Quality evaluation and biological properties of Algerian commercial honeys labeled as Rosemary, Tamarisk, Thistle and Multiflora

Kheira Moufida Mouffok

Dissertation submitted to Escola Superior Agrária de Bragançca to obtain the Degree of Master in Biotechnological Engineering under the scope of the double diploma with Université Moulay Taher in Algeria

Supervised by

Miguel José Rodrigues Vilas Boas

Soraia Isabel Domingues Marcos Falcão

Ziani Kadour

Bragança

2021

ACKNOWLEDGEMENTS

With the elaboration of this work, I can say that another great stage of my life has been completed. I want to thank all the people who directly or indirectly contributed to this dream becoming real. Therefore, I´m grateful to Allah, I would also thank my parents and sisters, for the power transmitted and encouragement, for all the effort they made so that I could get here because without them I could not achieve this master's goal.

First, I want to thank Professor Miguel Vilas Boas for dragging me to this great school, this great institution (IPB), for all the knowledge he has transmitted to me, for patience, dedication, permanent availability, support, advice, friendship, and good disposition. I thank you, for the theme that I proposed to develop, which made me interested in the world of the honeybee, for the essence of honey that I somehow did not know, although bees are not my best friends. A thank you very much!

I would also like to thank my advisor Dr. Soraia Falcão for her wise advice and suggestions, for her patience, and her support in all the guiding moments of this work. Thank you very much!

I would also like to thank Professor Paulo Russo-Almeida from *LabApis*[UTAD], for its collaboration in the melissopalynological analysis.

I would also thank my supervisor in Algeria Dr. Kadour Ziani for all his commitment, professionalism, understanding, patience, and support demonstrated throughout this work.

I also thank Andreia Tomás for the availability shown in the laboratory and for the transmission of knowledge, companionship, help, guidance, patience, advice, encouragement, goodwill, friendship and understanding demonstrated throughout the work.

To every CIMO lab team that has always made themselves available to assist in whatever was necessary, for having integrated me and for the knowledge they have transmitted to me.

It is with great satisfaction and pride that I thank all those who have contributed in some way to the accomplishment of this work. To all who have supported me, helped, encouraged, and accompanied me during the last few years on this journey and who are not mentioned here. Thank you so much for your friendship.

TABLE OF CONTENTS

ABSTRACT

Honey is considered a natural sweet substance produced by honeybees, from the nectars of plant flowers and honeydews. Honey has always been regarded as a food that is beneficial for human health with several therapeutic qualities described. The quality of honey is still a top concern for experts as no good method has been defined so far for the simultaneous detection of different types of honey. Consequently, the development of easy, quick, precise analytical tools that may give data for assessing honey authenticity, is important. Because of that, it is essential to inform consumers of the mislabeling of honey with lower quality. This study aimed to evaluate the physicochemical characteristics and to assess the quality of Algerian honey from different botanical and geographical origins. For that, ten samples of honey with different marked botanical origins were analyzed, including three samples from rosemary honey, three from tamarisk honey, three from milk thistle honey and one multiflora honey. The quality of the samples was determined through different parameters. Melissopalynological and physicochemical analyses (color, moisture, pH, acidity, electrical conductivity, diastase index, proline, 5-hydroxymethylfurfural, mineral content, proteins, carbohydrates, energy, and ash) were performed, as well as the profile evaluation of sugar and phenolic compounds. Antioxidant activity (reducing power and DPPH free radical scavenging activity) antitumor and anti-inflammation activity were also evaluated. Finally, the presence of antibiotics, recurrent residues in honey, such as tetracyclines and sulphonamides were screened using the multi-analyte receptor assay system Charm II.

The melissopalinological analysis showed the presence of 10 major types of pollen grains, with *Rosmarinus officinalis*, *Cytisus stratitus* and *Centaurea* sp. pollens as the most abundant. Furthermore, since no honeydew elements were detected, all the samples were classified as nectar honeys. Samples R1, R2, and R3 were classified as rosemary monofloral honey; samples T1, T2 and MF were classified as *Cytisus striatus* honey; CH1-CH3 were classified as *Centaurea* sp. and T3 as multifloral, which not always agreed with the labeled botanical origin.

Generally, honey samples presented values of moisture, free acidity, 5-HMF, proline content, and diastase index within the limits of the legal requirements, suggesting that the honey was extracted at a correct ripeness stage. The results showed that almost all honey

samples have light amber color, except the rosemary honeys which presented an extra white amber color. Although exhibiting a normal diastase index, the R2 and R3 samples presented a 5-HMF value higher than the admitted in the legislation, suggesting that less adequate heat treatments and/or conservation methods might have been employed. The most common minerals were potassium, sodium, calcium, manganese, while copper and Manganese were present in some samples in minor quantities and the heavy metals (cadmium and lead) are absent from all samples. The sugar profile, analyzed by high pressure liquid chromatography with refractive index detection (HPLC-RI), showed that fructose and glucose were the most abundant compounds, representing more than 60% of total sugars. Other sugars, such as turanose, maltulose and maltose were also detected in a lower proportion. Regarding the phenolic profile, nineteen compounds (eight phenolic acids and seven flavonoids), two isoprenoid compounds (*trans, trans*- and *cis, trans*- abscisic acid), one spermidine and one phenolic diterpene were identified. T2 sample showed a higher amount of phenolic acids than flavonoids. However, the most abundant compounds were the benzoic acid derivative which was detected in all samples. Concerning the evaluation of the antitumor activity and anti-inflammatory activity the samples showed a significant potential. Finally, concerning the antibiotics screening, not all the samples showed negative results.

Keywords: honey**,** rosemary, tamarisk, milk thistle, physicochemical parameters, nutritional value, phenolics compounds, antioxidant activity, anti-inflammatory activity, antitumor activity, antibiotics.

RESUMO

O mel é considerado uma substância doce natural produzida pelas abelhas, a partir dos néctares das flores das plantas e de meladas. O mel sempre foi considerado um alimento benéfico para a saúde, com várias qualidades terapêuticas descritas. A sua qualidade ainda é uma das principais preocupações para os especialistas, pois não há um método ideal para a classificação simultânea de diferentes tipos de mel. Consequentemente, é importante o desenvolvimento de ferramentas analíticas simples, rápidas e precisas que possam fornecer dados que permitam avaliar a autenticidade do mel. Por esse motivo, é essencial informar os consumidores da incorreta rotulagem de méis com baixa qualidade. O objetivo deste estudo foi avaliar as características físico-químicas e desse modo aferir a qualidade de méis argelinos com diferentes origens botânicas e geográficas. Para isso, foram recolhidas dez amostras de méis rotulados com diferentes origens botânicas, nomeadamente: três de mel de alecrim, três de mel de tamarino, três de mel de cardo e um de mel multifloral. A qualidade dos méis foi aferida através de diferentes parâmetros. Foram realizadas análises melissopalinológicas e físico-químicas (cor, humidade, pH, acidez, condutividade elétrica, índice diastático, prolina, 5-hidroximetilfurfural, conteúdo em minerais, proteínas, hidratos de carbono, energia e cinzas), bem como a avaliação do perfil em açúcares e compostos fenólicos. Também foi avaliada a atividade antioxidante (poder redutor e poder bloqueador de radicais livres) e atividade antitumoral e antiinflamatório. Finalmente, a presença de antibióticos, resíduos recorrentes no mel, como tetraciclinas e sulfonamidas, foram investigados através do sistema de despistagem Charm II.

A análise melissopalinológica mostrou a presença de 10 tipos de grãos de pólen maioritários, sendo os pólenes de *Rosmarinus officinalis*, *Cytisus stratitus* e *Centaurea* sp. os mais abundantes. Além disso, e como não foram detetados elementos de melada, as amostras analisadas foram classificadas como méis de néctar: as amostras R1, R2 e R3 foram classificadas como mel monofloral de alecrim; as amostras T1, T2 e MF foram classificadas como mel de *Cytisus striatus*; CH1-CH3 foram classificados como de *Centaurea* sp. e T3 como multifloral, nem sempre coincidindo com a classificação utilizada no rótulo.

De uma forma geral, as amostras de mel apresentaram valores de humidade, acidez livre, 5-HMF, teor de prolina e índice de diástase dentro dos limites requeridos legalmente, sugerindo que os méis foram extraídos no nível de maturação correto. Os resultados mostram que quase todas as amostras de mel apresentaram uma color âmbar clara, exceto o mel de

alecrim que apresentou uma color âmbar extra clara. Apesar de apresentarem um índice de diástase normal, as amostras R2 e R3 apresentaram um valor de 5-HMF superior ao admitido na legislação, sugerindo a utilização de tratamentos térmicos e/ou métodos de conservação menos adequados. Os minerais mais comuns identificados foram o potássio, sódio, cálcio, enquanto cobre e manganês estiveram presentes em algumas amostras em quantidades menores e os metais pesados (cádmio e chumbo) estão ausentes em todas as amostras. O perfil dos açúcares, analisado por cromatografia líquida de alta pressão com deteção de índice de refração (HPLC-RI), mostrou que a frutose e a glucose foram os compostos mais abundantes, representando mais de 60% dos açúcares totais. Outros açúcares, como a turanose, a maltulose e a maltose, também foram detetados em menor proporção. Em relação ao perfil fenólico, foram identificados dezanove compostos (oito ácidos fenólicos e sete flavonóides), dois compostos isoprenóides (ácido *trans, trans*- e *cis, trans*-abscísico), uma espermidina e um diterpeno fenólico. No que diz respeito à avaliação da atividade antitumoral e atividade anti-inflamatória, as amostras apresentam potencial significativo. Finalmente, após o estudo de deteção de antibióticos verificou-se que nem todas as amostras estão isentas de resíduos.

Palavras-chave: mel, alecrim, tamarino, cardo, parâmetros fisicoquímico, valor nutricional, compostos fenólicos, atividade antioxidante, atividade anti-inflamatória, atividade antitumoral , antibióticos.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AGAE- Antioxidant Gallic Acid Equivalents

CETOF-MS- Capillary Electrophoresis-Time-Of-Flight Mass Spectrometry

CH- Thistle honey

DHSTR- Dihydrostreptomycin

DPPH- 2, 2-diphenyl-1-picrylhydrazyl-hydrate

EC- European Commission

Ec- Electrical conductivity

EC_{50}- Half maximal Effective Concentration

EU- European Union

LD_{50}- Median lethal dose

HPLC-RI- High-pressure Liquid Chromatography Coupled to a Refractive Index Detector.

IHC- International Honey Commission

IC_{50}- Half maximal inhibitory concentration

GAE- Gallic Acid Equivalents

GI_{50}- Half maximal of growth inhibition

MF- Multiflora honey

MRL- Maximum Residue Level

PTFE- Polytetrafluoroethylene

QE- Quercetin Equivalent

R-Rosemary honey

T- Tamarisk honey

W/W- Weight/Weight

5-HMF- 5-Hydroxymethylfurfural

INTRODUCTION

Honey was the first and most consistent sweetener used by human beings. As a source of energy, the beneficial features of honey are its great nutritional value and the fast absorption of its carbohydrates during consumption (Feás et al., 2010). Furthermore, in many areas of daily life, the importance of honey has been recognized for centuries and across civilizations for its good qualities and benefits. In fact, Hippocrates, the father of medicine, emphasizes that the nutritional and pharmaceutical value of honey is not accidental. Many researchers have stated honey to be a useful alternative for healing wounds and burns, and for oral health; others have discovered its important role in cancer care and its antimicrobial characteristics; as a natural, unprocessed and easily digested food, honey can be seen as an important part of our diet (Feás et al., 2010). For these reasons, honey still saves this natural representation, and consumption increase can be attributed to the global increase in living standards which makes people want to know more about its natural and beneficial health substances. Honey quality control is directly connected to the authenticity parameters stablished by the legislative requirements. Codex Alimentarius (Codex, 2001) and European regulation (Commission Regulation, 2006) legislation are set to act for the minimum marketing value of the product and the need for consumer safety through correct denominations (Feás et al., 2010).

Algeria is the second-largest country on the African continent, it has an area of about 2.4 million km^2 with circa 33.3 million populations. As the country is separated in the north by the Tell Atlas Mountains, which is parallel to the Mediterranean coast, and by the Saharan Atlas in the South, different environmental and geologic conditions exist. The Tell Atlas region enjoys a Mediterranean climate in the coastal areas and is very good for beekeeping. The main honey flow is in April, May, and early June. There are several trees, cultivated crops and wild plants, like Rosemary (*Rosmarinus officinalis*), tamarisk (*Tamarix gallica* L.) and milk thistle (*Silybum marianum*), which offer nectar and pollen for the bees. Also, natural forests, incorporating pine trees, are suitable sources for the bees and it is possible to have honey all year round. Second, the center part of the country contains high plateaus with plains and some agriculture, while, in the south, we encounter a desert climate. In the Saharan desert, constituting 80% of the country area, the date palm is cultivated but the conditions for

beekeeping are unfavorable. The conditions in the north part of the country plays a potential role for this activity: there is migratory beekeeping for honey production, but bees are usually not transported for pollination. It is estimated that the yearly honey production reaches 800 tones. Good quality honey is highly appreciated by the consumer but due to the limited knowledge of beekeepers, a high standard products is not always achieved (Makhloufi et al., 2010).

In Algeria, honey is used both for nutritive and healing purposes, and its price reaches quite great levels, while the information on the product is still deprived, and the quality control of local and imported honey is insufficient. This situation does not guarantee sufficient safety to the consumer and leads to possible frauds. Indeed, on the scientific plane, only a few pieces of information are available, so, to contribute more for the knowledge on Algerian honeys, in the present study we evaluate the quality of ten samples labelled as different botanical honey type (three samples from rosemary honey, three from tamarisk honey, three from milk thistle honey and one multiflora honey) supplied from local producers from Algeria, and verify its compliance with the standards of Codex Alimentarius (Codex, 2001) and the European regulation (Commission Regulation, 2006).

Chapter I : LITERATURE REVIEW



1. Literature review

1.1. Definition of honey

Honey is a natural product obtained by *Apis mellifera* bees by sucking nectar and exudates from plant excretions. They collect and combine these liquids with specific products of their metabolism and then stock them up in the comb to ripen and mature (Feás et al., 2010). Following honey origin, it is categorised into blossom, honeydew, monofloral and multifloral honeys. Blossom honey is obtained mainly from the nectar of flowers while honeydew or forest honey is produced by bees after they collect "honeydew" from plant saps. Monofloral honey is arising predominantly from a single botanical origin with above 45% of total pollen content from the same plant species, and is named after that plant, such as citrus, manuka and acacia honey. Multifloral honey is also known as polyfloral honey. It has several botanical sources where none is predominant (Ling Chin & Sowndhararajan, 2020).

1.2. Honey composition

1.2.1. Sugars

Honey mainly incorporates simple sugars or monosaccharides, including fructose and glucose (<65%). Additionally, there are small percentages of disaccharides present in honey composition (Bhandari et al.,1999). The percentage of sugars present influences its viscosity due to the strong impact of the sugar's molecular chains (Bhandari et al.,1999). The monosaccharides fructose (32–44%) and glucose (23–38%) are the major honey sugars, while sucrose (1 %), maltose (7%), and other trace sugars are present in smaller amounts (Machado De-Melo et al., 2017). In nectar honey, fructose percentages are frequently higher than glucose (Zafar et al., 2008). The sum of fructose and glucose, fructose/glucose ratio, and glucose/water ratio are also essential factors associated with the quality of honey. The fructose/glucose ratio shows the ability of honey to crystallize. Honey that has a high amount of fructose, has less tendency for crystallization, while honey rich in glucose frequently crystallizes directly after harvesting or sometimes inside the comb cells (Dyce, 1931 and Maurizio, 1962). Previous studies on honey samples produced in different regions of Algeria (Makhloufi et al., 2007) revealed a sugar content in agreement with the international standards, with only two samples showing a level of fructose + glucose lower than 60 %, probably due to the presence of some

honeydew (Makhloufi et al., 2007).

1.2.2. Protein content

The protein content in honey can be attributed to the presence of enzymes, some of which are introduced by bees themselves, and others are thought to be derived from the nectar also influenced by time of storage (Saxena et al., 2010). The amount of protein in honey ranges from 0.1 to 0.5%, however, some honey such as ling heather (*Calluna vulgaris*) show a higher protein amount (1–2%) (Chua et al., 2013). Previous studies on market Algerian honey showed protein values up to 4g/kg (0.4%) which are in the range normally found for honey around the world (Khalil et al., 2012).

1.2.3. Vitamins

The main vitamins present in honey are the B group vitamins and vitamin C (León-Ruiz et al., 2013). The content of water-soluble vitamins is higher than the quantity of fat-soluble vitamins, because honey hardly contains lipidic substances (Machado De-Melo et al., 2017). The use of commercial filtration procedures and the presence of hydrogen peroxide, which naturally occurs in honey (Ciulu et al.,2011) may contribute to the decrease of vitamin C levels in honey. In Algerian honey, the levels of ascorbic acid (vitamin C) were reported around 160 mg/kg (Khalil et al., 2012).

1.2.4. Mineral content

The mineral content of honey has a significant linear relationship with its electrical conductivity and ash content and is influenced by the botanical origin and the type of soil in which the nectar plants were situated. Besides, it can also provide information about ecological pollution (Anklam, 1998). Honey with higher mineral content is generally darker (González-Miret et al., 2005) due to the formation of colorful compounds between transition elements and some organic complexes in honey (Amri & Ladjama, 2013). A high value of acidity is also correlated with honeys with high mineral content. The mineral content in honey can varied from 0.02 to 1.0g/100 g (Bogdanov, 2016). The minerals mainly found in honey are magnesium,

calcium, sodium, and potassium, while the less abundant minerals are manganese, copper, iron and in minor quantities trace elements like, nickel, phosphorus, sulfur, silicon and boron (Doner, 2003).

Recently, a study involving 22 multiflora Algerian honey samples described a mineral content that ranged from 0.02 to 0.5% (Amri & Ladjama, 2013).

1.2.5. Phenolic content

Phenolic acids and their derivatives are the major bioactive substances found in honey, with concentrations varying from 5 to 1300 mg/kg (Alvarez-Suarez et al., 2012). The phenolic compounds are related to the geographical and botanical source of the flowers in which the bees collect the nectar. The healthy honey characteristics are linked to the presence of the phenolic acids and flavonoids (Da Silva et al., 2016). Some beneficial actions of flavonoids such as prevention of cardiovascular diseases (Cianciosi et al., 2018), makes honey a tool in alternative health treatment, known as apitherapy (Vit et al., 2004). The content of phenolic compounds is associated directly with the color, having the darker honey higher content in phenolic compounds, sensory features, and antioxidant activity (Da Silva et al., 2016). According to previous studies concerning Algerian honey, where the phenolics were estimated by a modified spectrophotometric method, the honey samples present a phenolic content around 460 ± 2 mg gallic acid equivalents/kg and flavonoids in concentrations around 54.2 ± 0.6 mg catechin equivalents/kg (Ouchemoukh et al., 2007).

1.2.6. Organic acids

Organic acids, which are connected to honey flavor (Suárez-Luque et al., 2002), are present in small percentages in honey (0.5%). They have impact in the honey acidity, which can be used as a quality parameter for the evaluation of deterioration linked to storage, aging or for authenticity measure (Suárez-Luque et al., 2002). The acidity of honey helps the preservation against spoilage by microorganisms (El Sohaimy et al., 2015). Diverse organic acids were described to be present in honey, including citric, lactic, acetic, malic, butyric, pyroglutamic, succinic and oxalic acid (Machado De-Melo et al., 2017), which can be used to characterize different honey types. The concentration of citric acid is useful as a factor to distinguish between two types of honey: floral and honeydew honey (Soares et al., 2017) The citric acid values found in floral honeys ranged from 36.5 to 1454.2 mg/kg, and the

values found in honeydew honey ranged from 447.6 to 3019.8 mg/kg (Mato et al., 1998).

1.3. Honey botanical origin

The traditional method used to determine the botanical origin of honey is the melissopalynology, which consists of pollen identification by microscopy. Honey contains a lot of pollen grains and honeydew elements giving a good fingerprint of the ecological area of the honey. However, this method presents some limitations, such as the longtime of analysis, the availability of a comprehensive collection of pollen grains, and the need of experts with adequate experience to identify the different pollen morphologies (Von Der Ohe et al., 2004).

Several advanced approaches have been proposed aiming at accurately assessing the botanical and geographical origins of honey, by targeting certain minor compounds in honey, such as phenolic acids, sugars, amino acids, and other constituents, through the use of gas-chromatography coupled to mass spectrometry (GC-MS), liquid chromatography coupled with mass spectrometry (LC-MS), capillary electrophoresis-time-of-flight mass spectrometry (CETOF-MS), matrix-assisted laser desorption/ionization-time of fly ionization mass spectrometry (MALDI-TOF MS), and nuclear magnetic resonance spectroscopy (NMR) (Schievano et al., 2013). Nevertheless, the assessment of these chemical markers can be affected by beekeeping practices, environmental conditions, and climate changes, leading frequently to an unreliable determination of its floral or geographical origin (Madesis et al., 2014). So far, melissopalynological analysis is kept as the basic techniques of the botanical determination of honey, however, the physicochemical and sensory diagnosis are also important for an appropriate analysis of the botanical origin (Von Der Ohe et al., 2004). In Algeria, there are several types of monofloral honey such as rosemary honey, tamarisk honey, milk thistle honey, multiflora honey and honeydew has been characterized.

1.3.1. *Rosmarinus officinalis*

Commonly known as rosemary, is a woody, perennial herb with fragrant, evergreen, needle-like leaves and white, pink, purple, or blue flowers, Figure 1. It is native to the Mediterranean and Asia but is reasonably hardy in cool climates, surviving even in the lack of water for lengthy periods. In temperate climates, the plant flowering period is between spring and summer; however, the plant can be in constant bloom in warm climates. Rosemary also has a propensity to flower outside its normal flowering season, it has been recognized to flower as

late as December, and as early as mid-February (Amin & Hamza, 2005).

Rosemary honey from Algeria is a light color honey. In general, has low conductivity and acid content and values of fructose higher than glucose (Homrani et al., 2020). The physicochemical parameters of Algerian rosemary honey are represented in Table 1.

TABLE 1- Characteristics of one sample of Algerian rosemary honey (Homrani et al., 2020)

Parameter	Value	Parameter	Value
Humidity (%)	16	Glucose (%)	30.1
Ec (mS/cm)	0.33	Sucrose (%)	nd
pH	3.8	Maltose (%)	3.3
Color (mm Pfund)	13	Turanose (%)	3.1
Diastase Index (Ghote)	6.4	Raffinose (%)	0.3
HMF (mg/100 g)	0.9	Polyphenol (mg/100 g)	26.5
Fructose (%)	38.1	Flavonoid (mg/100 g)	1.0

Figure 1-*Rosmarinus officinalis* (Marion, 2017)

1.3.2. *Tamarix gallica* L.

