UNIVERSITY OF THE
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Department of Food Science & Nutrition

DISSERTATION STUDY

Determination and Comparison of Antioxidant Activity and Phenolic Load of Lemnos honey, Samothrace honey and Manuca Honey.

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Determination and comparison of antioxidant activity and phenolic load of Lemnos honey, Samothrace honey and Manuka honey

Dissertation study

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ABSTRACT

Apart from being food, honey has also therapeutic and medicinal properties, known since antiquity. These properties differ between honeys with different plant origins and are largely due to substances such as polyphenols and especially flavonoids.

In this dissertation study, 3 types of honey were studied: Lemnos honey from thyme, Samothrace honey from the plant known as Greek strawberry tree (*Arbutus andrachne*) and Manuka honey. Total phenolics, antioxidant capacity and total flavonoids were determined. It turned out that Samothrace honey, for which there are no bibliographic references, is superior in both antioxidant activity and total phenolics. However, the index of its total flavonoids is not so high, which is worth discussing.

Chapter 1 Introduction

In recent years, the trend for healthy and less processed foods has been increasing. A typical example is honey. Honey is a natural product known since antiquity for its therapeutic and nutritional properties **(Fatin Aina Zulkhairi Amin et al, 2018)**.

There are about 320 different varieties of honey that come from various botanical sources. The taste, the colour and the smell of a particular type of honey depend on the various sources of nectar and honeydew of the flowers and plants visited by the bees. The different types of honey are affected by temperature, rainfall and seasonal and climatic changes. The colour of honey ranges from light brown to dark brown **(Sultan Ayoub Meo,Saleh Ahmad Al-Asiri et al, 2017)**.

Honey is not only considered a nutritious food but also stands out as a functional food. Depending on the type of honey, substances that give its antioxidant and antimicrobial activity have been identified in different quantities, such as polyphenols, terpenes, hydrogen peroxide, etc. **(Viuda-Martos et al, 2008).**

One of the honeys examined in this dissertation is Samothrace honey which is produced from the wild arbutus plant known as Greek strawberry tree (*Arbutus andrachne*) and has not been studied before. In order for its properties to become known, it is compared to Manuka honey, produced in Oceania from the plant *Leptospermum scoparium*, known for its many therapeutic properties against many diseases, as well as to the honey of Lemnos, which comes mainly from the plant thyme (*Thymus capitatus*), the plant called the lavender-leaved anthyllis (*Anthillis hermanniae*) and from secondary botanical sources of the endemic flora of the island.

The purpose of the dissertation is to determine the chemical characteristics (total phenolics, antioxidant capacity and flavonoids) of all three types of honey (Lemnos honey from thyme, Samothrace honey from Greek strawberry tree and Manuka honey).

Chapter 2- Literature review

2.1 Honey

Honey is a natural substance produced and stored in honeycombs by bees with carbohydrates that make up about 95 to 97% of the dry weight of honey. Fructose and glucose are the predominant sugars present and they are responsible for most of the physical and nutritional characteristics of honey. Volatile compounds found in honey include alcohols, ketones, aldehydes, acids, esters, and terpenes. Phenolic acids (benzoic and cinnamic acids) and flavonoids (flavonones, flavanols) contribute to the healing capacity of honey, which varies greatly depending on its botanical origin, significantly **(Yalemwork Ewnetu, Wossenseged Lemma et al, 2013)**.

Research has shown that the reason for the different colour, taste and functional properties of honey is mainly due to its phenolic composition and not to its differentiation in relation to other components e.g. carbohydrates, proteins, etc. **(Fatin Aina Zulkhairi Amin et al, 2018)**.

The consumption of honey has a very long history among people. It has been used in countless foods and beverages as a sweetener and flavouring agent. Since ancient times, honey has been known for its nutritional and therapeutic values. Honey is produced all over the world. Historically, the Egyptians suggested honey for fertility. In addition, many cultures have traditionally consumed honey to enhance the vitality of males. Honey has been suggested to men with impotence problems and to women with infertility-related problems, including irregular ovulation **(Sultan Ayoub Meo,Saleh Ahmad Al-Asiri et al, 2017)**.

Its high caloric value (300 kcal per 100g) makes it suitable for athletes as it contains easily digestible glucose, which is converted into energy in a short time **(Fatin Aina Zulkhairi Amin, Suriana Sabri et al, 2018)**.

2.1.1 Components of honey

Carbohydrates are the main components of honey. The monosaccharides fructose and glucose are the dominant fraction and account for 85-95% of honey sugars, while the rest is represented by a number of different di- and trisaccharides. Fructose is reported as the most abundant sugar found in honey representing about 31-39% **(Bogdanov et al, 1996)**.

In addition, honey contains amino acids, trace elements B, Vitamin B6, Vitamin C, niacin, folic acid, minerals, iron, zinc and antioxidants **(Sultan Ayoub Meo,Saleh Ahmad Al-Asiri et al, 2017)**.

