Quality evaluation of Algerian honeys: Eucalyptus, Jujube, Euphorbia and multiflora

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DEDICATION

This thesis is dedicated to:

- The sake of Allah, my Creator and my Master
- My great teacher and messenger, Mohammed (May Allah bless and grant him), who taught us the purpose of life.
- My homeland Algeria, the warmest womb that I miss it a lot, and I am just waiting impatiently to breathe its fresh air again after a year and six months absence
- My great parents, who never stop giving of themselves in countless ways, I miss you so much and forgive me for the long absence
- My beloved brothers and sisters
- My friends who encourage and support me
- All the people in my life who touch my heart, I dedicate this research.

Abstract

This study was intended to evaluate the quality parameters of ten honey samples, from various regions in semi-arid region of Algeria. Different parameters such as the melissopalynological and the physicochemical properties of the honeys (moisture, color, electrical conductivity, 5-hydroxymethylfurfural, pH, acidity, proline, and diastase activity) were determined, as well as the evaluation of sugars, minerals and phenolic compounds. Nutritional composition, antioxidant activity (reducing power and DPPH free radical scavenging activity), anti-inflammatory and cytotoxicity were also evaluated. Finally, antibiotics residues such as sulphonamides and tetracyclines antibiotics residues were screened.

The melissopalynological results identified ten types of pollen, with *Cytisus striatus* pollen being identified as the most abundant, present in all samples with percentages between 26.0 % and 83.8 %. EC1, MF1 and MF2 (Sidi Belabes region) were classified as monofloral of *Cytisus striatus* honey. Additionally, although samples J1 to J3 were not considered as Jujube monofloral, they showed a high percentage of *Ziziphus* pollen. The remaining samples (EC2, EF1, EF2, and EF3) were classified as multifloral.

Regarding the physicochemical parameters, no significant differences were found in the color of the samples which ranged between amber, light amber and extra light amber. Moisture content was found to be between 13.6% (EF1) and 18.3% (EC1), while pH values ranged between 4.2 and 5.1. Electrical conductivity values varied between 270 and 410 $\mu S.cm^{-1}$, while 5- hydroxymethylfurfural content showed values between 0 and 36.5 $mg.kg^{-1}$ and diastase values between 8.8 DN and 13.3 DN. Concerning the proline content, the samples showed proline levels between 2.2–4.7 mg/kg, indicating a good maturity of the honeys and absence of adulteration. All the honeys meet the standard required by the European legislation with exception of the diastase index. The sugar profile, analyzed by high pressure liquid chromatography with refractive index detection (HPLC-RI), showed that all samples have higher fructose content than glucose, being the total more than 88.70 %, allowing the classification of all the samples as nectar honeys.

Within the minerals, potassium was quantitatively the most important mineral (72.93% of total minerals quantified), having an average content 730.59mg/kg, followed by sodium, calcium and magnesium, with 17.05%, 4.43% and 4.22%, respectively, while cadmium and lead had the lowest concentration, 0.003 % and 0.04% respectively.

The total phenolic content of the analyzed honey samples ranged between 0.7 mg GAE/g, for samples EF and J and 1.4 mg GAE/g, for samples EC, with an average of 0.9 mg GAE/g. The total flavonoid content varied from 0.03 to 0.09 mg QE/g with the highest levels observed in J honey samples. The values obtained for DPPH ranged from 0.02 to 0.04 mg/mL, without significant differences between the samples.

The analysis of the phenolic profile was performed by UPLC/DAD/ESI-MS^n, where nineteen phenolic compounds were identified, including six phenolic acids, nine flavonoids, two isoprenoids (abscisic acid isomers), one phenolic diterpenoid (carnosol) and one spermidine (N^1, N^5, N^{10}-tri-*p*-coumaroyespermidine). The major quantity of phenolic compounds was found in sample EC1 with 202 mg/100 g, while sample EF3 showed the lowest amount with 59.85 mg/100 g.

Concerning the anti-tumoral evaluation, all the studied extracts presented good activity, with MF1 showing the highest cytotoxicity, followed by EF1. Also, all the extracts under study showed anti-inflammatory capacity, with IC_{50} values between 8 and 400 µg/mL.

Regarding the antibiotics residues, its presence was found in three of the samples (MF1 EF1 EF3) showed positive results for sulphonamides residues.

Keywords: honey, melissopalynological analysis, physicochemical parameters, antioxidant activity, anti-inflammatory activity, cytotoxicity, antibiotic residues

Resumo

Este estudo teve por objetivo avaliar os parâmetros de qualidade de dez amostras de mel, de várias regiões da região semiárida da Argélia. Neste âmbito foram determinadas as características melissopalinológicas e os parâmetros físico-químicos dos méis (humidade, cor, condutividade elétrica, 5-hidroximetilfurfural, pH, acidez, prolina e diástase), bem como efetuada a avaliação do perfil de açúcares, minerais e compostos fenólicos. A presença de resíduos de antibióticos como sulfonamidas e tetraciclinas foi também verificada. Paralelamente foi estudada a composição nutricional dos méis e a sua bioatividade através da atividade antioxidante (DPPH e poder redutor), anti-inflamatória e citotoxicidade.

Os resultados melissopalinológicos identificaram dez tipos de pólen, sendo o pólen de *Cytisus striatus* o mais frequente, estando presente em todas as amostras com percentagens entre 26,0% e 83,8%. As amostras EC1, MF1 e MF2 (região de Sidi Belabes) foram classificados como méis monoflorais de *Cytisus striatus*. Já as amostras J1, J2 e J3, não tenham sido consideradas monoflorais de Jujube, apresentaram uma alta percentagem de pólen de *Ziziphus*. As restantes amostras (EC2, EF1, EF2 e EF3) foram classificadas como méis multiflorais.

Em relação aos parâmetros físico-químicos, não foram encontradas diferenças significativas na cor das amostras que variaram entre âmbar, âmbar claro e âmbar extra claro. Os resultados do teor de humidade encontrados ficaram entre 13,6% (EF1) e 18,3% (EC1), enquanto os valores do pH variaram entre 4,2 e 5,1. Os valores da condutividade elétrica variaram entre 270 e 410 $\mu S.cm^{-1}$, enquanto o conteúdo de 5-hidroximetilfurfural apresentou valores entre 0 e 36,5 $mg.kg^{-1}$ e a diástase variou entre 8,8 DN e 13,3 DN. Quanto ao conteúdo de prolina, as amostras apresentaram níveis de prolina entre 2,2–4,7 mg/kg, indicando boa maturidade dos méis e ausência de adulteração. Todos os méis presentaram valores dentro do requerido pela legislação europeia, com exceção do índice de diástase. O perfil de açúcares, analisado por cromatografia líquida de alta pressão com deteção de índice de refração (HPLC-RI), confirmou um maior teor de frutose do que glucose, sendo o total superior a 88,7%, permitindo a classificação de todas as amostras como méis de néctar.

O potássio foi o mineral encontrado em maior quantidade (72,93% dos minerais totais quantificados), tendo um teor médio de 730,59mg/kg, seguido do sódio, cálcio e magnésio com17,05%, 4,43% e 4,22% respetivamente), enquanto o cádmio e o chumbo apresentaram a concentração mais baixa, 0,003% e 0,04%, respetivamente.

O conteúdo fenólico total das amostras variou entre 0,7 mg GAE/g, para as amostras EF e J e 1,4 mg GAE/g, para as amostras CE, apresentando uma média de 0,9 mg GAE/g. O teor de flavonóides totais variou entre 0,03 e 0,09 mg QE/g, sendo as amostras J as que apresentaram um valor mais elevado. Os valores obtidos para o DPPH variaram entre 0,02 e 0,04 mg/mL, sem diferenças significativas entre as amostras.

A análise do perfil dos compostos fenólicos foi realizada por UPLC/DAD/ESI-MS^n, onde foram identificados dezanove compostos fenólicos, incluindo seis ácidos fenólicos, nove flavonóides, dois isoprenóides (isómeros do ácido abscísico), um diterpenóide fenólico (carnosol) e uma espermidina (N^1, N^5, N^{10}-tri-*p*-coumaroyespermidina). A amostra EC1 apresentou a maior quantidade de compostos fenólicos com 202 mg/100g, enquanto a amostra EF3 apresentou a menor quantidade com 59,85 mg/100 g.

Quanto à avaliação anti-tumoral, todos os extratos estudados apresentaram atividade, sendo o MF1 o que apresentou maior citotoxicidade, seguido do EF1. Além disso, os extratos apresentaram capacidade anti-inflamatória, com valores de IC_{50} entre 8 e 400 µg/mL.

Em relação aos resíduos de antibióticos verificou-se a presença de três das amostras (MF1, EF1, EF3) com resultados positivos para resíduos de sulfonamidas.

Palavras-chave: mel, análise melissopalinológica, parâmetros físico-químicos, atividade antioxidante, atividade anti-inflamatória, citotoxicidade, resíduos de antibióticos

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Abbreviations List

$[M-H]^-$ - Ion product

5-HMF - 5-Hydroxymethylfurfural

Abs – Absorbance

AFB- American foulbrood

DN- Diastase index

GAE- Gallic acid equivalents

EU- European Union

HPLC– High pressure liquid chromatography

IHC– International Honey Commission

IR - Refractive index

LC- Liquid chromatography

LC-MS- Liquid chromatography coupled to mass spectrometry

MS- Mass spectrometry

m/z- mass to charge ratio

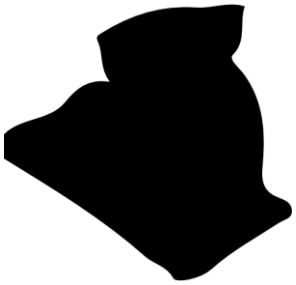
QE- quercetin equivalents

rpm- Rotation per minute

SPE- Solid phase extraction

T_R - Retention time

UPLC/DAD/ESI-MS^n- Ultra-pressure liquid chromatography with photodiode detection coupled to tandem mass spectrometry with electrospray ionization.


Chapter I- Introduction

Introduction

Algeria has a rich variety of melliferous plants, which is distributed in different bioclimatic zones. It has a potentially large beekeeping production area, but honey production remains low. This weakness is due to the lack of expertise of intensive production techniques on the part of beekeepers, but also due to climate change and absent of transhumance.

In Algeria, the agricultural sector set up during the year 2000 an operational strategy for agricultural development (national agricultural development plan PNDA) extended from 2002 to the rural domain in favor of new attributions entrusted by the government to the ministry of agriculture and rural development. In this context, attention was given to beekeeping production and in particular to the establishment of modern hives and the production of honey (Adjlane**,** Doumandji and Haddad, 2012).

Honey is the world's primary sweetener and nature's original sweetener prepared by honeybees. Honey has been used as a food and medicine for at least 6000 years. The demand for high quality honey is attracting great attention because of its health benefits (Alvarez-Suarez et al., 2010) derived from its diversity and has been shown to have biological properties, such as antimicrobial, antiviral, antiparasitic, anti-inflammatory, antioxidant, antimutagenic and antitumor effects (Bogdanov, Jurendic, Sieber, & Gallmann, 2008). Diseases prevention through consumption of honey is probably due to the presence of more than 181 substances, such as amino acids, enzymes, proteins, vitamins, minerals, ash, organic acids and phenolic compounds (Ouchemoukh et al., 2007; Ferreira et al., 2009). Its composition varies with the floral source used by the bees, the harvest period and the geo-climatic conditions of the regions concerned (Mbogning et al., 2011). In Algeria, several studies on honey characterization have been carried out; we can cite the studies of: (Chefrour, 2007), (Ouchemoukh et al, 2007), (Makhloufi et al 2010), (Zerrouk et al 2011), (Zerrouk et al, 2014), (Nair, 2014), (Draiaia et al, 2015) and (Haouam et al, 2016).

1.1. Objectives

Algerian beekeepers who have constantly attempted to rescue and guarantee the common characteristics of honey hope to discover different markets from local ones. For that, an extensive study of the Algerian honey is needed, having in mind the establishment of quality and authenticity guidelines and regulations. The aim of the present study is to evaluate the quality of Algerian honey and verify its compliance with the established standards of Codex. For that, ten samples with different botanical and geographical origin were analyzed

regarding the following physicochemical parameters: melissopalynological analysis, color, moisture, acidity, pH, ash content, electrical conductivity, diastase index, proline, 5-hydroxymethylfurfural (HMF), nutritional composition and mineral content. Phenolic compounds were evaluated through spectrophotometric methods and liquid chromatography coupled with mass spectroscopy (LC-MS). Antioxidant activity (reducing power, DPPH free radical scavenging activity), cytotoxicity and anti-inflammatory activities were also evaluated. Finally, the presence of antibiotics, recurrent residues in honey, such as tetracyclines and sulphonamides were screened to attest its safety.

1.2. Honey bees and bee products

1.2.1. *Apis mellifera*

Apis mellifera naturally occurs in Europe, the Middle East, and Africa. This species has been subdivided into at least 20 recognized subspecies (Mortensen, Schmehl and Ellis, 2013). Like all Hymenopterans, honeybees have haplo-diploid sex determination. Unfertilized eggs develop into drones (males), and fertilized eggs develop into females. Female larvae, which taken care with a standard food regimen of pollen, nectar, and brood nourishment become grown-up worker bees. Female larvae fed with a rich food regimen of royal jelly, pollen, and nectar become queen (Mortensen, Schmehl and Ellis, 2013**).** Worker honeybees are non-reproductive females. They are the smallest in physical size of the three ranks and their body is designed specifically for pollen and nectar collection **(Fig.1.A**)**.** Queen honeybee **(Fig.1.B)** is the only reproductive female in the colony. Her head and thorax are similar in size compared to that of the worker, while the abdomen is more extended and plumper**.** Drones are the male cast of honeybees. Drone's head and thorax are bigger than those of the females, (**Fig.1.C**) (Mortensen, Schmehl and Ellis, 2013).

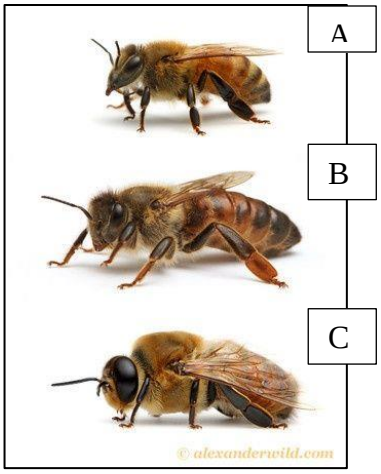


Figure 1. (A) Worker European honeybee, *Apis mellifera* Linnaeus. (B) A Queen. (C) Drone (male) European honeybee, *Apis mellifera*. Photograph by Alexander wild https://www.alexanderwild.com/Insects/Stories/Honey-Bees/i-3DtbsJ.

1.2.2. Bee Products

1.2.2.1. Beeswax

Beeswax is an extremely inert common material that is secreted by worker bees from the wax glands (Avshalom and Yaacov, 1996). Bees use beeswax to grow their larvae and construct honeycomb cells where pollen and honey are stored. When secreted by bees, beeswax is white, but in the honey combs rapidly obscures due to the contact with the bees and also the pollen and honey (Avshalom and Yaacov, 1996).

1.2.2.2. Propolis

The word propolis comes from the Greek «pro» = in front, «polis» = city, and means a substance with a protecting role for the bee colony (Bogdanov, 2014). Bees gathered resinous exudates from leaf buds, shoots and petioles of leaves from different plants with their mandibles, which once introduced into the hive, are mixed with wax and salivary secretions, in order to produce propolis, which is used as a building and defense material within the hive. Propolis has a very complex composition which is dependent on the plant origin (Bankova and De Castro, 2000). The main chemical classes and most bioactive compounds found in propolis are the phenolic compounds, which are responsible for most of the bioactivities (Bankova and De Castro, 2000).

1.2.2.3. Royal jelly

Royal jelly is a bee product secreted by the hypopharyngeal and mandibular glands of the nurse working bees (Zahran et al., 2016), between the 6^{th} and 12^{th} day of their life cycle. This bee product is a white-yellow colloid with a pH between 3.6–4.2, with a variable composition which depends on the metabolic and physiologic condition of the worker bees, bee specie and on the seasonal and local conditions (Scorselli and Donadio, 2005).

1.2.2.4. Bee pollen and bee bread

Pollen grains are microscopic structures, male gametes located in the anthers of stamens, indispensable for the fertilization of the female sexual organ of the flower (Krell, 1996). Pollen is extremely important for the hive, it is the main source of food for the larvae providing them with important nutrients for their development such as proteins, and carbohydrates, lipids, vitamins and minerals (Luz et al.,2010).

1.2.2.5. Bee venom

Bee venom (BV) is an odorless and transparent liquid produced by female worker bees containing a hydrolytic mixture of proteins with acid pH (4.5 to 5.5) that bees often use as a defense tool against predators. One drop of BV consists of 88% of water and only 0.1 µg of dry venom (Bellik, 2015)

1.2.2.6. Honey

The Codex Alimentarius defined honey as a natural sweet substance, produced by honeybees from the nectar of plants, secretions of their living parts, or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature (Codex Alimentarius, 2001). The definition of honey under European Union (EU) legislation is very similar, with the difference that it stipulates the bee species as being *Apis mellifera* (Directive 2001/110/EC).

1.3. Honey categories concerning origin

1.3.1. Nectar honey

This type of honey is produced by bees after they harvest the nectar of the flowers. Nectar is a sugar-rich liquid produced by plants in glands called nectaries, and mainly exist to encourage pollination by insects and other animals. About 95% of the dry substance are sugars, the rest are amino acids (0.05 %), minerals (0.02-0.45 %) and restricted amounts of organic acids, nutrients, and vitamins (Bogdanov, 2014). According to their botanical origin, nectar honeys can be classified as monofloral honeys, if they are produced from a single family or plant species, or as multifloral honeys when there is no floral species that stands out. This assessment is often carried out through an analysis of pollen grains that are present in honey, considering that when collecting nectar in the flower, bees transport pollen grains that they will inadvertently introduce into honey (Bear, 2009).

1.3.2. Honeydew honey

Honeydew honey is formed from secretions of living parts of plants or from the excretions of sucking insects (Hemiptera, mostly aphids) (Terrab et al., 2003). These insects break the plant cell and ingest the sap. The excess is excreted as droplets of honeydew, which are gathered by the bees (Bogdanov, 2014). Honeydew is a solution

with varying sugar concentration (5-60 %), containing mainly sucrose, besides higher sugars (oligosaccharides). There are also smaller amounts of amino acids, proteins, minerals, acids and vitamins. Besides, honeydew contains cells of algae and fungi (Bogdanov, 2014).

1.4. Honey chemical composition

Honey is composed mainly by sugars, glucose and fructose, and in a less amount water and other components like minerals, vitamins, proteins and amino acids, **Table 1.**

Table 1. Honey composition after (Bogdanov, 2009) values in g/100g.

