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CHEMISTRY, BIOLOGY AND POTENTIAL APPLICATIONS OF HONEY BEE PLANT-DERIVED PRODUCTS

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Chemistry, Biology and Potential Applications of Honeybee Plant-Derived Products

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FOREWORD

This book is about relationships: complicated, sophisticated, intriguing, sometimes mysterious but always fascinating and rewarding. These are the relationships between the society of honeybees, the plant world and the human society.

The book is dedicated to bee products of plant origin: honey, bee pollen and propolis (bee glue). The plant-derived bee products are a result of the co-evolution of honeybees and flowering plants, of the activities of two very different types of genetic inheritance, leading to mutual benefits. Much later, a third party appeared in this relationship: humanity, and contributed further to the benefits by beekeeping. Also, curiosity and scientific research have become a part of the relationship, and so the present work is also among the results.

Plant-derived bee products have specific characteristics which set them apart and make their study different, if not more demanding, compared to the study of the bee-synthesized products. Bee products of plant origin deserve such special attention because their study requires a multidisciplinary approach. First of all, there is the phytochemical aspect, revealing the chemical features of the source plant and the product; including the specific phytochemical methods and approaches to bee products. The study of their pharmacological properties, aimed to prove or disprove the numerous anecdotal data about health benefits and healing properties of honey, pollen and propolis, is another important aspect. The combination of the knowledge acquired by these two lines of research leads to the possibility to standardize plant-derived bee products for different purposes. And last but not least, although the use of these products might seem traditional, finding new potential for their innovative applications requires imagination, ingenuity and skills. This book is an attempt to cover the most recent advances in all those aspects.

Being invited to write this Foreword is a great honor. I have spent my professional life studying bee glue and fascinated all these years by the ability of bees to find the chemicals that suit them best in the complex environment they inhabit, in almost every geographic region of the Earth. The authors that have contributed to this book share this fascination; they are devoted and well known experts in their respective fields. The combination of distinct approaches and view points is a merit of the work. All this makes the book particularly valuable to researchers: bee scientists, pharmacologists, phytochemists, but also to anyone interested in bees, bee-plant relationship and apitherapy.

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PREFACE

Domestic bees produce several products including honey, propolis, pollen, royal jelly, beeswax and bee venom, which are essential to their survival and development. Honeybee products have also been used in folk medicine since ancient times and presently constitute one of the most applicable groups of natural products for Humans. While beeswax, bee venom, and royal jelly are chemically synthesized by the bees themselves, the remaining products result from bee´s engineering modification action on plant-derived samples.

The primary usefulness of honeybee plant-derived products to Man is largely based on their utilities in the hive, *i.e.* honey and pollen are stable food sources for bees, with the first being particular enriched in sugars and the latter being an excellent supplement of minerals, vitamins and proteins, whereas propolis is used for the hive protection.

The specific composition of each product is rather variable, depending on the plants found around the hive, as well as on the geographic and climatic characteristics of the site, thus affecting specific biological properties and possible applications. Still, worldwide the usage of such products has been increasing exponentially because of the believed health-benefits of those products. The investigation of the chemical composition and associated biological properties of honey, pollen and propolis has been imperative in elucidating their specific composition and respective potential health benefits, being as well a open door for standardization of the products for distinct usages.

This eBook comprises of comprehensive review on the chemical composition of honey, pollen and propolis of worldwide, complemented with the contribution of distinct analytical techniques for this topic. It also summarizes the current information of relevant biological properties and applications of honey, pollen and propolis, which overall contribute for added-value to these bee plant-derived products. We deeply believe that the present volume is not only important for scientific community, but also for beekepers and readers in general.

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Section I

As mentioned in the preface, the first three chapters of this volume are particularly devoted to the description of the chemical composition of honey, propolis and pollen of wordwilde. The authors focused not only in the main nutritional characteristics of these products, as well as in other non-nutritional constituents that are also relevant for the overall properties of these bee products.

CHAPTER 1

Chemical Characterization of Honey

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Abstract: The chemical composition of honey has been widely investigated around the world. Studies have shown how geographical and botanical origin of honey influences its physical-chemical properties, being a useful tool for evaluation of authenticity and differentiation. In this chapter, a review has been performed in order to describe the chemical composition and the most important nutritional properties of honeys from several countries and diverse botanical origins. Reported data of honeys for water, sugars, ash, minerals, color conductivity, aminoacids and quality indicators, such free acidity, enzymes and hydroxymethylfurfural (HMF) are mentioned and also compared to the established limits given by the Codex Alimentarius and the International Honey Commission.

Keywords: Apiculture, Authenticity, Bioactive compounds, Biological activity, Carbohydrates, Chemical markers, Classification, Color, Differentiation, Electrical conductivity, Flavonoids, HMF, Honey enzymes, Minerals, Moisture, Nutrition, Origin, Phenolic compounds, Quality, Volatile compounds.

1. INTRODUCTION

The Codex Alimentarius defines honey as "the natural sweet substance produced by honey bees from the nectar of plants or from secretions of living parts of plants 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 the honey comb to ripen and mature" [1].

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Susana M. Cardoso (Ed)

Honey market is currently growing worldwide, mainly because consumers have recognized this food as a natural product that provides health benefits. In 2012, honey production was higher than 1.5 million tons and its production in the world is distributed as following: Asia (38.5%), Americas (24.1%), Europe (23.9%), Africa (11.3%) and Oceania (2.2%). The main producers are China, United States, Argentina and Turkey [2].

Honey is not a new product. Since very early in human history, honey was the only source of concentrated sugar and, even, it was already recognized for its therapeutic significance. Many of the myths of the traditional medicinal use of honey have continued even today [3]. Due to the recognition and value-added of honey, unfortunately it is common to find the existence of counterfeit or adulterated products, which have become a constant concern among beekeepers. As a strategy to overcome this problem, several studies have been conducted in order to evaluate, from different points of view, the quality and authenticity of honey [4 - 6].

