtained at 415 nm (A_{415}) using deionized water as blank solution and the TFC was calculated from a calibration curve, constructed with quercetin as the calibration standard. TFC was expressed as micrograms of quercetin equivalents (QE) per gram of dry propolis weight.

Statistical Analysis

Data were summarized by estimating absolute and relative frequencies (percentages %), measures of central tendency (mean values ± Standard Error-SE), measures of variability (minimum and maximum values) and measures of association (Spearman's *rho* rank correlation coefficient). The association between the three variables AA, TPC and TFC was graphed and studied by inspecting the corresponding scatter plots. The best fitted line was plotted using the Loess method.[70] Comparisons among groups of samples (e.g., areas, seasons and color) were performed according to the following method: for the two groups' comparison, relative to the distribution of AA, TPC and TFC, the Mann–Whitney test was performed; for comparisons among multiple groups, pair-wise Mann–Whitney (M–W) tests were performed, but only when statistically significant result from an omnibus Kruskal–Wallis (K–W) test were obtained. The inherent variability of the samples according to their qualitative characteristics (AA, TPC and TFC) was depicted by plotting (1×2 factorial plan) the Principal Components Analysis (PCA) results. PCA was performed without the rotation of the factorial axes. For exploring the possibility of a 'blind' grouping of the samples based on their three qualitative characteristics, the Hierarchical Cluster Analysis method was applied on the *z*-scores of the three variables (AA, TPC and TFC). The squared Euclidean distance was chosen as a dissimilarity measure among the samples in combination with the Ward's criterion/method for cluster merging.[71,72] The contribution of each of the three variables in cluster formation was identified by examining the magnitude and the statistical significance of the corresponding η^2 (eta squared) coefficients estimated by the application of a series of one-way ANOVAs; cluster membership was used as the independent variable and the values of AA, TPC and TFC as the dependent variables. The value of η^2 indicates the percentage of variance of the examined variable accounted by the differences between the clusters.[73] In all hypotheses-testing procedures (K–W and M–W

tests), the observed significance level (*P* value) was estimated by the Monte–Carlo simulation method, utilizing 10,000 random samples.[74] This method leads to safe inferential conclusions even in cases where the methodological assumptions of the non-parametric tests are not fulfilled (i.e., large samples, random samples, independent measurements, symmetrical distributions, absence of 'heavy' outliers). The significance level in all statistical tests was predetermined at $a = 0.05$ (or $P \leq 0.05$). All statistical analyses were done with the IBM SPSS v24.0 software enhanced with the module Exact Tests (for the implementation of Monte–Carlo simulation method).

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Author Contribution Statement

A.P. and E.K. performed the experiments at the apiaries and analyzed propolis samples. A.P., E.K. and I.M. wrote the manuscript. G.M. performed statistical analysis. K.G. designed the experiment with A.P. and I.M. and performed preliminary analyses.

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