	Nectar honey g/100g		**Honeydew honey g/100g**	
	Average	Min-Max	Average	Min-Max
Water content	17.2	15-20	16.3	15-20
Fructose	38.2	30-45	31.8	28-40
Glucose	31.3	24-40	26.1	19-32
Sucrose	0.7	0.1	0.5	0.1-4.7
Other disaccharides	5.0	4.8	4.0	16
Melezitose	<0.1	-	4.0	0.3-22.0
Erlose	0.8	-	1.0	0.16
Other oligosaccharides	3.6	0.56	13.1	0.1-0.6
Total sugars	79.7	0.5-1	80.5	-
Minerals	0.2	0.1-0.5	0.9	0.6-2
Amino acids and proteins	0.3	0.2-0.4	0.6	0.4-0.7
Organic acids	0.5	0.2-0.8	1.1	0.8-1.5
pH	3.9	3.5-4.5	5.2	4.5-6.5

1.4.1. Sugars

Sugars are the main constituents of honey, comprising about 95 % of honey dry weight (Bogdanov, 2014). The monosaccharides glucose and fructose are the main sugars found in honey, which are the building blocks of the more complex sugars and are the resulting products of the disaccharide sucrose hydrolysis (White, 1980). The main oligosaccharides in nectar honeys are disaccharides: sucrose, maltose, turanose, erlose. Honeydew honey also contains the trisaccharides melezitose and raffinose. Trace amounts of tetra and pentasaccharides have also been isolated, including isomaltotetraose and isomaltopentaose (Bogdanov, 2014).

1.4.2. Water content

Water is the second largest constituent of honey, and its content is also related to the maturity of this product. The moisture content can be influenced by floral and geographical origin, climatic factors, season of the year, processing and storage conditions, as well as the degree of maturity achieved in the hive (Gallina et al., 2010). It has significant impact on the physical properties of honey, such as, viscosity and crystallization, but also taste, color, flavor, solubility, conservation and specific gravity and also in the shelf life of the product. According to the Codex Alimentarius Committee on Sugars, the moisture content in honey should not exceed 20 g /100 g (Codex Alimentarius, 2001). If the moisture content is higher, the honey is more likely to ferment due to the presence of yeasts and osmophilic microorganisms. Since honey is hygroscopic, the moisture in honey can also increase during the processing operations of the product, as well as the inadequate storage conditions (White, 1980).

1.4.3. Proteins and amino acids

Proteins and amino acids in honey are originated from both bees (salivary glands), and plants (nectar, honeydew and mainly pollen). About 20 different non-enzymatic proteins have been identified in honey (De-Melo et al., 2018). The quantity of proteins can vary from 0.1 to 0.7%, **Table 1**. Overheated or long-time stored honeys show a reduction or absence of protein content (De-Melo et al., 2018). Around 26 amino acids have been detected in honey, such as proline, glutamic acid, alanine, phenylalanine, tyrosine, leucine, among others (Cotte and Giroud, 2004). The most abundant amino acid found in honey is proline, ranging from 50 to 85% of the total. The proline content in honeys should be

higher than 200 mg/kg (Bogdanov, 2002). When the values of this amino acid are significantly lower than 180 mg/kg, the minimum value that has been agreed for genuine honey, it indicates sugar adulteration. Proline can be seen as quality criteria for honey ripeness (Von-der, Dustmann, 1991).

1.4.4. Enzymes

The degrees of enzymes present in honey are sometimes used as an indicator for honey quality, freshness and overheating. Enzymes in honey are originates from the honey bees or from the plant visited by the bees. Diastase (α- and β-amylase) digests starch to maltose and is relatively stable to heat and storage and invertase (glucosidase) catalyzes mainly the conversion of sucrose to glucose and fructose, but also many other sugar conversions (Raude, 1994). Also, glucose oxidase and catalase regulate the production of H_2O_2, one of the honey antibacterial factors (Bogdanov, 2014). The enzyme content also depends on temperature, honey botanical origin, nectar abundance flow, state and strength of the colony, seasonal activity of the bee, bee specie, diet, age and physiological stage of the bee (De-Melo et al., 2018).

Diastase activity is a physicochemical parameter usually investigated as marker of honey freshness (Fechner et al., 2016; Flores et al., 2015). It can be expressed in Schade, Göthe or diastase units and honey generally should present a diastase activity of at least 8 Schade units, which is the minimum value accepted by regulatory organizations (Codex Alimentarius Commission, 2001). Similar to 5-HMF, the diastase activity can be used as an indicator of aging and increase temperature because it may be reduced during storage or when the product is subjected to heating above 60 ^{o}C (Fechner et al., 2016; Flores et al., 2015).

1.4.5. 5-Hydroxylmethylfurfural (5-HMF)

5-HMF is a product of the decomposition of monosaccharides such as fructose, **Fig. 2.** The reaction occurs slowly and naturally during the storage of honey, and much more quickly when honey is heated. The 5-HMF amount present in honey is the reference used as a guide to the amount of heating that has taken place; the higher the 5-HMF value, the lower the quality of the honey (Bear, 2009). However, 5-HMF alone cannot be used to determine the severity of the heat treatment, because other factors can influence the levels of 5-HMF, such as the sugar profile, presence of organic acids, pH, moisture content, water activity and floral source. Therefore, the 5-HMF content gives only an indication of overheating or inadequate storage conditions (Bogdanov, 2014). As indicated by the

Codex Alimentarius and EU standards, the 5-HMF maximum is 40 mg/kg for the mixture or processed honey, and a maximum of 80 mg/kg for honeys with a tropical origin. (Bogdanov, 2014).

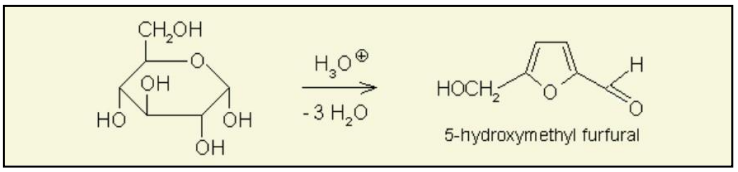


Figure 2. 5-HMF formation resulting from a sugar decomposition reaction (Bogdanov, 2014)

1.4.6. Organic acids

Honey contains organic acids, in equilibrium with the corresponding lactone, representing less than 0.5% of total solids. They are important for honey taste, aroma, color, acidity and honey preservation, making it difficult for microorganisms to grow (Bogdanov, 2014). Organic acids in honey have different sources, while some acids can come directly from nectar or honeydew, the majority, are produced from sugars by the action of enzymes secreted by bees during ripeness and storage (De-Melo et al., 2018). Gluconic acid is the main honey organic acid, representing the 70–90% of the total (Bogdanov, 2014). It comes from glucose by the action of glucose oxidase. In addition to gluconic acid, more than 30 different non-aromatic organic acids were found in honey. Legally, organic acids should not exceed 50 meq/kg. For honey intended for industry, the tolerated limit is of 80 milliequivalents (Lequet, 2010).

1.4.7. Vitamins

Honey has small amounts of vitamins, which come mainly from the pollen grains in suspension (Matzke and Bogdanov, 2003). Vitamins found in honey include thiamine (B1), riboflavin (B2), nicotinic acid (B3), pantothenic acid (B5), pyridoxine (B6), biotin (B8), folic acid (B9) and also vitamin C. Those vitamins present in honey are preserved due to the low pH of honey. The commercial filtration of honey may cause a reduction in vitamin content due to the almost complete removal of pollen. Also, the loss of vitamins in honey can happen due to the oxidation of ascorbic acid by the hydrogen peroxide produced by glucose oxidase (Ciulu et al., 2011).

1.4.8. Mineral content

Mineral composition in honey is generally low, ranging between 0.02 and 0.3% in nectar honeys, while in honeydew honeys can reach 1% of the total (Felsner et al., 2004).

Its content can vary with the soil and climatic conditions, as well as the chemical composition of the nectars originated from the different botanical sources. Also, the harvesting and the beekeeping techniques can have influence in the honey mineral (Felsner et al., 2004) content. The main minerals found in honeys are potassium, sodium, calcium and magnesium and in lesser amounts iron, copper and, manganese. In minor quantities, as trace elements, are found boron, phosphorus, sulfur, silicon and nickel, among others (Doner, 2003). Generally, dark honeys contain more minerals than the light ones, being higher in honeydew honeys (Bear, 2009). The mineral content is correlated with the ash percentage and the electrical conductivity (Da Silva et al., 2016).

1.4.9. Volatile compounds

Researchers began the study of honey aromatic substance in the mid of 1960. Honey volatiles are the substances responsible for the honey fragrance. Most of them are derived from plants, but also some are included by the honey bees. Until now around 600 compounds have been identified in the volatile fraction of honey, and some are used as markers of monofloral honeys, such as 3,9-epoxy-1-*p*-mentadieno, t-8-*p*-menthan-oxide-1,2-diol and cis- rose, which have been proposed as markers of lemon honey; diketones, sulfur compounds and alkanes are characteristic of eucalyptus honey, while hexane and heptanal are the main compounds in the aroma of lavender honeys (Castro-Vázquez et al., 2007). Other volatiles from different chemical families are present in honey at very low concentrations, such as monoterpenes, C13-norisoprenoid, sesquiterpenes, benzene derivatives and, to a lower content, superior alcohols, esters, fatty acids, ketones, terpenes and aldehydes (Pontes et al., 2007).

1.4.10. Phenolic compounds

Phenolic compounds are plant-derived secondary metabolites. These compounds have been used as chemotaxonomic markers in plant systematics. They have been recommended as potential markers for the determination of botanical origin of honey and for the differentiation between monofloral and multifloral honeys. In honey, as well as from pollen or propolis they are mainly derived from plants (Ferreres, Ortiz and Silva, 1992), being present in a range of 5–1300 mg/kg (Gheldof and Engeseth, 2002). According to the phenolic structural features, polyphenols are divided into two main groups, phenolic acids and flavonoids (Tomás- Barberan et al., 2001). Flavonoids aglycones are the mainly polyphenols found in honey. The loss of the sugar moiety of the

glycosides present in nectar is due to the hydrolysis by bee saliva enzymes (Tomás-Barberán et al., 2001). Dark honeys usually contain a higher quantity of phenolic compounds than the light ones. Dark honeys have been reported to contain more phenolic acid derivatives but less flavonoids than light ones (Tomás-Barberan et al., 2001).

1.5. Other physicochemical parameters

1.5.1. Color

Honey color can vary from practically colorless to brown dark, sometimes with green or reddish reflexes. These variations in the color of honey can related to its flavor: honey with lighter color have a gentle flavor while the darker honeys have a stronger flavor (Marchini, Sodré and Moreti, 2004). The color of honey depends on its floral origin, climate factors during nectar flow, soil conditions and the temperature at which the honey matures in the hive. Also, pollen, sugars, carotenoids, xanthophylls, anthocyanins, minerals, amino acids and phenolic compounds, mainly flavonoids (Bogdanov et al., 2004). Furthermore, honeydew honey is darker than bloom honey primarily because of mineral and phenolic substance and other components (Can et al., 2015).

1.5.2. Electrical conductivity

Electrical conductivity is a property related to the ability of a material to lead an electric flow. Honey contains minerals and acids, serving as electrolytes, which can conduct the electrical current, thus, the higher their content, the higher the resulting conductivity. It is an indicator often used in the quality control of honey that can be used to distinguish floral honeys from honeydew honeys. At present it is the most useful quality parameter for the discrimination between floral honeys and honeydew honeys. As this parameter is directly related to the ash content, it was included in the Codex Alimentarius Standards, replacing the determination of the ash in honey. The standards recommend a maximum value of 0.8 mS cm^{-1} (Codex Alimentarius, 2001; Bogdanov, 2014).

1.5.3. pH and acidity

The pH of honey ranges between 3.5 and 5.5 depending on its floral and geographical source, the pH of nectar, soil or plant association, and the amount of different acids and minerals (Crane, 1985). While pH analysis is useful as an auxiliary variable to estimate the quality of the product and as a parameter for evaluating total acidity, it is not directly related to free acidity due to the actions of the buffer acids and

minerals present in honey (Pereira et al., 2009). The acidity of honey can be assessed as free, lactonic, and total (free + lactonic) acidity (Navarrete et al., 2005). Free acidity is a parameter related to the deterioration of honey, being its limit established as 50 meq kg^{-1} (Codex Alimentarius, 2001; EU Commission, 2002). Higher values may be indicative of fermentation of sugars into organic acids (Almeida et al., 2013).

1.6. Antibiotic residues in honey

According to Regulation (EC) No 470/2009, no veterinary medicinal product is permitted in beekeeping products. In fact, no antibiotic has ever had an MRL (Maximum residue limits) in honey (Cara et al., 2012). However, some countries, like Switzerland, UK, and Belgium, have established action limits for antibiotics in honey, which generally lies between 0.01 to 0.05 mg/kg for each antibiotic group (Al-Waili et al., 2012). Some antibiotics have the potential to produce toxic reactions in consumers directly while some other can produce allergic or hypersensitivity reactions (Velicer et al., 2004). Antibiotic residues consumed along with food and honey can produce resistance in bacterial populations. Antibiotic resistance is a global public health problem and continues to be a challenging issue (Al-Waili et al., 2012). Two main approaches are used to determine the content of antibiotic residues in honey: screening tests and multi-stage analytical methodologies. The simple tests provide qualitative information, enabling determination of a single target analyte. With multi-stage methods, a fairly broad spectrum of analytes can be determined in one analytical run. (Barganska, Slebioda and Namiesnik, 2011).

1.7. Biological properties of honey

Honey has been found to contain significant antioxidant compounds including glucose oxidase, catalase, ascorbic acid, flavonoids, phenolic acids, carotenoid derivatives, organic acids, amino acids and proteins (Beretta et al., 2005). Research showed a correlation between color and antioxidant capacity, with the darker honeys providing the highest levels of antioxidants (Jaganathan and Mandal, 2009).

Phenolic content in honey is responsible for anti-inflammatory effect (Al-Waili, Boni, 2003). These phenolic and flavonoids compounds cause the suppression of the pro-inflammatory activities of cyclooxygenase-2 (COX-2) and/or inducible nitric oxide synthase (iNOS) (Viuda, Ruiz, Fernandez, 2008). Furthermore, ingestion of diluted natural honey has produced reductions on concentrations of prostaglandins such as PGE2

(prostaglandin E2), PGF2α (prostaglandin F2a) and thromboxane B2 in plasma of normal individuals (Reyes, Segovia and Shibayama, 2007).

1.8. Beekeeping in Algeria

Beekeeping in Algeria is practiced mainly in the north of the country, where the floral diversity is ensured almost all the year. The honeybees need to be adapted to the desert climate and to be resistant to unfavorable environmental conditions such as high temperatures and strong prevailing winds. Hives which are best suited or adapted to the desert conditions must be used. Traditional hives made from rocks and muds are known from ancient times in Algerian deserts. Nowadays, Langstroth hive type is used in Algeria**,** **Fig.3**, with modifications due to the hot weather (Moustafa, 2001).

A **B**

Figure 3. (A) The Langstroth hive and (B) the Langstroth hive different parts (John, 2014).

In 2010, the Algerian Beekeeping Organization, counted around 1.2 million colonies **Fig.4.A,** and 20,000 beekeepers. The development of honey production shows a clear increase from 2002 to 2010**, Fig.4.B** (Adjlane et al., 2012).

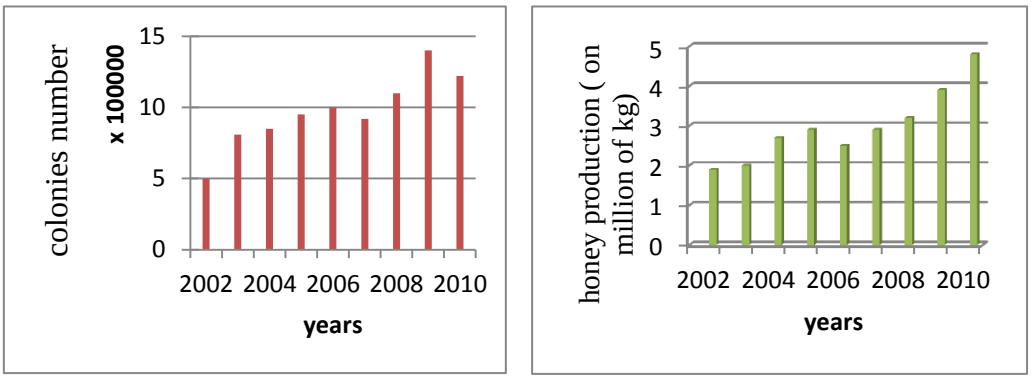


Figure 4. Number of honeybee colonies in Algeria from 2002 to 2010. (B) Honey production in Algeria from 2002 to 2010. Source: Ministry of Agriculture and Rural Development: MADR (2009-2010) (Adjlane, Doumandji and Haddad N. al., 2012).

In Algeria, there are two main bee subspecies. The Tellian bee (*Apis mellifera intermissa*)**, Fig.5-(A)**, is native of the region located between the atlas and the Mediterranean which is known by the name of Tell. It is characterized by its black abdomen and its agressivity. The main advantages of this bee are its longevity, remarkable ability to harvest pollen and a high production of honey which can reach up to 100 kg per colony provided that modern beekeeping methods are applied (Fresnay, 1981).

The Saharan (desert) bee (*Apis mellifera sahariensis*), **Fig.-5(B)**, better known as the Sahara bee, or locally the yellow bee. It is recognized for its many advantageous features such as the high breeding, the precocity, the extraordinary aptitude for nectar and pollen harvesting and good adaptability under difficult climatic conditions (Kessi, 2013).

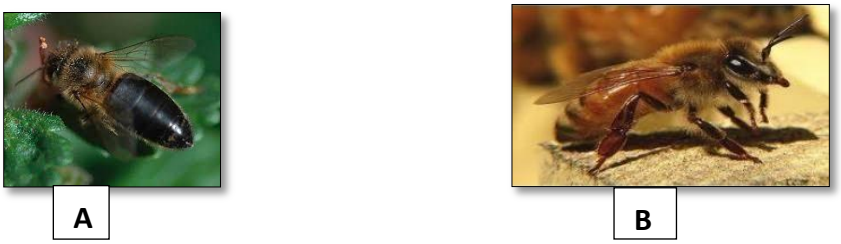


Figure 5. Images showing (A): *Apis mellifera intermissa* bee and a (B): *Apis mellifera Sahariensis* bee (Tlemcani, 2013).

1.9. Algerian honey

In this research, representative Algerian honeys such as, Euphorbia (*Euphorbia bupleuroides*), jujube (*Ziziphus lotus*), Eucalyptus (*Eucalyptus globulus*) and multifloral honeys will be focused.

1.9.1. Eucalyptus honey

The eucalyptus tree is a large, fast-growing evergreen that is native from Australia and Tasmania. The tree can grow to 125-160 meters. Eucalyptus belongs to the Myrtaceae family and more than 300 species of eucalyptus are described as *Eucalyptus globulus*, **Fig 6.A**, which is the most common and well-known (Catherin, 2020). Many of which produce enough nectar for honey bees to yield appreciable amounts of honey (Catherin, 2020; Persano, Baldi and Piazza, 2004). The main physicochemical parameters are shown in, **Table2**. It is a honey with a clear amber color, a wet wood, very intense and persistent aroma, a sweet with a slight acid note and a medium tendency for crystallization with fine crystals (Orantes et al., 2018).