80% of honey consists of sugars while 17% of it is water and the remaining 3% consists of vitamins, enzymes, amino acids, etc. **(Fatin Aina Zulkhairi Amin, Suriana Sabri et al, 2018)**.

2.1.2 Properties of honey

There are numerous studies which constantly prove that honey has very important medicinal and therapeutic properties. The existence of a 100% natural product, without further processing and additions, with such a strong therapeutic effect, attracts the interest of both scientists and the public.

The therapeutic properties of most types of honey probably depend on their natural origin. Honey has excellent antibacterial, anti-inflammatory and bacteriostatic properties for wounds and sunburns **(Fatin Aina Zulkhairi Amin, Suriana Sabri et al, 2018)**. The practice of covering wounds with honey is gaining popularity in modern medicine as a result of its antimicrobial function. In addition, some specific types of honey develop a broad-spectrum antimicrobial role against bacterial pathogens which are resistant to antibiotics **(Sultan Ayoub Meo,Saleh Ahmad Al-Asiri et al, 2017)**.

Studies have shown that honey can act as a very important factor in the treatment of chronic pathological conditions e.g. cancer, due to its excellent anti-inflammatory activity **(Badolato et al, 2017), (Ewnetu et al, 2013), (Aina et al, 2018), (Meo et al, 2017)**. It is worth noting that the consumption of honey increases the absorption rates

of magnesium and calcium resulting in the structural strengthening of bones and teeth **(Fatin Aina Zulkhairi Amin, Suriana Sabri et al, 2018)**.

Honey has been proven to have positive effects both in the treatment of ophthalmological diseases e.g. blepharitis, corneal injury and conjunctivitis, and in fertility by enhancing the corresponding hormones **(Fatin Aina Zulkhairi Amin, Suriana Sabri et al, 2018)**.

2.1.3 Antibacterial activity

The antibacterial activity of honey depends on:
1) osmotic stress due to high sugar concentration
2) low pH (between 3.2 and 4.5)
3) the presence of hydrogen peroxide (H_2O_2) which increases the activity of 2 enzymes, glucose oxidase and catalase.

It is believed that the latter is mainly responsible for the antibacterial activity of honey. However, after the neutralization of H_2O_2 by catalase, some honeys maintain high levels of antibacterial activity, referred to as non-peroxide activity (NPA). NPA was first observed in Manuka honey of New Zealand. Active honeys of Manuka type from New Zealand and Australia have now been proven to have significantly higher levels of NPA than honeys from other plant sources. This is due, in part, to the high concentrations of the natural chemical methylglyoxal (MGO) contained in honeys from this plant species **(Daniel Bouzo, Nural N. Cokcetin, Liping Li, Giulia Ballerin et al, 2020)**.

An additional factor that has been proven by recent studies to be responsible for the antibacterial activity of honey is the minimum inhibitory concentration (MIC), i.e. the minimum concentration of honey required to inhibit microbial growth **(Matthew Johnston, Michael McBride et al, 2018)**.

2.1.4 Antioxidant activity

In addition to the antibacterial effects of honey, its antioxidant properties are also considered important. The antioxidant activity of honey is mainly attributed to its polyphenols (e.g., flavonoids and phenolic acids), antioxidant enzymes (e.g., catalase

and peroxidase), vitamins (e.g., vitamin C), Maillard reaction products (e.g., melanoids), and carotenoids and amino acids (e.g., proline). Several studies have reported that the antioxidant compounds of honey can prevent pathological conditions caused by oxidative stress **(Stagos D., Soulitsiotis N. et al, 2018)**.

Examples of phenolic acids responsible for the antioxidant and anti-inflammatory activity of honey are gallic acid and quercetin **(Laura M. Porcza,Claire Simms and Mridula Chopra 2016).**

It is a fact that foods containing antioxidants help promote health. According to literature, honey contains strong antioxidants. As an antioxidant, honey has many preventive properties against many clinical conditions such as inflammatory disorders, coronary artery diseases, neurological deterioration, aging, and cancer **(Sultan Ayoub Meo,Saleh Ahmad Al-Asiri et al, 2017)**.

2.1.5 Manuka honey

Manuka honey is produced throughout New Zealand and Australia by bees that collect the nectar of the *Leptospermum scoparium* / Tea tree shrub. Published evidence on Manuka honey proves that it is one of the best natural antibiotics in the world with very specific therapeutic properties **(Sarfarz Ahmed and Nor Hayati Othman ,2013), (Gopal Shankar,Krishnakumar[a] et al, 2020)**.

The composition of Manuka honey consists of carbohydrates, minerals, proteins, fatty acids, phenolic and flavonoid compounds. What sets Manuka honey apart from other types of honey is an unusually high level of methylglyoxal (MGO) formed of dihydroxyacetone (DHA) and associated with its antibacterial activity. Also, the appearance of leptosperin is another unique element of Manuka honey **(Sherlock O., Dolan A., Athman R. et al. 2010),(Matthew Johnston, Michael McBride et al, 2018),(Daniel Bouzo, Nural N et al, 2020)**.