Tamarix gallica L is a high perennial shrub/little tree, densely ramified, 2-10 m high. The purple-brown bark is initially smooth with huge, elongated lenticels, after developing shallow splits and becoming rough when full-grown. The small, scale-like, 1-3 mm, long leaves are grey-green or green. The tiny flowers have 5 lavender-pink or white petals 1.5-2 mm, Figure

2. The fruits are tiny dry capsules that have tiny cottony seeds. The capsules are conical, trigonous, tapering, and pale pink. Flowering begins around March and ends until May. In the central Sahara it has been shown in full bloom in June (Cooperation, 2005). Tamarisk honey which is collected from Iran is intermedium-colored honey with reddish tones and a taste of malt with overtones of citrus, with a slightly bitter after taste. The chemical composition is also characterized by low conductivity and medium acidity, with fructose and glucose values around 36 and 27 g/100g, respectively (Khalafi et al., 2016). The physicochemical properties of Iranian tamarisk honey are resumed in Table 2.

Table 2- Physicochemical properties of Iranian tamarisk honey (Khalafi et al., 2016).

Parameter	Value	Parameter	Value
Moisture (g/100g honey)	15.1	Diastase activity (Gothe)	13.8
pH	4.1	HMF (mg/kg)	2.2
Ash (g/100g honey)	0.052	Total phenolic content (mg/100g honey)	24.6
Electrical conductivity (mS/cm)	0.16	Total flavonoid content (mg/100g honey)	2.1
Fructose (g/100 g honey)	35.9	Antioxidant activity (%)	46.7
Glucose (g/100 g honey)	26.7	Color intensity	0.34
The ratio of fructose/ glucose	1.3		

Figure 2- *Tamarix gallica* (Urfi et al., 2016).

1.3.3. *Silybum marianum*

Silybum marianum is an annual/biennial plant, more than 2 meters high. The stem is 20-150 cm high, rarely smaller, slightly downy, or glabrous, branched and erect in the superior part. The leaves are alternate, wide, white-veined, glabrous with strongly spiky margins. The inflorescences are wide and circular capitula, solitary at the top of the stem or its branches, bordered by thorny bracts. The florets are hermaphrodite, tube-shaped with a red-purple corolla, Figure 3. The fruits are hard-skinned achenes 6 to 8 mm long, usually brownish with white silk-like pappus at the top. The fruits are harvested in May - June, after blooming (Cooperation, 2005).

Table 3- Physicochemical parameters in Croatian milk thistle honey (Mandic et al., 2006).

Parameter	Value	Parameter	Value	
Electrical conductivity (mS/cm)	0.22	Glucose/Water (%)	2.38	
pH	3.72	Ksilose (%)	0.53	
Free Acidity (meq/kg)	19.9	Maltose (%)	1.02	
Water (%)	16.7	Melezitose (%)	0	
Diastase (DN)	16.8	Raffinose (%)	0.05	
HMF (mg/kg)	7.2	Total sugars (%)	78.34	
Fructose (%)	36.4	Visual assessment	**Colour intensity:** medium **Colour tone:** bright yellow	
Glucose (%)	39.8	Olfactory assessment	**The intensity of odor:** medium **Description:** floral-fresh fruit	
Sucrose (%)	0.5	Tasting assessment	**Sweetness:** medium to strong	**Acidity:** Absent
Fructose+Glucose (%)	76.2		**Bitterness:** absent	**The intensity of aroma**: weak
Fructose/Glucose (%)	2.38		**Persistence/aftertaste:** absent	
		Physical characteristics	**Crystallization rate:** slow	

Milk thistle honey collected from *Silybum marianum* in Croatia has a color ranging from pale yellow to deep amber, with a mild taste and sweet flavor reveal a slight bitterness and astringent aftertaste. It has a fresh floral aroma and slightly woody or mossy. The physicochemical parameters of milk thistle honey show slightly higher values of conductivity and acid content when compared with the former honey, with high values of fructose and glucose (Mandić et al., 2006). The physicochemical parameters in the analysis of Croatian milk thistle honey are represented in Table 3.

Figure 3- *Silybum marianum* (Poppe, 2017).

1.4. Quality and physicochemical parameters of honey

To ensure the quality of honey, different international institutions such as the International Honey Commission (IHC), the Codex Alimentarius and the European Commission suggests parameter levels and methodologies of analyses to assure the authenticity of honey (Draiaia et al., 2015). Within those regulations we can find the following parameters:

1.4.1. Color

The color of honey is a parameter closely linked with the consumer acceptance of a particular sort of honey (González-Miret et al., 2005). The color can vary from colorless to dark-brown (Codex, 2001), and according to Belay et al (2015), it correlates with the flavor. Honey with light colors have a mild flavor, while dark honey has an extra pronounced flavor (Bertoncelj et al., 2011). Also, throughout storage or under heating for an extended period, honey can change due to non-enzymatic browning reactions, like the Maillard reactions (Oroian

& Ropciuc, 2017). These reactions produce substances like furfural and 5-hydroxymethylfurfural (5-HMF), associated with the browning of honey (Da Silva et al., 2016). Previous work on Algerian honey reported honey from dark amber to light amber color (Khalil et al., 2012).

1.4.2. Moisture content

The moisture content of honey is correlated with the climatic and harvesting conditions and affects the physicochemical parameters of honey such as viscosity and crystallization, with consequences in the quality (Gallina et al., 2010). Generally, the water content of honey is less than 20%, except for heather honey, where the maximum can be up to 23% (Council Directive 2001/110/EC and FAO 2001). According to previous works on Algerian honey, most of the samples showed relatively low levels of moisture content (average value 16.5%), with only one sample above the limit of 20% defined by the international standards (Makhloufi et al., 2007). High values of water can lead to fermentation and, consequently, reducing the shelf life. These high levels can be related to premature honey harvesting or inadequate storage conditions (Makhloufi et al., 2007).

1.4.3. Ash and electrical conductivity

The electrical conductivity of honey is correlated with the minerals, proteins, and organic acids, and so, is directly linked with the ash content. Usually, it is a characteristic estimated within the nutritional evaluation. Besides, it is a useful parameter for the differentiation of different botanical origin of honey (Krauze & Zalewski, 1991). Concerning the Algerian honey, the literature reported a high electrical conductivity within the analyzed samples with an average value of 0.65 mS/cm. The international standards recommend a limit of 0.8 mS/cm for all nectar honey (Makhloufi et al., 2007).

1.4.4. pH and acidity

The acidity of honey is due to the existence of organic acids, mostly gluconic acid (Terrab et al., 2004), and can be accessed by the evaluation of the free acids present in the sample together with the lactonic acidity, defined as the existing acidity when the honey is turned to alkaline (Terrab et al., 2003). Honey with low pH inhibits the existence and development of microorganisms. This factor is highly important during the storage and

extraction of honey and is related to its stability, texture, and shelf life (Terrab et al.,2004). Published reports show that honey pH ought to be between 3.2 and 4.5 and can be used to distinguish between nectar and honeydew honey (Bogdanov et al.,1997). Free acidity limits are specified in European legislations as lower than 50 meq/kg, representing the non-existence of unwanted fermentation (Feás et al., 2010). Multifloral Algerian honey from different regions was reported to have a pH in a range of 3.3 to 4.6 (Amri & Ladjama, 2013).

1.4.5. 5-Hydroxymethylfurfural (5-HMF)

Hydroxymethylfurfural is commonly identified as a freshness parameter for honey samples. Several parameters influence the formation of 5-HMF, like storage conditions (e.g., temperature) and floral origin. It is known that honey heating originates 5-HMF, which is synthesized throughout acid-catalyzed dehydration of hexoses, like glucose and fructose. According to the Codex Alimentarius and EU standards, the 5-HMF maximum level is 40 mg/kg (Codex, 2001) (Commission Regulation, 2006). Beekeeping organizations of some countries, e.g. Germany, Italy, Finland, Switzerland have set the highest limit of 15 mg/kg for, particularly labeled “quality” or “virgin” honey (Bogdanov, 2016). Regarding Algerian honey, the literature revealed values for HMF around 18.5 mg/kg, on average, with 4 samples over the limit of 40 mg/kg (Makhloufi et al., 2007). The building of HMF from a hexose sugar is represented in Figure 4.

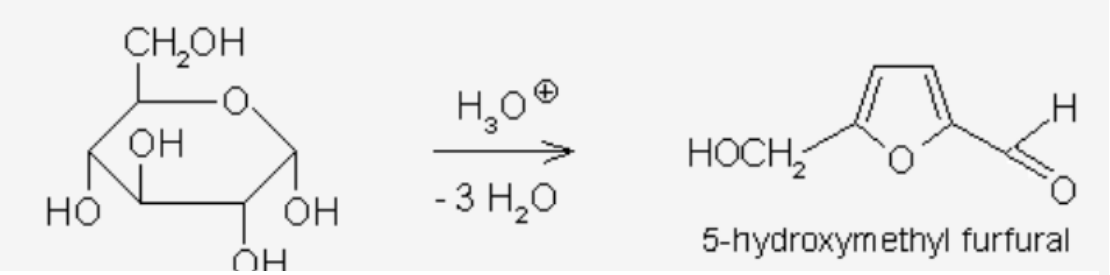

Figure 4 -Building of 5-HMF from a hexose sugar (Bogdanov, 2016).

1.4.6. Diastase activity

Diastases are a group of enzymes that comprise α-and β-amylase, which are naturally present in honey. It is a parameter usually assign for honey freshness and can be quantified in Schade, Göthe, or diastase units (Fechner et al., 2016). A minimum level of 8 diastase units is set by the Codex Alimentarius and the European honey directive (Bogdanov, 2016). For that reason, honey with diastase activity under the permitted limits is linked to long storage periods

and/or heating throughout its storage or processing (Fechner et al., 2016). The activity of diastase also depends on the honey botanical origin (Pascual-Maté et al., 2018), so that citrus and rosemary honeys, among others, are known to have low natural enzyme contents (Machado De-Melo et al., 2017) .

Algerian honey samples were reported to have the mean value of 17.4 ± 9.0 ranging from 4 to 40 Schade units (Makhloufi et al., 2007).

1.4.7. Proline content

Proline is the most abundant free amino acid in honey varying from 50 to 85% of the total (Pascual-Maté et al., 2018). It generally comes from salivary secretions of honeybee through the conversion of honeydew or nectar into honey (Bergner & Hansjörg, 1972), and so is not a good indicator of the botanical origin of honey. However, high amount of proline was mainly found in honeydew honey (Machado De-Melo et al., 2017). Proline could be related to the content of enzymes, because of its important role in the regulation of nectar enzymatic transfer, generally, the secretions of invertase during the transformation of nectar in honey and the main content of proline in honey should be more than 200 mg/kg (Bogdanov & Pascale, 2001). Some researchers analyze proline as a quality indicator for ripeness of honey, and as a criterion of sugar adulteration, particularly when the levels of this amino acid are less than 180 mg/kg, the minimum level that has been established for genuine honey (Bogdanov et al., 1999). Proline was detected in a higher amount (1692–2712 mg/kg) in all Algerian honey samples (Khalil et al., 2012).

1.5. Bioactivity of honey

Honey has a natural antioxidant activity, and it has proved to prevent food spoilage due to oxidative reactions (Gheldof & Engeseth, 2002). In vitro studies have shown that honey intakes block the oxidation of lipoproteins of human serum (Al-waili, 2003). This antioxidant potential of honey is due to the number of compounds that exist on it, both enzymatic (e.g., peroxidase, glucose oxidase, and catalase) and non-enzymatic compounds (e.g., phenolic acids, carotenoids, α-tocopherol, proteins, amino acids, flavonoids, Maillard reaction products) (Gheldof & Engeseth, 2002). The amount and sort of these antioxidants are related to the floral source, and the antioxidant activity is related to phenolic content (Gheldof & Engeseth, 2002).

1.5.1. Antioxidant activity

1.5.1.1 DPPH

The radical scavenging activities of honey samples is measured using the DPPH radical scavenging assay. DPPH is a stable nitrogen-centred radical that has been extensively used to test the free radical scavenging ability of various samples. In evaluating the radical-scavenging potential of honey, the DPPH assay is frequently used because the antioxidant potential of honey is directly associated with its phenolic and flavonoid contents: high DPPH scavenging activity confers the superior antioxidant activity of the sample (Khalil et al., 2012). One study on Algerian honey within 26 samples of multifloral honey has shown a mean value for radical scavenging activity of 30.6% using 6 x 10^{-5} M of DDPH solution (Homrani et al., 2020).

1.5.1. 2. Reducing power

Reducing power assay method is based on the principle that substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric–ferrous complex that has an absorption maximum at 700 nm (Bhalodia et al., 2013). One study about multifloral Algerian honey has published an amount of reducing power between 20 and 30 mg AGAE/100g (Mouhoubi-Tafinine et al., 2016).

1.5.2. Anti-inflammation activity

The anti-inflammatory effect of honey has been pointed out for the last 30 years. In addition, it has also been observed in inflammatory bowel disease. The anti-inflammatory effect has been linked to the reduction of free radicals produced at the site of inflammation, antibacterial potential, and direct anti-inflammatory potential (Khan et al., 2017). Also, it has been observed on studies in animal models a reduced number of white blood cells. As a result of reduced inflammation, edema and exudates are prevented by honey, which subsequently decreases pain through the reduction in the prostaglandin mediated by the inflammatory process. A wound causes the production of protease activity which can eliminate the healing process. The anti-inflammatory action of honey eliminates the process and promotes healing. In addition, the anti-inflammatory activities of honey are linked with the reduction of bacterial load, promoting debriding, and ultimately preventing the inflammatory reaction. The anti-inflammatory effect of honey has been linked with different flavonoids that inhibit the

development of inflammation. One of the important flavonoids is galangin which is capable of inhibiting cyclooxygenase (COX) and lipo-oxygenase activity, reducing the expression of cyclooxygenase-2 (COX-2) and limiting the action of polygalacturonase. Another compound is caffeic acid phenethyl ester (CAPE) which showed the anti-inflammatory effect through inhibiting the production of arachidonic acid from the cell membrane causing suppression of cyclooxygenase-1 (COX-1) and inhibits COX-2. Chrysin, a flavonoid present in honey, exhibited an anti-inflammatory effect by suppression of pro-inflammatory activities of COX-2 and inducible nitric oxide synthase (iNOS). (Khan et al., 2017). A former study in multifloral Algerian honey has shown a mean value for IC50 between 1.7 and 7.4 mg/mL of anti-inflammatory activity (Zaidi et al., 2019).

1.5.3. Antitumor activity

The activity of honey on cancer has been studied both in terms of prevention, progression, and treatment. Most of the research is in vitro has been carried out on several types of cell lines with different sorts of honey. The antitumoral potential is generally attributed to diverse mechanisms, like the stimulation of apoptosis, cell cycle blocking, the controlling of oxidative stress, the improvement of inflammation, the stimulation of mitochondrial outer membrane permeabilization (MOMP), and the blocking of angiogenesis, Apoptosis is a programmed cell death process that eliminates damaged cells. Through the up-regulation of some proapoptotic proteins, such as caspase 3, 8, 9, Bax, p53, and the downregulation of other antiapoptotic proteins, such as Bcl2 and poly (ADP-ribose) polymerase (PARP), honey is considered a good inducer of apoptosis. Another mechanism for honey in acting against cancer cells is the arrest of the cell cycle, by modulation of some molecules, such as cyclooxygenase and some kinases, or the induction of MOMP, promoted especially by flavonoids, which cause the release of intramembrane proteins into the cytosol, resulting in cell death. Indeed, the permeabilization of mitochondrial membrane is an early event that leads to the activation of the intrinsic mitochondrial pathway, which induces several processes, including the release of certain proteins such as cytochrome C (cytC), potentially cytotoxic, causing cell death. The role of ROS and oxidative stress in cancer is still controversial since it is unknown if it has a stimulatory or inhibitory effect. However, the inhibition of tumor growth is still linked to the antioxidant properties of honey. Finally, honey can counteract chronic inflammatory processes, which increase the risk of cancer. Two important factors of inflammatory pathway in cancer

are nuclear factor kappa B (NF-kB) and mitogen-activated protein kinase (MAPK), which are involved in the up-regulation of some and proapoptotic effects on both cell lines. Acacia honey has demonstrated anti-tumor activity in lung cancer cells (NCI-H460), inhibiting cell proliferation by stopping the cycle in the G0/G1 phase, stimulating cytokines and downregulating Bcl2 and p53, thus acting as a proapoptotic. Morales, and Haza studied the effect of three different types of Spanish honeys, two monofloral (Heather and Rosemary) and one polyfloral in human leukemia cell line (HL-60). Monofloral honeys, particularly Heather honey, demonstrated a greater cytotoxic effect, mainly due to the induction of apoptosis through a ROS-independent pathway (Cianciosi et al., 2018) as shown in Figure 5. One study for Algerian honey has shown a mean value of LD50 more than 1000 µg/mL for different cell lines including MCF-7 (the human breast adenocarcinoma), MDA-MB-231 (the human mammary gland adenocarcinoma), Hela PC3 (the human epithelial adenocarcinoma) and K562 (the human prostate cancer PC3) (Bakchiche et al., 2020).

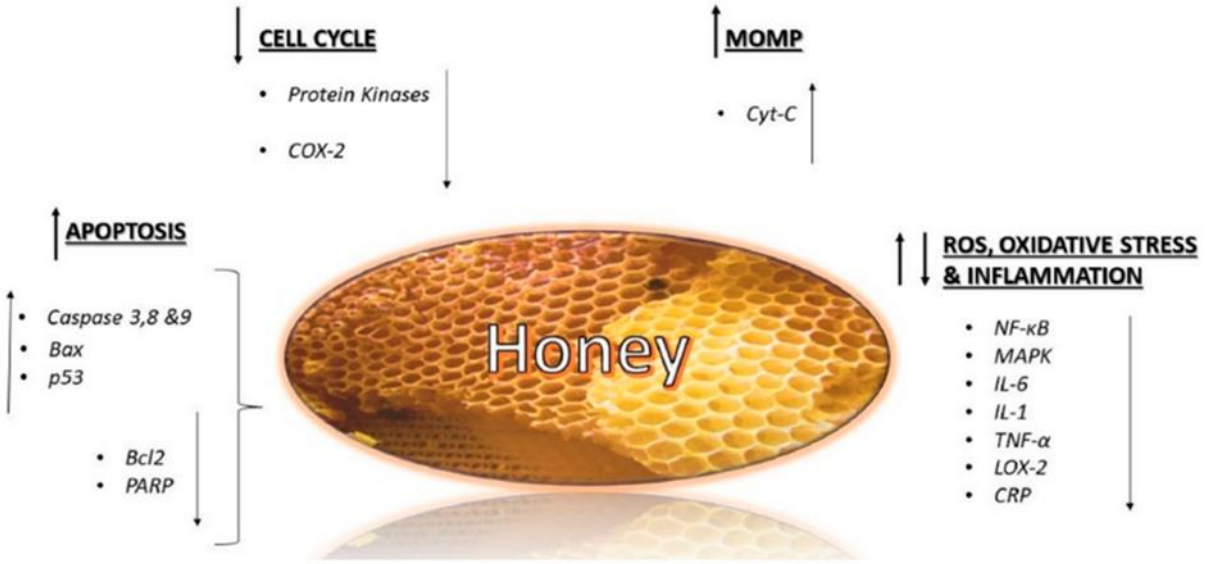

Figure 5 Molecular mechanism involved in anticancer effect of honey (Cianciosi et al., 2018).

1.6. Antibiotics in honey

Antibiotics drugs are used by beekeepers to fight foulbrood diseases in honeybee colonies and so, they may contaminate honey if those colonies are used in production. Also, the contamination of honey might occur during the regular application of antibiotics like streptomycin and its derivative dihydrostreptomycin which is frequently joint with tetracycline (Draiaia et al., 2015). According to the Codex Alimentarius and Council Directive of the

European Union, these bactericides are completely banned from honey (Van Bruijnsvoort et al., 2004), with no maximum residue level (MRL) established for tetracycline. This means that the existence of tetracycline residues in honey is not permitted. Although this determination, some countries have set action tolerated or limits amounts for tetracycline in honey. Currently, in Belgium, the limit for the group of tetracycline has been fixed at 20 µg kg^{-1}, France sets a non-conformity limit for tetracycline in the honey of 15 µg.kg^{-1}. Also, the published limit in Great Britain is 50 µg.kg^{-1}, however the tolerance amount in Switzerland is 20 µg.kg^{-1} (Cara et al., 2012). Besides the fact that antimicrobials drug residues in honey can cause a potential danger to human health (Draiaia et al., 2015), it harms the consumer's perception of honey as a natural product. In one study concerning the quality evaluation of 36 samples of different honey types supplied by local producers from Algeria, it was shown two samples present very low concntrations of oxytetracycline, with no residues of streptomycin or tetracycline (Draiaia et al., 2015).

1.7. Objectives

Quality of honey is regulated by different international institutions, like the International Honey Commission, the Codex Alimentarius and the European Commission suggest methods of analysis to make sure that honey is authentic in respect to the legislative requirements. The production of Algerian honey is less than the needs of local consumption while it is said to be at the origin of a huge export. This low production affects the price and makes it remain high. For that reason, consumption remains as low as production. This absence of production is the result of many causes like the lack of national regulation, absence of a professional organization, and insufficient quality control laboratories. Even so, Algerian researchers and scientists try to make an appropriate denomination that makes sure of a minimum marketing value of the product.

In order to contribute more to the knowledge of Algerian honey, the aim of this work is to determine the quality of selected commercial monofloral Algerian honeys, such as rosemary, tamarisk, thistle and multiflora, in terms of physicochemical properties and verify their compliance with the international standards. The presence of antibiotics, recurrent residues in honey, such as tetracyclines and sulphonamides will be screened using the multi-analytic receptor assay system Charm II. Other methodologies to access the botanical origin of these honeys will be explored such as the determination of phenolic compounds through LC-MS but also its bioactivity will be evaluated.

CHAPTER II: MATERIALS AND METHODOLOGY



2. Material and methods

2.1. Sampling

The present work included the analysis of ten commercial honey samples, three samples labeled as rosemary honey, three samples labeled as tamarisk honey, and three samples labeled as milk thistle honey and one multiflora honey, from two geographic regions in Algeria, produced in 2019, Figure 6.