1.9.2. Euphorbia honey

Euphorbia is one of the largest flowering plant in the spurge family (Euphorbiaceae). With over 2,000 species, euphorbias can range from tiny annual plants to large and long-lived trees and look completely different. In the deserts of Africa and Madagascar, euphorbia adapted its physical characteristics becoming similar to cacti of America, although they are not cacti (Cherif et al., 2011). Recent inventory of native plants in Algeria identify over 51 species of Euphorbiaceae, where *E. bupleuroides***, Fig.6.B,** is the main species used by bees to produce honey (Le Houèrou, 1995; Quezel and Médail, 2003).

Table 2. Physicochemical properties of Jujube, Euphorbia, Eucalyptus honeys of arid and semi-arid zones in north Africa (Cherif et al., 2016; Cherif et al., 2016); (Makhloufi et al., 2010)

Botanical origin	pH	Electrical conductivity s/cm	Water content %	Diastase Schade unit	Sucrose %	5- HMF mg/kg	References
Ziziphus	4.4	673	16.65	15.63	0.61	8.71	**(Cherif et al., 2016)**
Euphorbia	4.2	411	17.06	12.67	0.97	12.08	**(Cherif al., 2011)**
Eucalyptus	4.2	769	16.5	9.64		25.63	**(Makhloufi et al., 2010)**

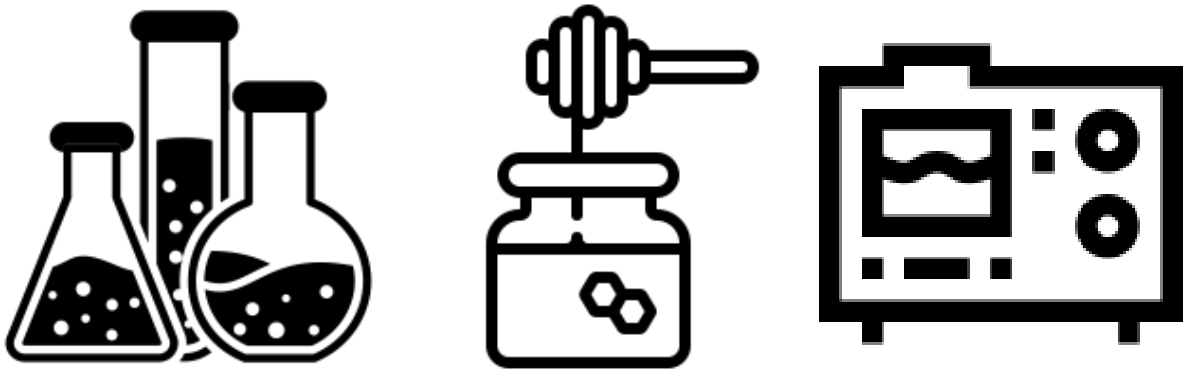
The main physicochemical parameters are shown in **Table 2.** It is a honey with golden yellow to dark amber color, with a sweet, pinch in the throat with a typical light bit back flavor and with a spicy almost peppered aroma and pungent flavor (Cherif et al., 2011).

Figure 6. (A) Eucalyptus plant (Orantes, Gonell, Torres et al., 2018). (B) Euphorbia plant. (C) Jujube plant (Photograph by Andrii Salomatin, https://www.shutterstock.com/fr/g/Andrii%2BSalomatin retrieved on 24-05-2

1.9.3. Jujube honey

Ziziphus lotus L. belongs to the family Rhamnaceae, which consist of about 135 species. The trees are medium-sized, growing 7-10 meters high, with shiny green leaves about 5 cm long. The edible fruits are a globose dark yellow drupe with 1–1.5 cm diameter, **Fig.6.C.** The wild jujube *Ziziphus lotus* is a species found in many habitats of arid and semiarid regions of the Mediterranean area, throughout Libya to Morocco and Algeria **(**Benammar et al., 2010).

Jujube honey is a highly demanded product in Algeria and worldwide, being considered one of the most expensive honeys. Despite the commercial interest, this honey type has been scarcely described (Cherif et al., 2016). The main physicochemical parameters of jujube honey are shown in **Table2**. Its color is varied from light-amber to amber.



Chapter II- Materials and methods

2.Material and methods

2.1. Honey samples

This work was carried out with ten Algerian monofloral and multifloral honey samples, obtained from local beekeepers and harvested in 2019, **Fig.7**. The honey samples were stored in the original containers at room temperature.

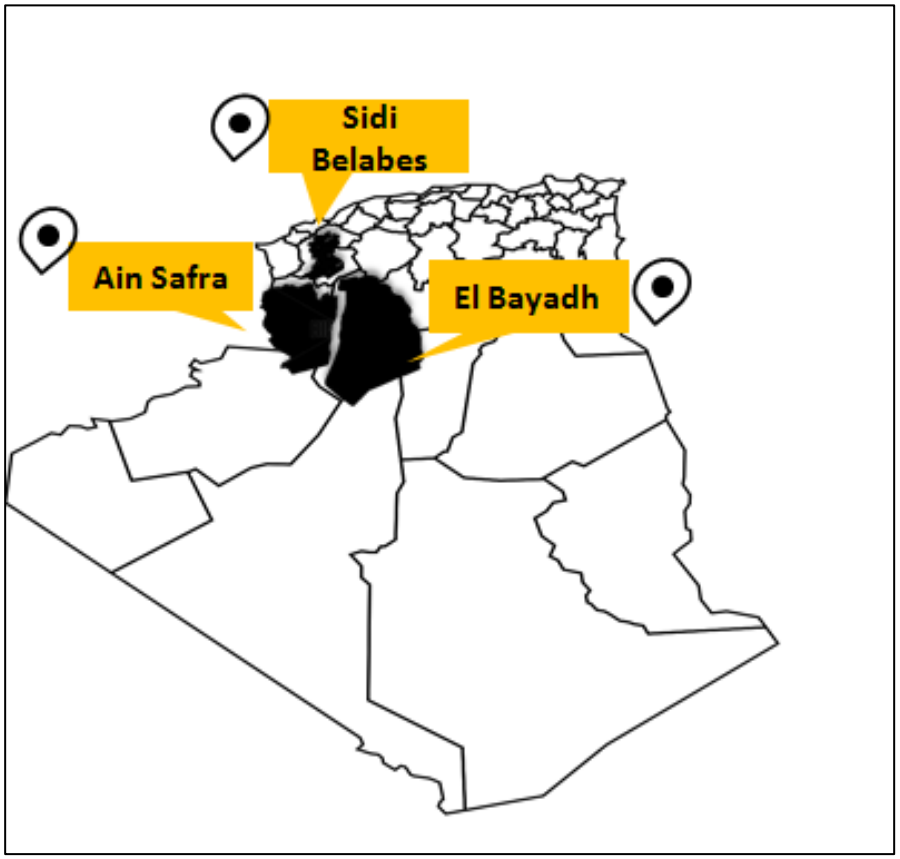


Figure 7. Geographic origin of the honey samples.

In Table 3, there is information regarding the honey samples used throughout this work, namely their geographical origin, year of production and other relevant information on the label. Also the probable floral origin, given by the label, is shown in the Table 3.

Table 3. Geographic origin and other information from honey samples.

Samples	Floral origin on the label	Geographic origin	Collection year
EC1	Eucalyptus	Sidi Belabes	2019
EC2	Eucalyptus	Sidi Belabes	2019
MF1	Multifloral	Sidi Belabes	2019
MF2	Multifloral	Sidi Belabes	2019
J1	Jujube	Ein Safra	2019
J2	Jujube	Ein Safra	2019
J3	Jujube	Ein Safra	2019
EF1	Euphorbia	El bayed	2019
EF2	Euphorbia	El bayed	2019
EF3	Euphorbia	El bayed	2019

2.2. Honey analysis

The honey characterization was carried out through the identification of their floral origin by pollen analysis and by the evaluation of the physicochemical parameters, defined by the International Honey Commission (IHC) (International Honey Commission. 2009). Also, the composition of proteins, phenolic compounds and antioxidant activity was evaluated. All parameters were evaluated in triplicate.

2.2.1. Pollen analysis

For pollen analysis, 10 g of honey, for each sample, 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 40 ^{o}C. 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 (Von Der et al, 2004). This work was done in collaboration with *LabApis*^{UTAD}.

2.2.2. Physicochemical analysis

2.2.2.1. Color

The color intensity of honey samples was measured according to the Pfund scale. Briefly, homogeneous honey samples were transferred into a cuvette with a 10 mm light path until the cuvette was approximately full. Then, the cuvette was inserted into a C221 colorimeter (Hanna Instruments, Woonsocket, RI, USA). color grades were expressed in millimeter (mm) Pfund grades, compared to an analytical-grade glycerol standard.

2.2.2.2. Moisture content

Moisture content was determined using a hand refractometer (Digit-5890, Ref: 8100.5890), expressing the results in percentages.

2.2.2.3. Electrical conductivity

A honey solution was prepared by diluting 20 g of anhydrous honey in 25mL of deionized water, and the respective electrical conductivity was measured with the help of a calibrated Consort C868 conductivity meter (Hanna Instruments, Woonsocket, RI, USA), **Fig. 8.** The results are expressed in mS.cm^{-1}.

Figure 8. Conductivity meter.

2.2.2.4 pH, free and lactonic acidity

Free acidity, pH, lactone acidity and total acidity measurements were performed according to IHC (the International Honey Commission (Bogdanov, 2002). Briefly, 5 g of honey were dissolved in 25 mL of deionized water, which were pipetted into a beaker where the pH electrode was immersed and the initial pH value was read. This solution was titrated with 0.119 M sodium hydroxide, NaOH. The volume spent to reach the equivalence point (pH=7) was recorded, and the obtained value allowed the determination of the free acidity. Immediately, an additional volume of 0.119 M NaOH to complete 10 mL was added, and without delay, back-titrated with 0.022 M sulfuric acid, H_2SO_4, to pH 7, and so obtaining the lactonic acidity. Total acidity results were obtained by adding free and lactone acidities. The results are expressed in meq.kg^{-1} of honey. The titrations were done using a HI902 potentiometer titrator (Hanna instruments, pH 211 microprocessor pH meters), **Fig. 9.**

Figure 9. Potenciometer titrator.

2.2.2.5. Proline

The proline content in honey samples was measured weighting 0.5 g of honey into a volumetric flask and dissolved in about 10 mL deionize distilled water. Then, 0.5 mL of diluted honey solution was placed in a test tube, 0.5 mL of deionized water (blank test) into a second tube, and 0.5 mL of proline standard (0.032 M) solution into a third tube. After, 0.5 mL of deionized water, 1 mL of formic acid (98%) and 1 mL of ninhydrin solution (3%) were added to each tube. The tubes were capped carefully and shaken vigorously. After, they were placed in ultrasound for 15 min followed into a water bath at 100°C for 15 min and then transferred to a water bath at 70°C for 10 min. Finally, 5 mL of 2-propanol (50%) was added and the tubes were capped immediately. After the tubes were allowed to cool down for 45 min, the absorbance was measured at 510 nm using a UV/Vis spectrophotometer (Specord 200 spectrophotometer, Analytikjena, Jena, Germany). Proline content of honey, in mg/kg, was calculated according to following equation:

Equation 1. Proline= ((Abs sample)/(Abs standard)) × ((Weight standard)/(Weight sample)) ×80

2.2.2.6 5-Hydroxymethylfurfural (5-HMF)

For the 5-HMF quantification, 5 g of honey were weighted and dissolved in 25 mL of deionized water and transferred quantitatively into a 50 mL volumetric flask. Then, 0.5 mL Carrez solution I and Carrez solution II were added, completing the final volume of 50 mL with deionized water. The solution was filtered through Waltman paper, rejecting the first 10 mL of filtrate. The filtrate was pipetted into each of two test tubes. To one of the tubes, 5 mL of distilled water (sample solution) was added and to the other 5 mL of sodium bisulphite solution, $NaHSO_3$, 0.2% (reference solution). The absorbance was measured at 284 nm and 336 nm in a spectrophotometer (Specord 200 spectrophotometer, Analytikjena, Jena, Germany), and the 5-HMF value was expressed in mg/kg and determined according to the following equation:

Equation 2. HMF= $(Abs_{284}-Abs_{336})$ ×149.7× (5/ (sample weight))

2.2.2.7. Diastase activity

For the measurement of the diastase index the Phadebas method (Bogdanov, 2002) was used. For that, 0.1g of honey was weighed, quantitatively transferred to a 10 mL volumetric flask and completed the volume with 0.1M acetate buffer (pH=5.2). After preparing the solution, 5 mL were added to a test tube (sample) and placed in a water bath of 40 °C, together with a second tube (reference solution) containing instead 5 mL of 0.1 M acetate buffer solution (pH 5.2). Then, the Phadebas tablets were added to the two tubes, which, after mixing, were kept at 40ºC for 15 minutes, After this time, The absorbance was measured at 620 nm using a spectrophotometer (Specord 200 spectrophotometer, Analytikjena, Jena, Germany). The result was presented as diastase index (DN), in Schade units, corresponding to a unit of diastase and the enzymatic activity of 1 g of honey capable of hydrolyzing 0.01 g of starch at 40ºC in one hour. The formulas used to calculate the DN value were as follows:

Equation 4. $DN= 28.2*Abs_{620} + 2.64$, if $DN > 8$

Equation 3. $DN= 35.2*Abs_{620} – 0.46$ if $DN<8$

2.2.3. Sugar analysis

For sugars analysis, about 2.5 g of honey was mixed with 20 mL of deionized water and 12.5 mL of methanol and 1 mL of xylose (internal standard, 30mg/mL) and the resulting solution was diluted to a final volume of 50 mL with deionized water. Afterwards, the sample was passed through a 0.2 μm filter and analyzed by high performance liquid chromatography coupled to a refractive index detector (HPLC-RI). HPLC-RI was performed on an integrated Knauer system with pump (Smartline 1000), a degasser (Smartline 5000), a UV detector (Knauer Smartline 2300) and an autosampler (Jasco, AS-2057). Data acquisition and remote control of the HPLC system was done by Clarity Chrom software (Knauer, Berlin, Germany). The chromatographic separation was achieved using a Eurospher 100-5 NH2 (4.6 × 250 mm, 5 mm, Knauer) column at 30 ˚C. The mobile phase was composed by acetonitrile/water, 80:20 (v/v) at a flow rate of 1.3 mL/min. The identification of sugars was obtained by comparison of retention time between samples and standards. Quantification was achieved using calibration curves of Table 4.

Table 4. Calibration curve for sugars.

Sugars	**Calibration curve**	R^2
Fructose	y = 82.665x + 75.806	0.9900
Glucose	y = 60.65x + 154.24	0.9903
Sucrose	y = 66.558x + 58.629	0.9907
Trehalose	y = 86.976x + 0.7149	0.9900
Turanose	y = 129.76x - 10.213	0.9983
Maltulose	y = 71.156x + 1.4642	0.9976
Maltose	y = 65.454x - 2.224	0.9996
Melezitose	y = 58.269x + 18.123	0.9903
Raffinose	y =53.431x + 12.721	0.9941
Melibiose	y = 32.126x +6.8297	0.9903
Kojibiose	y= 95.399x + 1.8282	0.9981
Erlose	y = 60.749x + 9.616	0.9913
Isomaltose	y = 57.638x - 1.958	0.9968

2.2.4. Minerals

For the test of 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) cadmium (Cd) and lead (Pb) was done using atomic absorption spectrophotometry thought graphite chamber via a Perkin Elmer PinAAcle 900 spectrophotometer.

2.2.4.1. Sample Digestion

A sample of 1g was weighted 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 200ºC. The continuous of these conditions were sustained for another 15 minutes. After that, it was cooled and quantitatively transferred into a volumetric flask of 50 mL.

2.2.4.2. Sample Analysis

The quantification of the different minerals required a previous preparation for specific solutions and standards according to the following procedures:

2.2.4.2.1. 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 5), for presenting the calibration standards as follows.

Table 5. The calibration standards used in the spectrophotometer for the determination of potassium and sodium.

Standard	V(sample)/mL	V_f/mL
P1/4	0.25	50
P1/2	0.25	
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 5 mL CsCl buffer in a final volume of 50 mL). For the analysis of potassium, a digested supplement solution of 5 mL, buffer solution of 1 mL and 4 mL of deionized water were added. For the analysis of sodium, 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.

2.2.4.2.2. 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 1L 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 6).

Table 6. The calibration standards used in the spectrophotometer for the determination 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	0.25	0.25	
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	

The standards applied in the spectrophotometer calibration to determine the content of Ca are 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 were done from the thirty-three-fold dilution of these standards (1.50mL solution of each standard and 5 mL of solution La to a final volume of 50mL). 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 quantification, a digested solution of 10 mL and lanthanum solution of 1 ml was added. To determine the Ca and Mg the recommended condition according to the equipment was followed.

2.2.4.2.3. Iron

Matrix modifier: diluted 1.7mL 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 50mL 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. 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.

Table 7. The calibration standards used in the spectrophotometer for the determination 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

2.2.4.2.4. 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 10mL 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, according to Table 8.

For the sample analysis, 20µL of the sample was pipetted with a 5 µL of matrix modifier. The instrumental conditions for the analysis of lead were used.

Table 8. The calibration standards used in the spectrophotometer for the determination of lead

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

2.2.4.2.5. 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 final volume of 10 mL with deionized water. Two standards for manganese were done, one diluting 0.50 mL of standard solution (1000 ppm) to a final volume of 50 mL of deionized water and another by the dilution of 0.20 mL of the previous solution to a final volume of 50 mL of deionized water (standard 2). For copper, a modified matrix resulted from the dilution of 1.0 mL of palladium solution, Pd, 10 g/L, and 0.1mL 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 0.50mL of the previous solution to a final volume of 50mL (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 the second, by 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, according to (Table 9). To analyze all the samples, 20 µL of sample and 5 µL of the modified matrix were pipetted with the application of the recommended instrumental conditions for each one of the analyses.

Table 9. The calibration standards used in the spectrophotometer for the determination of manganese, copper, and cadmium.

Standard	V(P_2)/mL	V(matrix)/mL	V (H_2O)/µL
P1/4	5	5	15
P1/2	10	5	10
P1	15	5	5
P2	20	5	0

2.2.3. Nutritional parameters

2.2.3. Ash content

The ash content was determined, in triplicate, indirectly through its calculation, according to the defined in the literature (Sancho el al, 1992) using the following formula:

Equation 6. % Ash= (conductivity/1000)-0.14/1.74

2.2.3.2. Protein content

For the determination of the protein content,1 g of honey sample was weighed into a 250 mL test tube, 2 catalyst tablets (9% $CuSO_4$) and 15 mL concentrated sulphuric acid (98%) were added. The blank was prepared with all chemicals and without sample; 5mL of distilled water was used instead of sample. Samples were digested for 70 minutes at 400 °C. Before distillation and titration, the test tubes were let to cool down to 50-60 °C, then 25 mL of distilled water was added to the mixture. The samples were distilled according to the following parameters; HCl (0.2M) as titrant solution, NaOH (32 %): 50 mL, H_3BO_3 (4 % with indicators): 30 mL. For the conversion of nitrogen content into total protein, a factor of 6.25 was used, expressing the results in g/100 g of honey.