The additional factor contributing to the antibacterial activity of Manuka honey is glycoside called leptosine, which has recently been identified **(Gopal Shankar,Krishnakumar[a] et al, 2020)**.

According to **Maria J. Fernandez-Cabezudo et al. (2013)**, it has been studied the potential effect of Manuka on cancer cell proliferation. The results showed that in vitro treatment of cancer cells with low Manuka concentrations resulted in significant inhibition of cell proliferation.

2.1.6 Greek honeys

Greece is a country with rich flora and a high percentage of endemic plants. Given this, it includes a wide variety of types of honey derived from thyme, pine, fir and numerous other plants. Following studies, **(AnnaV.Tsiapara[a],MariJaakkola[b] et al. 2009)** it has been shown that some varieties of Greek types of honey, especially thyme honey, are very rich in compounds known to have anti-cancer properties, such as polyphenols and phenolic acids.

Greece produces 13,000-15,000 tons of honey per year, 60-65% of which is pine honey, 10% thyme honey, 10% citrus honey and 5-10% fir honey. Chestnuts, heather, oak and cotton honey are produced in smaller quantities. At present, the only honey classified as a PDO in Greece is that of Menalon vanilla honey from Arcadia in southern Greece **(Karabagias et al. 2014).**

It is worth noting that about 80% of the honey produced in Greece is from honeydew (pine, fir, etc.). Honeydews are some sugary juices that are created on some plants after insect parasitism and which are a first-class food for bees, just like nectar. It has also been reported that the antioxidant and antibacterial activity of honeydew is higher compared to nectar **(Karabagias et al. 2020).**

Chapter 3- Methodology

3.1 Materials

Three types of honey were studied, each from a different origin (figure 1):

-Lemnos honey: Collection on the island of Lemnos, by the Agricultural Beekeeping Cooperative of Lemnos, Summer 2019.

-Samothrace honey: Collection on the northeast side of the island of Samothrace, Spring 2019.

-Manuka honey: Commercial sample of the company Manuka Health MGO 30+, from New Zealand (year of collection 2019)

The honeys were compared to syrup, a preparation-control created by mixing 40% of glucose and 30% of fructose (the main sugars in honey) in water.

Figure 1. The three honeys studied. A: Samothrace honey, B: Manuka honey, C: Lemnos honey.

There were also used the following:

2.2-diphenyl-picrylhydrazyl (DPPH) from Scientific Industries Inc. (New York, USA).

Trolox and gallic acid derived from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany).

Folin-Ciocalteau from Merck (Darmstradt, Germany) and AlCl3 from Fisher (Fair Lawn, NJ).

Finally, there were used the following:

The ONDA SPECTROPHOTOMETER V-10 PLUS (VIS) photometerandthe MICROPLATE READER TECAN SPARK (SPARKCONTROL agent).

3.2 Methods

3.2.1 Determination of antioxidant capacity with the DPPH method

For the determination of antioxidant capacity, it was used the 2.2-biphenyl-1-picryl-hydrazylth free radical (DPPH) binding method **(Stagos et al. 2018)**. After appropriate dilutions (figure 2) so that the readings of the photometer are within limits (DPPH value near the unit), 25μL of sample were mixed with 975μL of DPPH reagent. Test-tube racks with eppendolf tubes were used, 3 for each sample, which were left in a dark place for 30 minutes after stirring with vortex .

Figure 2. Preparation of DPPH solution

Then, it was performed photometry at 515nm using cells and the results were recorded. In addition, photometry was also carried out on a control sample, which contains only the DPPH reagent without the addition of a sample. The results are expressed in mM Trolox Equivalents/L solution (TRE) (Trolox -analogue of vitamin E- with the ability to bind the free radical DPPH*), through the equation:

AA (mM TRE) = 0.016- %SA* 0.034

which resulted from linear regression (figure 3), after correlation of
%SA of Trolox reagent (Sigma-Aldrich Chemie GmbH, Germany) and its concentration (0.1-1.6 mM Trolox) with R^2 =0.998, where:

%SA = (Ablank-Asample)/Ablank * 100.