Figure 6- Geographical origin for honey samples

Table 4 shows the information on honey samples used throughout this work, in particular their geographical origin and year. All honey samples were stored at room temperature, in the original packaging until they were analyzed. The samples were coded as showing in table 4 (R: rosemary honey, CH: milk thistle honey, T =tamarisk honey and MF: multifloral honey)

Table 4- Geographical origin and year of collection of honey samples

Sample Code	Botanical origin on the label	Geographic origin	Month/year of collection
R1	*Rosmarinus officinalis* L.	Sidi Belabbes	June 2019
R2	*Rosmarinus officinalis* L.	Sidi Belabbes	June 2019
R3	*Rosmarinus officinalis* L.	Sidi Belabbes	June 2019
CH1	*Silybum marianum*	El Bayedh	June 2019
CH2	*Silybum marianum*	El Bayedh	June 2019
CH3	*Silybum marianum*	El Bayedh	June 2019
T1	*Tamarix gallica L.*	El Bayedh	June 2019
T2	*Tamarix gallica L.*	El Bayedh	June 2019
T3	*Tamarix gallica L.*	El Bayedh	June 2019
MF	*Tamarix gallica L.*	Sidi Belabbes	June 2019

2.2. Honey analysis

The characterization of honey samples was performed by identifying their floral origin by pollen analysis and by evaluating the physicochemical parameters, defined by the

International Honey Commission (IHC) (Bogdanov et al.,1997). In addition to these parameters, the assessment of phenolic compounds, flavonoid compounds, antioxidant activity, the screening of antibiotics, cytotoxicity, and anti-inflammation potential was also done. All parameters were evaluated in triplicate.

2.2.1. Melissopalynology analysis

For pollen analysis, 10 g of each sample honey were dissolved in 20 mL of distilled water and centrifuged at 3500 rpm for 10 min. After discarding the supernatant liquid, 2 mL of glacial acetic acid were added and vortexed. The tube was centrifuged in the same conditions and the supernatant discarded. Then, 2 mL of the acetolysis solution (acetic anhydride: sulphuric acid, 9:1) were added and the solution vortexed. The tube was placed in a boiling water bath for 3 min. After cooling and centrifuged, the supernatant was discarded and 4 mL of 50% glycerol solution was added followed by another step of centrifugation and removal of the supernatant. A volume of liquefied glycerol-gelatin was added and immediately vortexed. Then, 17 µL of the mixture were pipetted and spread on a slide at 40ºC. The slides were allowed to rest, at room temperature, in an invert position. After sealing the coverslips with nail varnish, the slides were observed under an optical microscope, at 1000X magnification, 500-1000 pollen grains per sample and complete lines were counted and identified at random in the coverslip area (Louveaux et al., 1978). This work was done in collaboration with *LabApis*^{UTAD}.

2.2.2. Color

The color analysis was carried out by placing honey samples in a preheated (45ºC) ultrasonic bath (Escuredo et al., 2021). Then, the evaluation of the color of the samples was performed by their classification according to the Pfund scale, by direct reading on a C221 colorimeter (Hanna Instruments, Woonsocket, RI, USA), Figure 7.

Figure 7- Colorimeter

2.2.3. Moisture content

The moisture content was determined, in triplicate, through a refractometer (Digit-5890, Ref:8100.5890, Netherlands), Figure 8, and the results were expressed in percentage (%) (Bogdanov et al., 1997).

Figure 8 - Refractometer

2.2.4. Electrical conductivity

The conductivity was carried out according to a previously reported method (Bogdanov et al., 1997). The solution of honey was prepared by diluting 5 g of honey in 25 mL of distilled water, and its electrical conductivity was measured by using a consort C868 conductivity meter, Figure 9, previously calibrated. The results were expressed in $mS.cm^{-1}$.

Figure 9- Conductivity meter

2.2.5. pH, free and lactonic acidity

To evaluate the acid properties of honey, three different parameters were evaluated: pH value of the initial honey solution, free acidity, and lactonic acidity (Bogdanov et al., 1997). Titration was performed with an automatic titrator, Figure 10, (Hanna instruments, pH 211 Microprocessor pH meter, Woonsocket, USA).

Figure 10- Automatic titrator

For the identification of free acidity, the procedure reported by the IHC was performed (Bogdanov et al.,1997). Initially, a solution was prepared by dissolving 10 g of honey in 100 mL of deionized water. Then 25 mL of this solution was put into a beaker where the pH electrode was placed, recording the initial pH value, and then titration of the solution with sodium hydroxide (NaOH) 0.1 mol.dm^{-3}. The base volumes consumed to reach the equivalence point (pH 7) were recorded. The obtained value allows determining the free acidity which is measured by titration with sodium hydroxide (NaOH) up to the equivalence point (pH 7).

To determine the lactonic acidity, after reaching the equivalence point, the base was added until reaching the final volume of 10 mL, then a re-titration of the excess base with sulfuric acid (H_2SO_4) 0.025 mol.dm^{-3} until reaching the equivalence point again (pH 7). The difference in NaOH consumed in the two titrations allows the calculating of the lactation acidity and the total acidity (free + lactonic). The results are expressed in meq.kg^{-1}.

2.2.6. Proline

Proline evaluation was performed by spectrophotometric methods (Bogdanov et al., 1997) using an aqueous honey solution obtained by dilution of 0.5 g of honey in 10 mL distilled water. For the analysis, 0.5 mL of the honey solution was placed in a test tube (sample), 0.5 mL of distilled water in a second tube (white), and 0.5 mL of standard proline solution (0. 032 mg. mL) in triplicate in other tubes (standard), together with the same volume of water. To each of the 10 tubes, 1 mL of formic acid (98%) was added with 1 mL of ninhydrin solution (3%) and stirred vigorously for 15 minutes. After that time, the tubes were placed in a boiling water bath for 15 minutes, and then in another bath at 100 ºC for an additional 15 minutes. In the end, 5 mL of propan-2-ol was added to each test tube and after being closed, the tubes were cooled for 45 minutes and then reading the absorbance at 510 nm using a spectrophotometer (Specord 200 spectrophotometer, Analytikjena, Jena, Germany). The proline content was calculated using the following equation, and the results expressed in mg.kg^{-1}.

$$\text{Proline} = (\text{Abs}_{\text{Sample}}/\text{Abs}_{\text{standard}}) \times (\text{mass}_{\text{standard}}/\text{mass}_{\text{Sample}}) \times 80 \qquad \text{(Equation nº1)}$$

2.2.7. 5- Hydroxymethylfurfural

The 5-HMF content was determined spectrophotometrically according to Bogdanov et al. (1997). Two solutions were prepared by dissolving 5 g of honey in 25 mL of distilled water. The solutions were transferred to a 50 mL volumetric flask, to which 0.5 µL of Carrez I solution and 0.5 µL of Carrez II solution were added, and 24 mL of distilled water. Then the solution

was filtered, collecting 5 mL for each test tube. 5 mL of distilled water (sample) was added to one of the tubes and the other 5 mL of a sodium bisulfite solution ($NaHSO_3$) 0.2%, then the absorbance was read at 284 and 336 nm in a spectrophotometer.

The value of 5-HMF is expressed in $mg.kg^{-1}$ and determined according to the following equation:

$HMF = (Abs_{284} – Abs_{336}) x14.7 x (5/mass\ of\ the\ sample)$ (Equation nº2)

2.2.8. Diastase activity

The analysis of the diastase activity was performed by the Phadebas method (Bogdanov et al.,1997). This spectrophotometric method is performed by preparing an aqueous honey solution obtained by dilution of 1 g of honey in a 100 mL volumetric flask. After preparing the solution, 5 mL was transferred to a test tube and placed in a bath at 40 ºC, together with a second tube (blank) containing 5 mL of acetate buffer solution 0.1 M (pH 5), each sample was put in 3 tubes (5 mL). The Phadebas tablets were then placed in the three tubes at 40 ºC for 15 minutes. Subsequently, 1 mL of sodium hydroxide (NaOH) 0.5 M was put on and filtered. The absorbance was measured at 620 nm in a spectrophotometer. The result is obtained as a diastase index (DN) in Schade units, equivalent to the unit of diastase and the enzymatic activity of 1g of honey capable of hydrolyzing at an hour, 0.01 g of starch at 40 ºC. The equations used for the calculation of the value of DN were as follows:

$DN= 28.2*Abs_{620} + 2.64$ if $DN>8$ (Equation nº 3)

$DN= 35.2*Abs_{620} – 0.46$ if $DN<8$ (Equation nº 4)

2.2.9. Sugars

The determination of the sugar content in the samples was performed by liquid chromatography coupled to a refraction index detector (HPLC-RI), using a calibration by internal standards. For the analysis, a solution was prepared by dilution of 2.5 g of honey in 20 mL of deionized water. Then, 12.5 mL of methanol was pipetted, and the diluted honey solution was transferred to a 50 mL volumetric flask, then 1mL of xylose was added (internal standard), making the total volume with deionized water. Subsequently, the sample was filtered in 0.2 µm nylon filters before injecting it into the chromatograph. The chromatography system used consisted of a pump (Knauer, Smartline 1000 system), a degassing (Smartline 5000), an automatic sampler (AS-2057 Jasco), and an IR detector (Knauer Smartline 2300). Data analysis was performed with clarity 2.4 (DataApex) software. For chromatographic separation, a 100-5

NH_2 Eurospher column (4.6 x 250 mm, 5 mm, Knauer) was used operating at 30 ºC (Grace 7971 R oven). As a mobile phase, a mixture of acetonitrile/water 80:20 (v/v) was used, with a flow rate of 1.3 $mL.min^{-1}$. The identification of sugars was obtained by comparing the retention times of the peaks of the samples with those of standards, namely fructose, glucose, sucrose, turanose, maltulose, maltose, trehalose, melezitose, raffinose, melibiose, erlose, isomaltose, and kojibiose. For each of these standards, a calibration line was established by the internal standard method, using a range of concentrations according to the expected levels for each sugar, Table 5. The obtained values by the samples were calculated from the peak area and are presented in g/100 g of honey. The analysis of the sugar profile was also considered in terms of fructose+glucose, fructose/glucose and glucose/water ratio, to assess the tendency to crystallization of the honey.

Table 5- Range of concentrations for each standard, and respective calibration equation

Sugar	Concentration range (mg. mL $^{-1}$)	Calibration equation	R^2
Fructose	1.56- 60	Y=82.665x + 75.806	0.9996
Glucose	1.17 - 45	Y=60.65x + 154.24	0.9994
Sucrose	0.9 - 15	Y=154.68x + 1.613	0.9997
Turanose	0.14 – 4.5	Y=135.18x + 1.0489	0.9996
Malutose	0.14 – 4.5	Y=154.85x – 5.333	0.9904
Maltose	0.14 – 4.5	Y=85.487x – 17.581	0.9989
Trealose	0.14 – 4.5	Y=145.94x – 7.7245	0.9994
Melezitose	0.14 – 4.5	Y=22.329x – 2.3994	0.9996
Rafinose	0.14 – 4.5	Y=119.13x + 9.7327	0.9992
Melibiose	0.14 – 4.5	Y=108.3x – 2.7603	0.9997
Isomaltose	0.32 – 5.1	Y=42.552x – 1.8933	0.9999
Kojibiose	0.08 -1.35	Y=95.399x + 1.8282	0.9981
Erlose	0.15 – 2.5	Y=36.292x – 0.034	0.9997

2.2.10. Protein content

For the determination of the protein content, the Kjeldahl method was applied, which consists of indirect determination based on the quantification of total organic nitrogen (Pascual-Maté et al., 2018). This process began with the digestion of 1 g of honey by the addition of 15 mL sulfuric acid and a metallic catalyst that accelerates the oxidation process of organic matter in a digester at 400 ºC for 70 minutes. After the degradation of the sample and transformation of nitrogen into ammonium sulfate, a process of neutralization, distillation, and finally titration of released ammonia is followed. For the conversion of nitrogen content into total protein, a conversion factor of 6.25 was applied, expressing the results in g/100 g of honey.

2.2.11. Ashes

The ash content was determined in triplicate, indirectly through its calculation, according to what is defined in the literature (Sancho et al., 1992) from the following formula:

$$\%Ashes = \frac{\left(\frac{condutivity}{1000}\right) - 0{,}14}{1{,}74} \quad \text{(Equation nº 7)}$$

2.2.12. Carbohydrates

The carbohydrate content of the honey samples was obtained by differential calculation considering the following expression defined in the literature (Shugaba, 2012):

% Carbohydrates = 100 - % moisture - (% ash + % protein + % lipids) (Equation nº 5)

2.2.13. Energy

The energy value expressed in kcal was calculated in 100 g of honey, using the following equation (Shugaba, 2012):

Energy value (kcal/100g) = 4 x (% protein + % carbohydrates) + 9 x (% lipids) (Equation nº 6)

2.2.14. Mineral content

To check the minerals content, the following elements were assessed: magnesium (Mg), calcium (Ca), sodium (Na), and potassium (K), via the spectrophotometer of flame atomic absorption (Pye Unicam PU9100X). The detection of manganese (Mn), copper (Cu) and cadmium was done using atomic absorption spectrophotometry thought graphite chamber via a Perkin Elmer PinAAcle 900 spectrophotometer (AOAC International, 2016).

2.2.14.1. Sample Digestion

1g of sample was balanced into a PTFE digestion tube then 10 mL of concentrated nitric acid (HNO_3) was added. The sample was digested in a microwave via the following temperature gradient sequencer: a power of 1200 W during 15 minutes until 200 ºC. The continuous of these conditions were sustained for another 15 minutes. After that, samples were left to cool and quantitatively transferred into a volumetric flask of 50 mL. The quantification of the different minerals needs a previous preparation for specific solutions and standards according to the following technique:

2.2.14.3. Potassium And Sodium

For the quantification of the sodium and potassium elements, a cesium chloride buffer (10 g/L) and the preparation of different standard solutions were done according to the following requirement: solution 1: 10 mL of the potassium standard (1000 ppm) and 5 mL of sodium standard (1000 ppm) were pipetted into a flask of 20 mL and the volume completed with deionized water. Then the dilution of this stock solution was done further, according to **Table 6**, for presenting the calibration standards as follows.

Table 6- The calibration standards used in the spectrophotometer for the determination of the amounts of potassium and sodium.

Standard	V(sample)/mL	Vf/mL
P1/4	0.25	50
P1/2	0.50	
P1	1.00	
P2	2.00	
P3	3.00	
P4	4.00	
P5	5.00	

The calibration standards were done in the spectrophotometer resulted from the ten-fold dilution of these standards (5.0 mL solution of each standard and 5mL CsCl buffer in a the final volume of 50 mL). For the analysis of potassium in the supplement, a digested supplement solution of 5 mL, buffer solution of 1mL and 4 mL of deionized water were added. For the analysis of sodium in the supplement, 10 mL of the digested supplement solution, 1 mL of the buffer solution were added. The recording of the result was taken according to the conditions suggested for the tools.

2.2.14.4. Calcium and Magnesium

For the detection and quantification of calcium and magnesium, a solution (10 g/L) of lanthanum was prepared by diluting 13.15 g of La $(NO_3)_2$ in 1 L of deionized water. Also, a Ca standard solution (1000 ppm, solution 2) and an Mg standard solution (1000 ppm, solution 3) was set in 10 mL of deionized water. Also, from stock solutions 2 and 3 a series of standard solutions were set according to the following, Table 7.

The standards applied in the spectrophotometer calibration to determine the content of Ca was done from the ten-fold dilution of these standards (5.0 mL solution of each standard and 5 mL of solution La to a final volume of 50 mL). The standards applied in the spectrophotometer calibration to determine the content of Mg was done from the thirty-three-

fold dilution of these standards (1.50 mL solution of each standard and 5 mL of solution La to a final volume of 50 mL). To detect the content of potassium in the supplement, a digested supplement solution of 5 mL, buffer solution of 1 mL and 4 mL of deionized water were added.

For the examination, a digested solution of 10 mL and lanthanum solution of 1 mL was added. To determine the Ca and Mg a recommended condition according to the equipment was performed.

Table 7- The calibration standards used in the spectrophotometer for the quantification of calcium and magnesium.

Standard	**V (sol 2)/mL**	**V (sol 3)/mL**	**V_f/mL**
P1/4	0.25	0.25	50
P1/2	0.50	0.50	
P1	1.00	1.00	
P2	2.00	2.00	
P3	3.00	3.00	
P4	4.00	4.00	
P5	5.00	5.00	

2.2.14.5. Iron

Matrix modifier: diluted 1.7 mL of magnesium nitrate solution, Mg $(NO_3)_2$, 10 g/L to 10 mL of solution with deionized water.

Standard 1: diluted 0.50 mL of 1000 ppm standard solution to 50 mL with deionized water.

Standard 2: diluted 0.50 mL of standard solution to 50 mL with deionized water.

The standards used to construct the calibration curve resulted from the automatic dilution of standard 2 according to the Table 8.

Table 8- The calibration standards used in the spectrophotometer for the determination of amount of Iron.

Standard	V(P2) /µL	V(Matrix)/µL	V (H_2O) /µL
P1/4	5	5	15
P1/2	10	5	10
P3/4	15	5	5
P1	20	5	0

For sample analysis, 20 µL of the sample was pipetted from a 5 µL matrix modifier.

The instrumental conditions recommended for iron analysis were used.

2.2.14.6. Lead

Matrix modifier: 0.10 mL of magnesium nitrate solution, Mg $(NO_3)_2$, and 1.0 mL of 10% monobasic ammonium phosphate solution were diluted to 10 mL of solution with deionized water.

Standard 1: 0.50 mL of 1000 ppm standard solution was diluted to 50 mL with deionized water.

Standard 2: 0.70 mL of standard 1 solution was diluted to 50 mL with deionized water.

The standards used to construct the calibration curve resulted from the automatic dilution of standard 2, as stated above, Table 8. For the sample analysis, 20 µL of the sample was pipetted with a 5 µL of matrix modifier. The instrumental conditions for the analysis of lead were used.

2.2.14.7. Manganese, Copper and Cadmium

To determine the content of manganese, a modified matrix was applied by the dilution of 1.7 mL of a magnesium nitrate solution, Mg $(NO_3)_2$, 10 g/L, to a final volume of 10 mL with deionized water. Two standards solutions for manganese were prepared diluting 0.50 mL of standard solution (1000 ppm) to a final volume of 50 mL of deionized water and 0.20 mL of the previous solution to a final volume of 50 mL (standard 2). For copper, a modified matrix resulted from the dilution of 1.0 mL of palladium solution, 10 g/L, and 0.1 mL of magnesium nitrate solution, Mg $(NO_3)_2$, to a final volume of 10 mL of solution in deionized water. After that, the preparation of two copper standards was done by the dilution of 0.50 mL of the 1000 ppm standard solution (Vf = 50 mL deionized water, standard 1) and the dilution of 0.50 mL of the previous solution to a final volume of 50 mL (standard 2).

To determine the cadmium content, preparation of modified matrix was done by the dilution of 0.10 mL of magnesium nitrate solution, Mg $(NO_3)_2$, and 1.0 mL of 10% monobasic ammonium phosphate solution, $NH_4H_2PO_4$, in 10 mL of deionized water. The preparation of two standard solutions was then done, the first by the dilution of 0.25 mL of standard solution (1000 ppm) to 50 mL with deionized water (standard 1) and a second, the dilution of 0.10 mL of the above solution to 50 mL with deionized water (standard 2). The standards applied for the construction of the calibration curve resulted from diluting standard 2, as stated above, Table 8. To analyze all the samples, 20 µL of sample and 5µL of the modified matrix were pipetted with the application of the recommended instrumental conditions for each analysis.

2.2.15. Total phenolic content

Total phenolic content was determined according to a previously described method (Feás et al., 2010), with some modifications. Initially, a solution was prepared to weigh 1 g of honey in 10 mL of methanol. Then 0.5 mL of sample solution (or blank or standard) was mixed with 0.5 mL of Folin-Ciocalteu reagent. After 3 minutes, 1 mL of saturated sodium carbonate solution (Na_2CO_3) (10% w/v) and 3 mL of deionized water were added. The final solution was kept in the dark at room temperature for 1 hour. The absorbance was then read at 760 nm using a spectrophotometer (Analytik Jena, Jena, Germany). Gallic acid was used (0.005–0.15 mg/ml) as the standard solution. The following calibration curve (y = 8.0586x + 0.0027; R^2 = 0.992) was used for quantification with the total phenolic content expressed in milligram of gallic acid equivalent per gram of sample (mg GAE/g).

2.2.16. Total Flavonoid content

The total flavonoid content was recorded spectrophotometrically according to a previously described method (Falcão et al.,2013), with some modifications. An aliquot (1 mL) of the ethanolic extract (0.1 mg/mL) was mixed with 0.5 mL of aluminium chloride solution (5% aluminium chloride). The mixture was left in the dark for 30 min at room temperature, then the absorbance was recorded at 415 nm. Quercetin was applied for the calculation of the standard curve (y=4.4625x+0.0031; R2=0.9992). The total flavonoid content value of samples was expressed as milligram of quercetin equivalent per gram of sample (mg QE/g).

2.2.17. Antioxidant activity

2.2.17.1. DPPH free radical scavenging effect

The ability to block free radicals from DPPH (2,2-diphenyl-1- picrylhydrazyl) was evaluated in triplicate according to the methodology described in the literature with some modification (Ferreira et al., 2009). Briefly, 10 µL of methanol extract was mixed with different concentrations (0.003 - 0.03 mg. mL^{-1}), with 0.15 mL of a methanolic solution containing DPPH radicals (0.024 mM). After 60 minutes in the dark at room temperature, DPPH radical scavenging activity was measured by monitoring the decrease in absorbance at 515 nm in a microplate reader (ELX800 Microplate Reader Bio-Tek Instruments, Inc.). The DPPH radical scavenging activity was calculated as a percentage using the following equation (Equation nº 8), in which Abs corresponds to the absorbance of the solution with the sample extract and ABS DPPH to the initial absorbance of the DPPH solution.

$$\% \text{ Inhibition} = [(Abs_{DPPH} - Abs_{SAMPLE})/Abs_{DPPH}] \times 100 \quad \text{(Equation nº 8)}$$

The results were expressed using the EC_{50} value, corresponding to the concentration of extract that blocks 50% of the DPPH radicals present in the initial solution. For comparison, a standard solution of gallic acid whose mean value of EC_{50} is 1.22 mg. mL^{1} was applied.

2.2.17.2. Reducing power

To evaluate the reducing power, a previously described methodology was applied, with some modifications (Ferreira et al.,2009). 125 µL of a honey solution was mixed with a concentration of 0.010 $g.mL^{-1}$ with 1.25 mL of a phosphate buffer solution (pH 6.6) and 1.25 mL of trihydrated potassium ferrocyanide (0.2 M). After addition, the mixture was vigorously stirred and incubated at 50 °C for 20 minutes. After this period, 1.25 mL of trichloroacetic acid (at 10%) was followed by a centrifuge process at 3000 rpm for 10 minutes (Centurion K24OR-2003). In the end, 1.25 mL of the supernatant was put in another test tube, and 1.25 mL of distilled water and 0.25 mL of 0.1% of ferric chloride was added. The absorbance was then read at 700 nm using a spectrophotometer. For the blank, the solution was prepared with 125 µL of methanol instead of the honey sample. Gallic acid (0.0004-0.025 mg/mL) was used as standard ($y = 46.415x - 0.0275$; $R^2 = 0.993$), and the results were expressed as milligram of gallic acid equivalent per gram of sample (mg GAE/g).