2.2.3.3. Total Carbohydrates:

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

Equation 7. % Total carbohydrates = (100% -Moisture)- (% ash+%protein+%lipids)

2.2.3.4. Energy

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

Equation 8. Energy value (kcal/100g) =4× (%protein+%carbohydrates) +9× (%lipid)

2.3. Spectrophotometric analysis of the phenolic compounds

2.3.1. Total phenolic content

For the total phenolic content, 1 g of honey sample was diluted with 10 mL methanol. Then, an aliquot of 0.5 mL of the solution was mixed with 0.5 mL of the Folin–Ciocalteu reagent and 1 mL of a 20% sodium carbonate solution. Deionized water was added to a final volume of 5 mL. Following the incubation of 1 hour, the absorbance of the reaction mixture was measured at 760 nm using a spectrophotometer (Analytik Jena, Jena, Germany). Gallic acid was used (0.005–0.15 mg/mL) as the standard solution and the values expresses as milligram of gallic acid equivalent per g of sample (mg GAE/g).

2.3.2Total flavonoid content

Total flavonoid content was determined using the aluminum chloride ($AlCl_3$) colorimetric method (Alothman, Bhat and Karim, 2009). The Al^{3+} cations form stable complexes with free hydroxyl groups of flavonoids this causes the extension of the conjugated system a shift of the absorption maxima to a longer wavelength region, allowing quantification in a spectrophotometer at 415 nm (Buriol et al, 2009). The honey solutions were prepared at the concentration of 0.1 g/mL. One milliliter of the stock solution was diluted with 10 mL of methanol and then mixed with 0.5 mL of a 5% aluminum chloride solution (2% aluminum chloride in 5% acetic acid/methanol) and the volume adjusted to 5 mL with 5% acetic acid/methanol. Following incubation for 30 min, in the dark at room temperature, the absorbance was measured at 415 nm using a spectrophotometer (Analytik Jena, Jena, Germany). Quercetin was used to calculate the standard curve (0.0016-0.5 mg/mL) and the results were expressed as mg of quercetin equivalents per g of sample (mg QE/g).

2.4. Phenolic compounds

2.4.1. Extraction

Extraction of polyphenols from honey is generally accomplished using either liquid–liquid extraction (LLE) or solid-phase extraction (SPE). In both methods, the first step is to separate the sugars, which make up the great majority of the honey mass. In our case SPE followed by LLE were used. For that, 25 g honeys were mixed with 125 mL of acidified water (pH 2 with HCl) until completely fluid and filtered through cotton to remove solid particles. The extraction was conducted in a glass column (25 cm x 2 cm) fitted with an

opening valve and a fritted glass support. The column was packed with 25 g of Amberlite®XAD®-2 in methanol, Figure 10. The phenolic compounds remained in the column, while sugars and other polar compounds eluted with the water. After passing the honey solution, the column was washed with the acidified water and then with deionized water. Then, the phenolic fraction was eluted with methanol and the solution evaporated under reduced pressure at 40 °C. The residue was re-dissolved in 5 mL of water and extracted with diethyl ether (5 mL x 3). The resulting extracts were combined, concentrated under reduced pressure and re-dissolved in methanol for further LC-MS analysis.

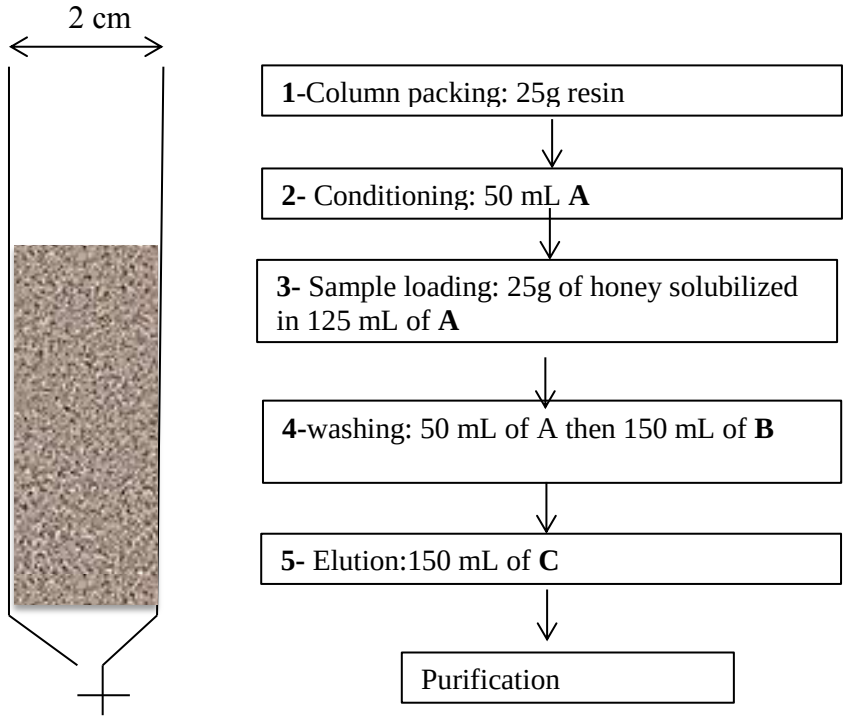


Figure 10. Phenolic compounds extraction stages; acidified water (pH 2) (A), deionized water (B), and methanol (C).

2.4.2. Phenolic profile by UPLC / DAD / ESI-MS^n

The phenolic compounds characterization was made through UPLC / DAD / ESI-MS^n performed on a Dionex UPLC 3000 equipment (Thermo Scientific, USA) (Figure 11) equipped with a photodiode detector and coupled to a mass detector. The chromatographic system consisted of a quaternary pump, an automatic sampler maintained at 5°C, a degasser, a photodiode array detector and an automatic thermostatic column compartment. The

chromatographic separation was performed on a U-VDSpher PUR C18-E 100 mm x 2.0 mm i.d. column, with particle size of 1.8 µm (VDS Optilab, Germany), maintained at 30ºC. 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 with 0.22 µm porosity. A linear gradient with a flow rate of 0.3 mL/min was used: 0.0-1.0 min 20% B ; 1.0-11.1 min 20-95% (B); 95% (B) for 2 min; 13.1-13.3 min 95-20% (B); and 20% (B) for 5 min. The injection volume was 3 µl. Spectral data for all peaks were detected in the range 190-600 nm. Each sample was filtered through a 0.2 µm nylon membrane (Whatman). Mass analysis was performed on a LTQ XL mass spectrometer (Thermo Scientific, CA, USA), in negative mode, equipped with an ESI electro spray ionization source: spray voltage, 5 kV; capillary voltage, -20V; capillary tube voltage, -65V; capillary temperature, 325 ° C; gas flow and auxiliary gas (N_2), 50 and 10 (arbitrary units), respectively. Mass spectra were acquired in the mass range 100-1000 *m/z.* Mass spectra were acquired by full range acquisition covering 100–1000 *m/z*. For the fragmentation study, a data dependent scan was performed by deploying collision-induced dissociation (CID). The normalized collision energy of CID cell was set at 35 (arbitrary units). Data acquisition was performed using the Xcalibur® software (Thermo Scientific, CA, USA). Quantification was performed with standard substance calibration curves for p-hydroxybenzoic acid ($y = 4x10^6x-134082$; $R^2 = 0.9988$), caffeic acid ($y = 3x10^6x-12895$; $R^2 = 0.9998$), *p*-coumaric acid ($y = 4x10^6x-13435$; $R^2 = 0,9999$), 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 behavior, UV spectra and mass profile with that obtained for commercial standards and also with the information obtained in the literature when these were not available.

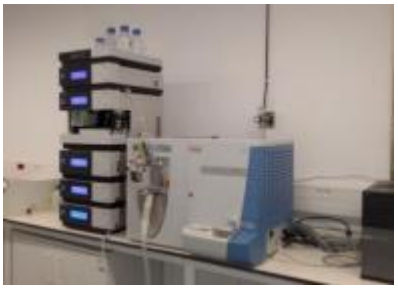


Figure 11. UPLC / DAD / ESI-MS^n equipment

2.5. Antioxidant activity

2.5.1. $DPPH^{\cdot}$ assay

The antiradical activity of the honey samples was estimated using the 2, 2-diphenyl-1-picrylhydrazyl hydrate radical ($DPPH^{\cdot}$). For that, 1g of honey was dissolved in 10 mL of methanol 20 %. Using a microplate, sample solution, methanol and DPPH were added as described in the Table10. The absorbance was read at 515 nm using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc.). Different sample concentrations were used in order to obtain antiradical curves for calculating the EC_{50} values, according to the following equation:

$$\%\text{Inhibition} = [(\text{Abs DPPH} - \text{Abs sample})/\text{Abs DPPH.}] \times 100$$

For comparison a standard solution of gallic acid was used with an average value of EC_{50} of 1.22 mg/mL.

Table 10. DPPH assay steps.

Well	Volume (µL)
A	10 µL Sample solution +140 µL methanol+150 µL DPPH *3
B	20 µL Sample solution+130 µL methanol+150 µL DPPH *3
C	40 µL Sample solution +110 µL methanol+150 µL DPPH *3
D	60 µL Sample solution + 90 µL methanol+150ul DPPH *3
E	80 µL Sample solution +70 µL methanol+150 µL DPPH *3
F	100 µL Sample solution +50 µL methanol+150 µL DPPH *3
G	Blanc (150 µL methanol+150 µL DPPH) *3

2.5.2. Reducing power activity

The reducing power of honey samples was measured by the ferricyanide prussian blue assay. Through this assay the capacity to convert Fe^{3+} into Fe^{2+} is determined, measuring the absorbance at 700 nm (Ferreira et al., 2009). A volume of 0.125 mL of honey sample (0.1g/mL) was mixed with 1.125 mL of phosphate buffer (0.2 mol/L, pH 6.6) and 1.250 mL of 1% potassium ferricyanide. The mixture was incubated in a water bath at 50 ºC for 20 min at 100 rpm. Then, 1.250 mL of 10% trichloroacetic acid was added to the mixture and centrifuged at 3000 rpm (Centurion K2R series) for 10 min. The supernatant (1.250 mL) was mixed with deionized water (1.250 mL) and $FeCl_3$ (0.250 mL, 0.1%), and the absorbance was measured at 700 nm. Gallic acid was used as standard (0.001-0.01 mg/mL), and results were expressed as milligram of gallic acid equivalent per 100 g dry of sample (mg GAE/100 g).

2.6. Cytotoxic potential

The following human tumor cell lines were used: AGS (gastric adenocarcinoma), CaCo (colorectal adenocarcinoma), MCF-7 (breast adenocarcinoma), and NCI-H460 (lung carcinoma). A non-tumor cell line, Vero (African green monkey kidney), was also tested. All of them were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 mg/mL), with the exception of Vero, that wasmaintained in DMEM medium supplemented with fetal bovine serum (10%), glutamine and antibiotics. The culture flasks were incubated in an incubator at 37ºC and with 5% CO_2, under a humid atmosphere. The cells were used only when they had 70 to 80% confluence. A known mass of each of the extracts (8 mg) was dissolved in H_2O (1 mL), in order to obtain the stock solutions with a concentration of 8 mg/mL. From which successive dilutions were made, obtaining the concentrations to be tested (0.125 - 8 mg/mL). Each of the extract concentrations (10 µL) were incubated with the cell suspension (190 µL) of the cell lines tested in 96-well microplates for 72 hours. The microplates were incubated at 37ºC and with 5% CO_2, in a humid atmosphere, after checking the adherence of the cells. All cell lines are tested at a concentration of 10,000 cells/well, except for Vero in which a density of 19,000 cells/well was used. After the incubation period, the cells were corrected: TCA (10% w/v; 100 µL) was previously cooled and plates were incubated for 1 hour at 4ºC, washed with water and, after drying, a SRB solution (0.057%, m/v; 100 µL) was added, left to stand at room temperature for 30 minutes. To remove non-adhered SRB, plates were washed three times with a solution of acetic acid (1% v/v) and placed to dry. Finally, an

adhered SRB was solubilized with Tris (10 mM, 200 μL) and the absorbance at a wavelength of 540 nm was read in the Biotek ELX800 microplate reader. The results are expressed in terms of the concentration of extract with the ability to inhibit cell growth by 50% - GI_{50}. As a positive control ellipticin was used.

2.7. Anti-inflammatory activity

The extracts were dissolved in H_2O in order to obtain a final concentration of 8 mg/mL. From which successive dilutions were carried out, obtaining the concentrations to be tested (0.125 - 8 mg/mL). The RAW 264.7 mouse macrophage cell line, obtained from DMSMZ - Leibniz - Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, was grown in DMEM medium, supplemented with heat-inactivated (SFB) fetal serum (10%), glutamine and antibiotics, and kept in an incubator at 37ºC, with 5% CO_2 and under a humid atmosphere. Cells were detached with a cell scraper. An aliquot of the cell suspension of macrophages (300 μL) with a cell density of 5 x 105 cells/mL and with a proportion of dead cells below 5% according to the Trypan blue exclusion test, was placed in each well. The microplate was incubated for 24 hours in the incubator with the conditions previously indicated in order to allow an adequate adherence and multiplication of the cells. After that period, the cells were treated 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. Stimulation was performed with the addition of 30 μL of the liposaccharide solution - LPS (1 mL/mL) and incubated for an additional 24 hours. Dexamethasone (50 mM) was used as a positive control and samples in the absence of LPS were used as a negative control. Quantification of nitric oxide was performed 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) prepared in a 96-well plate. The nitric oxide produced was determined 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 calculated through the graphical representation of the percentage of inhibition of nitric oxide production versus the sample concentration and expressed in relation to the concentration of each of the extracts that causes the 50% inhibition of nitric oxide production - IC_{50}.

2.8. Detection of antibiotics residues

The Charm II test uses an antibody (as a binder) with specific receptor sites that bind all of the target antibiotics. The binder is added to a sample extract followed by addition of an exact amount of H^3 or C^{14} labeled antibiotics (as a tracer). Firstly, the unknown antibiotics in the sample combines with the receptor sites and then the radio labeled antibiotics occupy the remaining sites. After this reaction is complete, a scintillation fluid is added and the concentration of either H^3 or C^{14} associated with the binder is measured in counts per minute (CPM) using the Charm II system (Charm LSC 7600, Charm Science Inc., USA), Figure 12. Samples with high counts are considered negative (tracer antibiotics are largely bound to the binder) and samples with low counts are considered positive (tracer antibiotics are largely free in solution). Thus, the greater the counts, the lower the original antibiotic concentration in the samples (Kwon et al, 2011).

Figure 12. Charm LSC 7600

The detection of tetracycline and sulphonamide followed the operator' s manuals attached to the device.

2.8.1.Tetracycline residues

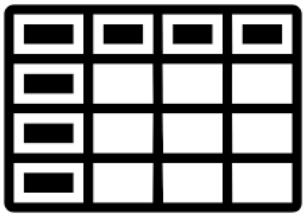
The charm II tetracycline test for honey is a rapid immunoreceptor assay for the detection of tetracyclines in honey at 10 to 20 ng/g or parts per billion (ppb). For that, 5 g of sample were weighted into a centrifuge tube and mixed vigorously with 20 mL of distilled water. In an empty test tube the green tablet was added with 300 µL of water and mixed 10 seconds to break the tablet. Then, 0.5 mL of the sample or control solution was added and mixed immediately. After incubation (45 C° for 5 min), the orange tablet was added, and the solution was mixed immediately. After a second incubation (45 C° for 5 min), the black tablet was added and the solution was mixed immediately and centrifuged for 5 min at 5000 rpm. Meanwhile, new test tube was labeled and the white tablet with 300 µL of water is added. The supernatant from the first tube were poured into the new labeled test tube and

mixed immediately. After incubation (45 C° for 5 min), the solutions were centrifuged for 5 min at 5000 rpm. Finally, the supernatant was removed and additional 300 µL of water was added to the tube and mixed thoroughly to break up the pellet. After, 3 mL of scintillation fluid was added into the tube, which was shaken until the mixture has with a uniform cloudy appearance. CPM (count per minute) were read on [^{3}H] channel by using (Charm LSC 7600, Charm Science Inc., USA).

2.8.2. Sulphonamide residues

The sensitivity of Charm II sulfa drug test for honey is set to detect sulphonamide at 10 ng/g or ppb. 5 g of sample were weighted and mixed vigorously with 20 mL of distilled water. An extraction procedure is required to free sulfa drugs bounded to the sugars in honey and to eliminate interference from sulfa drug analogs, filtrating the solution followed by SPE extraction in C18 cartridge. After extraction, a white tablet was added to an empty test tube than mixed well with 300 µL of water, and followed by the addition of 5 mL of extracted solution. A pink tablet was then added to the tube and mixed immediately. After incubation (85C° for 3 min) the solution was centrifuged for 3 min at 3400 rpm. Supernatants were poured off, fat rings were removed, and test tubes were wiped with swabs to avoid disturbing the pellet. Finally, 300 µL of water were added into the tube and mixed thoroughly to break up the pellet. 3 mL of scintillation fluid was added into each tube and shaken until the mixture has a uniform cloudy appearance. CPM (count per minute) were read on [^{3}H] channel by using (Charm LSC 7600, Charm Science Inc., USA).





Chapter III- Results and discussion

3. Results and discussion

3.1. Melissopalynological analysis

Pollen analysis of honey, or mellissopalynology, is of great importance for quality control. Honey always includes numerous pollen grains (mainly from the plant species foraged by honey bees) and honeydew elements (like wax tubes, algae and fungal spores) that altogether provide a good fingerprint of the environment where the honey comes from. Pollen analysis can therefore be useful to determine and control the geographical and botanical origin of honeys even if sensory and physicochemical analyses are also needed for a correct diagnosis of botanical origin. Moreover, pollen analysis provides some important information about honey extraction and filtration, fermentation (Russmann, 1998), adulteration types (Kerkvliet et al., 1995) and hygienic aspects such as contamination with mineral dust, soot, or starch grains (Louveaux et al., 1978).

Multifloral honeys have in their composition percentages of pollen from various floral species, while monofloral honeys are characterized by honeys obtained mainly from a single plant species (≥ 45% of the same pollen type), although this value may vary according to the plant's ability to produce pollen. (Estevinho et al, 2012).