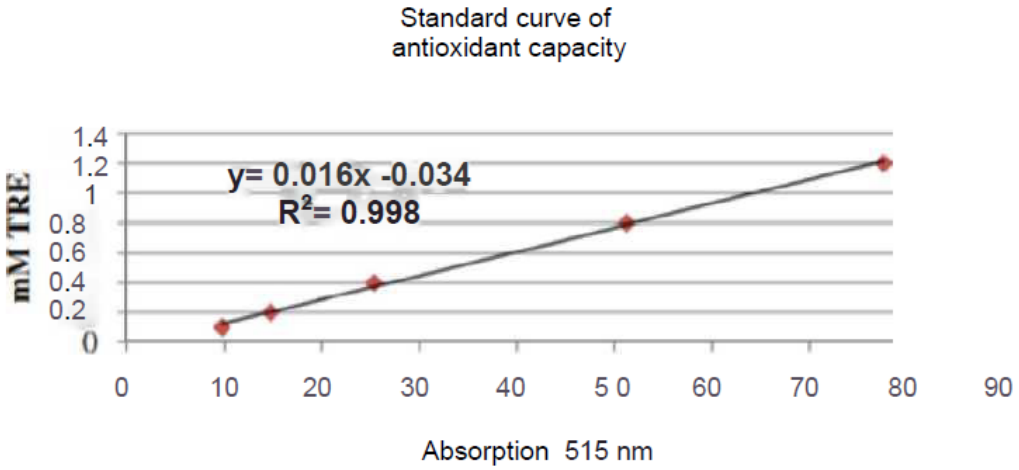

Figure 3. Standard curve of antioxidant capacity (Trolox equivalents)

3.2.2 Determination of antioxidant capacity with the FRAP method

The FRAP method was used to determine antioxidant capacity. The FRAP analysis was conducted according to Benzie & Strain (1996). The FRAP reagent was prepared by mixing acetate buffer (0.3mol / L), TPTZ (10mmol / L) and $FeCl_3,6H_2O$ (20mmol / L) in a ratio of 10:1:1 **(Alessandro C, MartinsLaisBukman et al,2013)**.

For the measurement, 20μL of a sample of appropriate dilution and 80μL of FPAP were added to the plate. Subsequently, the samples were measured in a UV-Vis spectrophotometer at 595nm after 30 minutes in a dark place **(Alessandro C, MartinsLaisBukman et al,2013)**. Before the measurement, the blank sample was reset to zero and placed in the back position of the photometer. The extraction solvent of the food or water were used as a blank sample.

3.2.3 Determination of total phenolic compounds

For the determination of total phenolic compounds it was used the Folin - Ciocalteau method **(Meda, A., Lamien et al, 2005)**. Initially, 2370μL of distilled water was mixed with 30μL of extract properly diluted to keep the indications within the standard curve. After shaking, 50μL of Folin – Ciocalteau reagent were added. Stirring followed again and after 1min 450μL of saturated sodium carbonate solution (Na_2CO_3 20% w/v) were added. The mixture was shaken and stored in a dark place at room temperature for 2 hours. The reaction product was measured at 750nm. In the blank sample prepared for zeroing it was placed methanol in the place of the sample **(Socha, R., Juszczak, 2009), (Magalhaes LM, Santos F, 2010),(Isabel C.F.R.Ferreira EdmurAiresJoão C.M.Barreira, 2009)**. The concentration of polyphenols in the sample is calculated using a Gallic acid reference curve (figure 4) and expressed as gallic acid equivalents via the equation with R^2 =0.9913:

TPC (mg GAE/L) = 0.0011* ASample – 0.0129

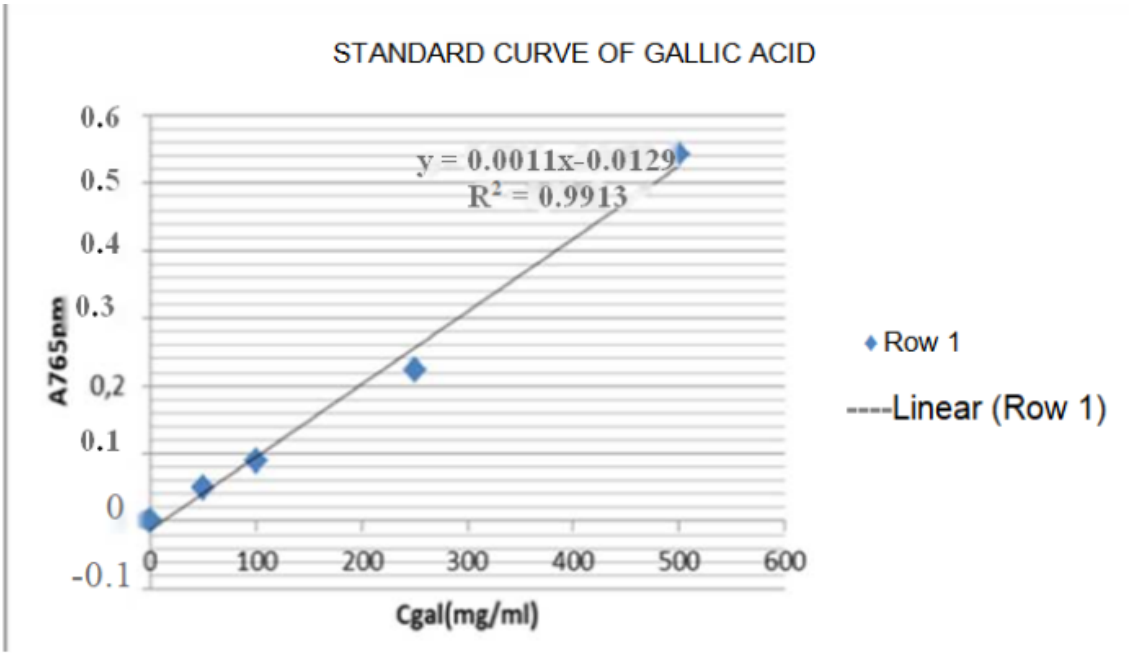

Figure 4 Standard curve of total phenolic compounds (Gallic acid equivalents)