2.2.18. Phenolic profile

2.2.18.1. Extraction

For the quantification and determination of the phenolic profile, honey samples were extracted in triplicate, weighing 25 g of honey in 125 mL of acidified water (pH 2, HCl). The solution was then filtered to remove any solid particles. The filtered solution was passed through an Amberlite® XAD®-2 column, which can selectively retain phenolic compounds. To remove sugars and other polar compounds, a wash was carried out with the passage of acidified water at pH 2 (50 mL). Subsequently with deionized water (150 mL). The phenolic fraction was eluted with methanol (150 mL) and taken to dryness under reduced pressure (40 ºC). The residue was redissolved in 5 mL of water and extracted with diethyl ether (5 mL). The ether extracts were combined, concentrated under reduced pressure, and re-dissolved in 0.5 mL of methanol for UPLC/DAD/ESI-MS^n analysis.

2.2.18.2. Analysis by UHPLC/DAD/ESI-MSn

UHPLC/DAD/ESI-MS^n analysis was performed on a Dionex UPLC 3000 equipment (Thermo Scientific, USA), Figure11, equipped with a photodiode array detector coupled to a mass detector. The chromatographic system consisted of a quaternary pump, an automatic sampler maintained at 5 ºC, a degassing, a photodiode array detector, and an automatic thermostatic column compartment. Chromatographic separation was performed with a U-VDSpher PUR C18-E 100 mm x 2.0 mm, 1.8µm column, with a particle size of 1.8 µm (VDS Optilab, Germany) maintained at 30 ºC. The mobile phase was composed of (A) 0.1% (v/v) formic acid in water and (B) 0.1% (v/v) formic acid in acetonitrile, previously degassed and filtered using a nylon membrane filter with 0.22 µm porosity.

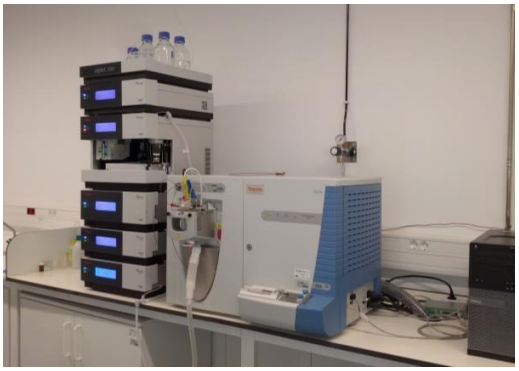

Figure 11- UHPLC/DAD/ESI-MSN equipment

For the run, a linear gradient with a flow rate of 0.3 mL.min^{-1} was used: 0.0- 1.0 min 20% B; 1.0-11.1 min 20-95% (B); 95% (B) for 2 min; 13.0-13.3 min 95- 20% (B); and 20% (B) for 5 min. The injection volume was 3 µL. Spectral data from all peaks were collected in the range of 190-600 nm. Each sample was filtered through a 0.2 µm (Whatman) nylon membrane.

Mass analysis was performed on an LTQ XL mass spectrometer (Thermo Scientific, CA, USA), in negative mode, equipped with an ESI electrospray ionization source: spray voltage, 5 kV; capillary voltage, -20 V; capillary tube voltage, -65 V; capillary temperature, 325 °C; gas flow and auxiliary gas (N2), 50 and 10 (arbitrary units), respectively. The mass spectra were acquired in the mass range of 100- 1000 *m/z*. The collision energy used in the MS^n experiments was 35 (arbitrary units). Data acquisition was performed using Xcalibur software® (Thermo Scientific, CA, USA). Quantification was performed with standard substance calibration curves for *p*-hydroxybenzoic acid (y = 4x10^{6}x-134082; R^2 = 0.9988), caffeic acid (y = 3x10^{6}x-12895; R^2 = 0.9998), *p*-coumaric acid (y = 4x10^{6}x-13435; R^2 = 0,9999), quercetin (y = 893859 x-11231; R^2 = 0.9999), chrysin (y = 5x10^6 x-32533; R^2 = 0.9990), naringenin (y =

$5x10^6 + 14548$, $R^2 = 0.9996$) and abscisic acid ($y = 2x10^7x-4x10^6$; $R^2 = 0.9988$). When standards were not available, the compounds were expressed by equivalents of the structurally more similar phenolic compound. The elucidation of the structure of phenolic compounds was carried out by comparing their chromatographic behaviour, UV spectra and mass profile with that obtained for commercial standards and with the information obtained in the literature, when these were not available.

2.2.19. Antitumor activity

The human tumor cell lines explored were the following: Caco (colorectal adenocarcinoma), AGS (gastric adenocarcinoma), NCI-H460 (lung carcinoma), MCF-7 (breast adenocarcinoma). A non-tumor cell line, Vero (African green monkey kidney), was also experimented. All of them were conserved in RPMI-1640 medium enriched with 10% fetal bovine serum, glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 mg/mL), except for Vero cells, conserved in DMEM medium enriched with fetal bovine serum (10%), glutamine and antibiotics. The incubation of the culture flasks was done in an incubator at 37 ºC and with 5% CO_2, under a humid atmosphere. The cells were applied only when the confluence reach 70 to 80%. An identified mass of each of the extracts (8 mg) was dissolved in H_2O (1 mL), to obtain the stock solutions with a concentration of 8 mg/mL. From this, several sequential dilutions were prepared, in the following range, 0.125 - 8 mg/mL. The incubation of each of the extract concentrations (10 µL) were done with the cell suspension (190 µL) of the cell lines examined in 96-well microplates for 72 hours. The incubation of the microplates was done at 37 ºC and with 5% CO_2, in a humid atmosphere, after ensuring the adherence of the cells. All cell lines were examined at a concentration of 10,000 cells/well, except for Vero in which a density of 19,000 cells/well was applied. After a period of the incubation, the cells were adjusted: TCA (10% w/v; 100 µL) was cooled before and the incubation of the plates was done for 1 hour at 4 ºC, rinsed with water and, once being dry, the addition of SRB solution (0.057%, m/v; 100 µL) was done, kept at room temperature for 30 minutes. To eliminate non-adhered SRB, plates were rinsed three times with a solution of acetic acid (1% v/v) and left to dry. Finally, an adhered SRB was solubilized with Tris (10 mM, 200 µL) and the reading of the absorbance at a wavelength of 540 nm was done in the Biotek ELX800 microplate reader. The expression of results was in terms of the concentration of extract through the ability to inhibit cell growth by 50% - GI_{50}. As a positive control of an ellipticin was used.

2.2.20. Anti-inflammatory activity

The dissolution of the extracts was performed in H_2O to get a final concentration of 8 mg/mL. From which sequential dilutions were carried out, obtaining (0.125 - 8 mg/mL) concentrations to be tested. The RAW 264.7 mouse macrophage cell line, gotten from DMSMZ - Leibniz - Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, was maintained in DMEM medium, enhanced with heat-inactivated (SFB) fetal serum (10%), glutamine and antibiotics, and the incubation was done in an incubator at 37 ºC, with 5% CO_2, under a humid atmosphere. Cells were removed with a cell scraper. An aliquot of the cell suspension of macrophages (300 µL) with a cell density of 5 x 10^5 cells/mL and with several dead cells below 5% according to the Trypan blue exclusion test, was placed in each well. The microplate was kept for 24 hours in the incubator with the conditions previously mentioned to allow a suitable adherence and growth for the cells. Subsequently, the treatment of cells was done with different concentrations of extract (15 µL, 0.125 - 8 mg/mL) and incubated for one hour, with the range of concentrations tested being 6.25 - 400 µg/mL, and then stimulated by the addition of 30 µL of the liposaccharide solution - LPS (1 mL/mL) and incubated for 24 hours. Dexamethasone (50 mM) was applied as a positive control and samples in the absence of LPS were considered as a negative control. Quantification of nitric oxide was done using a Griess reagent system kit (nitrophenamide, ethylenediamine, and nitrite solutions) and through the nitrite calibration curve (100 mM sodium nitrite at 1.6 mM) set in a 96-well plate. The determination of the nitric oxide produced was performed by reading absorbances at 540 nm (ELX800 Biotek microplate reader, Bio-Tek Instruments, Inc., Winooski, VT, USA) and by comparison with the standard calibration line. The results were assessed using the graphical representation of the inhibition´s percentage of nitric oxide production versus the sample concentration and expressed with the concentration of each of the extracts that responsible for 50% inhibition of nitric oxide production - IC_{50}.

2.2.21. Screening of antibiotics

For the detection of antibiotics residues (sulphonamides and tetracycline) in honey, the methodology described in the literature (Serra Bonvehí & Lacalle Gutiérrez, 2009) was applied, with some modifications. The honey extract with active reagents were added in consecutive and competitive assay formats at many temperatures incubation improved for detection of drugs. The extraction technique was as defined in the operator´s manual Charm II sulpha drug test for honey (Operator's Manual, 2011). The tests took 12–20 minutes for tetracycline, while for sulphonamides assay used more complex acid hydrolysis and reverse-phase preparation to

remove p-aminobenzoic acid (PABA) and change carbohydrate-SAs into free form (total time 1 hour).

2.2.21.1. Sulphonamides

Approximately 5 g of honey was weighted into a 50 mL centrifuge conical tube and combined with 20 mL of 1 M HCl. followed by the incubation of the solution at room temperature for 1 hour. Then, 2 mL of 30% NaOH were combined to the sample solution, after that, the pH was set at 7.7–8.0, drop by drop, with 30% NaOH. After a previouse filtration, the solution was passed through a Bond Elute C18 cartridge (500 mg, 3 mL), previously activated with 5 mL of methanol and 5 mL of distilled water. The solution was set at a flow rate of 1–2 drops per second, and then the cartridges were rinsed with 5 mL of distilled water. The elution of the bounded compounds from the column was done with 1 mL methanol, and then the eluate was evaporated until dryness on a hot plate (40–60 ˚C). The reconstitution of the dry residue was made with the addition of 4 mL of Zero Control Standard solution and cooled in ice for 10 min. After cooling, the solution was first mixed with the binding reagent tablet SMMSU-22U (previously dissolved in 300 µL of distilled water), and then mixed with the tracer reagent tablet SMMSU-022C, followed by incubated at 85 ˚C for 3 min. After incubation, the samples were homogenized and centrifuged (3400 rpm for 3 min, Heraeus centrifuge). The supernatant was then discarded, and the residue redissolved in 300 µL of distilled water and 3mL scintillation fluid (Opti-fluor). Immediately, the solution was measured on [^{3}H] channel of Charm analyzer in counts per minute (CPM) and compared with a control point, which is the cut-off between a negative (in this case 980) or positive result (in this case 1629). Any antimicrobial agent present in the sample extract competes for the binding sites with the tracer, thus, the greater the CPM measured, the lower the antimicrobial drug concentration in the samples and vice-versa. Samples with high counts are considered negative (tracer antimicrobials are largely bound to the binder) while those with low counts are considered positive (tracer antimicrobials are largely free in solution). (Mukota et al., 2020). A positive and negative control was made in every series of assays, as control measure.

2.2.21.2. Tetracycline

Sample preparation for tetracyclines was restricted to a simple dilution step after labeling of 50 mL tube, by dissolving 5 g of honey in 20 mL of distilled water. In a glass tube, the green tablet containing the tracer reagent was first suspended in 300 µL of distilled water,

followed by the addition of 5 mL of the diluted honey solution, and incubated at 45 ˚C for 15 min. After incubation, the samples were homogenized and mixed with the orange tablet (second tracer) followed by a new incubation stage for 5 min at 45 ºC. Finally, the black tablet also containing a tracer reagent is added, mixed and centrifuged (5000 rpm for 5 min, Heraeus centrifuge). The supernatant was then transferred to a new tube containing the white tablet with the binding reagent (previously dissolved with 300 µL of water) which was incubated for 5 minutes in 45 ºC. Finally, the supernatant was poured off and the residue was dissolved with 300 µL distilled water and 3 mL scintillation fluid (Opti-fluor). Immediately, the solution was measured on the [^{3}H] channel of the Charm analyzer (CPM) and compared with the control point, in this case the negative point is 1661 and the positive point is 1090. A positive and negative control was made in every series of assays, as control measure.

CHAPTER III: RESULTS AND DISCUSSION



3.Results and discussion

3.1. Honey quality assessment parameters

Honey is considered the most important primary product of beekeeping, being a nutritional food. The strict control of the quality of food products, particularly honey has progressively been required. Therefore, ten Algerian honey samples were evaluated in terms of its quality parameters, namely melissopalynological profile, humidity, pH, acidity, conductivity, color, 5-HMF, diastase activity, proline, nutritional parameters, and antibiotics residues. Besides, the samples were also characterized for the total phenolics, flavonoid content, phenolic profile, antioxidant activity, antitumor, and anti-inflammatory activity.

3.1.1 Melissopalynological analysis

Honey can result from a wide variety of plant species due to the collection of nectar from botanical sources available around the apiary. Generally, honey is considered monofloral when no less than 45% of pollen grains come from one floral species (Soares et al., 2017). There are some exceptions, especially honey with underrepresented pollen grains, such as the case of lavender honey, where only 15% of the pollen grains are needed to classify it as monofloral honey. For overrepresented pollen grains, like chestnut and eucalyptus, the honey must display 70% to 90% of pollen abundancy (Pires et al., 2009). If none of the identified pollen can be considered predominant the honey is classified as multifloral (Pires et al., 2009). Within the studied samples, forty-three different pollen types were identified, with the most frequent ones summarized in Table 9. The main pollen families were Asteraceae, Fabaceae, and Lamiaceae. *Cytisus striatus* pollen type was present in 7 samples, in a percentage ranging between 7 % and 60 %, with the MF sample from the Sidi Belabbes region presenting the highest value. Also, *Rosmarinus officinalis* and *Centaurea* sp. pollen types ranged from 56-64% and 54-71%, respectively. *Brassica napus* and *Carlina racemosa*, pollen types were also found, but less frequently. Figure 12 shows the dominant pollen found in some honey samples.

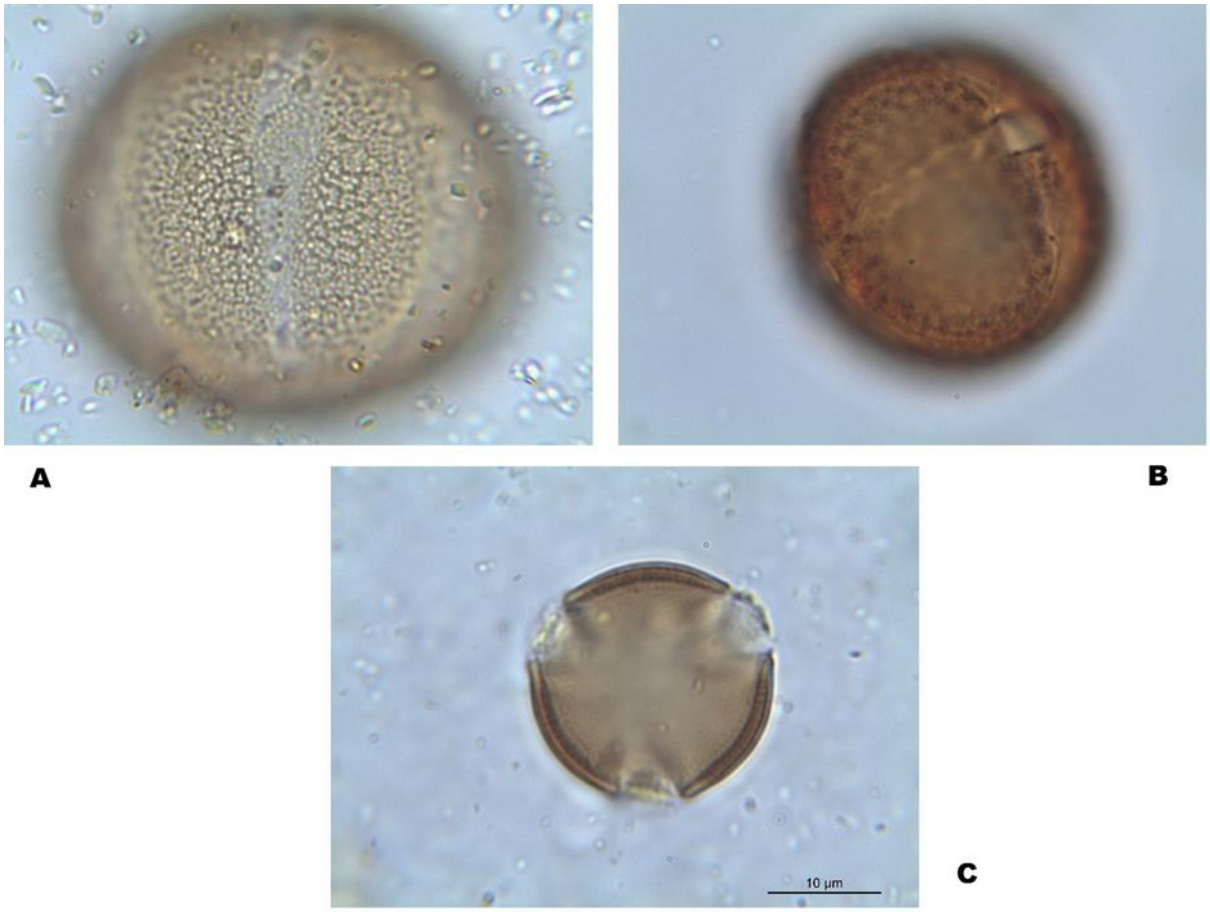

Figure 12 Dominant pollen found on each type of honey on these ten samples:A (*Rosmarinus officinalis*), B (*Centaurea* sp.) and C (*Cytisus striatus*) (Pictures made by Paulo Russo ,LabApis - UTAD)

To produce monofloral honey, beekeepers place the beehives in area where the bees have access mainly to a specific type of flower. However, bees have innate movement that cannot be controlled by the beekeepers. This situation may induce to mislabel the honey. Indeed, in the present study, the pollen analysis for six samples do not confirmed the label from the honey jar. Considering these results, the samples CH1, CH2, and from the El Bayedh region were classified as Centaurea monofloral honey since the pollen of *Centaurea* sp. represent more than 45% of total pollen, Table 9, with CH1 presenting the highest pollen percentage with a value of 71.2%. In other samples, such as T1, T2, and T3 (El Bayedh region), the presence of *Tamarix gallica* pollen is observed but not above 45%, leading to the classification of these honeys as hairy-fruited broom monoflorals, since pollen of *Cytisus striatus* floral species represent more than 45% of the total pollen, with the sample a T1 and T2 with a higher percentage of this type of pollen (48.5 %). Besides those, also the labeled

multifloral sample MF (Sidi Belabbes region) is indeed a monofloral honey of *Cytisus striatus* with the pollen percentage at 60.2%.

Table 9- Melissopalynological profile of the honey samples.

Sample	Floral origin on the label	Origin	D	A	I
R1	*Rosmarinus officinalis*	Sidi Belabbes	*Rosmarinus officinalis* (62.15%)	*Brassica napus* (17.5 %)	Apiaceae (9%)
R2	*Rosmarinus officinalis*	Sidi Belabbes	*Rosmarinus officinalis* (56.11%)	*Brassica napus* (15.5%)	-
R3	*Rosmarinus officinalis*	Sidi Belabbes	*Rosmarinus officinalis* (64.40 %)	*Brassica napus* (16.7 %)	Apiaceae (6.0%) Liliaceae (6.2%)
T1	*Tamarix gallica* L.	El Bayedh	*Cytisus striatus* (48.5 %)	*Tamarix* (23.5%)	Apiaceae (5.9 %), *Carlina recemosa* (3.7 %), *Ononis* (2.5%), Liliaceae (4.9%).
T2	*Tamarix gallica* L.	El Bayedh	*Cytisus striatus* (48.5 %)	*Tamarix* (24.2%)	Apiaceae (5%), *Carlina recemosa* (5 %), *Ononis* (2.9%), Liliaceae (5.1 %).
T3	*Tamarix gallica* L.	El Bayedh	*Cytisus striatus* (43.7 %)	*Tamarix* (24,0%)	Apiaceae (5%), *Carlina recemosa* (3.2 %),*Ononis* (5 %),Liliaceae (4.6 %)
CH1	*Silybum marianum*	El Bayedh	*Centaurea* sp. (71.2%)	-	*Type Cytisus striatus* (7.1%)
CH2	*Silybum marianum*	El Bayedh	*Centaurea* sp. (53.5%)	*Cytisius striatus* (16 %)	*Ailanthus altissima* (8.7%)
CH3	*Silybum marianum*	El Bayedh	*Centaurea* sp. (56.6 %)	*Cytisius striatus* (22.4%)	-
MF	Multifloral	Sidi Belabbes	*Cytisus striatus* (60.2 %)	*Tamarix* (16.5 %)	*Carlina racemosa* (4.4 %)

Note: D- dominant pollen (45% or more); A- accompanying pollen (15% - 45%) and I- important pollen (3% - 15%).

For the labeled rosemary samples, the *Rosmarinus officinalis* pollen emerged as the majority in al samples (Sidi Belabbes), with an average of 60.9 %, allowing its classification as monofloral rosemary honey (Perez-Arquillué et al., 1994).

3.2. Physicochemical characterization

3.2.1. Color

Color is related to the botanical origin, climate, and soil conditions. Some authors have reported that pollen, sugars related products, carotenoids, xanthophylls, anthocyanins, minerals, amino acids and phenolic compounds, mainly flavonoids, influence the honey color (Machado De-Melo et al., 2017). The colorimetric examination of the honey samples understudy was achived using the Pfund scale by direct reading on the colorimeter.

The results show that almost all honey samples have light amber color, except the samples for rosemary honey which has extra white amber color, Table 10. Rosemary honey color varied from 42 mm to 49 mm Pfund, which is above the value previously described for this type of honey collected from Algeria (13 mm Pfund) (Homrani et al., 2020). However, the values are similar to the ones cited previously for Moroccan rosemary honey, where the values varied from 28 to 51 mm Pfund (Chakir et al., 2016). The samples T1, T2, and T3 showed a light amber color varying from 77 mm to 79 mm Pfund. Also, CH1 CH2 and CH3 honey presented light amber color, as well as MF sample, Table 10, which is similar to the previously reported results for this type of honey (Homrani et al., 2020).

3.2.2. Moisture content

The moisture content is a very significant feature in the honey analysis, being associated with many factors like the geographical and botanical origin of nectar, the soil, the climatic conditions, the intensity of nectar flow, the season of harvesting, the manipulation by beekeepers during harvesting, as well as the conditions of extraction, storage, processing, and the degree of maturation (Machado De-Melo et al., 2017). This parameter affects other features of honey, like viscosity and its tendency of crystallization, taste, color, conservation, and solubility (Olaitan et al., 2007).