Honey samples EC1 and EC2 from Sidi Belabes region had *Cytisus striatus* type as the dominant pollen and accompanying pollen respectively, in fact EC1 and EC2 which were labeled as eucalyptus and showed low percentages of eucalyptus pollen, Table 11. *Cytisus striatus* was also the dominant pollen type of MF1 and MF2, which represented a minimum of 83.3% to a maximum of 83.8% of the total pollen content, Table 11. Honey samples EF, from El Bayadh region, had *Cytisus striatus* type as the dominant pollen for the three samples, with an average of 79.8%, instead of *Euphorbia* pollen which were indicated in the commercial label. Regarding to this pollen type, it can be either the *Cytisus striatus* type or another within the same genus, like *C. arboreus*, *C. triflorus, C. purgains*, *C. pinifolius, C. fontanesii, C. monspessulanus, C. arboreus*, which were previously reported as present in the areas of the apiaries (Quezel and Santra, 1962). Honey samples J1, J2 and J3 from Ain Safra region, contained pollen grains from *Ziziphus* sp. in percentages ranging between 38.4% and 40.5%. Thus, the pollen of this species is nearly dominant, suggesting that this plant is the main source of pollen in these honeys, Table 11**.** *Cytisus striatus* pollen type was present in a total of 10 samples and it considered dominant in 7 of them.

Table 11. Pollen characteristics of the analyzed honey samples.

Sample	Floral origin on the label	D	A	I
EC1	*Eucalyptus* sp.	*Cytisus striatus* type *(47.3%)*	*Brassica napus* type *(18.4%)*	*Eucalyptus sp. (5.5%); Sesamoides sp. (5.9%); Rhamnus alaternus (13.9%)*
EC2	*Eucalyptus* sp.	-	*Cytisus striatus* type *(39.3%); Brassica napus* type *(12.6%); Rhamnus alaternus (26.8%);*	*Eucalyptus sp. (5.1%); Sesamoides sp. (9.1%)*
MF1	Multifloral	*Cytisus striatus* type *(83.3%)*	-	*Calina racemosa (5.0%)*
MF2	Multifloral	*Cytisus striatus* type *(83.8%)*	-	*Calina racemosa (5.0%)*
EF1	*Euphorbia* sp.	*Cytisus striatus* type *(82.3%)*	-	*Centaurea sp. (4.5%); Brassica napus* type *(6.3%)*
EF2	*Euphorbia* sp.	*Cytisus striatus* type *(76.9%)*	-	*Centaurea sp. (5.6%); Brassica napus* type *(7.9%)*
EF3	*Euphorbia* sp.	*Cytisus striatus* type *(80.2%)*	-	*Centaurea sp. (6.4%); Brassica napus* type *(5.8%)*
J1	*Ziziphus* sp.	-	*Ziziphus* sp. *(39.5%); Eucalyptus sp. (16.2%); Cytisus striatus* type *(25.8%)*	*Echium sp. (4.9%)*
J2	*Ziziphus* sp.	-	*Ziziphus* sp. *(40.5%); Cytisus striatus* type *(28.6%)*	*Eucalyptus sp. (12.6%); Echium sp. (3.9%)*
J3	*Ziziphus* sp.	-	*Ziziphus* sp. *(38.4%); Cytisus striatus* type *(26.0%)*	*Eucalyptus sp. (15.1%); Echium sp. (3.4%)*

D: Dominant pollen (≥ 45%); A: Accompanying pollen (15% - 45%); I: Important pollen (3% - 15%).

3.2. Physicochemical parameters

3.2.1. Color

The color of honey is closely linked to its botanical origin and is an important parameter for evaluating honey quality. Honey color is generally related to its sensory properties such as flavor and odor and can give information on its floral source, mineral content, and storage conditions. The colorimetric analysis of the honey was performed using the Pfund scale by the direct reading in the colorimeter. The color ranged from (extra light

amber until amber), Table 12, EC1 honey presented the darker color and EF1 and EF3 showed the clearest color. Honey samples EC1 and EC2 showed amber color, with values of 89mm and 88 mm Pfund, respectively, while MF1 and MF2 presented a light amber color, 79 and 77 mm Pfund, respectively. All these results are in accordance with the last study on multifloral honey samples of Morocco (Chakir et al., 2016), and were similar to those obtained by (Homrani et al., 2020) on Algerian honeys. J1, J2 and J3 honey samples showed extra light amber color in which values ranged between 51 and 55 mm Pfund. The results obtained were very near to those obtained on *Citrus* and *Retama* honeys from Algerian semi-arid region (Homrani et al., 2020). The three EF samples (EF1, EF2, EF3, and EF4) gave the same color, extra light amber, which were in accordance with those obtained previously (Homrani et al., 2020).

Table 12. Physicochemical parameters: color, moisture content and conductivity.

Samples	Color (mm Pfund)	Moisture content (%)	Conductivity ($\mu S.cm^{-1}$)
EC1	89 ± 0 (Amber)	18 ± 0	410 ± 0.02
EC2	88 ± 0 (Amber)	18 ± 0	410 ± 0.02
MF1	79 ± 0 (Light Amber)	15 ± 0	270 ± 0.01
MF2	77 ± 0 (Light Amber)	15 ±0	300 ± 0.06
J1	55 ± 0 (Extra Light Amber)	15 ± 0	370 ± 0.01
J2	55 ± 0 (Extra Light Amber)	15 ± 0	370 ± 0.01
J3	55 ± 0 (Extra Light Amber)	15 ± 0	370 ± 0.01
EF1	51 ± 0 (Extra Light Amber)	14 ± 0	360 ± 0.01
EF2	52 ± 0 (Extra Light Amber)	14 ± 0	360 ± 0.01
EF3	51 ± 0 (Extra Light Amber)	14 ± 0	360 ± 0.00

3.2.2. Moisture content

Moisture is a parameter related to the maturity degree of honey and temperature. In the present study, the moisture values varied between 14% (EF1) and 18% (EC1), which

were within the limit of 20% stablished by the European Community regulations (The Council of the European Union, 2002), Table 12. EC honey samples have the highest water content around 18 %, which were in accordance with the values found in *Hedysarum coronarium* and *Eucalyptus* honeys from Bejaia region *(*Ouchemoukh et al., 2006) and higher than those obtained in Morocco (Chakir et al., 2016). The moisture content of MF samples were in the order of 15%, which were similar to results previously report in *Capparis* and multifloral honeys from Bejaia region (Ouchemoukh et al., 2006). However, when comparing the results with Morocco multifloral honey samples (Chakir et al., 2016), the latest presented higher water content (17.8%) comparing to our samples. In another hand, the water content of *Ziziphus* samples, around 15%, are directly in line with the results previously reported by Algerian *Ziziphus* honeys (Latifa et al, 2013). The water content of EF samples was 14%, consistent with what has been previously found in Algerian *Euphorbia* honey (Latifa et al, 2013) harvested in the semi-arid region of Algeria.

3.2.3. Electrical conductivity

Electrical conductivity (EC) is closely related to the concentration of mineral and organic acids and shows great variability according to the floral origin. The sample with electrical conductivity values higher than 800 $\mu S.cm^{-1}$ are considered honeydew honeys. While those that express values below 800 $\mu S.cm^{-1}$ are considered nectar honey or mixtures of different nectars (Bogdanov, 2011). All analyzed honeys presented values less than 800 $\mu S.cm^{-1}$, ranging between 270 and 410 $\mu s.cm^{-1}$, being considered nectar honeys. EC samples showed the higher values among our honey samples 410 $\mu S.cm^{-1}$, Table 12. Those values were within the values found in Algerian honeys (between 410 and 630 $\mu S.cm^{-1}$) (Djamila B, Paul S, 2010) and less than those found in Moroccan honeys (768.78 $\mu S.cm^{-1}$) reported by (Chakir et al., 2016). MF honey samples showed values between 270 (MF1) and 300 (MF2) μScm^{-1}. Hadia et al., (2017) found similar results (100 and 370.5 $\mu S.cm^{-1}$) in multifloral honey harvested in the east of Algeria. The EC average value for Z honey was 370 $\mu S.cm^{-1}$.The values are lower than those given for *Z. lotus* of Morocco (673.42 $\mu s.cm^{-1}$) previously reported (Chakir et al., 2016) and near to those given for *Z. lotus* of Algeria (478.25 $\mu S.cm^{-1}$) reported by (Latifa, 2013).

The EC average of EF honeys was 360 $\mu s.cm^{-1}$. Our results are similar to the findings previously reported by (Latifa, 2013) and lower than those obtained by (Chakir et al., 2016) on Moroccan *Euphorbia* samples.

3.2.4. pH, free, lactonic and total acidity

Ibrahim Khalil et al., (2012), indicated that honey is naturally acidic regardless of its geographical origin, which may be due to the presence of organic acids that contribute to its flavor and stability against microbial spoilage. Nectar honeys usually have low pH values (3.3 to 4.6). Honeydew honeys have, due to their higher buffering salt content, higher average pH values (Bogdanov, 1995).

The results obtained in this study show that all the analyzed honeys are acidic and within the standard limit (Codex Food, 2001), ranging from 4.2 to 5.1, Table 13. EF samples are the most acidic with (pH=4.37), followed by MF samples (pH=4.44), the lower acidity was detected in the honey samples from Ziziphus (4.93 in average), while EC honeys showed values between 4.4 and 4.9. The pH of samples from Algerian semi-arid regions (Media, Djelfa, El aghouat) was 3.61 to 4.16 and 3.49 to 4.44 (Zerrouk et al. 2011 and Zerrouk and Bahloul. 2020), respectively.

The pH values of nectar honeys vary between 3.5 and 4.5 and honeydew honeys have higher average pH values between 4.5 and 5.5 (Gonnet, 1986). We could say that the honeys studied are of the nectar type.

The acidity of honey is mainly due to gluconic acid (Vaillani and Mary, 1988), which results from the oxidation of glucose by the enzyme glucosidase from the bee (Russo, 1997). Rogulja et al., (2009) suggested that honeys with lighter color are characterized by a low content in organic acids, while darker honeys generally appear richer in acidity. Free acidity gives information about the origin of honey and influencing its stability (Pataca et al., 2007). The values obtained for free acidity in our study were between 11and 18.3 $meqkg^{-1}$ and between 5.8 and 43.9 $meqkg^{-1}$ at the two equivalence points (pH=7 and pH=8.3), respectively. All the honeys analyzed are within the required standard of the Codex Alimentarius (1998), which is 50 $meqkg^{-1}$, indicating an absence of unwanted fermentation in our samples. The results are also in accordance with previous work carried out on Algerian honeys. Zerrouk et al. (2011) found values ranging between 14.91 and 40.33 $meqkg^{-1}$, while Makhloufi (2010) report values between 17.97– 49.1 $meqkg^{-1}$.

Lactonic acidity is considered as an acidity reserve when honey becomes alkaline (Gonnet, 1982). The values obtained in our lactonic acidity study are between 5.7 and 36.1 $meqkg^{-1}$. Total acidity is the sum of free and lactonic acidity, it is a quality criterion, and our results showed values between 20.1 and 64.7$meqkg^{-1}$, and these results indicate that all the honeys analyzed comply with the standard required by the codex. Our results are higher than

those given by Hadia (2020) ranged between 17.12 to 34.29 meqkg^{-1} on north of Algeria and are similar to those given on Morocco honeys from semi-arid regions reported by (Chakir et al 2016) ranged between 11.94–58.03 meqkg^{-1}

Table 13. pH and acidity of the honey samples analyzed.

Sample	pH	Free acidity pH=7 (meqkg^{-1})	Free acidity pH=8,3 (meqkg^{-1})	Lactonic (meqkg^{-1})	Total (meqkg^{-1})
EC1	4.4	18.3 ± 0.3	12.2 ± 1.1	17.5 ± 0.6	24.7
EC2	4.9	18.1 ± 0,1	13.0 ± 2.0	15.5 ± 0.7	22.9
MF1	4.2	18.3 ± 0.0	12.7 ± 1.5	17.2 ± 0.5	24.3
MF2	4.6	18.3 ± 0.1	21.9 ± 0.5	31.5 ± 0.4	46.1
J1	4.9	11.5 ± 1.1	22.6 ± 1.6	28.5 ± 0.2	43.2
J2	5.1	12.1 ± 0.8	17.6 ± 0.4	22.8 ± 0.6	35.6
J3	4.8	11.0 ± 0.8	21.7 ± 0.4	35.8 ± 0.5	50.6
EF1	4.4	17.2 ± 0.1	43.9 ± 0.4	27.1 ± 0.1	58.3
EF2	4.3	17.3 ± 0.0	41.6 ± 1.8	36.1 ± 0.3	64.7
EF3	4.4	17.2 ± 0.1	5.8 ± 0.1	5.7 ± 0.1	20.1

3.2.5. Proline

Proline is an important amino acid that originates mostly from the salivary secretions of *Apis mellifera* during the conversion of nectar into honey (Bergner and al, 1972). Proline content is an indication of honey ripeness and, in some cases, sugar adulteration. Some authors have reported that high concentrations of proline are also typical for honeydew honeys. Indirectly, proline levels also reflect botanical origin (*Cotte and al, 2004*). Previous studies found that the proline content of honey was associated with its floral and geographical origin (Kečkeš et al, 2013). The proline concentration should be above 0.180 mg/g, lesser values could mean that the honey is possibly corrupted by sugar addition (Bogdanov. 2002).

The studied honey samples have good proline levels (2.2 – 4.7 mg/g), higher than the minimum limit proposed by Bogdanov et al. (2002), indicating the maturity of the honeys and absence of adulteration. The proline content in EC ranged between two values 3.4 (EC1) and 3.6 (EC2) mg/g, two times higher than those found in Algerian honeys given by (Ouchemoukh and al 2006). As well, the proline average of MF samples was around 3.3 mg/g, ranging between 3.2 (MF1) and 3.4 (MF2) mg/g. These values are two times higher than those found in Algerian honey given by Ouchemoukh 2006 and similar to those given by (Latifa, 2013). In the J samples, proline value was around 3.6 mg/g, ranging between 2.7 (J1) and 4.2 (J3) mg/g. Concerning EF, the proline average is around 3.6 mg/kg ranged between 2.22 (EF1) and 4.7 (EF2) mg/g.

3.2.6. 5-HMF

The presence of 5-HMF in honey result from the slow degradation of fructose which, in an acidic environment, breaks down and loses three water molecules. This process is accelerated by heating. The high acidity and water content promote this transformation (Hadia and Ali, 2017).

HMF is an indicator of the freshness and overheating of honey. According to White (1978), the level of HMF is a quality criterion of several varieties of food (Nozal et al., 2001) such as honey, which can provide all the necessary information regarding the heat exposure of any honey. There are differences between floral and honeydew honeys, between honeys of various botanical origins and also it depends on the variations in pH and acidity (Hadia and Ali, 2017). Freshly harvested honey contains virtually no HMF. On the other hand, in the case of hot storage, this value increases (Bogdanov, 1988; Mendes et al., 1998). The European legislation (European Honey Directive, 2001) established the limit of 40 $mg.kg^{-1}$, with the exception for honeys from tropical countries or regions where the maximum value may reach 80 $mg.kg^{-1}$.

The results in this study, Table 14, are between 0 and 36.5 $mg.kg^{-1}$, being within the standard required by the European legislation. The HMF average of EC honeys is around 35 $mg.kg^{-1}$ and are similar to those given by (Djamila B and Paul S, 2010) and beyond those found in Morocco honeys (between 3.25 and 43.87 $mg.kg^{-1}$) by (Chakir et al., 2016). The HMF average of MF honeys is around 26 $mg.kg^{-1}$, while for J samples its around 2 $mg.kg^{-1}$. Those latter are also within those found by (Latifa 2013) in *Ziziphus* Algerian honey (between 0 and 6 $mg.kg^{-1}$). The HMF average of EF honeys is around 20 $mg.kg^{-1}$ range between three values 19.2 (EF1), 18.7(EF2), 21.0 (EF3) $mg.kg^{-1}$. Our results were similar to

those reported by (Chakir et al., 2016) in Moroccan honey harvested in semi-arid region (between 12.08 and 20.32 $mg.kg^{-1}$).

Table 14. Physicochemical parameters of honey: 5- HMF, diastase and proline.

Sample	HMF ($mgkg^{-1}$)	Diastase (DN)	Proline (mgg^{-1})
EC1	34.2 ± 3.1	9.3 ± 0.1	3.6 ± 0.1
EC2	36.5 ± 2.3	10.1 ± 0.5	3.4 ± 0.0
MF1	25.8 ± 1.8	9.4 ± 0.0	3.2 ± 0.1
MF2	27.7 ± 1.1	9.4 ± 0.4	3.4 ± 0.0
J1	5.9 ± 0.7	9.7 ± 1.0	2.7 ± 0.1
J2	0.0 ± 2.4	8.8 ± 1.1	3.7 ± 0.1
J3	0.0 ± 2.0	12.8 ± 0.4	4.5 ± 0.3
EF1	19.2 ± 1.4	13.8 ± 0.4	2.2 ± 0.2
EF2	18.7 ± 1.7	12.4 ± 0.4	4.7 ± 0.5
EF3	21.0 ± 1.6	12.2 ± 0.4	3.9 ± 0.0

3.2.7. Diastase activity

Diastase content depends on the floral and geographical origins of the honey. Diastase enzymes are sensitive to heat and consequently is able to indicate overheating of the product and the degree of preservation (Ligia et al, 2020).

The results of our honeys were between 8.8 DN and 13.8 DN, they were in accordance with the minimum of 8 DN established by the European Community Regulation (The Council of the European Union, 2002). EF samples have the higher values; however J samples have the lower diastase index. The diastase results are lower than those given in Moroccan honeys (14.45 DN in average) reported by (Chakir et al, 2016), as well as Tunisian honeys (17.6 DN in average) reported by (Jilani et al, 2008)

3.3. Sugar analysis

Honey is a supersaturated sugar solution in which major compounds are monosaccharides (fructose and glucose), which represent about 75% of the sugars found in

honey. The percentage of glucose and fructose for nectar honeys should not be less than 60%, and for honeydew honeys it should be a minimum of 45% (Decree-Law nº 214/2003). The sugar profile also gives information on the origin of honey, with honeydew honeys having higher levels of trisaccharides (melezitose or erlose).

All samples under study revealed higher fructose content than glucose, Table 15, with these two monosaccharides representing more than 88%, which allows to classify them, in accordance with international legislation, as nectar honeys. The analyzed samples do not have sucrose which is indicative of no unadulterated honeys. The sugar profile of the different samples showed a similar composition, with values ranging between 37.8 - 43.4 g/100 g and 29.9 – 36.5 g/100 g for fructose and glucose, respectively. The EF samples, showed the highest values of glucose and fructose, 43.26 g/100g of fructose and 36.16 g/100 g of glucose, while MF samples showed the lowest values of fructose and glucose, 37.9 g/100 g and 29.9 g/100 g, respectively. The values are in accordance with the Algerian honey levels of fructose which were found to vary between 33.40 and 48.60 g/100 g and glucose levels to vary between 26.67 and 38.42 g/100 g (Ouchemoukh et al, 2010).