3.2.4 Determination of total flavonoids

For the determination of total flavonoids, initially 120μL of AlCl3 solution (2% AlCl3 in methanol/acetic acid mixture, 95/5, v/v) and 1680μL of 5% methanolic acetic acid solution were added successively in 1.2mL of extract of suitable dilution. The absorption of the formed complex was measured at 415nm with reference solution after 30 min left at room temperature to react. Correction of the absorption values was performed by removing the initial absorption (415nm) of a corresponding solution in the reaction environment in the absence of the reagent **(Meda, A., Lamien, 2005), (Isabel C.F.R.Ferreira EdmurAiresJoão C.M.Barreira, 2009)**. The results are expressed in mM of quercetin (figure 5) extract through the equation with R^2 =0.9979:

TFC (mM quercetin) = 0.0111 * ASample + 0.0178

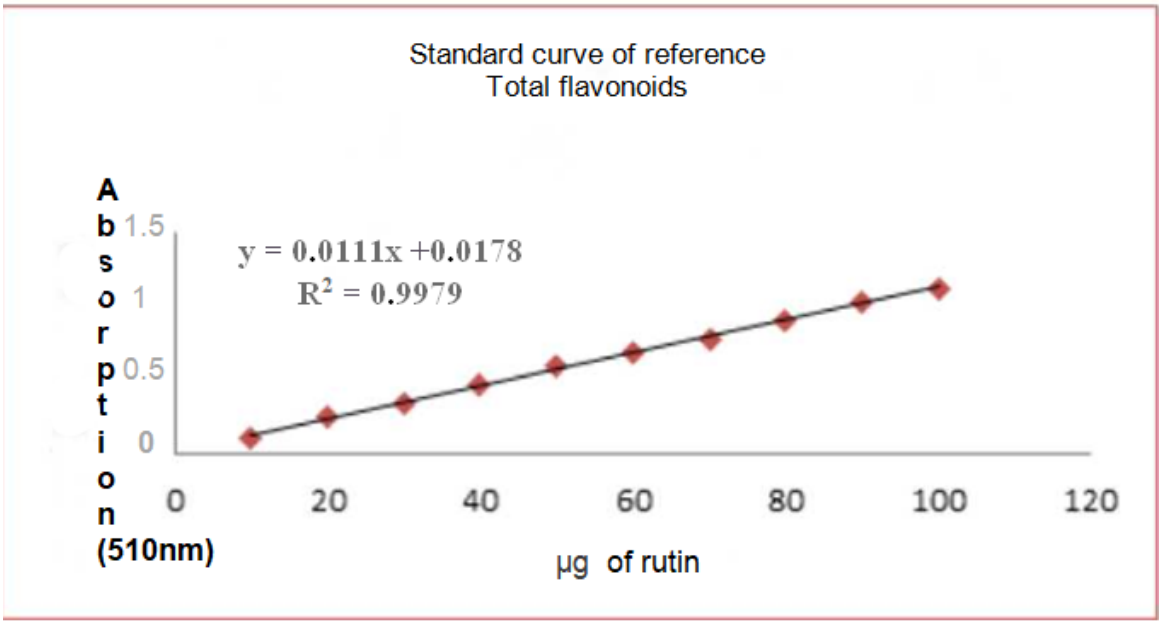

Figure 5: Standard curve of total flavonoid compounds (querquetin equivalents)

3.2.5 Statistical processing of the results

The results were processed with the statistical package GraphPad v3. Measurement average values were compared using One-Way ANOVA.

Chapter 4- Results and Discussion

4.1 Comparison of antioxidant activity using DPPH

The results from the analysis of the honeys for their antioxidant activity using DPPH are presented in Table 1.

Table 1. Values shown by the honeys under study during the check of their antioxidant activity using DPPH (mmol trolox equiv./g honey)

	N	Mean	St. Error of Means	Standard	Median
Samothrace	**3**	64.545	0.2525	0.1458	64.600
Lemnos	**3**	7.389	0.2133	0.1232	7.470
Manuka	**3**	37.661	2.318	1.338	37.496
Control	**3**	-56.948	1.129	0.6517	-56.948

The comparison of average values performed using One-Way ANOVA (table 2) showed that there were significant statistical differences between all types of honey with a significance level of $p<0.0001$ (Figure 6).

The honey that showed higher antioxidant activity was Samothrace honey (mean=64.54, sd=0.25). Manuka honey (mean=37.66, sd=2.31) and Lemnos honey (mean=7.38, sd=0.21) followed next. Finally, not only did not the control cause a decrease in the oxidative activity of DPPH but it also increased it instead (mean=-56.94, sd=1.12).