Table 10- physicochemical parameters: color, humidity, and conductivity include: (mean +/- SD).

Samples	Color (mm Pfund)	Moisture content (%)	Conductivity ($mS.cm^{-1}$)
R1	49 ± 0 (Extra white Amber)	13 ± 1	0.09 ± 0.01
R2	43 ± 0 (Extra white Amber)	14 ± 1	0.10 ± 0.01
R3	42 ± 0 (Extra white Amber)	13 ± 1	0.11 ± 0.01
T1	77 ± 0 (Light Amber)	16 ±1	0.11 ± 0.01
T2	76 ± 0 (Light Amber)	16 ± 1	0.34 ± 0.05
T3	79 ± 0 (Light Amber)	16 ± 1	0.33 ± 0.04
CH1	61 ± 0 (Light Amber)	14 ± 1	0.24 ± 0.04
CH2	60 ± 0 (Light Amber)	15 ± 1	0.25 ± 0.01
CH3	72 ± 0 (Light Amber)	15 ± 1	0.25 ± 0.01
MF	60 ± 0 (Light Amber)	15 ± 1	0.28 ± 0.02

Moisture content normally ranges between 13 and 25%, considering that honey with moisture level above 18% have a great propensity for fermentation (Machado De-Melo et al., 2017) and according to codex Alimentarius (Codex, 2001) the maximum content established for the moisture level of honey is 20%, except industrial use honey and heather honey (*Calluna* sp.) which may have levels that can reach 23%. The moisture level in the studied samples varied between 13% and 16%, Table 10, all respecting the maximum value established by codex Alimentarius (Codex, 2001). Honey samples T1, T2, and T3 from El Bayedh revealed a higher moisture content (16%) as opposed to the R1 and R3 (rosemary) samples from Sidi Belabbes with a value of 13%. The moisture content of these samples was below to the previously reported for Algerian rosemary honey, where the mean value was 16% (Homrani et al., 2020). However, these values are similar to Portuguese rosemary honey with 13.6% (Mendes et al., 1998). The sample MF (Sidi Belabbes), classified as multiflora honey, present moisture values (15 %) within the defined intervals for this honey which vary between 14% and 19.5% (Dahmani et al., 2020). For the samples CH1, CH2 and CH3, the

values ranged from 14% to 15%.

The moisture values obtained suggest that the honey samples were extracted adequately and present an adequate degree of maturation for honey.

3.2.3. Electrical conductivity

The electrical conductivity of honey is associated with the protein content, organic acids, acidity, and ash content (Yucel & Sultanoglu., 2013), being a significant feature for the identification of the honey botanical origin, specifically to discriminate between nectar honey and honeydew. Honey with electrical conductivity levels above 0.8 $mS.cm^{-1}$ are indicative of honeydew honey or, exceptional nectar honeys with high conductivity such as chestnut honey, while those that have levels under 0.8 $mS.cm^{-1}$ are defined as nectar honey or mixtures of various nectars (Codex, 2001).

In the analyzed honey samples, conductivity values were recorded between 0.09 $mS.cm^{-1}$ and 0.34 $mS.cm^{-1}$, Table10, being similar to what is described in the literature for Algerian multiflora honey with values ranging from 0.110 to 0.930 $mS.cm^{-1}$ (Makhloufi et al., 2010). For rosemary honeys, the values ranged from 0.09 to 0.11 $mS.cm^{-1}$, which were below comparing to the results reported for Algerian rosemary honey whose mean value was 0.330 $mS.cm^{-1}$ (Homrani et al., 2020). However, this value is within the range of Moroccan rosemary honey with values ranging from 0.11 to 0.14 $mS.cm^{-1}$ (Chakir et al., 2016). All the analysed samples presented conductivity values lower than 0.80 $mS.cm^{-1,}$ suggesting that they were nectar or nectar mixture.

3.2.4. pH and acidity

Acidity is one of the most significant features of honey responsible for its conservation and stability and helps in the prevention of microorganism's development and correlated with its flavor. The free acidity of honey is affected by the organic acids that are present in equilibrium with the corresponding esters, lactone, and some inorganic ions like phosphates, chlorides, and sulfates (Finola et al.,2007). To evaluate the acidic features of honey, three parameters were evaluated: the pH level of the initial solution; the free acidity; and the lactone acidity. The free acidity level is obtained by titration with sodium hydroxide to the equivalence point pH 7. Lactone acidity is measured by the addition of an excess sodium

hydroxide that is titrated with hydrochloric acid. To determine the total acidity the sum of free acidity and lactation are done.

Even though the Codex Alimentarius commission (Codex, 2001) does not establish a limit on the pH level in honey, it should vary between 3.2 and 4.5 for the inhibition of the most of microorganisms (Doner, 2003). The studied honey samples have pH values ranging between 3.98 and 4.67, Table 11, in agreement with the values frequently found for Algerian honey (Dahmani et al., 2020). The results for free acidity, determined at the equivalence point (pH 7) ranged from 7 to 31.2 meq.kg^{-1} being within the limit of 30 meq.kg^{-1} defined for honey in Algeria (Dahmani et al., 2020). The CH2 and CH3 samples showed the highest values of free acidity, 31.2 and 28.5 meq.kg^{-1}, respectively, thus suggesting possible contribution of chemical species responsible for the acidity of honey to its conductivity. For these samples, conductivity may massively affect by the presence of another compound, such as inorganic matter. Rosemary honey (coded by R) exhibited a value of free acidity ranging from 12.2 to 13 meq.kg^{-1} which are lower than the value described in the literature for Spanish rosemary honey (Perez-Arquillué et al., 1994). However, this value are similar to the value described for Portuguese rosemary honey of 13.9 meq.kg^{-1} (Mendes et al., 1998).

The lactone acidity values of the samples varied between 5.7 and 36.1 meq.kg^{-1}**,** Table 11. All samples present acidity values close to the stipulated maximum limit, which is reflected in the total acidity values between 20.1 meq.kg^{-1} and 64.7 meq.kg^{-1}, Table 11.

Table 11- pH and acidity of the honey samples include: (mean +/- SD).

Sample	pH initial	Free for pH=7 (meqKg^{-1})	Free for pH=8.3 (meqKg^{-1})	Lactonic (meqKg^{-1})	Total (meqKg^{-1})
R1	4.56	7.2 ± 0.3	12.2 ± 1.1	17.5 ± 0.6	24.7
R2	4.55	7.4 ± 0,7	13.0 ± 2.0	15.5 ± 0.7	22.9
R3	4.67	7.0 ± 0.2	12.7 ± 1.5	17.2 ± 0.5	24.3
T1	4.27	14.5 ± 0.2	21.9 ± 0.5	31.5 ± 0.4	46.1
T2	4.31	14.7 ± 0.4	22.6 ± 1.6	28.5 ± 0.2	43.2
T3	4.55	12.8 ± 0.1	17.6 ± 0.4	22.8 ± 0.6	35.6
CH1	4.32	14.9 ± 0.6	21.7 ± 0.4	35.8 ± 0.5	50.6
CH2	3.98	31.2 ± 0.1	43.9 ± 0.4	27.1 ± 0.1	58.3
CH3	3.99	28.5 ± 0.8	41.6 ± 1.8	36.1 ± 0.3	64.7
MF	4.23	14.4 ± 0.4	5.8 ± 0.1	5.7 ± 0.1	20.1

3.2.7. Proline

The amount of proline (free amino acid) in honey is very high and, as a quality parameter, may be linked to the maturation stage of honey and possible adulterations. However, it should be taken into account that there is considerable proline variation, depending on the honey type (Bogdanov et al., 1999). Low proline level indicates inadequate processing and storage conditions due to the reaction of this amino acid with reducing sugars, like glucose and fructose, in terms of Maillard reactions. Despite that, the proline level is not regulated in the Algerian legislation, but it is recognized that genuine honey should have a proline content of more than 0.18 mg. g^{-1} (Bogdanov,2002).

In this study, it was observed that the values obtained for the proline content ranged between 0.4 mg. g^{-1} and 2.8 mg. g^{-1}, Table 12. The obtained values indicated a high proline content indicative of unadulterated honey and an excellent degree of maturation, and are in accordance with the previously reported for Algerian multiflora honey where the proline contents range between 0.20 and 18.77 $mg.g^{-1}$ (Dahmani et al., 2020). Another study on Tunisian rosemary honey showed a mean value of proline content of 0.4 ± 0.02 $mg.g^{-1}$ (Boussaid et al., 2018) which are similar for the rosemary honey samples in the present study where the value ranged from 0.4 to 0.7 $mg.g^{-1}$.

3.2.5. 5-Hydroxymethylfurfural

The content of 5-HMF is an indicator of the quality of honey, and its presence means a certain deterioration of the honey. Directly after the process of the extraction, the 5-HMF is almost absent in honey. Nevertheless, during the process, which may involve thermal treatments and long storage period, its level tends to increase gradually due to degradation reactions of sugars, like glucose and fructose, in acidic medium (Castro-Vázquez et al.,2003), and Maillard reactions between some amino acid residues and reducing sugars (Soares et al.,2017). The formation of 5-HMF is affected by many features, specifically floral origin, the presence of organic acids, sugar profile, pH, storage condition, aging, and temperatures (Fallico et al.,2006). The Codex Alimentarius (Codex, 2001) sets a limit for the HMF of 40 $mg.kg^{-1}$, with the exception of honeys from tropical regions, where the highest amount may reach 80 $mg.kg^{-1}$.

For the analyzed samples, the 5-HMF values ranged between 11.2 $mg.kg^{-1}$ and 35.8

mg.kg^{-1}, Table 12, except for sample R2 and R3 from the region of Sidi Belabbes, which presented a value of 65.6 mg.kg^{-1} and 79.8 mg.kg^{-1} respectively, being above the established value. However, the value of R2 and R3 is similar to the previously reported Portuguese rosemary honey with 62.5 mg.kg^{-1} (Mendes et al., 1998). The high value of 5-HMF recorded in these samples may be due to different factors such as poor sample storage, exposure to high temperatures, or may also be indicative of counterfeiting by adding inverted syrup (Capuano & Fogliano., 2011). The samples labeled as multifloral honey MF presented 5-HMF content of 11,2 mg.kg^{-1}, Table 12, which is following the values described in some studies for this type of honey where the values are ranging between 0.50 and 123.98 mg.kg^{-1} (Makhloufi et al., 2010). The samples T1 to T3 showed concentrations of 5-HMF between 19.5 and 27.7 mg.kg^{-1}, respectively. Also, CH1, CH2 and CH3 samples had values ranging from 12.6 to 15.4 meq.kg^{-1}.

Table 12- Physicochemical honey parameters: 5-HMF, diastase index, and proline include: (mean +/- SD)

Sample	HMF (mg.kg^{-1})	Diastase index (DN)	Proline (mg. g^{-1})
R1	65.6 ± 0.8	2.7 ± 0.2	0.6 ± 0.1
R2	79.8 ± 1.1	2.1 ± 0.1	0.4 ± 0.1
R3	35.8 ± 0.7	3.9 ± 0.8	0.7 ± 0.1
T1	27.7 ± 0.6	9.1 ± 0.2	2.8 ± 0.1
T2	18.2 ± 0.3	9.8 ± 0.3	2.0 ± 0.1
T3	19.5 ± 0.4	7.9 ± 0.5	2.5 ± 0.1
CH1	15.4 ± 0.5	14.0 ± 0.1	1.8 ± 0.1
CH2	12.6 ± 0.8	14.7 ± 0.5	1.6 ± 0.1
CH3	19.6 ± 0.5	12.9 ± 0.4	2.6 ± 0.1
MF	11.2 ± 1.1	8.6 ± 0.8	2.2 ± 0.3

3.2.6. Diastase index

Diastase is a set of enzymes (α and β-amylase) secreted by the bee in honey, and usually used as an indicator of honey aging, since they have a high sensitivity to heat. According to the current quality standards (Codex, 2001), the minimum diastase activity content is 8 units of Schade (DN) or 3 DN for low natural enzyme honeys, such as citrus honey.

The diastase activity values of the samples, Table 12, varied between 2.1 and 14.7

DN**,** Table 12. R1, R2, and R3 presented a lower diastase index, ranging between 2.1 and 3.9 DN. Taking in account the high 5-HMF content of these samples, these values seem to indicate the occurrence of a slight fermentation resulting from a possible heating process or a less adequate storage. Also, the floral origin can be correlated with that, since rosemary honeys are recognized for their low enzyme content (Machado De-Melo et al., 2017), which is in accordance with the reported for Portuguese rosemary honey (Mendes et al., 1998).

Nevertheless, the values recorded for the other samples analysed are following the Codex Alimentarius and EU standards, with the value of the T1, T2, and T3 samples ranging from 7.9 to 9.8 DN, and the samples CH1, CH2 and CH3 with values ranging from 12.9 to 14.7 DN, which are in range to previously published works in Algerian multiflora honey (Makhloufi et al., 2010).

3.2.8. Protein content

The amount of proteins in honey is related to the enzymes that are derived both from the plant (nectar and pollen) and bees (secretions from the salivary glands) (Machado De-Melo et al., 2017). The total protein content influences the aroma, which is considered typical to each type of honey, due to the occurrence of the Maillard reactions.

Table 13- Nutritional parameters: ashes, proteins, energy, and carbohydrates include: (mean +/- SD)

Sample	Ash (g/100g)	Protein content (g/100g)	Energy (Kcal)	Carbohydrates (mg/100g)
R1	-	0.29 ± 0.01	347.3	86.5
R2	-	0.29 ± 0.03	344.5	85.8
R3	-	0.36 ± 0.03	347.7	86.6
T1	-	0.39 ± 0.01	335.7	83.5
T2	0.11 ± 0.03	0.64 ± 0.02	334.7	83.1
T3	0.10 ± 0.00	0.47 ± 0.02	335.2	83.3
CH1	0.07± 0.02	0.31 ± 0.02	342.6	85.3
CH2	0.06 ± 0.01	0.34 ± 0.01	341.0	84.9
CH3	0.06 ± 0.01	0.52 ± 0.03	340.6	84.7
MF	0.08 ± 0.01	0.41 ± 0.05	339.3	84.4

The total protein content of samples ranged from 0.29 to 0.64 g/100 g, Table 13. The obtained results showed remarkable similarity with the protein amounts recorded in Algerian

multiflora honey (0.4-0.9 g/100g.) (Ouchemoukh et al., 2007). For the rosemary honey, it was recorded a value ranging between 0.29 and 0.36 g/100g which is above the values recorded in a previous study about Tunisian rosemary honey (0.13 ± 0.02 g/100g) (Boussaid et al., 2018).

3.2.9. Ashes

The Codex Alimentarius (1999) provide values for ashes parameter and establishes that must have a maximum of 0.6 % for nectar honey and 1.2 % for honeydew honey or a mixture of honeydew honey with blossom honey or chestnut honey. The obtained results in this study for the ash content were between 0.06 and 0.11%, Table 13, being within the recommended values for nectar honey. Also, the samples showed similar values to those described for Algerian multiflora honey of whose values are between 0.09 and 0.54% (Ouchemoukh et al., 2007). For rosemary honey, the values were not analytically significant. For the CH1, CH2 and CH3 samples, the values ranged from 0.06% to 0.07 %, while the highest ash value was found in samples T2 (0.11%), and T3 (0.10%) from El Bayedh. These samples were also those that had presented the highest electrical conductivity values, evidencing a positive correlation between these parameters that are frequently reported in the literature (Yücel & Sultanoglu., 2013). MF sample showed 0.08% of ashes, which is in accordance with previously reported values for Algerian multiflora honey with values ranging from 0.02% to 0.52 % (Amri & Ladjama, 2013).

3.2.10. Total carbohydrates and energy

Energy value and carbohydrate content have no regulation of the limits but are important parameters for nutritional assessment, and frequently mandatory on labelling. The results showed that the honey samples had similar total carbohydrates and energy values ranging from 83.0 to 86.6 g/100g and 334.7 and 347.7 kcal, respectively, Table 13. The energy value obtained in the samples of the present study is identical to that defined in the previous study for monofloral European honey with a mean value of 321.4 ± 5.8 kcal (Escuredo et al., 2013).

3.2.11. Sugars

Honey is a super-concentrated solution of sugars whose main compositions are fructose and glucose. Monossacarides (fructose and glucose) are about 75% of the total sugars found in honey, follow by dissacarrides (maltose, maltulose, turanose, melibiose, kojibiose, isomaltose and trehalose) and in a smaller percentage trissacarrides (melizitose, raffinose and erlose) (Machado De-Melo et al., 2017). The proportion of glucose and fructose for nectar honey should be more than 60%, and for honeydew should have at least 45% (Machado De-Melo et al., 2017). Honeydew honey has a higher value of trissaccharides (melezitose or erlose), because of the activity of many enzymes added by the sucking of the insects. A high amount of sucrose in honey may indicate an adulteration due to the bees artificial feeding by the syrup of sucrose, or an early honey harvesting, in which sucrose decomposition into monosaccharides has not been done yet , with a maximum amount of 5% in nectar honey (Codex, 2001).

The sugar profile of the present samples has a similar composition, with a high occurrence of fructose and glucose monosaccharides, and in smaller content turanose, maltulose, maltose, trehalose, and raffinose. Figure 13 shows a typical chromatogram, obtained by HPLC-RI.

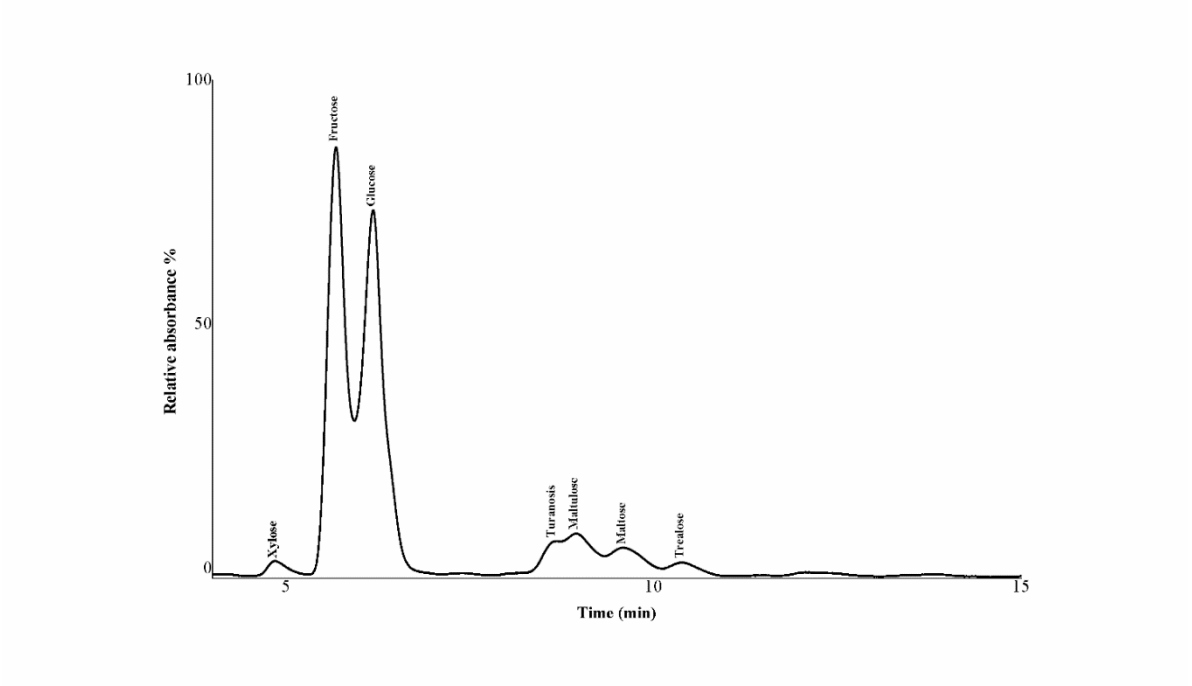

Figure 13 Sugar profile of a honey sample (R2).

All the analysed samples had a fructose amount greater than glucose, representing

these two monosaccharides, together, more than 60%, which according to international legislation may classify them as nectar honey. Samples R1, R2 and R3, described in this study as rosemary monofloral honey, present a mean value of F/G 1.1, Table 14, which is similar to a previously study conducted for Algerian rosemary honey where the F/G value was 1.2 (Homrani et al., 2020). For the MF sample, the value of fructose to glucose ratio is 1.1, which is in accordance with the results present in the literature for the same type of honey (Makhloufi et al., 2010). The tested samples did not contain sucrose which is indicative of unadulterated honey and correct ripening period.

Crystallization is a process that happens naturally in honey and is associated with its content in sugars, moisture, and honey type. The F/G (fructose/glucose) and G/H (glucose/humidity) ratios show evidence of how long a honey sample takes to crystallize. The ratio of fructose and glucose (F/G) is related largely to the source of nectar (Machado De-Melo et al., 2017). Some researchers state that the fructose and glucose ratio have a mean value of 1.2 for honey, reporting that if the level is higher than 1.3 a slow crystallization may occur and more than 1.5 imply zero crystallization (Escuredo et al., 2014). Also, values below 1.1 mean that crystallization is fast, and this happens because of glucose being less soluble in water. The speed at which glucose crystallization happens is also related to the G/H ratio. According to the literature, honey crystallization is slow or null when the G/H ratio is under 1.7 and fast when the ratio is above 2.2 (Escuredo et al.,2014). Table 14 results showed that the F/G values ranged between 1.1 and 1.2. This value demonstrate that all samples have a tendency of crystallization. The G/H values range from 2.3 to 2.8, also pointing to an average propensity for crystallization.