The sugars in honey are responsible for its viscosity, hygroscopicity and crystallization. The distribution between the different sugars will provide valuable information that will allow predicting the rate of crystallization and the stability of the structure of honey (Pourtallier et al, 1970). Crystallization is occurring naturally in honey depending on its composition in sugars and moisture and that appears related to the type of honey. The ratios of F/G (fructose/glucose) and G/H (glucose/moisture) provide information on predicting the time that a honey sample takes to crystallize. The ratio of fructose to glucose depends largely on the source of nectar. Many researchers report that the fructose and glucose ratio have an average value of 1.2 for honey, stating that values greater than 1.3 imply a slow crystallization, above 1.5 indicates that honey does not crystallize and less than 1.1 indicates that crystallization is rapid. This process occurs because glucose is a sugar more insoluble in water than fructose. The speed at which glucose crystallization occurs also depends on the G/H ratio. According to the literature (Escuredo et al 2014), the crystallization of a honey is slow or null when the G/H ratio is less than 1.7 and fast when the ratio is greater than 2 (Escuredo et al,2014). In Table 15, the samples analyzed at the F/G ratio have values between 1.2 and 1.3 which can be said that all samples have a slow tendency to crystallize, and the values of G/H oscillate between 1.7 and 2.6 indicating that the samples have an average propensity to crystallize.

Table 15. Sugar profile, obtained by HPLC-RI, of the studied honey samples (values expressed in g/100g of honey).

Sample	Fructose	Glucose	Turanose	Maltulose	Maltose	Trealose	Rafinose	F+G	F/G	G/H
EC1	39.9 ± 0.6	30.3 ± 0.6	0.6 ± 0.0	3.0 ± 0.6	1.8 ± 0.7	0.6 ± 0.0	N/D	70.2	1.3	1.7
EC2	40.2 ± 0.5	30.6 ± 0.5	0.7 ± 0.0	2.9 ± 0.8	1.5 ± 0.6	0.6 ± 0.0	N/D	70.8	1.3	1.7
MF1	37.8 ± 0.7	29.9 ± 1.1	0.9 ± 0.0	3.8 ± 0.9	2.0 ± 0.2	0.4 ± 0.0	0.8 ± 0.0	67.7	1.3	2.0
MF2	38.0 ± 0.7	29.9 ± 0.0	0.9 ± 0.0	3.6 ± 0.8	1.9 ± 0.1	0.4 ± 0.0	0.8 ± 0.0	67.8	1.3	2.0
J1	40.1 ± 0.7	31.8 ± 0.4	0.2 ±0.0	6.5 ± 0.7	4.8 ± 0.4	1.3 ± 0.3	1.4 ± 0.5	71.9	1.3	2.1
J2	39.9 ± 0.7	31.9 ± 0.3	0.6 ± 0.0	6.6 ± 0.6	4.9 ± 0.4	1.2 ± 0.2	1.5 ± 0.6	71.8	1.3	2.1
J3	40.2 ± 0.3	31.5 ± 0.2	0.6 ± 0.0	5.9 ± 0.4	4.5 ± 0.1	1.5 ± 0.2	1.1 ± 0.0	71.7	1.3	2.1
EF1	43.2 ± 0.6	36.1 ± 1.3	0.9 ± 0.0	3.8 ± 0.9	2.0 ± 0.2	0.4 ± 0.0	0.8 ± 0.0	79.3	1.2	2.6
EF2	43.2 ± 0.6	36.5 ± 1.5	0.9 ± 0.0	3.6 ± 0.2	2.5 ± 0.2	0.5 ± 0.0	0.4 ± 0.1	79.6	1.2	2.6
EF3	43.4 ± 0.7	35.9 ± 0.6	0.9 ± 0.1	3.5 ± 0.0	2.4 ± 0.2	0.5 ± 0.1	0.4 ± 0.1	79.3	1.2	2.6

3.4. Minerals

Honey contains diversified amounts of mineral substances, ranging from 0.02 to 1.03g/100g (White, 1975). Potassium, with an average of about one third of the total, is the main mineral element (Feller-Demalsy et al., 1989; Gonzalez-Miret et al., 2005). The amount of different minerals in honey is largely dependent on the soil composition, as well as various types of floral plants (Anklam 1998). In addition to these factors, the beekeeping practices, environmental pollution, and honey processing may also contribute to the diversified mineral content present in honey (Pohl, 2009).

The contents of each mineral found in our honeys expressed in mg/kg are shown in Table 16. The potassium was quantitatively the most important mineral, 72.93% of total minerals quantified, having an average content 730.60 mg/kg. Sodium, calcium and magnesium were present in moderate amounts in the honeys (17.05% and 4.43% and 4.22% of total minerals, respectively), while cadmium and lead were below the detection limit. Magnesium content (42.31 mg kg^{-1} in average) was above the limit 25 mg kg^{-1} for Mg, iron (11.4 mg kg^{-1} in average) and copper (0.33 mg kg^{-1} in average) concentrations were less than the maximum limit set by the codex Alimentarius [15 mg kg^{-1} for iron and of 5 mg kg^{-1} for copper] (Yaiche and Khali, 2014; Codex Alimentarius 2001).

Lead and cadmium are released into the environment through its use in various industrial processes, and enters the food chain from uptake by plants from contaminated soil or water. Moreover, Cd and Pb are considered bioindicators for honey contamination (Licata et al. 2004). The regulations establish a maximum level of 300 μg kg^{-1}, recommended by FAO/WHO/1984 (Al-Eed et al. 2002) while for Cd the European legislation and the Codex Alimentarius, 2001 fixed a maximum of 0.05 mg kg^{-1}, nevertheless our results did not reveal its presence. Z samples showed the highest values of potassium, sodium and calcium however *Euphorbia* labeled samples showed the highest values of magnesium, while EC samples presented the highest values of manganese and MF samples showed the highest values of iron, lead and cadmium.

Table 16. Minerals contents, obtained by using flame atomic absorption spectrophotometer (values expressed in mg/100 kg of honey).

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)
J1	979.9±12.6	285.6±40.2	40.4±4.0	31.8±2.6	0,4±0,0	0.3±0.0	<0.03	8.7±0.1	<0.4
J2	863.7±6.8	243.0±60.3	40.2±5.0	31.9±2.9	0.4±0.0	0.4±0.0	<0.03	8.6±0.4	<0.4
J3	737.2±7.7	84.3±2.2	43.5±9.0	29.9±5.5	0.4±0.0	0.4±0.0	<0.03	8.8±0.5	<0.4
EF1	462.5±27.1	142.7±5.6	46.6±1.5	50.8±2.5	0.9±0.0	0.3±0.0	<0.03	13.0±1.1	<0.4
EF2	684.4±7.4	229.0±1.0	48.0±2.4	54.2±7.2	0.9±0,0	0.3±0.0	<0.03	12.6±2.2	<0.4
EF3	518.2±2.5	169.3±0.7	32.6±1.4	49.9±5.0	0.9±0,0	0.3±0.0	<0.03	12.8±1.7	<0.4
EC1	937.7±1.4	157.6±2.8	51.1±6.8	47.6±1.2	1.9±0,7	0.3±0.1	<0.03	10.7±0.4	<0.4
EC2	884.2±4.7	177.6±2.8	41.4±6.8	49.3±1.2	0.5±0.1	0.4±0.1	<0.03	11.3±0.4	<0.4
MF1	744.2±3.4	123.8±1,0	85.3±3.1	40.5±2.3	1.8±0,6	0.3±0.0	<0.03	14.9±1.6	<0.4
MF2	494.1±3.5	93.8±1.0	14.7±3.9	37.2±2.3	0.5±0,0	0.3±0.0	<0.03	12.6±1.6	<0.4

3.5. Nutritional parameters

Honey is considered of high nutritional value. Its ash content is related to color and flavor, and it is often observed that honeys with higher ash content are also those that have a darker color and a stronger flavor (Escuredo et al, 2013). In addition, the ash content also contributes to the electrical conductivity of honey, with a positive correlation between these two parameters. The Codex Alimentarius (Codex Alimentarius Commission, 1981) does not provide values for this parameter. Some studies have shown an average value of 0.17% (w/w) in honey (Chakir et al, 2011). The results obtained in this study for the ash content, varied between 0.07 and 0.16%, being within the recommended values for nectar honey, Table 17.

According to Anklam (1998), the proteins in honey are related to plant nectar, bees enzymes and pollen. The quantity of proteins can vary from 0.1 to 0.7 g/100 g Anklam (1998). Overheated or long-time stored honeys show a reduction or absence of protein content (De-Melo et al., 2018).

Table 17. Nutritional values of honey: Ash, energy, proteins and carbohydrates.

Sample	Ash (g/100 g)	Protein (g/100 g)	Energy (kcal)	Carbohydrates (mg/100g)
EC1	0.16±0.01	0.7 ± 0.00	327±0.0	81.0±0.0
EC2	0.16±0.01	0.6 ± 0.0	326±0.0	80.9±0.0
MF1	0.07±0.01	0.5 ± 0.1	340±0.0	84.5±0.1
MF2	0.11±0.04	0.5 ± 0.2	340±0.0	84.5±0.2
J1	0.13 ± 0.0	0.7 ± 0.0	340±0.0	84.3±0.0
J2	0.13 ± 0.0	0.7 ± 0.0	339±0.0	84.2±0.0
J3	0.13± 0.0	0.7 ± 0.0	341±0.0	84.5±0.0
EF1	0.13 ± 0.0	0.6 ± 0.0	345±0.0	85.7±0.0
EF2	0.13 ± 0.0	0.6 ± 0.1	344±0.0	85.5±0.1
EF3	0.13 ± 0.0	0.6 ± 0.0	344±0.0	85.3±0.0

The results obtained in this study vary between 0.5 and 0.7g/100g, Table 17. This variation can be associated to the type of flora and the diets of the bees (El Sohaimy et al., 2015). Sample J1 is the richest in protein, with a rate of 0.7 g/100g. This is the sample that comes mainly from Ziziphus, moderately rich in pollen. Sample MF1 is the poorest in proteins with a content equal to 0.5g/100g. The range of protein observe in our results are similar to the results obtained by Ouchemoukh and his collaborators in (2007) who found values between 0.37 and 0.94 g/100g in the Bejaia (City in the north of Algeria) honeys. Also, the protein content of most Tunisian honeys was between 0.13 and 0.16 mg / 100g of honey (Boussaid et al., 2014).

As with the mineral and protein content, there is also no legislation that regulates the limits for the energy value and carbohydrate content present in the different honeys. The honey samples studied showed similar values of carbohydrates, ranging from 80.9 to 85.7 g/100g, and of energy value, with values between 326 and 345 kcal, Table17**.**

3.6. Total phenolics and total flavonoids contents

Polyphenols are a class of important secondary metabolites with multiple phenolic hydroxyl groups in which the main sources are plant secretions, and includes flavonoids, phenolic acids, stilbenes, and tannins (hydrolysable and condensed), which are mainly synthesized by the phenylpropanoid metabolic pathway (Kumar and Goel, 2019). They possess various pharmacological activities, such as anti-cardiovascular, anti-oxidation, anti-inflammatory, and anti-tumor effects (Olas B. (2020). Among the structures identified in honey: phenolic acids (benzoic and cinnamic acids), flavonoids (flavones and flavanones) are the major compounds detected in variable proportions (Al Mamary et al., 2002 cited in Yahia Mahammed, 2015). A correlation between the antioxidant activity and total phenolic content is frequently established in literature [Al, M.L et al, 2009- Aljadi et al, 2004]. The high levels of flavonoids, phenolic acids, ensure a high level of antioxidants in honey which is the hallmark of its effect as a natural medical product (Madhavi and Kailash, 2014).

According to Anklam (1998), a careful evaluation of polyphenol content could probably give an indication of the botanical, geographic and climatic origin of honey and the conditions of plant sources in the region likewise it allows to differentiate between honeydew, and nectar honey. Darker honeys are richest in phenolic compounds when comparing with lighter color honeys (Campus et al., 1983).

Table 18. Total phenolic and total flavonoid contents and antioxidant activity of honey samples.

Sample	Total phenolic content (mg/GAE.g^{-1})	Total flavonoid content (mg/QE.g^{-1})
EC1	1.4 ± 0.0	0.07 ± 0.00
EC2	1.2 ± 0.0	0.05 ± 0.00
MF1	0.8 ± 0.2	0.08 ± 0.01
MF2	0.9 ± 0.2	0.07 ± 0.04
J1	0.7 ± 0.0	0.05 ± 0.00
J2	0.7 ± 0.0	0.03 ± 0.00
J3	0.8 ± 0.1	0.04 ± 0.02
EF1	1.1 ± 0.1	0.06 ± 0.02
EF2	0.7 ± 0.0	0.06 ± 0.00
EF3	0.7 ± 0.0	0.09 ± 0.01

The total phenolic content values obtained in our work vary from 0.7 mg GAE/g honey (EC1) to 1.4 mg GAE/g honey (EF and J), with an average of 0.9 mg GAE/g honey, Table 18. Our results are higher than those obtained by Khalil et al., (2012), who reported values between 0.459 ± 0.0015 mg GAE/g honey for Algerian samples. Douka et al., (2014), reported values between 1.66 to 4.27 mg GAE /g honey in some honeys from western Algeria.

The total flavonoid content of honey samples (mg of QE/100 g) varied from 0.03 to 0.09 mg QE/g, Table 18, with the highest levels observed in J honeys. The mean values for total flavonoids were 0.06 mg QE/g, which were similar to those obtained previously (Khalil et al, 2012).

3.7. Phenolic compounds by UPLC / DAD / ESI-MS^n

Nowadays, new analytical technologies, such as the analysis of the profile of phenolic compounds, are used to characterize and evaluate the authenticity of honeys associated with particular botanical origins. The profile of phenolic compounds was

evaluated by UPLC/DAD/ESI-MS^n, after the extraction of these compounds from the honey samples. The methodology allowed the elucidation of the phenolic compounds by comparing their chromatographic profile, UV spectrum and mass spectrometry information, with reference compounds. When standards were not available, structural information was confirmed with the combination of UV data and MS fragmentations described in the literature. ESI-MS^n in the negative mode was used due to the great sensitivity that this mode presents in the detection of the different classes of phenolic compounds (Falcão et al, 2013). Table 19 shows the various compounds identified in each sample, with the respective retention time, maximum absorbance bands and mass spectrometry information.

Table 19. Phenolic compounds and abscisic acid identified by UPLC/DAD/ESi-MSn in the honey samples under study.

Nº	Compound	T_R (min)	λ_{max} (min)	$[M-H]^-$	$[M-H]^2$
1	Benzoic acid derivative[b,c]	1.25	284	121, $[M+46]^-$:167	
2	*p*- Hidroxybenzoic acid[a,b]	1.87	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[b]	6.38	276	345	161(100), 285(91), 309(21), 327(24)
7	*trans, trans*- Abscisic acid[a,b,d]	6.88	265	263	154(100), 153 (69), 220 (36)
8	*p*- hydroxybenzoic derivitave[b]	7.05	219, 203	199	155(100), 137(20)
9	*cis, trans*- Abcisic acid[a,b,d]	7.46	265	263	154(100), 153(69), , 220(36)
10	Isorhamnetin rhamnoside[b]	7.57	254, 354	461	315
11	Pinobanksin-5-methyl-ether[b,f]	7.67	287	285	267 (100), 239 (29), 252 (13)
12	Quercetin[a,b]	7.76	256, 370	301	179(100), 151(69)
13	N^1, N^5, N^{10}-tri-*p*-coumaroyespermidine[b,e]	8.31	292, 308	582	462(100), 436(10), 342(7)
14	Pinobanksin[b,f]	8.33	292	271	253(100), 225(20), 151(10)
15	Kaempferol[a,b]	8.45	269, 345	285	229(100), 151(93), 257(80)
16	Carnosol[b]	8.92		329	241 (100), 185 (65), 311 (58)
17	Chrysin[a,b]	10	269	253	253(100), 209(49), 225(17)
18	Pinocembrin[a,b]	10.13	290	255	213 (100), 151 (34) 253(100), 271(20)
19	Galangin[a,b]	10.22	265, 300sh, 358	269	269 (100), 241 (61), 227 (20), 151 (20)

[a]Confirmed with standard; [b]Confirmed with MS^n fragmentation; Confirmed with references: [c]Ouchemouck et al., 2016; [d]Bertoncelj et al., 2011; [e]Falcão et al., 2019; [f]Falcão et al., 2013;

In this study it was possible to identify nineteen phenolic compounds, which included nine flavonoids, six phenolic acids, two isoprenoids, one spermidine and one

phenolic diterpene. Among the identified phenolic acids, three are derived from benzoic acid (benzoic acid derivative, *p*-hydroxybenzoic acid, salicylic acid and *p*-hydroxybenzoic acid derivative) and two are derivatives of cinnamic acid (caffeic acid, *p*-coumaric acid. Of the nine flavonoids identified, five belong to the class of flavonols (syringetin, isorhamnetin rhamoside, quercetin and kaempferol), two to the flavone class (chrysin, galangin), one flavanone (pinocembrine) and two dihydroflavonols (pinobanksin-5-methyl- ether and pinobanksin). In addition two isoprenoids, which included two isomers of abscisic acid, have also been identified (cis, trans- and trans, trans-), as well as carnosol, which is a phenolic diterpene, and spermidine: N^1,N^5,N^{10}-tri-*p*-coumaroyespermidine. Among the compounds identified (Table 20), it can be seen that *p*-coumaric acid were presented only in EC samples while kaempferol, pinocembrin and galangin were presented only in J samples. The trans, trans isomer of abscisic acid was presented in both EC and J samples but it was presented in high concentration in J honeys than EC honeys. The compounds specific for one type of sample can be considered as marker compounds for that honey. In Table 20, it can be seen that the samples that presented the greatest amount of phenolic compounds are sample EC1 with 202 mg/100 g and with the lowest amount is sample EF3 with 60 mg/100 g. It can be seen that in relation to phenolic acids, the EC1 is the one with the highest amount of compounds derived from benzoic acid (92 mg/100g) and the EC2 sample stands out for the acids derivatives of cinnamic acid (58.6mg/100g). These phenolic compounds were already reported in Algerian honeys (Ouchemoukh et al, 2017). Moreover, it has been found by Can et al. (2015) that benzoic, caffeic and *p*-coumaric acids were present in differing amounts in all unifloral Turkish honeys.

The flavonoids found in honey come from pollen, propolis and nectar, with propolis being the richest source of flavonoids. Pinobanksin and its derivatives, pinocembrine, chrysin and galangin are compounds described as propolis derivatives (Falcão et al, 2013; Tomás et al, 2001). Pinobanksin is present in all samples in exception of EC1 and EC2 and pinocembrine is present in small amount only in samples J1, J2 and J3, with values ranging between 0.1-13.5mg/100 g and 003-0.2 mg 100g, respectively, Table 20.

Some authors (Tomás et al, 2001) report that the amount of flavonoids is higher in honeys harvested during dry seasons with high temperatures and that the darker honeys contain more derivatives of phenolic acids, while lighter honeys contain more flavonoids (De-Melo et al, 2017). Abscisic acid (two isomers) is an important phytohormone regulating plant growth, and has an essential role in multiple physiological processes of plants.