Table 2. Comparison of the honeys for their antioxidant activity using DPPH

Comparison	Mean Difference	Q	P value
Samothrace vs Lemnos	57.156	76.167	***P<0.001
Samothrace vs Manuka	26.884	35.826	***P<0.001
Samothrace vs Control	121.49	161.90	***P<0.001
Lemnos vs Manuka	30.272	40.341	***P<0.001
Lemnos vs Control	64.337	85.737	***P<0.001
Manuka vs Control	94.609	126.08	***P<0.001

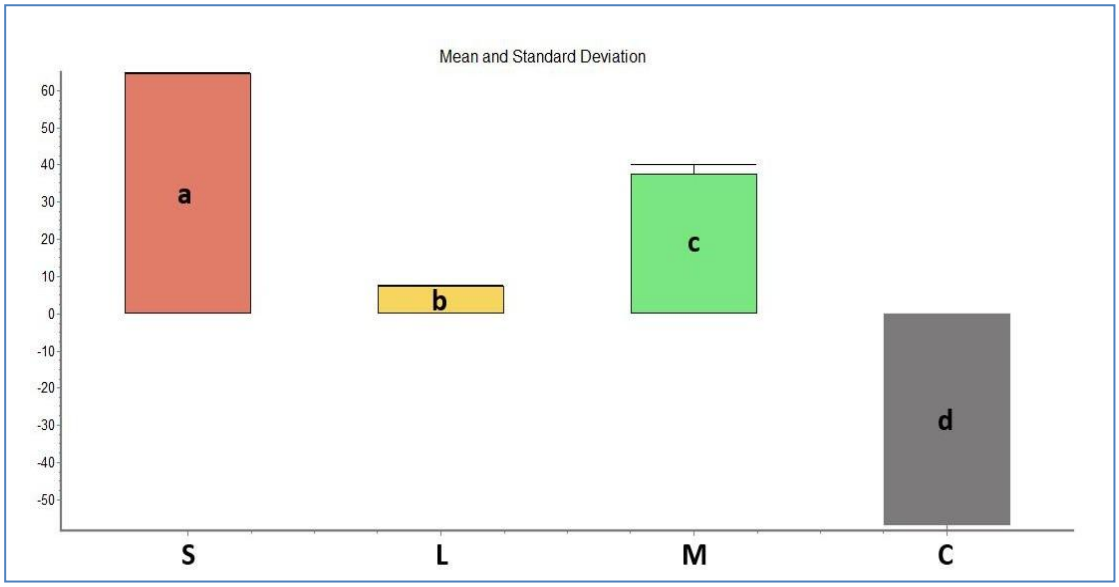

Figure 6. Comparison of average values and standard deviation of the antioxidant activity of the honeys studied using DPPH (S: Samothrace, L: Lemnos M: Manuka C: Control). Average values marked with a different index (a,b,c,d) differ statistically from each other.

4.2 Comparison of antioxidant activity using FRAP

The results from the analysis of the honeys for their antioxidant activity with the FRAP method are presented in Table 3.

Table 3. Values shown by the honeys under study during the check of their antioxidant activity using FRAP

	n	Mean	St. deviation	St. Error of Means	Median
Samothrace	9	1.194	0.1793	0.05978	1.171
Lemnos	9	0.4425	0.1147	0.03822	0.4258
Manuka	9	0.6964	0.1534	0.05113	0.7343
Control	3	0.1229	0.008421	0.004862	0.1202

The comparison of average values performed using One-Way ANOVA (table 4, figure 7) also showed that there were significant statistical differences with a significance level of $p<0.0001$.

Table 4. Comparison of the honeys for their antioxidant activity using FRAP

Comparison	Mean Difference	Q	P value
Samothrace vs Lemnos	0.7516	15.490	*** P<0.001
Samothrace vs Manuka	0.4976	10.257	*** P<0.001
Samothrace vs Control	1.071	15.611	*** P<0.001
Lemnos vs Manuka	-0.2539	5.234	** P<0.01
Lemnos vs Control	0.3196	4.657	* P<0.05
Manuka vs Control	0.5735	8.358	*** P<0.001

It was also shown in this method that Samothrace honey presented higher antioxidant activity (mean=1.194 & st=0.179), followed by Manuka honey (mean= 0.696 & sd=0.153) and finally, Lemnos honey (mean=0.442 & sd=0.114). Through the values of the control (mean=0.122 & sd=0.0084) we can understand how strong the antioxidant activity of Samothrace honey is.