Table 14- Sugar profile, obtained by HPLC-RI, of the studied honey sample (values expressed in g/100g of honey) include: (mean +/- SD)

Sample	Fructose	Glucose	Turanose	Maltulose	Maltose	Trealose	Raffinose	F+G	F/G	G/H
R1	42.9 ± 0.8	36.6 ± 1.0	1.4 ± 0.2	5.8 ± 0.3	4.6 ± 1.0	1.0 ± 0.1	N/D	79.5	1.2	2.8
R2	43.3 ± 0.1	39.9 ± 0.6	1.2 ± 0.4	5.1 ± 1.2	4.5 ± 1.2	1.4 ± 0.3	N/D	83.1	1.1	2.8
R3	40.7 ± 0.4	38.8 ± 1.0	1.3 ± 0.1	6.3 ± 0.7	4.7 ± 0.1	1.1 ± 0.3	N/D	79.5	1.0	3.0
T1	40.2 ± 1.2	36.8 ± 0.8	0.7 ± 0.1	3.3 ± 0.1	1.1 ± 0.7	0.4 ± 0.1	0.3 ± 0.1	77.0	1.1	2.3
T2	40.9 ± 1.0	37.0 ± 0.4	0.7 ± 0.1	3.3 ± 0.1	0.7 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	77.9	1.1	2.3
T3	40.0 ± 0.7	36.5 ± 1.0	0.7 ± 0.1	3.3 ± 0.1	1.9 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	76.6	1.1	2.3
CH1	40.8 ± 0.6	38.6 ± 0.6	N/D	1.5 ± 0.1	N/D	N/D	3.2 ± 0.4	79.4	1.1	2.8
CH2	40.0 ± 0.1	36.8 ± 1.0	N/D	1.4 ± 0.2	N/D	N/D	3.2 ± 0.1	76.8	1.1	2.5
CH3	39.9 ± 0.1	36.4 ± 0.6	N/D	1.5 ± 0.1	N/D	N/D	3.3 ± 0.4	76.3	1.1	2.4
MF	42.2 ± 0.4	37.0 ± 0.1	0.6 ± 0.1	2.6 ± 0.1	2.2 ± 0.1	0.1 ± 0.1	N/D	79.2	1.1	2.5

3.2.12 Mineral content

Sodium (Na), potassium (K), magnesium (Mg), and calcium (Ca) were determined using a flame ionization atomic absorption spectrophotometer, while the other minerals such as copper (Cu), manganese (Mn), iron (Fe), cadmium (Cd), and lead (Pb) were determined using a graphite chamber atomic absorption spectrophotometry. The results regarding the mineral content of honey samples are given in Table 15. In general, the most common minerals are potassium, sodium, calcium, and magnesium. Concerning minerals related to heavy metal contamination, the values of all samples appear below the quantification level of 0.03 mg/kg for cadmium and 0.4 mg/kg for lead.

Manganese is present in six samples (with value ranging from 0.7 to 0.4 mg/kg) while copper is present in minor quantities ranging from 0.5 to 1.7 except R2, CH1 and CH3 (with <0.3 mg/kg). Concerning the results of the present samples, the values are in accordance with what is described on literature of Algerian multiflora honey (Guiseppa et al., 2020; Achour et al., 2014), with the exception for sodium and magnesium which presented lower values (Guiseppa et al., 2020). This may be due to climatic condition, floral origin, environmental, geographical area, beekeeping practice and materiel used for storage as well as soil type (Bouhlali et al., 2019). Concerning the samples of Rosemary honey, the values are similar to the ones reported on the literature of Moroccan rosemary honey (Bouhlali et al., 2019) and also Tunisian rosemary honey (Boussaid et al., 2018), with the exception of manganese, magnesium and calcium. The value of iron was higher than the one showed in the literature for the same type of honey (Boussaid et al., 2018), which may be related to the soil characteristics (Guiseppa et al., 2020).

Table 15- Mineral composition of the honey samples include: (mean +/- SD).

Samples	Potassium (mg/kg)	Sodium (mg/kg)	Calcium (mg/kg)	Magnesium (mg/kg)	Manganese (mg/kg)	Copper (mg/kg)	Cadmium (mg/kg)	Iron (mg/kg)	Lead (mg/kg)
R1	209.0±0.9	13.0±0.1	39.2±0.8	31.2±4.5	0,4±0,1	0.8±0.2	<0.03	12.3±1.9	<0.4
R2	240.0±0.6	13.1±0.1	25.4±3.0	19.1±1.0	<0.3	<0.3	<0.03	11.8±0.3	<0.4
R3	927.9±4.7	274.7±1.6	55.1±1.7	46.4±3.5	0.5±0.1	0.5±0.2	<0.03	11.5±1.6	<0.4
T1	821.9±1.0	243.3±3.6	83.8±4.1	93.0±4.0	0.6±0.0	0.5±0.1	<0.03	11.8±0.3	<0.4
T2	815.7±1.0	238.1±3.6	38.4±1.2	58.8±7.9	0.7±0.0	0.5±0.1	<0.03	11.7±0.1	<0.4
T3	248.8±0.0	61.3±0.2	72.1±2.9	35.1±4.8	<0.3	<0.3	<0.03	11.7±0.2	<0.4
CH1	343.3±2.3	90.9±0.8	34.6±4.3	65.3±1.7	<0.3	0.5±0.1	<0.03	12.1±0.8	<0.4
CH2	368.3±9.2	89.8±0.8	34.7±3.6	51.8±2.2	<0.3	<0.29	<0.03	12.0±0.5	<0.4
CH3	604.8±4.4	160.5±0.3	37.4±7.9	44.9±3.2	0.6±0.1	0.5±0.1	<0.03	11.6±1.3	<0.4
MF	774.4±3.5	141.5±1.7	38.2±2.0	35.9±3.1	0.4±0.1	1.7±0.1	<0.03	12.3±2.9	<0.4

3.3. Total phenolic compounds and antioxidant activity

Nowadays, the bioactive properties of food are showing a high interest by the researchers, mostly concerning the antioxidant activity, which is usually related to phenolic compounds content. In this way, total phenolic and flavonoid content, and antioxidant activities of the honey samples were investigated in this study and the results are given in Table 16.

Table 16- Phenolic content and antioxidant activity of honey samples include: (mean +/- SD).

Sample	Total phenolic content (mg GAE.g^{-1})	Total flavonoid content (mg QE. g$^{-1)}$	Reducing power (mg GAE.g$^{-1)}$	DPPH (EC_{50} mg/mL)
R1	0.37 ± 0.04	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
R2	0.30 ± 0.03	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
R3	0.61 ± 0.07	0.10 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
T1	0.56 ± 0.06	0.18 ± 0.04	0.03 ± 0.01	0.03 ± 0.01
T2	0.76 ± 0.03	0.09 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
T3	0.62 ± 0.05	0.10 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
CH1	0.73 ± 0.03	0.11 ± 0.02	0.02 ± 0.01	0.05 ± 0.01
CH2	0.47 ± 0.07	0.14 ± 0.02	0.02 ± 0.01	0.05 ± 0.01
CH3	0.35 ± 0.03	0.13 ± 0.01	0.02 ± 0.01	0.05 ± 0.01
MF	0.62 ± 0.01	0.08 ± 0.01	0.02 ± 0.01	0.05 ± 0.01

3.3.1 Total phenolic compounds

The total phenolic content of the samples is shown in Table 16, ranged from 0.30 to 0.76 mg GAE.g^{-1}, with a maximum of 0.76 and 0.73 mg GAE.g^{-1} for samples T2 and CH1, respectively, and a minimum of 0.30 and 0. 35 mg GAE g^{-1} for samples R2 and CH3. It was obseved that the samples with higher phenolic content correspond to honey samples with darker color, while the samples with lower total phenolic content presented a low Pfund value. MF sample showed total phenolic contents of 0.62 mg GAE.g^{-1}, which is similar to that reported in previous studies with values in the range of 0.24-0.96 mg GAE.g^{-1} (Dahmani et al., 2020). Rosemary honeys presented values ranging from 0.30 to 0.61mg GAE.g^{-1}, which are similar to the value described for this type of honey (Homrani et al., 2020). For the T1, T2 and T3 samples the values ranged from 0.56 to 0.76 mg GAE.g^{-1}, while for CH1, CH2, and CH3 samples the values ranged between 0.35 to 0.73 mg GAE.g^{-1}. These results showed a dependence of the amount of the phenolics compounds with the influence of climate, season, and processing

effect, but mainly with the botanical origin of honey (Soares et al., 2017).

3.3.2. Total flavonoid content

The total flavonoid content, assessed by spectrophotometric methods, gave values between a minimum of 0.02 mg QE. g^{-1} for sample R1 and a maximum of 0.18 mg QE. g^{-1} for T1 sample, Table 16. As the opposite of total phenolics, dark honey has been described to have more phenolic acid derivatives but a lesser amount of flavonoids than lighter ones (Machado De-Melo et al., 2017). Samples T1, T2, and T3 presented values ranging from 0.09 to 0.18 mg QE. g^{-1}, followed by the samples CH1, CH2, and CH3 with values of 0.14 to 0.11 mg QE. g^{-1} and rosemary honey with values of 0.02-0.10 mg QE. g^{-1}. These results are supported by the literature where values of 0.01 mg QE. g^{-1} reported for Algerian Rosemary honey (Homrani et al., 2020). These results are in accordance with those obtained for other Algerian multiflora honey, where flavonoids are the minor constituents in the phenolic fraction (Khalil et al., 2012).

3.2.3 Reducing power.

Table 16 shows the reducing power activity of samples, with a variation between 0.2 and 0.3 mg GAE.g^{-1}. It is possible to observe that almost all the samples had presented the same amount of reducing power activity (0.02 mg GAE.g^{-1}). This results reflect the fact that reducing power is not specific to any particular antioxidant, showing the overall antioxidant capacity of the sample (Moniruzzaman et al., 2012).

3.2.4. DPPH radical scavenging activity

DPPH (2,2-Diphenyl-1-picrylhydrazyl) is a stable free radical, for which antioxidant substances transfer electrodes or hydrogen atoms, neutralizing their radical character. One of the analytical techniques to evaluate the antioxidant activity is by the capacity blocking free radicals, which can be expressed by the parameter EC_{50} (Rebiai et al., 2015). Therefore, the high level of EC_{50} showed by the honeys, less the capacity for neutralizing the radicals and thus the antioxidant activity. Samples CH1, CH2, CH3 and MF showed values of 0.05 $mg.mL^{-1}$, corresponding to a lower antioxidant activity, while R1 and R3 have the lowest values of EC_{50}, 0.02 $mg.mL^{-1}$, Table 16, corresponding to a higher antioxidant activity. Previous studies in multiflora Algerian honey revealed a mean values for EC_{50} of 26.19 ±14.52 $mg.mL^{-1}$ (Rebiai et

al., 2015), which was higher than present ones , revealing a lower antioxidant activity, which can be related to the possible different floral origin.

3.2.5. Phenolic compounds profile

Due to the process of nectar harvesting by bees, the resulting honey may be the result of nectars from different plant species. Monofloral honey has great commercial demand due to its organoleptic characteristics and specific biological properties. Nowadays, new analytical methodologies, such as the analysis of the phenolic compounds profile, are used in the characterization and evaluation of the authenticity of honey associated with botanical origins (Soares et al., ,2017).

The phenolic compound profile of samples was evaluated by UPLC/DAD/ESI-MS^n. Figure 14 shows the chromatographic profile obtained for one of the honey samples studied. The analysis allowed the elucidation of phenolic compounds by comparing their chromatographic profile, UV spectrum, and mass spectrometry information, with reference compounds. When patterns were not available, structural information was confirmed with the combination of UV data and MS fragmentations described in the literature.

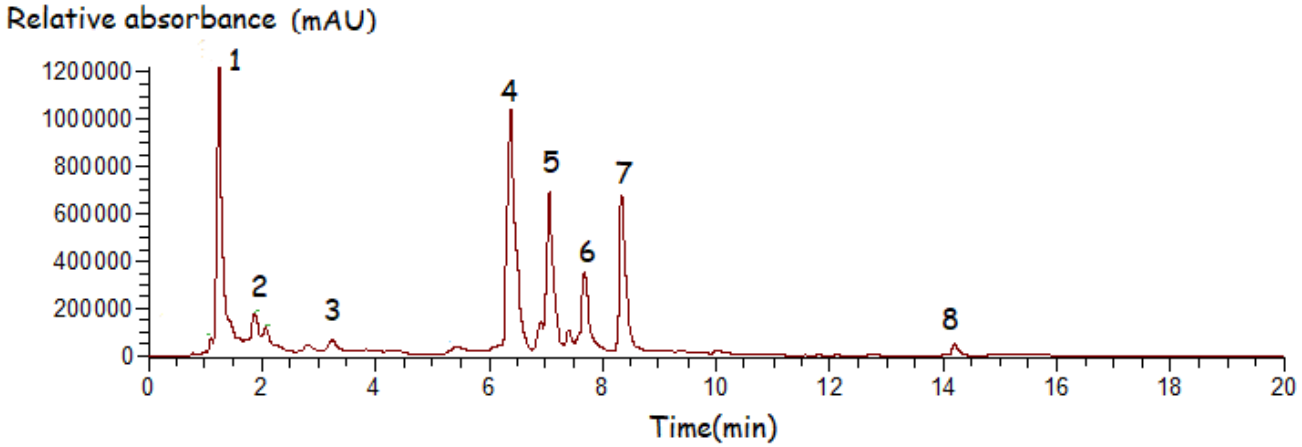

Figure 14- Chromatographic profile of MF sample. (The numbers in the figure represent the identified phenolic compounds in the sample, Table 17).

In the analysis of ESI-MS^n, the negative mode was used due to the great sensitivity that this mode represents in the measurement of different classes of phenolic compounds (Falcão et al.,2013). Table 17 shows the identified phenolic compounds in samples, with their retention time, maximum absorbance, and mass spectrometry information.

Table 17- Phenolic compounds profile of the honey samples.

Nº	Proposed compound	t_R (min)	λ_{max} (nm)	$[M-H]^-$	$[M-H]^{-2}$
1	Benzoic acid isomer[b,c]	1.24	284	121, $[M+46]^-$:167	
2	*p*-Hidroxybenzoic acid[a,b]	1.88	256	137	93
3	Caffeic acid[a,b]	2.07	292, 322	179	135
4	*p*-coumaric acid[a,b]	2.82	310	163, $[M+46]^-$:209	
5	Salicylic acid[a,b]	6.11	301	137	93
6	Syringetin[e]	6.38	276	345	161(100), 285(91), 309(21), 327(24)
7	*Trans*, *trans*-Abcisic acid[a,d]	6.88	265	263	154(100), 153 (69), 220 (36)
8	*p*- hydroxybenzoic derivitave[b]	6.91	219	199	137(20), 155(100)
9	*Cis*, *trans*- Abcisic acid[a,d]	7.06	265	263	153(69), 154(100), 220(36)
10	Pinobanksin-5-methyl-ether[b, c]	7.58	287	285	267(100), 239(29), 252(13)
11	Quercetin[a,b]	7.68	256, 370	301	179(100), 151(69)
12	N^1,N^5,N^{10}-tri-*p*-coumaroyespermidine[f]	7.86	292, 308	582	462(100), 436(10), 342(7)
13	Pinobanksin[b,c]	8.33	292	271	253(100), 225(20), 151(10)
14	Carnosol[g]	8.45	282, 358	269	171(23), 211(67), 229(100), 25(23), 293(32), 311(73)
15	Caffeic acid isoprenyl ester[b,c]	9.66	298, 325	247	135(14), 179(100)
16	Caffeic acid isoprenyl ester[b,c]	9.78	298, 326	247	135(14), 179(100)
17	Chrysin[a,b]	10	269	253	253(100), 225(17), 209(49)
18	Pinobanksin-3-*O*-acetate [b,c]	10.12	292	313	253(100), 271(20)
19	Galangin[a,b]	11	265, 300sh, 358	269	151(10), 197(54), 213(100), 227(49), 241(34), 269(16)

Note t_R, retention time of the compound; [M-H], Ion product; $[M-H]^2$, fragmentation of the product ion. [a]- Confirmed with a standard; [b]-confirmed with MS^n fragmentation; [c] - Confirmed with reference (Falcão et al.,2013); [d] - Confirmed with reference. (Bertoncelj et al.,2011); [e]-confirmed with reference (Barros et al., 2012); [f]-confirmed with reference (El Ghouizi et al., 2020); [g]-confirmed with reference (Sharma et al., 2020)..

In this study, it was possible to identify nineteen phenolic compounds, of which eight phenolic acids, seven flavonoids, two isoprenoids, one spermidine and one phenolic diterpene. Among the identified phenolic acids, four were derived from benzoic acid (benzoic acid derivative, *p*-hidroxybenzoic acid, salicylic acid, *p*-hydroxybenzoic derivative) and four were

derived from cinnamic acid (caffeic acid, *p*-coumaric acid, caffeic acid isoprenyl ester (isomer 1) and caffeic acid isoprenyl ester (isomer 2)). Of the seven flavonoids identified, three belong to the class of flavonols (syringetin, quercetin and galangin), three dihydroflavonols (pinobanksin-5-methyl-ether, pinobanksin, pinobanksin-3-*O*-acetate) and one flavone (chrysin). Also, two isoprenoids (*trans, trans*-abcisic acid and *cis, trans*- abcisic acid), one spermidine (N^1, N^5, N^{10}-tri-*p*-coumaroyespermidine) and one phenolic diterpene (carnosol), were identified.

The analysed honey samples show a similar phenolic composition, in which the different compounds are present in almost all samples, with some differences in their concentrations. Among the identified compounds, Table 18, it can be verified that the benzoic acid derivative and *p*-hydroxybenzoic derivatives are those that were found in most samples at higher concentrations, followed by *trans, trans*- abscisic acid and *cis, trans*- abscisic acid.

Phenolic acids are one of the compounds most often found in the composition of hive products and especially in honey. Compounds such as chrysin, galangin, and benzoic acid are described in honey with different floral origins from Algeria (Ouchemoukh et al., 2007). On the other hand, compounds such as pinobanksin and chrysin are typical for rosemary honey, (Arráez-Román et al., 2006). Concerning phenolic acids, the R2 sample is the one with the highest number of compounds derived from benzoic acid (56.6 mg/100 g) and the T3 sample stands out for the *p*-coumaric acid´s derivative, N^1, N^5, N^{10}-tri-*p*-coumaroyespermidine (35.3 mg/100 g), Table 18. The *cis, trans*- abscisic acid was only found in significant quantity in CH1 sample.

Flavonoids that are present in honey have their origin on pollen, propolis, and nectar. Pinobanksin and its derivatives, chrysin, and galagin are compounds described as derivatives of the propolis, which are present in honey by contamination of this resin (Falcão et al., 2013). Pinobanksin is present in all samples with values ranging from 7.8 mg/100g to 0.3 mg/100g, except for T1, T2, and T3.

The polyphenols profile and/or the identification of some individual components or a group of compounds are important tools for the characterization of both botanical and geographical origin of honeys, especially nectar-pollen-derived flavonoids which could be very useful for the honey botanical characterization, being the contribution of nectar more important than the contribution of pollen (Machado De-Melo et al., 2017).

Table 18- Phenolic compounds quantification in the honey samples include: (mean +/- SD).

Compound	Sample (mg/100g)									
	R1	**R2**	**R3**	**T1**	**T2**	**T3**	**CH1**	**CH2**	**CH3**	**MF**
Benzoic acid derivative	12.3±0.1	56.6±0.5	26.2±0.5	23.9±0.4	13.2±0.4	43.4±1.3	9.2±0.1	6.5±0.4	5.7±0.4	9.7±0.3
p-Hidroxybenzoic acid	1.5±0.1	3.3±0.1	2.8±0.1	9.9±0.2	5.3±0.1	16.3±0.2	2.1±0.0	2.2±0.1	1.2±0.1	5.1±0.1
Caffeic acid	0.5±0.1	1.5±0.1	1.6±0.1	1.0±0.1	0.2±0.1	3.8±0.1	1.5±0.0	0.9±0.1	0.7±0.1	0.9±0.1
p-coumaric acid	-	-	-	5.6±0.3	2.1±0.1	6.4±0.4	-	-	-	1.9±0.1
Salicylic acid	0.3±0.1	0.5±0.1	0.3±0.1	2.4±0.1	2.6±0.7	5.9±0.1	-	-	-	0.7±0.2
Syringetin	-	-	-	10.8±1.4	5.4±0.2	13.8±2.1	-	-	-	4.7±0.1
trans, trans – Abcisic acid	2.9±0.0	9.2±0.3	13.3±0.8	6.6±1.3	2.5±0.6	5.4±0.7	-	-	-	1.7±0.1
p-hydroxybenzoic derivitave	21.7±1.4	2.6±0.1	1.1±0.1	19.1±0.6	10.0±0.1	24.1±1.4	2.3±0.1	1.4±0.1	1.0±0.1	3.5±0.1
cis, trans- abcisic acid	6.1±0.1	14.2±0.1	6.4±0.1	4.2±0.3	2.3±0.2	6.8±0.1	16.9±0.3	11±0.6	11.0±2.0	4.7±0.2
Pinobanksin-5-methyl-ether	-	2.8±0.1	-	2.2±0.1	1.8±0.1	2.5±0.1	-	-	-	1.2±0.1
Quercetin	-	-	1.2±0.1	12.2±0.1	7.0±0.4	15.9±0.8	-	-	-	-
N^1,N^5,N^{10}-tri-*p*-coumaroyespermidine	0.3±0.1	0.3±0.1	0.2±0.1	22.3±1.1	16.9±4.9	35.3±1.5	1.1±0.1	0.6±0.1	0.9±0.1	-
Pinobanksin	0.3±0.1	1.80±0.1	0.7±0.1	-	-	-	7.8±0.3	4.9±0.1	5.2±0.4	5.2±0.1
Carnosol	-	-	-	14.0±0.1	7.4±1.2	14.8±2.8	-	-	-	-
Caffeic acid isoprenyl ester	-	-	-	-	-	-	0.7±0.1	0.5±0.1	0.6±0.1	-
Caffeic acid isoprenyl ester	-	-	-	-	-	-	0.8±0.1	0.5±0.1	0.6±0.1	-
Chrysin	1.8±0.1	4.8±0.1	1.8±0.1	0.5±0.1	0.1±0.1	-	1.6±0.1	1.0±0.1	1.3±0.1	0.7±0.1
Pinobanksin-3-*O*-acetate	-	-	-	-	-	-	0.1±0.1	0.04±0.10	0.1±0.1	-
Galangin	0.4±0.1	1.1±0.1	0.5±0.1	-	-	-	-	-	-	-

Some substances can be described as chemical markers (figure 15)such as the benzoic acid derivative, being present in higher concentration in the R samples, as well as *p*-coumaric acid. Carnosol and syringetin were only present in significant amount in T samples, and in the case of carnosol also in the MF sample, which showed in common a high percentage of *Cytisus striatus* pollen. Caffeic acid isoprenyl esters and pinobanksin-3-*O*-acetate were only present in the CH samples, which presented *Centaurea* sp. as main pollen. Also, galangin was only detected in *Rosmarinus officinalis* honeys.

Abscisic acid isomers (*trans, trans*- and *cis, trans*- abscisic acid) were within the main compounds of these honey samples, with a content ranging between 2.3 and 16.9 mg/100g, as well as benzoic acid derivative, Table 18. Although classified as isoprenoids, they show a phenolic similar chromatographic behavior, presenting a UV of 265 nm. The identification of these isomers was confirmed by the fragmentation profile of the molecular ion *m/z* 263 (Bertoncelj et al.,2011). Abscisic acid, which acts as a plant hormone, have functions of inhibiting growth, promoting dormancy and germination of seeds, and helping the plant tolerate water and environmental stress conditions (Bertoncelj et al., 2011). The floral species of the analyzed honey present a flowering period in the dry season, which justifies the presence of abscisic acid in monofloral and multi-floral samples. This plant hormone was previously identified in Algerian honey (Ouchemoukh et al., 2007). Therefore, this plant hormone can be considered as one of the most important phytochemical constituents for the authentication of this type of honey.