Abscisic acid controls downstream responses to abiotic and biotic environmental changes (Chen et al, 2020). Its content varied between 8.3 and 20.1 mg/100 g for isomer 1 (trans, trans- abscisic acid) and 6.2 and 25.7 mg/100 g for isomer 2 (cis, trans- abcisic acid), Table 20. Ouchemoukh and his collaborators, 2017 identified the two isomers in Algerian honeys.

Table 20. Quantification of phenolic compounds, expressed in mg/100 g honey.

Compound	EC1	EC2	MF1	MF2	J1	J2	J3	EF1	EF2	EF3
Benzoic acid derivative	26.3±0.2	19.7±0.1	24.2±1.3	33.4±0.4	5.9±0.2	4.9±0.0	7.8±0.0	9.7±0.1	14.7±0.2	7.2±0.0
p- Hidroxybenzoic acid	30.6±1.1	28.0±0.8	8.4±0.3	9.2±1.0	8.6±0.0	10.6±0.7	18.7±0.1	10.6±0.2	17.0±0.4	7.5±0.1
Caffeic acid	5.3±0.6	5.0±0.4	8.9±1.4	10.6±0.5	1.1±0.0	1.0±0.3	1.2±0.3	1.7±0.5	0.01±0.00	0.05±0.01
p-Coumaric acid	42.8±0.1	52.5±0.3	-	-	-	-	-	-	-	-
salicylic acid	1.9±0.1	2.1±0.2	1.1±0.1	4.0±0.7	2.3±0.1	3.3±0.0	5.0±0.1	1.6±0.2	1.0±0.1	0.1±0.0
Syringetin	14.6±0.1	16.5±1.7	17.3±2.2	24.9±1.4	9.9±0.2	12.0±1.6	7.1±0.1	35.9±0.1	54.2±0.8	19.6±0.0
trans, trans-abscisic acid	9.8±0.0	8.3±0.2	-	-	20.1±0.6	14.7±0.7	16.3±0.6	-	-	-
p- hydroxybenzoic derivitave	35.1±0.4	23.9±0.0	10.8±0.2	12.2±0.1	3.5±0.1	3.6±0.0	4.0±0.6	2.1±0.0	4.8±0.7	1.4±0.0
cis, trans- Abcisic acid	9.5±0.0	8.3±0.0	6.2±0.8	9.3±0.4	22.0±0.0	19.3±0.0	25.7±0.1	16.2±0.0	20.3±0.2	8.7±0.0
Isorhamnetin Rhamnoside	16.1±0.4	10.5±0.9	-	-	-	-	-	-	-	-
Pinobanksin-5-methyl- ether	0.4±0.0	0.2±0.0	0.4±0.0	0.4±0.2	0.4±0.1	0.1±0.0	0.1±0.0	0.4±0.1	0.8±0.1	0.3±0.0
Quercetin	5.2±0.3	1.9±0.0	2.7±0.5	3.9±0.0	9.7±0.2	3.4±0.5	4.5±0.2	9.5±0.2	16.7±1.5	6.7±0.5
N^1,N^5,N^{10}-tri-*p*-coumaroyspermidine	2.9±0.0	1.1±0.0	0.5±0.1	1.2±0.4	1.1±0.0	2.0±0.2	1.2±0.1	1.1±0.0	2.2±0.2	1.1±0.1
Pinobanksin	-	-	2.8±0.2	3.9±0.2	0.1±0.0	13.5±0.1	12.8±1.0	11.5±0.0	13.3±0.1	6.0±0.0
Kaempferol	-	-	-	-	7.9±0.0	16.5±2.3	3.4±0.1	-	-	-
Carnosol	1.0±0.0	0.5±0.0	-	-	0.7±0.1	0.9±0.1	1.1±0.1	-	-	-
Chrysin	0.7±0.0	0.9±0.1	0.5±0.0	0.9±0.1	3.4±0.2	2.9±0.2	3.2±0.1	2.4±0.1	3.6±0.1	1.2±0.0
Pinocembrin	-	-	-	-	0.03±0.00	0.2±0.0	0.2±0.0	-	-	-
Galangin	-	-	-	-	2.0±0.2	2.6±0.1	3.4±0.3	-	-	-

3.8. Antioxidant activity

3.8.1. DPPH

The scavenging activity of honey samples had been measured by DPPH assay. The unpaired electron of DPPH forms a pair with hydrogen donated by free radical scavenging antioxidant from honey and thus converting the purple colored odd electron DPPH to its reduced form in yellow. The lower the EC_{50} value the higher the scavenging capacity of honey, because it requires lesser amount of radical scavenger from the honey to reduce DPPH (Chua et al, 2013). The values obtained for DPPH in the analyzed samples are represented in Table 21 and ranged from 0.02 to 0.04 mg/mL, with higher antioxidant activity associated with EC and J honeys and a lower antioxidant activity associated with EF honeys. The values are correlated with the concentration of phenolic acids and flavonoids in the samples. Our results are lower than those obtained in a Moroccan study where the results of DPPH showed EC_{50} values ranged between 0.245 ± 0.009 mg/mL and 0.832 ± 0.069 mg/mL, meaning that, our honeys have a higher antioxidant activity than Moroccan samples (El Ghouizi et al, 2021).

Table 21. The antioxidant activity; reducing power and DPPH assay

Sample	Reducing power (mg/GAE.g^{-1})	DPPH (EC50 mg/mL)
EC1	0.03 ± 0.00	0.02 ± 0.00
EC2	0.04 ± 0.00	0.02 ± 0.00
MF1	0.04 ± 0.00	0.04 ± 0.00
MF2	0.04 ± 0.00	0.03 ± 0.00
J1	0.04 ± 0.00	0.03 ± 0.00
J2	0.04 ± 0.00	0.02 ± 0.00
J3	0.04 ± 0.00	0.02 ± 0.00
EF1	0.04 ± 0.00	0.03 ± 0.00
EF2	0.04 ± 0.00	0.04 ± 0.00
EF3	0.04 ± 0.00	0.03 ± 0.00

3.8.2. Reducing power

Fe (III) reduction is often used as an indicator of electron-donating activity. The presence of reducing agents in the honey reduced the ferric ions. This reduction is quantified by an absorbance measurement at 700 nm against a blank, with an increase in absorbance associated with high reducing power (Mouhoubi, 2016). Table 21 shows the values of the samples evaluated

by the reducing power, expressed in equivalents of gallic acid (mg GAE.g^{-1}). Results of the reducing power showed that there was no significant difference between our samples observing a variation between 0.03 and 0.04 mg GAE.g^{-1}. As described in the literature (Hatami et al, 2014; Lamuela and Rosa, 2018), it is possible to observe that samples with lower levels of total phenolic compounds were those that registered lower values of reducing power. Also, the presence of other non-phenolic compounds such as enzymes (glucose oxidase and catalase) and non-enzyme materials (vitamins and amino acids) may influence this activity (Aljadi and Kumaruddin, 2004).

3.9. Cytotoxic potential

The last decade has witnessed an astronomical increase in the amount of research investigating the role of honey in the treatment of various diseases, including cancer. These health benefits of honey in treating diverse diseases can be attributed to its various pharmacologically active constituents, especially flavonoids and phenolic constituents, which included anti-inflammatory, antioxidant, antiproliferative, antitumor, antimetastatic and anticancer Candiracci et al, 2012; Samarghandian et al, 2011).

The cytotoxicity of the Algerian honeys was evaluated in four human tumor cell lines (AGS-gastric adenocarcinoma, CaCo colorectal adenocarcinoma, MCF-7 breast adenocarcinoma, NCI H460- lung carcinoma) and a non-tumor cell line, Vero (African green monkey kidney). All the studied extracts inhibited the growth of the mentioned tumor cell lines. MF1 gave the highest cytotoxicity, followed by EF1, Table 22, presenting the lowest GI_{50} values against the tested tumor cell lines. The AGS cell line was the most sensible to the studied samples in the average, the MF1 extract was the most active (GI_{50} 8.1μg/mL; an excellent GI_{50} value in comparison with Portuguese Propolis extracts for example (Ricardo et al 2014). This activity could be related to the chemical composition of those samples. From the analysis of Table 20, it can be observed that samples EF1 and MF1 have significant concentrations of total phenolic and total flavonoids compounds. The EF3 sample showed the highest GI_{50} values for all the tested tumor cell lines with an average (375 μg/mL). This fact could be explained by its poor phenolic composition. These results can be explained also by the level of hydrogen peroxide of these samples. Hydrogen peroxide was reported to be responsible for the proliferative effect of honey in cancer cells (A. Henriques et al, 2006).

Despite the high cytotoxicity displayed by most of the honey samples against tumor cell lines studied, the samples also showed toxicity for non-tumor (normal) cell line, however they reporting higher GI_{50} values when compared to tumor cell lines.

Ricardo and his collaborators in 2014 found that total flavonoids were positively correlated (R^2 values higher than 0.5) with the cytotoxicity. However, the cytotoxicity was not correlated (R^2 values lower than 0.5) with flavonols, dihydroflavonols, and flavonoid esters.

The present data highlight the high cytotoxicity of Algerian honeys against tumor cell lines, being in agreement with Siti Noritrah et al. 2019, who reported a marked activity of Malaysian honey against human lung adenocarcinoma epithelial cell line (A549). As well, our results were similar to those obtained by Hamada et al, 2019 on Moroccan and Palestinian honeys rom different regions.

Table 21. Cytotoxicity potential (GI_{50} values, µg/mL). and anti-inflammatory activity (CI_{50} values, µg/mL).

Cell lines	**GI_{50}**										
	EC1	**EC2**	**MF1**	**MF2**	**EF1**	**EF2**	**EF3**	**EF4**	**J1**	**J2**	**J3**
CaCo	13.7±0.2	176±16	151±7	8.1±0.2	194±17	>400	>400	22.1±0.3	62±1	228±11	71±7
AGS	60±5	9±1	193±8	48±2	194±17	>400	>400	22.1±0.3	62±1	228±11	72±7
MCF-7	383±23	371±3	65±2	281±18	249±25	>400	>400	271±2	>400	>400	>400
NCl-H460	328±5	283±4	168±10	359±5	163±10	221±23	300±31	212±10	>400	>400	>400
VERO	254±7	245±8	>400	302±21	>400	>400	>400	237±7	>400	>400	>400
RAW	9.5±0.2	43±1	>400	12.7±0.1	57±3	82±4	150±4	9±1	9±1	8±1	8.5±0.3

3.10. Anti-inflammatory activity

Inflammation usually occurs when infectious microorganisms such as bacteria, viruses or fungi invade the body, reside in particular tissues and/or circulate in the blood (Artis and Spits, 2015; Isailovic et al,2015). Inflammation may also happen in response to processes such as tissue injury, cell death, cancer, ischemia and degeneration (Artis and Spits, 2015, Lucas et al, 2006). Mostly, both the innate immune response as well as the adaptive immune response are involved in the formation of inflammation.

The anti-inflammatory activity of our honey samples was assessed using the mouse macrophage (RAW 264.7) cell line. All honey extracts under study showed anti-inflammatory capacity, with IC_{50} values between 8 and 400 µg/mL. The highest activity was observed for sample J2, followed by the samples J1 and EC1, with an IC_{50} value of 9 µg/mL. In opposite the MF1 sample showed the highest IC_{50} values for the tested cell line more than 400 µg/mL Table 21. This fact could be explained by its poor phenolic composition. This is the first time, to the

best of our knowledge, that the effects of Algerian honey extracts on anti-inflammatory activity have been evaluated in vitro.

3.11. Screening of antibiotics residues

Tetracyclines are commonly applied in the treatment of many bacterial infections of the digestive system, the respiratory system and the skin. Also they are used as a growth stimulant in animals, in some countries its commonly use as additive in animal feed. The large-scale application of tetracyclines carries the risk of their residues appearing in food. For other side, sulphonamides has been used for treatment of American foulbrood (*Paenibacillus* larvae subsp, larvae) a deadly disease to honeybees. In 1940, sodium sulfathiazole was registered in the USA for the control of AFB (Moreno et al, 2009. In some countries outside Europe the use of tetracyclines, sulphonamides and other antibiotics is still legalized for the treatment of American foul brood (Reybroeck, 2002). Oxytetracycline is currently the only antibiotic registered for use by Canadian beekeepers to treat American foulbrood (AFB), a highly contagious bacterial disease of larvae, difficult to eradicate, caused by the rod-shaped bacteria *Paenibacillus larvae*). In Europe this is an illegal practice because ubiquitous administration of antibiotics may cause bacteria to become resistant to many drugs and spread antibiotic-resistant strains of bacteria (Żaneta et al, 2011). Antibiotic resistance has become a major concern due to overuse of antibiotics, leading to difficult to treat infections in humans and animals, with increased morbidity and mortality (Lekshmi et al, 2017). Because of that, the presence of residues of antibiotics in European honey is not permitted.

Table 22. Residues screening using CHARM II.

Sample	Sulfonamide (10 ppb)		Tetracycline (15 ppb)	
EC1	2205	Negative	2635	Negative
EC2	2183	Negative	2575	Negative
MF1	1525	Positive	2530	Negative
MF2	1751	Negative	2560	Negative
J1	2408	Negative	1980	Negative
J2	2552	Negative	1815	Negative
J3	2877	Negative	1839	Negative
EF1	1050	Positive	1663	Negative
EF2	2267	Negative	2523	Negative
EF3	1475	Positive	1677	Negative

The charm II test is a screening test used for different food matrix such as meat and milk. This has been adapted for honey testing (Bogdanov, 2003), allowing the detection of many antibiotics (penicillin, tetracycline, macrolides, sulfonamides, and aminoglycosides) by an immunocompetition reaction between the molecule to be sought and a molecule marked with C14 or H3 (Audigie et al, 1995). The results of the residues screening for sulfonamides and tetracyclines in our samples are summarized in Table 22. Out of this monitoring and screening data it could be concluded that the frequency of antibiotics residues agents in Algerian honeys from local beekeepers is very low, but still a concern if international trade is to be considered. In case of tetracycline residues all the results were negative; on the other hand, three of our samples (MF1, EF1, EF3) showed positive results for Sulfonamide residues.



Chapter IV- Conclusion and Future Perspectives

Conclusion

The results of the melissopalynological analysis show that the honey samples analyzed contain a great diversity of pollen grains, with no elements of honeydew being identified, which allows us to conclude that these are nectar honeys. Ten types of pollen were identified, *Cytisus striatus* pollen were the most abundant, being present in all samples with percentages between 26.0 % and 83.8 %, with samples EC1 (region of Sidi Belabes), MF1 and MF2 (region of Sidi Belabes) classified as monofloral *Cytisus striatus* honey. Although samples J1, J2 and J3 were not consider monofloral, they showed high percentages of *Ziziphus* pollen (greater than 39.5 %). The remaining samples were classified as multifloral. The results of the melissopalynological analysis seem to indicate that no samples of honey really correspond to the beekeeper classification. Thus, although food security is not at stake, the need to create additional mechanisms to ensure the authenticity of this type of food product becomes imperative.

There was a significant difference in color remarked between all studied samples of honey ranged between amber, light amber and extra light amber. Changes in color might be attributed to the beekeeper's interventions and different ways of handling the combs such as using of old honeycombs, contact with metals and exposure to either high temperatures or light. The higher Pfund and color intensity values might indicate higher phenolic compounds and flavonoids. The moisture content of the honey samples was within the limits established by the legal requirements, that is, less than 20%, which allows us to conclude that the honey will have been extracted with the appropriate degree of maturation. Regarding electrical conductivity, the honey samples analyzed showed values between 270 and 410 $\mu S.cm^{-1}$. In general, all samples showed conductivity values below 800 $\mu S.cm^{-1}$, which means that confirms the samples as nectar origin. The values established by Codex Alimentarius clearly confirm our results. The pH values were between 4.2 and 5.1 which again point out for the nectar origin. The values of free acidity were between 5.8 and 45.0 $meq.kg^{-1}$, being below the 50.0 $meq.kg^{-1}$ stipulated in the Codex Alimentarius, indicating the absence of undesirable fermentation processes for the quality of honey. The evaluation of the 5-HMF content and the diastase index provides important information about the quality of the honey, namely about the occurrence of heat treatments or inadequate storage conditions. The results were in accordance with the European legislation, ranging between 0 and 36.5 $mg.kg^{-1}$. Regarding diastase, the results ranged between 8.8 and 13.3 DN, being within the quality legal requirements. Honey samples presented high proline levels (2.2–4.7 mg/kg), indicating a good maturity of the honeys and absence of adulteration. For the proteins, the values varied between 0.5 and 0.7 mg/100 g. This

variation can be attributed to the type of flora and the diets of the bees.

All samples showed higher fructose than glucose content, with these two monosaccharides representing more than 89%, allowing the classification of the honeys as nectar honeys. The presence of sucrose was not detected, indicating unadulterated honeys.

Concerning the mineral content, the potassium was found to be the most important mineral (73% of total minerals quantified), followed by sodium, calcium and magnesium, with 17%, 4.4% and 4.2% of total minerals, respectively. Cadmium and lead where below the limit of detection.

The determination of the total phenolic compounds content by the Folin-Ciocalteau method showed values between 0.7 mg GAE/g honey (EF and J) and 1.4 mg GAE/g honey (EC). The total flavonoid content of honey samples varied from 0.03 to 0.09 mg QE/g honey, with the highest levels observed in jujube honeys. The scavenging activity of the honeys was evaluated by DPPH assay, with results ranging 0.02 to 0.04 mg/mL, with higher antioxidant activity associated with EC and J honeys and a lower antioxidant activity associated with EF honeys. Regarding the reducing power activity, results showed that there was no significant difference between our samples observing a variation between 0.03 and 0.04 mgGAE.g^{-1}.

The analysis of the phenolic compounds profile was performed by UPLC/DAD/ESI-MS^n, where was possible to identified nineteen phenolic compounds (six phenolic acids and nine flavonoids), two isoprenoid compounds (abscisic acid isomers), one phenolic diterpene (carnosol) and one spermidine (N^1, N^5, N^{10}-tri-*p*-coumaroyespermidine). The honey samples analyzed showed a similar phenolic composition, in which the different compounds are present in almost all samples, with some differences in their concentrations. Among the compounds identified, it can be seen that *p*-coumaric acid, syringetin as well benzoic acid are those that were detected in most samples in higher concentrations, followed by the two isomers of abscisic acid (*cis, trans*- and *trans, trans*- isomers). Sample EC1 presented the highest quantity of phenolic compounds, with 202 mg/100 g, while EF3 showed the lowest amount with 59.85 mg/100 g.

The anti-inflammatory activity of the samples was assessed using the mouse macrophage (RAW 264.7) cell line. All honey extracts under study showed anti-inflammatory capacity, with GI_{50} values between 8 and 400 µg/mL. The highest activity was observed for sample J2, followed by the samples J1 and EC1, with an GI_{50} value of 9 µg/mL. The cytotoxicity of the Algerian honeys was evaluated in four human tumor cell lines (AGS-gastric adenocarcinoma, CaCo-colorectal adenocarcinoma, MCF-7 breast adenocarcinoma, NCI H460- lung carcinoma) and a non-tumor cell line, Vero (African green monkey kidney). All the studied extracts inhibited the growth of the mentioned tumor cell lines. MF1 gave the highest cytotoxicity, followed by EF1.