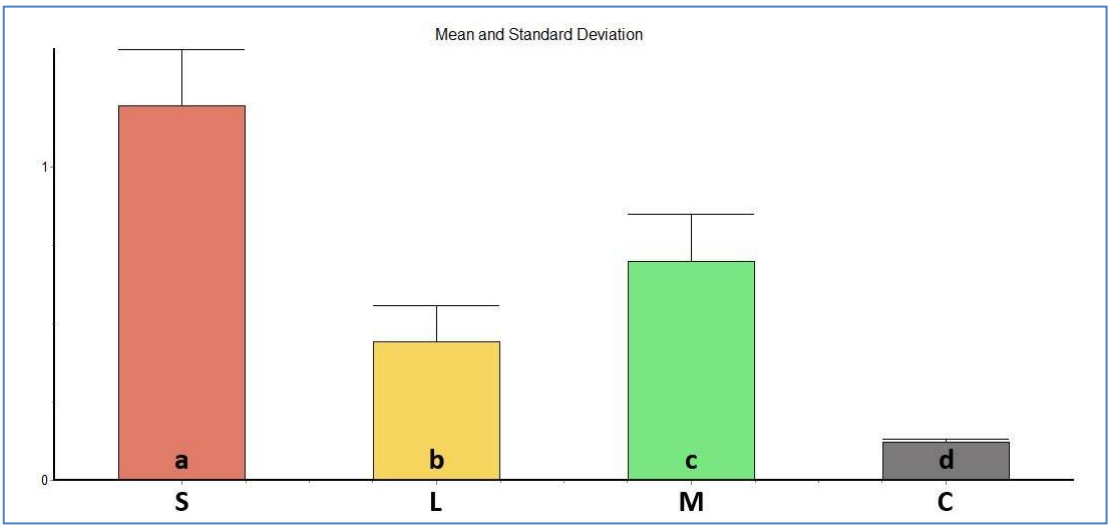

Figure 7. Comparison of average values and standard deviation of the antioxidant activity of the types of honey studied using FRAP (S: Samothrace, L: Lemnos M: Manuka C: Control). Average values marked with a different index (a,b,c,d) differ statistically from each other.

4.3 Comparison of total phenolic compounds

The results from the analysis of the honeys for total phenolics are presented in Table 5.

Table 5. Total phenolic values presented by the honeys under study (mg/L gallic acid)

	n	St.Error of Means	St.Deviation	Mean	Median
Samothrace	9	55.707	13.149	4.383	56.013
Lemnos	9	8.525	2.236	0.7453	7.516
Manuka	9	39.861	3.209	1.070	39.863

The comparison of average values performed using One-Way ANOVA (table 6, figure 8) also showed that there were significant statistical differences with a significance level of p<0.0001.

Table 6. Comparison of the phenolic load of the types of honey studied

Comparison	Mean Difference	Q	P value
Samothrace vs Lemnos	47.182	17.871	*** P<0.001
Samothrace vs Manuka	15.846	6.002	*** P<0.001
Lemnos vs Manuka	-31.336	11.869	*** P<0.001

It was shown that Samothrace honey once again won the first place in polyphenols (mean= 55.707 & sd=13.149). Then, Manuka honey also presented quite high values (mean= 39.861 & sd=3.209) while Lemnos honey conquered the last place (mean=8.525 & sd=2.236). No control was used in this test because the solution prepared for the use of the control did not contain phenolics or flavonoids. After all, its inclusion in this experiment could cause discrepancies in the results of the comparisons using ANOVA.

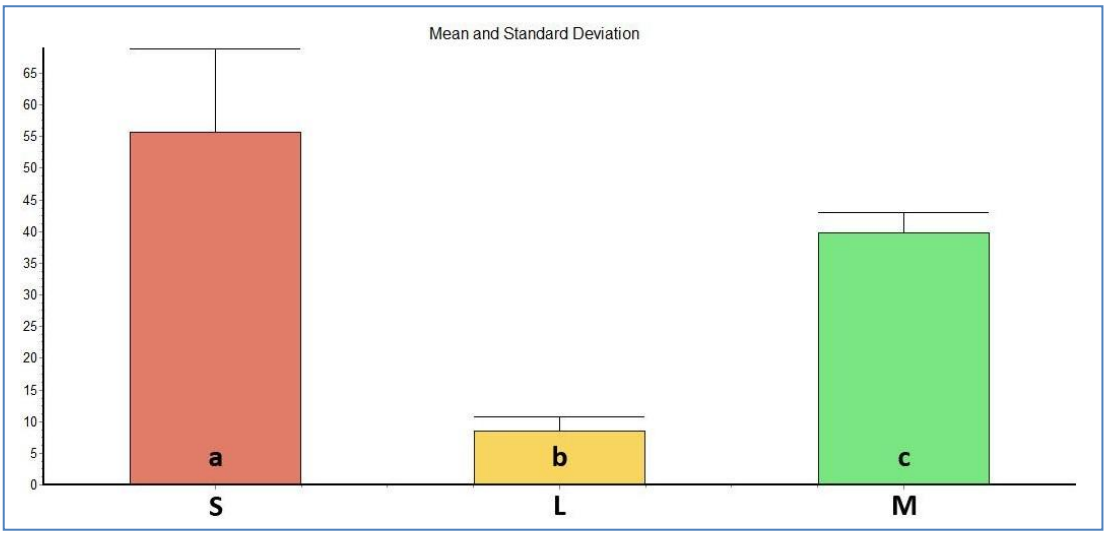

Figure 8. Comparison of average values and standard deviation of total phenols of the honeys studied (S: Samothrace, L: Lemnos M: Manuka). Average values marked with a different index (a,b,c) differ statistically from each other.