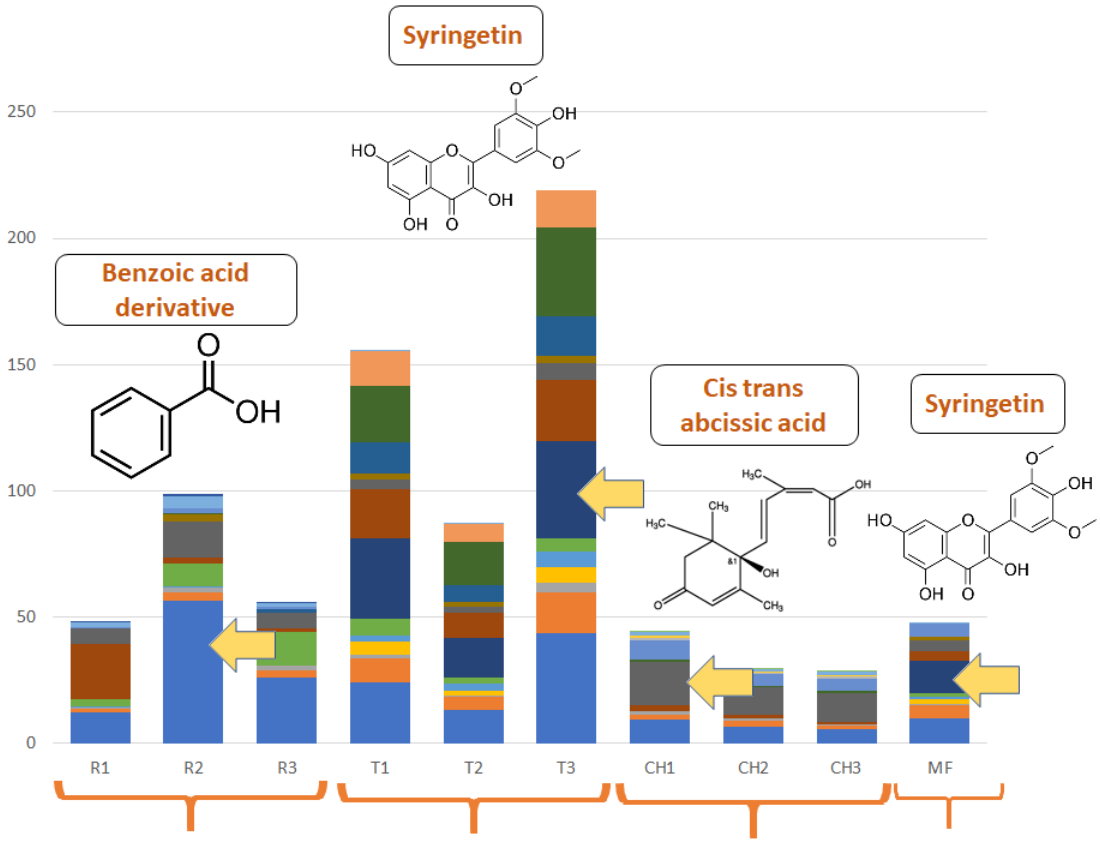

Figure 15 Chemical markers present in each honey sample

3.4. Antitumor activity

The growth inhibition of the tumor cells was observed in all the experiments, but particularly on sample MF, which had the lowest cytotoxicity GI_{50} values in four of the five cell lines (Table 19), followed by R1 with the best performance for colorectal adenocarcinoma and gastric adenocarcinoma cells.

Overall, growth inhibition GI_{50} results were higher compared to the ones reported by another study for Algerian honey (Bakchiche et al., 2020). However the values obtained for MCF-7 (the human breast adenocarcinoma) are lower than those obtained on another study for Malaysian acacia honey which was reported as 5.49 % (Mohd Salleh et al., 2017). The activity against AGS cell line is particularly interesting for samples MF and R1, with GI_{50} values of 11 µg/mL and 48 µg/mL, respectively, Table 19. On the opposite side is the sample T1, with the weakest performance against all the tumor cell lines tested. Although it showed a richer composition in total phenolic compounds than MF, the poor performance can be explained by the low concentration of specific bioactive compounds, such as those derived from the phenolic

acids. Regardless of the high cytotoxicity exhibited by the present honey samples against the studied tumor cell lines, three samples displayed some toxicity for non-tumor cell line. However, the values obtained for the tumor cells were always higher than the ones for non-tumor cells.

The reported bioactivity is most probably correlated with the phenolic composition of honey. Indeed, MF and R1 as the samples with an appropriate phenolic content were the ones with higher cytotoxicity. Several mechanisms of action are described for the interaction of phenolic compounds with tumor cells, including the process of apoptosis (caspase activation), arresting the process of differentiation and cell cycle (G2/M) or inhibitory effect on membrane tyrosine protein kinase (TPK) and cytosolic protein kinase C (PKC), such as the case for quercetin mentioned on previous review (Khan et al., 2017). This class of compounds, which includes flavonoids such as pinobanksin and chrysin observed in appropriate amounts in samples R1 and MF, were reported to play a key role in the bioactivity of honey samples, (Khan et al., 2017).

3.5. Anti-inflammatory activity

All honey samples under study showed anti-inflammatory capacity, with IC_{50} values between 7.5 and >400 µg/mL. The highest activity was observed for sample R1, which contains an appropriate number of bioactive compounds such as phenolic acid derivatives and flavonoids, followed by the sample MF, with an IC_{50} value of 12.5 µg/mL. It is worth mentioning that the great performance of sample R1, is due to the attribution of some compounds such as phenolic acid derivatives, flavonols, and dihydroflavonols derivatives. This also may explain the fact that, despite the low concentration of phenolic compounds in sample R2, its anti-inflammatory activity was almost similar to that of sample MF, which can be attributed to the higher concentration of dyhidroflavonols derivatives (pinobanksin-5-methyl-ether and pinobanksin), Table 18.

The values obtained in the present study are lower than those obtained on previous studies conducted for multifloral Algerian honey which exhibit a value of IC_{50} ranging from 1.72 to 7.43 mg/ml (Zaidi et al., 2019).

Table 19- Cytotoxicity activity (GI_{50} values, µg/mL) include: (mean +/- SD).

Cell lines	GI_{50}									
	R1	**R2**	**R3**	**T1**	**T2**	**T3**	**CH1**	**CH2**	**CH3**	**MF**
Caco	48±1	162±3	73±4	>400	>400	>400	232±23	201±12	>400	30±1
AGS	48±1	>400	22.3±0.3	>400	265±9	>400	144±14	157±10	>400	11±1
MCF-7	>400	>400	>400	>400	>400	82±2	83±2	70±7	281±41	98±1
NCl-H460	335±8	>400	>400	>400	>400	298±7	149±16	187±17	>400	109±4
VERO	248±2	>400	255±2	>400	>400	>400	>400	>400	>400	153±10
RAW	7.5±0.3	15±1	14±1	>400	376±19	>400	267±6	117±4	>400	12.5±0.2

3.6. Antibiotics

The contamination of honey by antibiotics can be due to several reasons such as agricultural practices (contamination of nectar with fruit trees treated with antibiotics, contamination the nectar due to degradation product of the herbicide), beekeeping practice (feeding bees with honey containing residues, treatment of bee diseases), environmental (biological production of streptomycin by some Streptomyces bacteria, contaminated honey consumed by bees from robbed colonies, contaminated water drunk by bees, migration of residues from polluted wax foundation) and/or fraud issues (mixing clean honey with contaminated honey) (Almeida-Muradian et al., 2020). According to the obtained results for antibiotic residues in the honey, three samples were positive for sulphonamide residues, while for the other seven samples the result was negative according to the control point, Table 20. Honey with very high hydroxymethylfurfural content can lead to false positive results (Serra Bonvehí & Lacalle Gutiérrez, 2009). The results of present study are in accordance with a previous study for multiflora Algerian honey samples, where no incidence of tetracycline residues was detected (Draiaia et al., 2015). Some antibiotics are metabolized or degradable in honey such as tetracycline, but sulphonamide cannot be degradable by the metabolism of the bees (Almeida-Muradian et al., 2020) which indicates why all the samples are negative (tetracycline residues), while for the sulphonamide three samples are positive. Concerning the European legislation regarding residues of tetracycline and sulphonamide in honey, no MRL was established for tetracycline and sulphonamides in honey (Commission Regulation, 2006),

which can be interpreted that the application of antibiotics by the beekeepers is not permitted (Almeida-Muradian et al., 2020).

Table 20- The number of traces of antibiotics in honey samples

Samples		**Sulfonamide (10 ppb)**		**Tetracycline (15 ppb)**
R1	1551	Positive	1728	Negative
R2	2449	Negative	1569	Negative
R3	2423	Negative	1491	Negative
T1	2090	Negative	1536	Negative
T2	1916	Negative	1360	Negative
T3	1771	Negative	1515	Negative
CH1	1351	Positive	1539	Negative
CH2	2021	Negative	1613	Negative
CH3	1662	Negative	1523	Negative
MF	897	Positive	1165	Negative

CHAPTER IV: CONCLUSION AND WORK PERSPECTIVE



4.1. Conclusion

The result of this study indicated that honey samples collected from the two regions of Algeria, were predominantly of good quality. However, some consideration can be given to the professional level of beekeepers which often does not allow high quality honey production and marketing in the country.

The melissopalynological analysis analyzed samples contain a great diversity of pollen grains, with *Cytisus striatus*, *Centaurea* sp. and *Rosmarinus officinalis* being the main pollens found. Furthermore, clear attention should be given to mislabelling, since several honey do not confirm the botanical origin mentioned on the jar.

The samples presented a light amber color, except for rosemary honey, which had an extra white amber color. All honeys presented a moisture content within the legal regulation and so safe to avoid fermentation. In addition, pH values were between 3.98 and 4.67, which also is an indicative of low possibility of microbial development and the occurrence of fermentative processes. Regarding the values of electrical conductivity, all the samples presented conductivity values below 0.80 $mS.cm^{-1}$ established in Codex Alimentarius (Codex, 2001) suggesting that they were nectar honeys. The analysis of the sugar profile of the honey samples showed the main presence of fructose and glucose, which in total make up more than 60% of the sugars found, while other sugars were still found with the absence of sucrose. The analysis of the sugar profile indicated that in general honey samples had an average tendency to crystallize, except sample R3, which showed an F/G value of 1.0 indicating a fast tendency for crystallization. Concerning the analysis of the minerals profile, the most common minerals were potassium, sodium, calcium, and magnesium. Regarding the heavy metals, honey samples were free from cadmium and lead. 5-HMF and the diastase activity was in accordance with the Codex Alimentarius, suggesting that honey samples were processed and stored appropriately, except R1 and R2 which have high 5-HMF content, that may be due to bad conservation procedures. All rosemary samples presented a low diastase index which can be explained by the fact that rosemary honey is recognized as having low enzymatic content, or it can be the result of e a less adequate processing or storage of this honey. Furthermore, the value of proline content was according to the legal requirements, indicating an adequate maturation status and the absence of possible adulteration.

Generally, the honey samples showed antioxidant activity, expressed in terms of their reducing power and radical scavenging activity, showing that the Algerian honey has an interesting antioxidant activity that may contribute in some way to the therapeutic properties. Through the phenolic profile determination, it was possible to identify nineteen phenolic compounds, of which eight phenolic acids, seven flavonoids, two isoprenoids, one spermidine and one phenolic diterpene. Some substances can be described as chemical markers such as benzoic acid derivative, being present in higher concentration in the R samples, as well as *p*-coumaric acid. Carnosol and syringetin were only present in significant amount in T samples, and in the case of carnosol also in the MF sample, which showed in common a high percentage of *Cytisus striatus* pollen. Caffeic acid isoprenyl esters and pinobanksin-3-*O*-acetate were only present in the CH samples, which presented *Centaurea* sp. as main pollen. Also, galangin was only detected in *Rosmarinus officinalis* honeys.

The antitumor activity towards four tumor cells lines (Caco, AGS, NCI-H460 and MCF-7) and non-tumor cell line (Vero) were also evaluated and showed significant potential towards those cells' lines. The anti-inflammation activity of the present samples also shows an important activity. These two activities are more related to the phenolics compounds which are responsible for the antioxidant activity of honey, and this makes those samples have effective therapeutic properties.

The screening of antibiotics residues (tetracycline and sulfonamides) showed that samples R1, CH1 and MF indicate a positive valor for sulfonamides and the remaining samples show a negative result for those drugs indicating an inappropriate beekeeping practice for those three samples, however for tetracyclines, all samples were negative.

4.2. Work prospects

This work aimed to contribute to the characteristic and evaluation of commercial honey labelled as rosemary, tamarisk, thistle, and multiflora. The results suggest that some of the samples do not correspond to monofloral tamarisk and thistle, thus, it would be important to confirm these results through the analysis of more samples of this honey. From this work, it is also evident the importance of using various analytical techniques to confirm the authenticity of honey.

In terms of future perspectives, it will be important to increase the number of samples to identify potential floral markers of tamarisk and thistle, particularly by assessing the profile of phenolic compounds, using statistical analysis techniques.

CHAPTER V: REFERENCES



5. References

Achour, H. Y., Universit, M. K., & Dahlab, S. (2014). Composition physicochimique des miels algériens. Détermination des éléments traces et des éléments potentiellement toxiques. *Afrique Science: Revue Internationale Des Sciences et Technologie*, *10*(2), 127–136.

https://www.researchgate.net/publication/285593248_Composition_physicochimique_des_miels_algeriens_Determination_des_elements_traces_et_des_elements_potentiellement_toxiques

Almeida-Muradian, L. B. de, Monika Barth, O., Dietemann, V., Eyer, M., De Freitas, A. da S., Martel, A., Marcazzan, G. L., Merchese, C. M., Mucignat-Caretta, C., Pascual-Mate, A., Reybroeck, W., Sancho, M. T., & Gasparotto Sattler, J. A. (2020). Standard methods for Apis mellifera honey research. *Journal of Apicultural Research*, *59*(3), 1–62.

https://doi.org/10.1080/00218839.2020.1738135

Alvarez-Suarez, J. M., Giampieri, F., González-Paramás, A. M., Damiani, E., Astolfi, P., Martinez-Sanchez, G., Bompadre, S., Quiles, J. L., Santos-Buelga, C., & Battino, M. (2012). Phenolics from monofloral honeys protect human erythrocyte membranes against oxidative damage. *Food and Chemical Toxicology*, *50*(5), 1508–1516. https://doi.org/10.1016/j.fct.2012.01.042

Al-waili, N. S. (2003). Short communication effects of daily consumption of honey solution on hematological indices and blood levels of minerals and enzymes in normal individuals. *Journal Of Medicinal Food*, *6*(2), 135–140.

https://doi.org/10.1016/0006-3207(72)90120-6

Amin, A., & Hamza, A. A. (2005). Hepatoprotective effects of Hibiscus, Rosmarinus and Salvia on azathioprine-induced toxicity in rats. *Life Sciences*, *77*(3), 266–278. https://doi.org/10.1016/j.lfs.2004.09.048

Amri, A., & Ladjama, A. (2013). Physicochemical properties and mineral content of honey samples from Vojvodina (Republic of Serbia). *Food Chemistry African Journal of Food Science*, *7*(July 2013), 168–173.

https://doi.org/10.1016/j.foodchem.2018.09.149

Amri, A., & Ladjama, A. (2013). Physicochemical characterization of some multifloral honeys from honeybees Apis mellifera collected in the Algerian northeast. *African Journal of Food Science*, *7*(7), 168–173.

https://doi.org/10.5897/ajfs13.0986

Anklam, E. (1998). A review of the analytical methods to determine the geographical and botanical origin of honey. *Food Chemistry*, *63*(4), 549–562. https://doi.org/10.1016/S0308-8146(98)00057-0

AOAC International. (2016). The Official Methods of Analysis of AOAC International.pdf. In G. W & L. Jr (Eds.), *The Official Methods of Analysis of AOAC International* (20th ed.). AOAC international.

https://www.techstreet.com/standards/official-methods-of-analysis-of-aoac-international-20th-edition-2016?product_id=1937367

Arráez-Román, D., Gómez-Caravaca, A. M., Gómez-Romero, M., Segura-Carretero, A., & Fernández-Gutiérrez, A. (2006). Identification of phenolic compounds in rosemary honey using solid-phase extraction by capillary electrophoresis-electrospray ionization-mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis*, *41*(5), 1648–1656. https://doi.org/10.1016/j.jpba.2006.02.035

Bakchiche, B., Temizer, İ. K., Güder, A., Çelemli, Ö. G., Yegin, S. Ç., Bardaweel, S. K., & Ghareeb, M. A. (2020). Chemical composition and biological activities of honeybee products from algeria. *Journal of Applied Biotechnology Reports*, *7*(2), 93–103. https://doi.org/10.30491/jabr.2020.109498

Barros, L., Dueñas, M., Carvalho, A. M., Ferreira, I. C. F. R., & Santos-Buelga, C. (2012). Characterization of phenolic compounds in flowers of wild medicinal plants from Northeastern Portugal. *Food and Chemical Toxicology*, 50(5), 1576–1582. https://doi.org/10.1016/j.fct.2012.02.004

Bath, P. K., & Singh, N. (1999). A comparison between *Helianthus annuus* and *Eucalyptus lanceolatus* honey. *Food Chemistry, 67*, 389–397. https://doi.org/https://doi.org/10.1016/S0308-8146(99)00132-6

Belay, A., Solomon, W. K., Bultossa, G., Adgaba, N., & Melaku, S. (2015). Botanical origin, colour, granulation, and sensory properties of the Harenna forest honey, Bale, Ethiopia. *Food Chemistry*, *167*, 213–219.

https://doi.org/10.1016/j.foodchem.2014.06.080

Bergner, K., & Hansjörg, H. (1972). Zum vorkommen und zur herkunft der freien aminosäuren in honig. *Apidologie*, *3*(1), 5-34.

https://doi.org/10.1051/apido:19720101

Bertoncelj, J., Polak, T., Kropf, U., Korošec, M., & Golob, T. (2011). LC-DAD-ESI/MS analysis of flavonoids and abscisic acid with chemometric approach for the classification of Slovenian honey. *Food Chemistry*, *127*(1), 296–302. https://doi.org/10.1016/j.foodchem.2011.01.003

Bhalodia, N., Nariya, P., Shukla, V., & Acharya, R. (2013). In vitro antioxidant activity of hydro alcoholic extract from the fruit pulp of Cassia fistula Linn. *AYU (An International Quarterly Journal of Research in Ayurveda)*, *34*(2), 209.

https://doi.org/10.4103/0974-8520.119684

Bhandari, B., D'Arcy, B., & Chow, S. (1999). Rheology of selected Australian honeys. *Journal of Food Engineering*, *41*(1), 65–68.

https://doi.org/10.1016/S0260-8774(99)00078-3

Birtić, S., Dussort, P., Pierre, F. X., Bily, A. C., & Roller, M. (2015). Carnosic acid. *Phytochemistry*, *115*(1), 9–19.

https://doi.org/10.1016/j.phytochem.2014.12.026

Bogdanov, S. (2016). Honey composition. *The Honey Book*, January, 1–10. https://doi.org/10.1016/j.sjbs.2016.12.010

Bogdanov, S., D'Arcy, B. R., Mossel, B., & Marcazzan, G. L. (1999). Honey quality and international regulatory standards : review by the International Honey International honey standards are specified in a European Honey. *Bee World*, *80*(2), 61–69.

https://doi.org/10.1080/0005772X.1999.11099428

Bogdanov, S., Jurendic, T., Sieber, R., & Gallmann, P. (2008). Honey for nutrition and health: A review. *Journal of the American College of Nutrition*, *27*(6), 677–689. https://doi.org/10.1080/07315724.2008.10719745

Bogdanov, S., Martin, P., Lullmann, C., Borneck, R., Flamini, C., Morlot, M., Lheritier, J., Vorwohl, G., Russmann, H., Persano, L., Sabatini, A. G., Marcazzan, G. L., Marioleas, P., Tsigouri, A., Kerkvliet, J., Ortiz, A., & Ivanov, T. (1997). Harmonised methods of the European Honey Commission. *Apidologie*, 28(SPEC. ISS.), 1–59.

https://www.researchgate.net/publication/292092907_Harmonised_methods_of_the_European_Honey_Commission/comments#fullTextFileContent

Bogdanov, S., & Pascale, B. (2001). Natürliche antibiotische Eigenschaften des Honigs. *Shweiz Bienen-Zeitung*, *124*(2), 18–21.

https://www.apitherapie.at/images/documents/Bogdanov_Nat_antibiot_Honig.pdf

Bouhlali, E. dine T., Bammou, M., Sellam, K., El Midaoui, A., Bourkhis, B., Ennasir, J., Chakib, A., & Filali-Zegrouti, Y. (2019). Physiochemical properties of eleven monofloral honey samples produced in Morocco. *Arab Journal of Basic and Applied Sciences*, *26*(26:1), 476–487.

https://doi.org/10.1080/25765299.2019.1687119

Boussaid, A., Chouaibi, M., Rezig, L., Hellal, R., Donsì, F., Ferrari, G., & Hamdi, S. (2018). Physicochemical and bioactive properties of six honey samples from various floral origins from Tunisia. *Arabian Journal of Chemistry*, *11*(2), 265–274. https://doi.org/10.1016/j.arabjc.2014.08.011

Capuano, E., & Fogliano, V. (2011). LWT - Food Science and Technology Acrylamide and 5-hydroxymethylfurfural (HMF): A review on metabolism , toxicity , occurrence in food and mitigation strategies. *LWT - Food Science and Technology*, *44*(4), 793–810. https://doi.org/10.1016/j.lwt.2010.11.002

Cara, M. C., Dumitrel, G. A., Glevitzky, M., & Perju, D. (2012). Stability of tetracycline residues in honey. *Journal of the Serbian Chemical Society*, *77*(7), 879–886. https://doi.org/10.2298/JSC111002214C

Castro-Vázquez, L., Pérez-Coello, M. S., & Cabezudo, M. D. (2003). Analysis of volatile compounds of rosemary honey. Comparison of different extraction techniques. *Chromatographia*, *57*(3–4), 227–233.

https://doi.org/10.1007/BF02491721

Chakir, A., Romane, A., Marcazzan, G. L., & Ferrazzi, P. (2016). Physicochemical properties of some honeys produced from different plants in Morocco. *Arabian Journal of Chemistry*, *9*, S946–S954.

https://doi.org/10.1016/j.arabjc.2011.10.013

Chua, L. S., Rahaman, N. L. A., Adnan, N. A., & Eddie Tan, T. T. (2013). Antioxidant activity of three honey samples in relation with their biochemical components. *Journal of Analytical Methods in Chemistry*, *2013*.