The use of antibiotics in beekeeping is an illegal practice in Europe because ubiquitous administration of antibiotics may cause bacteria to become resistant to many drugs. The frequency of antibiotics residues in Algerian honeys from local beekeepers is very low. For tetracycline residues, results were negatives while, three of the samples (MF1, EF1, EF3) showed positive results of sulfonamide.

Future perspectives

This study concerned the characterization and evaluation of samples from semi-arid regions in Algeria, and the verification of its compliance with the established legal standards. In the continuation of this work some recommendations for future research are given below:

- It would be important to confirm these results by analyzing more samples of these honeys, specially *Cytisus striatus*, considering that this is the first time that this type of mono flower honey from Algeria has been studied;
- A statistical analysis must be applied to obtain the correlation between different parameters and the influence of each parameter to another;
- Identify potential floral markers of the honeys of *Cytisus striatus*, namely through the evaluation of the profile in volatile compounds;
- A comparison between Algerian honeys and Portuguese honeys with same floral source should be studied.

Chapter V- References

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Chapter VI- Appendix

Appendix

Attached are two abstracts that resulted from two panel communications:

Seloua Kaid, Soraia I. Falcão, Andreia Tomás, Ziani Kaddour, Miguel Vilas-Boas. Physico-chemical evaluation of Algerian honeys: Eucalyptus, Jujube, and Spurge, multifloral. NPA (Natural Products Application: Health, Cosmetic and food), Online Edition 4-5 Feb 2021.

Seloua Kaid, Soraia I. Falcão, Andreia Tomás, Ziani Kaddour,Miguel Vilas-Boas. Characterization of Algerian honeys by phenolic compounds LC-DAD-ESI/MS^n analysis: Eucalyptus, Jujube, Spurge and multifloral.7 PYCHEM (Portuguese Young Chemists Meetings), 20-22 May 2021 Bragança

PHYSICO-CHEMICAL EVALUATION OF ALGERIAN HONEYS: EUCALYPTUS, JUJUBE, SPURGE AND MULTIFLORAL

Seloua Kaid,[1,2] Soraia I. Falcão,[1], Andreia Tomás,[1] Ziani Kaddour,[2] Miguel Vilas-Boas[1*]

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Arid and semi-arid zones represent nearly two-thirds of Algerian area. The immensity of these territories and the absence of systematic studies of the bee flora, make honeys from these regions poorly studied and poorly understood. The aim of the present study was to evaluate the quality of semi-arid Algerian honeys and verify its compliance with the established honey standards. For that, ten samples with different botanical and geographical origin, Eucalyptus (EC), Jujube (J), Euphorbia (EF) and multifloral (MF), were analyzed regarding the following physicochemical parameters: moisture, color, pH, free acidity, electrical conductivity, hydroxymethylfurfural (HMF), diastase index and proline. Concerning the moisture content, the samples presented values below the 20 % allowed by European Community regulations, ranging from 13.6% (EF) and 18.3% (EC). Eucalyptus honeys showed a darker color when comparing to the other samples All honey samples presented conductivity values lower than 0.8 $ms.cm^{-1}$, ranging between 0.27 (MF) and 0.41 (EC) $ms.cm^{-1}$ which are in accordance with the standard results for nectar honeys. The honeys pH values varied between 4.2 (MF) and 5.1 (J) with an average value equal to 4.6. For free acidity, tested at pH 8.3, the values where between 12.2 $meq.kg^{-1}$ (EC) and 43.9 $meq.kg^{-1}$ (EF). The HMF levels observed for the samples had a minimum of 0.53 (J) and a maximum of 36.5 (EC) $mg.kg^{-1}$, while diastase values ranged between 8.8 DN and 14.3 DN, being in accordance with the required by the European legislation (<40 $mg.kg^{-1}$ and not less than 8 DN). For proline, the values ranged between 2.2 and 4.7 mg/g indicating the maturity of the honeys and absence of adulteration. Generally, the samples were found to meet the requirements of the international honey standards and were within those found in previous studies about physicochemical properties of Algerian and Moroccan honeys [1].

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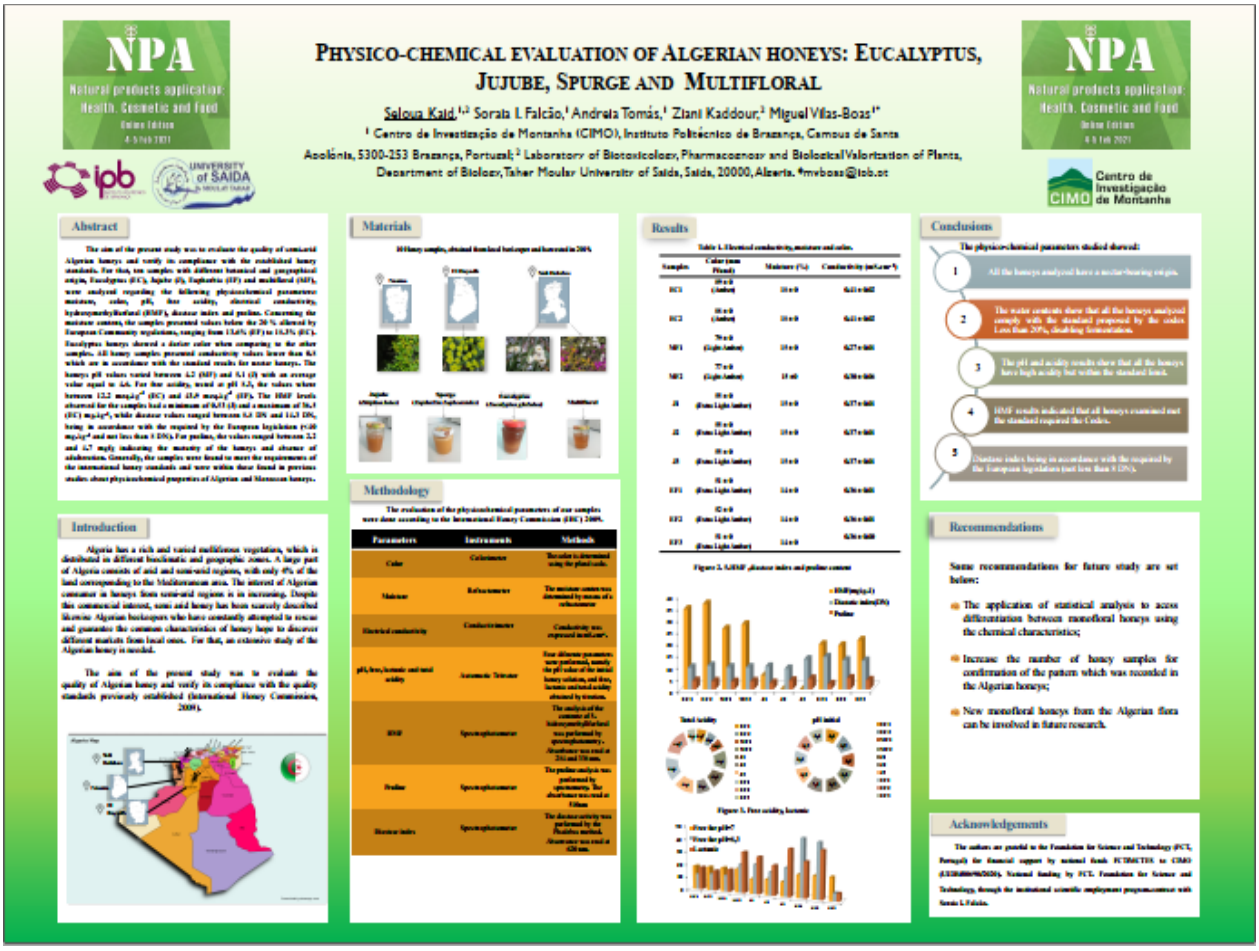

PHYSICO-CHEMICAL EVALUATION OF ALGERIAN HONEYS: EUCALYPTUS, JUJUBE, SPURGE AND MULTIFLORAL

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Abstract

The aim of the present study was to evaluate the quality of unifloral Algerian honeys and verify its compliance with the established honey standards. For this, ten samples with different botanical and geographical origin, Eucalyptus (EU), Jujube (J), Euphorbia (EP) and multifloral (MF), were analyzed regarding the following physicochemical parameters: moisture, color, pH, free acidity, electrical conductivity, hydroxymethylfurfural (HMF), diastase index and proline. Concerning the moisture content, the samples presented values below the 20 % allowed by European Community regulations, ranging from 13.6% (EP) to 18.3% (EU). Eucalyptus honeys showed a darker color when comparing to the other samples. All honey samples presented conductivity values lower than 0.8 which are in accordance with the standard results for nectar honeys. The honeys pH values varied between 4.2 (MF) and 5.1 (J) with an average value equal to 4.6. For free acidity, tested at pH 8.5, the values where between 12.2 meq.kg^{-1} (EC) and 43.8 meq.kg^{-1} (EP). The HMF levels observed for the samples had a minimum of 0.33 (J) and a maximum of 36.1 (EC) mg.kg^{-1}, while diastase values ranged between 6.1 DN and 14.3 DN, being in accordance with the required by the European legislation (>8 mg.kg^{-1} and not less than 8 DN). For proline, the values ranged between 3.2 and 4.7 mg/g indicating the maturity of the honeys and absence of adulteration. Generally, the samples were found to meet the requirements of the international honey standards and were within those found in previous studies about physicochemical properties of Algerian and Moroccan honeys.

Introduction

Algeria has a rich and varied melliferous vegetation, which is distributed in different bioclimatic and geographic zones. A large part of Algeria consists of arid and semi-arid regions, with only 4% of the land corresponding to the Mediterranean area. The interest of Algerian consumer in honeys from semi-arid regions is in increasing. Despite this commercial interest, semi-arid honey has been scarcely described likewise Algerian beekeepers who have constantly attempted to rescue and guarantee the common characteristics of honey hope to discover different markets from local ones. For that, an extensive study of the Algerian honey is needed.

The aim of the present study was to evaluate the quality of Algerian honey and verify its compliance with the quality standards previously established (International Honey Commission, 2009).

Materials

Methodology

The evaluation of the physicochemical parameters of our samples were done according to the International Honey Commission (IHC) 2009.

Parameters	Instruments	Methods
Color	Colorimeter	The color is determined using the pfund scale.
Moisture	Refractometer	The moisture content was determined by means of a refractometer
Electrical conductivity	Conductivimeter	Conductivity was expressed in mS.cm^{-1}.
pH, free, lactonic and total acidity	Automatic Titrator	Four different parameters were performed, namely the pH value of the initial honey solution, and free, lactonic and total acidity obtained by titration.
HMF	Spectrophotometer	The analysis of the content of 5-hydroxymethylfurfural was performed by spectrophotometry. Absorbance was read at 284 and 336 nm.
Proline	Spectrophotometer	The proline analysis was performed by spectrometry. The absorbance was read at 510nm
Diastase index	Spectrophotometer	The diastase activity was performed by the Phadebas method. Absorbance was read at 620 nm.

Results

Table 1. Chemical conductivity, moisture and color.

Samples	Color (mm Pfund)	Moisture (%)	Conductivity (mS.cm^{-1})
EC1	89 ± 0 (Amber)	18 ± 0	0.41 ± 0.02
EC2	91 ± 0 (Amber)	18 ± 0	0.41 ± 0.02
MF1	79 ± 0 (Light Amber)	18 ± 0	0.27 ± 0.01
MF2	77 ± 0 (Light Amber)	18 ± 0	0.30 ± 0.01
J1	35 ± 0 (Extra Light Amber)	18 ± 0	0.37 ± 0.01
J2	35 ± 0 (Extra Light Amber)	18 ± 0	0.37 ± 0.01
J3	35 ± 0 (Extra Light Amber)	18 ± 0	0.37 ± 0.01
EP1	51 ± 0 (Extra Light Amber)	14 ± 0	0.30 ± 0.01
EP2	52 ± 0 (Extra Light Amber)	14 ± 0	0.30 ± 0.01
EP3	51 ± 0 (Extra Light Amber)	14 ± 0	0.30 ± 0.01

Figure 2. HMF, diastase index and proline content

Figure 3. Free acidity, lactonic

Conclusions

The physico-chemical parameters studied showed:

1. All the honeys analyzed have a nectar-bearing origin.
2. The water contents show that all the honeys analyzed comply with the standard proposed by the codex. Less than 20%, disabling fermentation.
3. The pH and acidity results show that all the honeys have high acidity but within the standard limit.
4. HMF results indicated that all honeys examined met the standard required the Codex.
5. Diastase index being in accordance with the required by the European legislation just less than 8 DN%.

Recommendations

Some recommendations for future study are set below:

- The application of statistical analysis to acess differentiation between monofloral honeys using the chemical characteristics;
- Increase the number of honey samples for confirmation of the pattern which was recorded in the Algerian honeys;
- New monofloral honeys from the Algerian flora can be involved in future research.

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The authors are grateful to the Foundation for Science and Technology (FCT, Portugal) for financial support by national funds FCT/MCTES to CIMO (UIDB/00690/2020). National funding by FCT, Foundation for Science and Technology, through the institutional scientific employment program-contract with Sorata L Falcão.

Characterization of Algerian honeys by phenolic compounds LC-DAD-ESI/MS^n analysis: Eucalyptus, Jujube, and Spurge and multifloral

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Honey is a complex hive product produced by Apis mellifera bees, composed mainly by carbohydrates and containing small amounts of other constituents such as minerals, proteins, vitamins, organic acids, phenolic compounds, enzymes, and other phytochemicals [1]. The quality of a honey is correlated with its chemical composition and botanical origin. The phenolic profiles of honeys are determined by their phyto-geographical origin(s), and by the climatic conditions of the collection site [2]. Thus, identification and quantification of the phenolic compounds present in honey is of great interest for its origin assessment.

The aim of this research is to determine the phenolic composition of selected honeys collected from the semi-arid region of Algeria. For that, eleven honey samples, including three from eucalyptus, four from spurge, three from jujube and two from multifloral botanical origin. The phenolic compounds were extracted and analyzed trough liquid chromatography coupled to diode array detection and electrospray ionization mass spectrometry (LC-DAD-ESI/MS) operating in negative ion mode. The analysis of the UV spectra together with the molecular ion identification $[M-H]^-$ and MS^n fragmentation allowed the identification of twenty-two phenolic compounds, among which the most abundant were the abscisic acid isomers (*m/z* 263), *p*-hydroxibenzoic acid (*m/z* 137), *p*-coumaric acid (*m/z* 163), quercetin (*m/z* 301) and pinobanksin (*m/z* 271. The phenolics identified varied quantitatively depending on the botanical origin, with Eucalyptus honey showing the highest content of phenolic compounds.

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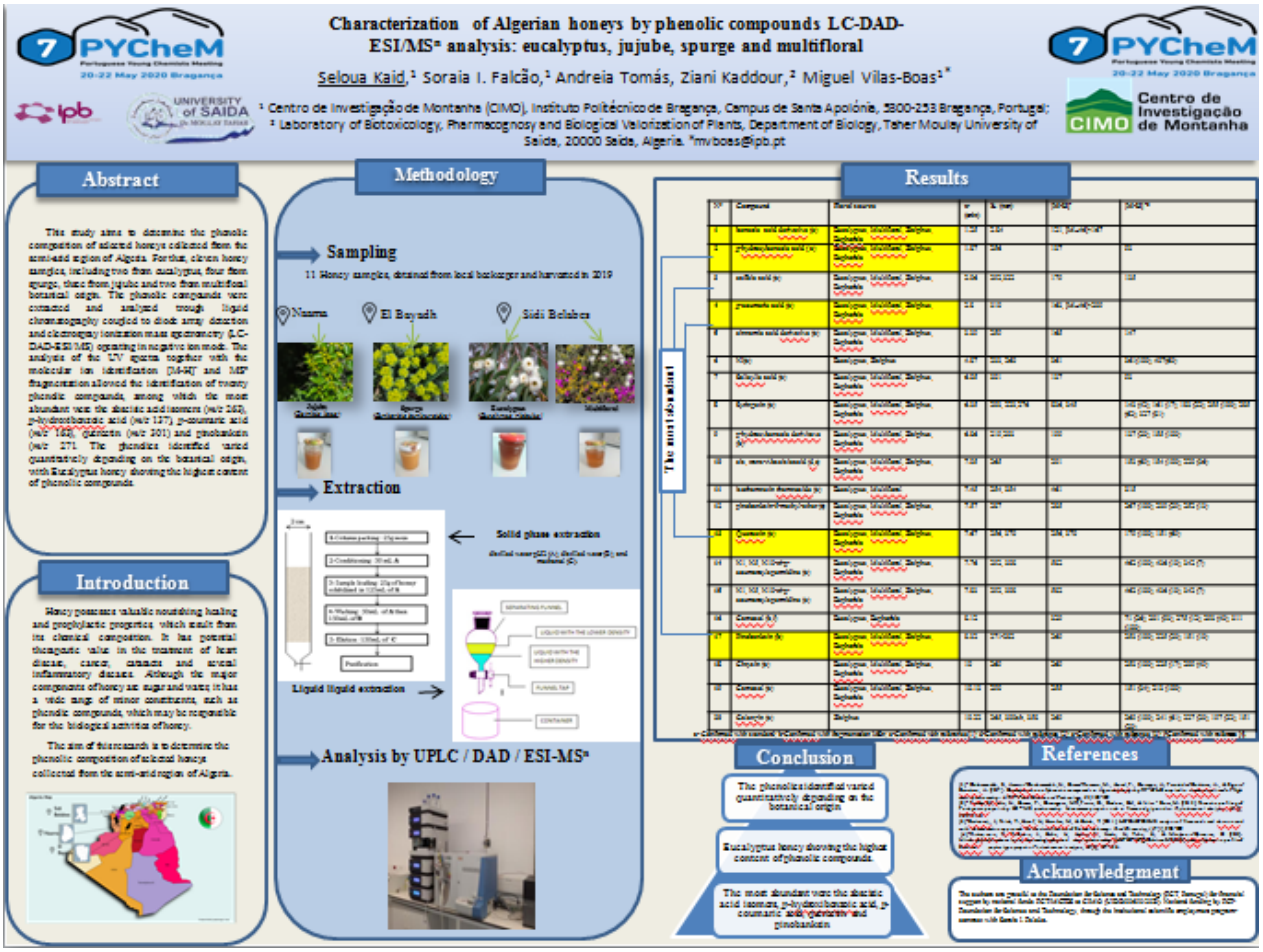
Characterization of Algerian honeys by phenolic compounds LC-DAD-ESI/MSⁿ analysis: eucalyptus, jujube, spurge and multifloral
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Abstract
Introduction
Methodology
Sampling
Extraction
Solid phase extraction
Liquid liquid extraction
Analysis by UPLC / DAD / ESI-MSⁿ
Results
The most abundant
Conclusion
References
Acknowledgment