4.4 Comparison of flavonoids

The results from the analysis of the honeys for flavonoids are presented in Table 7.

Table 7. Values of total flavonoid load presented by the honeys under study (mg quercetin / g honey)

	n	St. Error of Means	St.Deviation	Mean	Median
Samothrace	9	4.810	1.597	0.5322	4.316
Lemnos	9	3.264	1.222	0.4072	2.807
Manuka	9	6.471	1.244	0.4146	7.260

The comparison of average values performed using One-Way ANOVA (table 8, figure 9) also showed that there were significant statistical differences with a significance level of p<0.0002.

Table 8. Comparison of the flavonoid load of the types of honey studied

Comparison	Mean Difference	Q	P value
Samothrace vs Lemnos	1.546	3.399	ns P> 0.05
Samothrace vs Manuka	-1.660	3.650	* P<0.05
Lemnos vs Manuka	-3.207	7.048	*** P<0.001

The comparison of average values showed that Manuka honey (mean= 6.471 & sd=1.244) had the highest flavonoid load. It differed significantly both from Samothrace honey (mean=4.810 & sd=1.597) and from Lemnos honey (mean=3.264 & sd=1.222). It is worth noting that Samothrace honey, which had the second highest flavonoid load, did not differ significantly from the one from Lemnos (ns p>0.05).

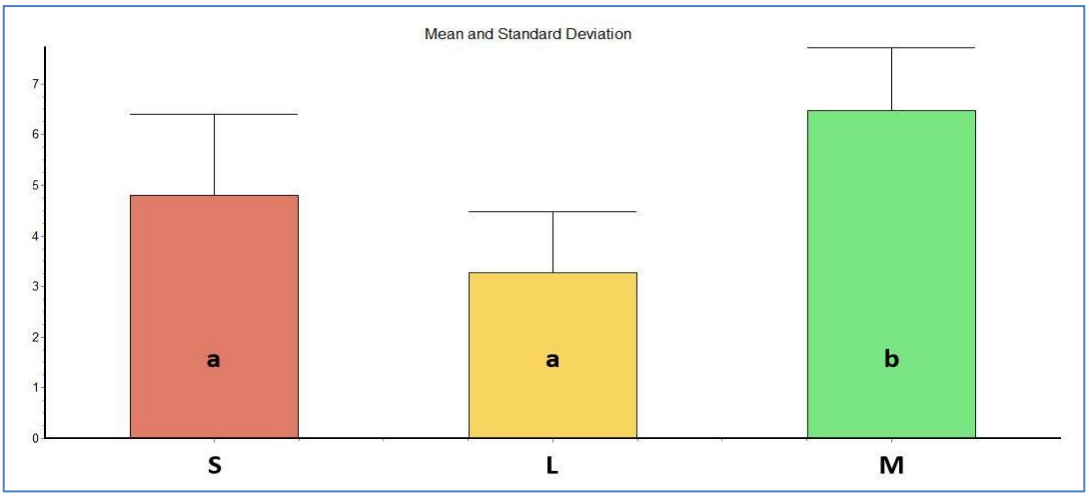

Figure 9. Comparison of average values and standard deviation of the flavonoids of the types of honey studied (S: Samothrace, L: Lemnos M: Manuka). Average values marked with a different index (a,b,c) differ statistically from each other.

An interesting observation is that while Samothrace honey showed higher antioxidant activity and phenolic load, it did not show a corresponding effect in the flavonoid load. It is known that flavonoids are phenolic substances with high antioxidant activity **(A. N. Panche, A. D. Diwan et al, 2016), (Pier-Giorgio Pietta, 2000)**. This leads to the conclusion that some other flavonoid species exist in this particular type of honey that have not been identified yet. According to **Papachristoforou et al, (2019)**, something similar was also observed in the red propolis found on the same island, whose strong antioxidant activity was not associated with the load of polyphenols and flavonoids that it contained.

Chapter 5-Conclusions

In conclusion, all three honeys studied were found to have antioxidant activity, a load of polyphenols and flavonoids, which can give them the classification of functional food.

The honey that showed the highest antioxidant activity and total phenolic index was Samothrace honey. Manuka honey followed next and last was Lemnos honey.

However, the index of flavonoids showed that Manuka honey had a higher concentration than Samothrace honey, which would be expected based on the previous results.

Therefore, Samothrace honey deserves to be further studied for additional polyphenolic or other substances (e.g. terpenic substances), which seem to have led to the specific results.

Chapter 6-References

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It is hereby certified that, to the best of the signer's knowledge and beliefs, this is a true and accurate translation of the attached Greek document into English.

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