https://doi.org/10.1155/2013/313798

Cianciosi, D., Forbes-Hernández, T. Y., Afrin, S., Gasparrini, M., Reboredo-Rodriguez, P., Manna, P. P., Zhang, J., Lamas, L. B., Flórez, S. M., Toyos, P. A., Quiles, J. L., Giampieri, F., & Battino, M. (2018). Phenolic compounds in honey and their associated health benefits: A review. *Molecules*, *23*(9), 1–20.

https://doi.org/10.3390/molecules23092322

Ciulu, M., Solinas, S., Floris, I., Panzanelli, A., Pilo, M. I., Piu, P. C., Spano, N., & Sanna, G. (2011). RP-HPLC determination of water-soluble vitamins in honey. *Talanta*, *83*(3), 924–929.

https://doi.org/10.1016/j.talanta.2010.10.059

Codex, A. (2001). Distribution of the Report of the Thirty-second Session of the Codex Committee on Food Additives and Contaminants. *Alinorm*, *01*(12), 185. http://www.fao.org/tempref/codex/Reports/Alinorm01/al01_12e.pdf

Commission Regulation (EC), 2006. (2006). Commission Regulation (EC) No 118/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union*, *364*, 5–24.

https://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:364:0005:0024:EN:PDF

Cooperation, I. U. for C. of N. and N. R. C. for M. (2005). *A Guide to Medicinal Plants in North Africa* (IUCN (ed.); IUCN). IUCN centre for miditereanean cooperation. https://portals.iucn.org/library/sites/library/files/documents/2005-093.pdf

Council, D. (2001). COUNCIL DIRECTIVE 2001/110/EC of 20 December 2001 relating to honey. *Official Journal of the European Communities*, *10*(110), 47–52. https://doi.org/10.1007/BF00348615

Da Silva, P. M., Gauche, C., Gonzaga, L. V., Costa, A. C. O., & Fett, R. (2016). Honey: Chemical composition, stability and authenticity. *Food Chemistry*, *196*, 309–323. https://doi.org/10.1016/j.foodchem.2015.09.051

Dahmani, K., Houdeib, J. B., Zouambi, A., Bendeddouche, B., Fernández-Muiño, M., Osés, S. M., & Teresa Sancho, M. (2020). Quality attributes of local and imported honeys commercialized in Algeria. *Journal of Apicultural Science*, *64*(2), 1–12. https://doi.org/10.2478/jas-2020-0019

De Almeida-Muradian, L. B., Stramm, K. M., Horita, A., Barth, O. M., Da Silva de Freitas, A., & Estevinho, L. M. (2013). Comparative study of the physicochemical and palynological characteristics of honey from Melipona subnitida and Apis mellifera. *International Journal of Food Science and Technology*, *48*(8), 1698–1706. https://doi.org/10.1111/ijfs.12140

Doner, L.W. (2003). Honey. Encyclopedia of food sciences and nutrition, 1(2), 3125–3130. https://doi.org/10.1016/B0-12-227055-X/00600-3

Draiaia, R., Chefrour, A., Dainese, N., Borin, A., Manzinello, C., Gallina, A., & Mutinelli, F. (2015). Physicochemical parameters and antibiotics residuals in Algerian honey. *African Journal of Biotechnology*, *14*(14), 1242–1251.

https://doi.org/10.5897/ajb2015.14456

Dyce, E. J. (1931). Crystallization of honey. *Journal of Economic Entomology*, *24*(31 june), 597–602.

https://agris.fao.org/agris-search/search.do?recordID=US201300676161

El Ghouizi, A., El Menyiy, N., Falcão, S. I., Vilas-Boas, M., & Lyoussi, B. (2020). Chemical composition, antioxidant activity, and diuretic effect of Moroccan fresh bee pollen in rats. *Veterinary World*, *13*(7), 1251–1261.

https://doi.org/10.14202/vetworld.2020.1251-1261

El Sohaimy, S. A., Masry, S. H. D., & Shehata, M. G. (2015). Physicochemical characteristics of honey from different origins. *Annals of Agricultural Sciences*, *60*(2), 279–287. https://doi.org/10.1016/j.aoas.2015.10.015

Escuredo, O., Rodríguez-Flores, M. S., Meno, L., & Seijo, M. C. (2021). Prediction of physicochemical properties in honeys with portable near-infrared (Micronir) spectroscopy combined with multivariate data processing. *In Foods* ,10, (2). https://doi.org/10.3390/foods10020317

Escuredo, O., Dobre, I., Fernández-González, M., & Seijo, M. C. (2014). Contribution of botanical origin and sugar composition of honeys on the crystallization phenomenon. *Food Chemistry*, *149*, 84–90.

https://doi.org/10.1016/j.foodchem.2013.10.097

Escuredo, O., Míguez, M., Fernández-González, M., & Carmen Seijo, M. (2013). Nutritional value and antioxidant activity of honeys produced in a European Atlantic area. *In Food Chemistry* ,138,(2–3). 851–856.

https://doi.org/10.1016/j.foodchem.2012.11.015

Falcão, S. I., Calhelha, R. C., Touzani, S., Lyoussi, B., Ferreira, I. C. F. R., & Vilas-Boas, M. (2019). In vitro interactions of moroccan propolis phytochemical's on human tumor cell lines and anti-inflammatory properties. *Biomolecules*, *9*(8). https://doi.org/10.3390/biom9080315

Falcão, S. I., Vale, N., Gomes, P., Domingues, M. R. M., Freire, C., Cardoso, S. M., & Vilas-Boas, M. (2013). Phenolic profiling of Portuguese propolis by LC-MS spectrometry: Uncommon propolis rich in flavonoid glycosides. *Phytochemical Analysis*, *24*(4), 309–318.

https://doi.org/10.1002/pca.2412

Fallico, B., Arena, E., Verzera, A., & Zappalà, M. (2006). The European Food Legislation and its impact on honey sector. *Accreditation and Quality Assurance*, *11*(1–2), 49–54. https://doi.org/10.1007/s00769-006-0128-6

Feás, X., Pires, J., Iglesias, A., & Estevinho, M. L. (2010). Characterization of artisanal honey produced on the Northwest of Portugal by melissopalynological and physico-chemical data. *Food and Chemical Toxicology*, *48*(12), 3462–3470. https://doi.org/10.1016/j.fct.2010.09.024

Fechner, D. C., Moresi, A. L., Ruiz Díaz, J. D., Pellerano, R. G., & Vazquez, F. A. (2016). Multivariate classification of honeys from Corrientes (Argentina) according to geographical origin based on physicochemical properties. *Food Bioscience*, *15*, 49–54. https://doi.org/10.1016/j.fbio.2016.05.002

Ferreira, I. C. F. R., Aires, E., Barreira, J. C. M., & Estevinho, L. M. (2009). Antioxidant activity of Portuguese honey samples: Different contributions of the entire honey and phenolic extract. *Food Chemistry*, *114*(4), 1438–1443. https://doi.org/10.1016/j.foodchem.2008.11.028

Finola, M. S., Lasagno, M. C., & Marioli, J. M. (2007). Microbiological and chemical characterization of honeys from central Argentina. *Food Chemistry*, *100*(4), 1649–1653. https://doi.org/10.1016/j.foodchem.2005.12.046

Islam, M. N., Khalil, M. I., Islam, M. A., & Gan, S. H. (2014). Toxic compounds in honey _ Enhanced Reader. *Applied Toxicology*, *34*(2014), 733–742.

https://doi.org/10.1002/jat.2952

Gallina, A., Stocco, N., & Mutinelli, F. (2010). Karl Fischer Titration to determine moisture in honey: A new simplified approach. *Food Control*, *21*(6), 942–944. https://doi.org/10.1016/j.foodcont.2009.11.008

Gheldof, N., & Engeseth, N. J. (2002). Antioxidant capacity of honeys from various floral sources based on the determination of oxygen radical absorbance capacity and inhibition of in vitro lipoprotein oxidation in human serum samples. *Journal of Agricultural and Food Chemistry*, 50(10), 3050–3055.

https://doi.org/10.1021/jf0114637

González-Miret, M. L., Terrab, A., Hernanz, D., Fernández-Recamales, M. Á., & Heredia, F. J. (2005). Multivariate correlation between color and mineral composition of honeys and by their botanical origin. *Journal of Agricultural and Food Chemistry*, *53*(7), 2574–2580.

https://doi.org/10.1021/jf048207p

Guiseppa, D. B., Patrizia, L., Angela, G. P., Rosalia, crupi, Vincenzo, N., Benameur, Q., Rossana, R., Giovani, B., Giacomo, D., & Vincenzo, L. T. (2020). Mineral content and physico-chemical paramters of honey from North regions of Algeria. *Natural Product Reserch*,1-8.

https://doi.org/10.1080/14786419.2020.1791110

Homrani, M., Rodríguez-Flores, M. S., Escuredo, O., Fatiha, D., Bouzouina Mohammed, Homrani, A., & Seijo, M. C. (2020). Botanical origin, pollen profile, and physicochemical properties of Algerian honey from different bioclimatic areas. *Foods*, *9*(938), 18.

www.mdpi.com/journal/foods

Khalafi, R., Goli, S. A. H., & Behjatian, M. (2016). Characterization and classification of several monofloral iranian honeys based on physicochemical properties and antioxidant activity. *International Journal of Food Properties*, *19*(5), 1065–1079. https://doi.org/10.1080/10942912.2015.1055360

Khalil, M. I., Moniruzzaman, M., Boukraâ, L., Benhanifia, M., Islam, M. A., Islam, M. N., Sulaiman, S. A., & Gan, S. H. (2012). Physicochemical and antioxidant properties of algerian honey. *Molecules*, *17*(9), 11199–11215. https://doi.org/10.3390/molecules170911199

Khan, R. U., Naz, S., & Abudabos, A. M. (2017). Towards a better understanding of the therapeutic applications and corresponding mechanisms of action of honey. *Environmental Science and Pollution Research*, *24*(36), 27755–27766. https://doi.org/10.1007/s11356-017-0567-0

Krauze, A., & Zalewski, R. I. (1991). Classification of honeys by principal component analysis on the basis of chemical and physical parameters. *Zeitschrift Für Lebensmittel-Untersuchung Und -Forschung*, *192*(1), 19–23.

https://doi.org/10.1007/bf01201436

León-Ruiz, V., Vera, S., González-Porto, A. V., & San Andrés, M. P. (2013). Analysis of Water-Soluble Vitamins in Honey by Isocratic RP-HPLC. *Food Analytical Methods*, *6*(2), 488–496.

https://doi.org/10.1007/s12161-012-9477-4

Ling Chin, N., & Sowndhararajan, K. (2020). A Review on Analytical Methods for Honey Classification, Identification and Authentication. *Honey Analysis - New Advances and Challenges*.

https://doi.org/10.5772/intechopen.90232

Louveaux. J.. Maurizio. A.. & Vorwohl. G. (1978). Methods of Melissopalynology. *Bee World*. 59(4). 139–157.

https://doi.org/10.1080/0005772X.1978.11097714

Machado De-Melo, A. A., Almeida-Muradian, L. B. de, Sancho, M. T., & Pascual-Maté, A. (2017). composition and properties of Apis mellifera honey : A reveiw. *Journal of Apicultural Research*, *57*(1), 5–37.

https://doi.org/10.1080/00218839.2017.1338444

Madesis, P., Ganopoulos, I., Sakaridis, I., Argiriou, A., & Tsaftaris, A. (2014). Advances of DNA-based methods for tracing the botanical origin of food products. *Food Research International*, *60*, 163–172.

https://doi.org/10.1016/j.foodres.2013.10.042

Makhloufi, C., Kerkvliet, J. D., D'Albore, G. R., Choukri, A., & Samar, R. (2010). Characterization of Algerian honeys by palynological and physico-chemical methods. *Apidologie*, *41*(5), 509–521.

https://doi.org/10.1051/apido/2010002

Makhloufi, C., Schweitzer, P., Azouzi, B., Persano Oddo, L., Choukri, A., Hocine, L., & Ricciardelli D'Albore, G. (2007). Some Properties of Algerian Honey. *Apiacta*,1(*42)*, 73-80.https://www.researchgate.net/publication/343141214_Some_Properties_of_Algerian_Honey

Mandić, M. L., Primorac, L., Kenjerić, D., Bubalo, D., Perl, A., & Flanjak, I. (2006). Characterisation of oak mistletoe and common thistle honeys by physicochemical, sensory and melissopalynology parameters. *Deutsche Lebensmittel-Rundschau*, *102*(6), 245–249.

https://www.researchgate.net/publication/277826064_Characterisation_of_Oak_Mistletoe_and_Common_Thistle_Honeys_by_Physicochemical_Sensory_and_Melissopalynology_Parameters

Operator Manual. (2011). Operator's Manual:Charm II Sulfa drug Test for honey. *Charm Sciences Inc*, *207*(013), 9.

https://www.charm.com/wp-content/uploads/2018/06/MRK-122.pdf

Marion, L. (2017). *Thesis: Le romarin, Rosmarinus officinalis L., une Lamiacée médicinale de la garrigue provençale*. Aix Maseille university ,Marseille (France). https://www.semanticscholar.org/paper/Le-romarin%2C-Rosmarinus-officinalis-L.%2C-une-Lamiac%C3%A9e-Leplat/73777945584aef719b165cac71ba13f0be4fc998

Mato, I., Huidobro, J. F., Cendón, V., Muniategui, S., Fernández-Muiño, M. A., & Sancho, M. T. (1998). Enzymatic determination of citric acid in honey by using polyvinylpoly-pyrro-lidone clarification. *Journal of Agricultural and Food Chemistry*, 46(1), 141–144. https://doi.org/10.1021/jf970418p

Maurizio, A. (1962). From the Raw Material to the Finished Product: Honey. *Bee World*, *43*(3), 66–81.

https://doi.org/10.1080/0005772x.1962.11096943

Mendes, E., Brojo Proença, E., Ferreira, I. M. P. L. V. O., & Ferreira, M. A. (1998). Quality evaluation of Portuguese honey. *Carbohydrate Polymers*, *37*(3), 219–223.

https://doi.org/10.1016/S0144-8617(98)00063-0

Mohd Salleh, M. A., Eshak, Z., & Wan Isamail, W. I. (2017). View of acacia honey induces apoptosis in human breast adenocarcinoma cell lines (MCF-7). *Journal Teknologi*, *79*(4), 9–16.

https://doi.org/10.11113/jt.v79.9882

Moniruzzaman, M., Khalil, M. I., Sulaiman, S. A., & Gan, S. H. (2012). Advances in the analytical methods for determining the antioxidant properties of honey: A review. *African Journal of Traditional, Complementary and Alternative Medicines*, *9*(1), 36–42. https://doi.org/10.4314/ajtcam.v9i1.5

Mouhoubi-Tafinine, Z., Ouchemoukh, S., & Tamendjari, A. (2016). Antioxydant activity of some algerian honey and propolis. *Industrial Crops and Products*, *88*, 85–90. https://doi.org/10.1016/j.indcrop.2016.02.033

Mukota, A. K., Gondam, M. F. K., Tsafack, J. J. T., Sasanya, J., Reybroeck, W., Ntale, M., Nyanzi, S. A., & Tebandeke, E. (2020). Primary validation of Charm II tests for the detection of antimicrobial residues in a range of aquaculture fish. *BMC Chemistry*, *14*(1), 1–15.

https://doi.org/10.1186/s13065-020-00684-4

Olaitan, P. B., Adeleke, O. E., & Ola, I. O. (2007). Honey: A reservoir for microorganisms and an inhibitory agent for microbes. *African Health Sciences*, *7*(3), 159–165. https://doi.org/10.5555/afhs.2007.7.3.159

Ouchemoukh, S., Louaileche, H., & Schweitzer, P. (2007). Physicochemical characteristics and pollen spectrum of some Algerian honeys. *Food Control*, *18*(1), 52–58. https://doi.org/10.1016/j.foodcont.2005.08.007

Oroian, M., & Ropciuc, S. (2017). Honey authentication based on physicochemical parameters and phenolic compounds. *Computers and Electronics in Agriculture*, 138, 148–156.

https://doi.org/10.1016/j.compag.2017.04.020

Pascual-Maté, A., Osés, S. M., Fernández-Muiño, M. A., & Sancho, M. T. (2018). Methods of

analysis of honey. *Journal of Apicultural Research*, *57*(1), 38–74. https://doi.org/10.1080/00218839.2017.1411178

Perez-Arquillué, C., Conchello, P., Ariño, A., Juan, T., & Herrera, A. (1994). Quality evaluation of Spanish rosemary (Rosmarinus officinalis) honey. *Food Chemistry*, *51*(2), 207–210.

https://doi.org/10.1016/0308-8146(94)90258-5

Pires J, Estevinho ML, Feas X, Cantalapiedra J, Iglesias A. (2009). Pollen spectrum and physico-chemical attributes of heather (*Erica* sp.) honeys of north Portugal. *Journal of the Science of Food and Agriculture*. 89(11):1862–70.

https://doi.org/10.1002/jsfa.3663

Poppe, L. (2017). *lavonolignan Biosynthesis in Silybum marianum – Potential Regulatory Mechanisms and Candidate Genes*. Marburg.

https://doi.org/10.17192/z2017.0674

Rebiai, A., Lanez, T., & Chouikh, A. (2015). Physicochemical and biochemical properties of honey bee products in south Algeria. *Scientific Study and Research: Chemistry and Chemical Engineering, Biotechnology, Food Industry*, *16*(2), 133–142.

https://www.researchgate.net/publication/281453791_Physicochemical_and_biochemical_properties_of_honey_bee_products_in_south_Algeria

Sancho, M. T., Muniategui, S., Sánchez, P., Huidobro, J. F., & Simal-Lozano, J. (1992). Evaluating soluble and insoluble ash, alkalinity of soluble and insoluble ash and total alkalinity of ash in honey using electrical conductivity measurements at 20 °C. *Apidologie*, *23*(4), 291–297.

https://doi.org/10.1051/apido:19920403

Saxena, S., Gautam, S., & Sharma, A. (2010). Physical, biochemical and antioxidant properties of some Indian honeys. *Food Chemistry*, *118*(2), 391–397. https://doi.org/10.1016/j.foodchem.2009.05.001

Schievano, E., Morelato, E., Facchin, C., & Mammi, S. (2013). Characterization of markers of

botanical origin and other compounds extracted from unifloral honeys. *Journal of Agricultural and Food Chemistry*, *61*(8), 1747–1755.

https://doi.org/10.1021/jf302798d

Serra Bonvehí, J., & Lacalle Gutiérrez, A. (2009). Residues of antibiotics and sulfonamides in honeys from Basque Country (NE Spain). *Journal of the Science of Food and Agriculture*, *89*(1), 63–72.

https://doi.org/10.1002/jsfa.3411

Sharma, Y., Velamuri, R., Fagan, J., & Schaefer, J. (2020). Full-spectrum analysis of bioactive compounds in rosemary (Rosmarinus officinalis L.) as influenced by different extraction methods. *Molecules*, *25*(20), 1–21.

https://doi.org/10.3390/molecules25204599

Shugaba, A. (2012). Analysis of Biochemical Composition of Honey Samples from North-East Nigeria. *Biochemistry & Analytical Biochemistry*, *2*(3). https://doi.org/10.4172/2161-1009.1000139

Soares, S., Amaral, J. S., Oliveira, M. B. P. P., & Mafra, I. (2017). A Comprehensive Review on the Main Honey Authentication Issues: Production and Origin. *Comprehensive Reviews in Food Science and Food Safety*, *16*(5), 1072–1100. https://doi.org/10.1111/1541-4337.12278

Suárez-Luque, S., Mato, I., Huidobro, J. F., Simal-Lozano, J., & Sancho, M. T. (2002). Rapid determination of minority organic acids in honey by high-performance liquid chromatography. *Journal of Chromatography A*, *955*(2), 207–214. https://doi.org/10.1016/S0021-9673(02)00248-0

Sun, L. P., Shi, F. F., Zhang, W. W., Zhang, Z. H., & Wang, K. (2020). Antioxidant and anti-inflammatory activities of safflower (Carthamus tinctorius L.) honey extract. *Foods*, *9*(8), 1–16.

https://doi.org/10.3390/foods9081039

Terrab, A., González, A. G., Díez, M. J., & Heredia, F. J. (2003). Characterisation of Moroccan unifloral honeys using multivariate analysis. *European Food Research and*

Technology, *218*(1), 88–95.

https://doi.org/10.1007/s00217-003-0797-x

Terrab, A., Recamales, A. F., Hernanz, D., & Heredia, F. J. (2004). Characterisation of Spanish thyme honeys by their physicochemical characteristics and mineral contents. *Food Chemistry*, *88*(4), 537–542.

https://doi.org/10.1016/j.foodchem.2004.01.068

Urfi, M. K., Mujahid, M., Badruddeen, Akhtar, J., Khalid, M., Khan, M. I., & Usmani, A. (2016). Tamarix gallica: for traditional uses, phytochemical and pharmacological potentials. *Journal of Chemical and Pharmaceutical Research*, *8*(1), 809–814.

https://www.researchgate.net/publication/306182825_Tamarix_gallica_For_traditional_uses_phytochemical_and_pharmacological_potentials

Van Bruijnsvoort, M., Ottink, S. J. M., Jonker, K. M., & De Boer, E. (2004). Determination of streptomycin and dihydrostreptomycin in milk and honey by liquid chromatography with tandem mass spectrometry. *Journal of Chromatography A*, *1058*(1–2), 137–142. https://doi.org/10.1016/j.chroma.2004.07.101

Vit, P., Medina, M., & Enríquez, M. E. (2004). Quality standards for medicinal uses of Meliponinae honey in Guatemala, Mexico and Venezuela. *Bee World*, *85*(1), 2–5. https://doi.org/10.1080/0005772X.2004.11099603

Von der Ohe, W., Oddo, L., Piana, M., Morlot, M., & Martin, P. (2004). Harmonized methods of melissopalynology. *Apidologie*, *35*(1), 18–25.

https://doi.org/10.1051/apido

Yücel, Y., & Sultanoğlu, P. (2013). Characterization of honeys from Hatay Region by their physicochemical properties combined with chemometrics. *Food Bioscience*, *1*, 16–25. https://doi.org/10.1016/j.fbio.2013.02.001

Zafar, A., Safdar, M. N., Siddiq, N., Mumtaz, A., Hameed, T., & Sial, M. (2008). Chemical analysis and sensory evaluation of branded honey collected from Islamabad and Rawalpindi market. *Pakistan Journal of Biological Sciences*, *21*, 86–91.

https://www.cabi.org/gara/FullTextPDF/2009/20093346450.pdf

Zaidi, H., Ouchemoukh, S., Amessis-Ouchemoukh, N., Debbache, N., Pacheco, R., Serralheiro, M. L., & Araujo, M. E. (2019). Biological properties of phenolic compound extracts in selected Algerian honeys—The inhibition of acetylcholinesterase and α-glucosidase activities. *European Journal of Integrative Medicine*, *25()*, 77–84. https://doi.org/10.1016/j.eujim.2018.11.008