Bees collect nectars from different flowers, and thus, honey comes from diverse floral sources [7]. According to this, honey can be associated to its botanical or geographical origin, where different kinds of characteristics, such climate or soil, determine the abundance of meliferous flora. Baroni *et al.* and Persano *et al.* [8, 9] suggested that floral origin have an important influence in honey quality. The main componets in honey are carbohydrates, but minor substances such organic acids, proteins, minerals or vitamins can also be found [3].

Several authors reported a moisture content in honey below 20%, reducing sugar content between 60-65% and a content of 1-10% sucrose [10 - 13], characteristics for which no differences can be drawn. Generally, those components present in low concentrations in honey are used to discriminate and detect potential fraud [14, 15].

The regulations of the European Community provides general definitions related to honey, including general and specific compositional characteristics such as hydroxymethylfurfural content, humidity and levels of pesticides, but these parameters have no relation to botanical or geographical origin [9, 16]. Other studies have found that laboratory markers such as volatile compounds, profile of sugars, flavonoids, phenolic compounds, minerals and electrical conductivity are useful to classify and differentiate honeys according to origin [17].

This chapter summarizes existing information regarding the physical-chemical properties, nutritional information and quality of honeys worldwide. Profiles of main components, used for honey characterization as recommended by the International Honey Commission, are described. In addition, the main bioactive compounds are presented. Due to lack of space and the vast amount of information published around the world regarding the composition of honey, it is impossible to report all available articles. Instead, this chapter focuses on the research performed mainly in the past three decades (1985–2014).

2. MAIN COMPONENTS OF HONEY

2.1. Water and Sugars

Honey is composed primarily of water and simple reducing sugars (mainly fructose and glucose), and non-reducing sugars (mainly sucrose and maltose). These parameters depend on many factors, such as the maturity achieved in the bee hive during the harvesting season, climatic and geographic factors, and other elements affecting floral abundance [18].

Water content for worldwide varieties of honey is given in (Table **1**). Bogdanov *et al.* [16] stated that "honey moisture is a quality criterion that determines the capability of honey to remain stable and to resist spoilage by yeast fermentation". Regulation from several countries, European Union and Codex Alimentarius agree to limit the water content in honey to not more than 20%, with some exceptions such as Heather honey for which moisture is allowed up to 23%. In general terms, honeys from around the world fulfill the requirement for moisture content no matter their floral origin.

For instance, Eucalyptus Uruguayan honey is reported to have low levels of water of near 6%; nevertheless, Nigerian honeys can present up to 30%, being more susceptible to spoilage. It is clear, then, this parameter more than a differentiation variable, is a primarily quality criterion.

CHAPTER 2

Latest Developments in Propolis Research: Chemistry and Biology

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Abstract: Propolis is a plant derived bee product which serves dual purposes in the honeybee colony: building material and protective substance. Propolis has been used as remedy in the traditional medicine of numerous nations, because of its antimicrobial, antioxidant, and many other beneficial pharmacological actions. In this chapter, the results of the newest (in the last 5 years) chemical studies of propolis from different geographic and plant origin are reviewed, together with the new identified source plants: 152 new constituents of propolis, being 57 new chemical entities, and 12 new chemical types of propolis are listed. The importance of propolis for the bee colony is discussed, with special attention to the activity of propolis and its constituents against bee pathogens and parasites. The review of recent propolis literature demonstrates its potential to serve as a source of new chemical structures and new bioactive compounds due to its chemical diversity. It also reveals the potential of propolis to be used for development of innovative products, mainly in the field of food industries, animal husbandry, and beekeeping. For this to happen, the combined efforts of researchers and technologists from different areas are necessary, in order to make better use of bee glue.

Keywords: *Apis mellifera*, *Ascophaera apis*, Bee health, New constituents, New sources, *Paenibacillus*, Plant sources, Propolis, Social immunity, *Varroa destructor*.

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Susana M. Cardoso (Ed)

1. INTRODUCTION

Propolis (bee glue) is a plant derived bee product that serves dual purposes in the honeybee colony. It is a building material, used by worker bees to cover the internal surface of the cavity they inhabit (the so-called "propolis envelope" in the nests of feral bees [1]), to block holes and cracks, to tighten the hive entrance in cold season, etc. On the other hand, propolis is a protective material, guarding the colony against infections: it contains putrefaction of insect, small animals – intruders killed in the hive and too large to be carried out. It is an important element of the social immunity of honeybees [2].

Bees collect resinous materials from different plant parts (lipophilic materials on leaves and leaf buds, mucilages, gums, resins, latices, etc.) [3], and mix these materials with wax to produce propolis. In order to come to the resinous materials, in some cases bees cut fragments of vegetative tissues to release the resin [4]. Bees incorporate in propolis protective plant excretions, which prevent vulnerable plant tissues from infection by harmful microorganisms. They make use of the biosynthetic potential of plants and apply the secondary plant metabolites in the resins for the same purpose as the source plant: for protection. Thus, the action against microorganisms is an essential property of propolis, and this fact has been recognized by human beings since times immemorial. Propolis has been used a remedy in the traditional medicine of numerous nations, mainly to treat burns and wounds, sore throat, stomach ulcer, *etc*. [5]. Modern science has revealed many other beneficial pharmacological properties of bee glue [6, 7].

The chemical composition of propolis varies significantly depending on the particular resin used by the honeybees in propolis production; at different locations with specific climatic and phyto-geographic conditions the chemistry of propolis differs dramatically. However, it took some time for researchers to realize this peculiarity of propolis. The paradigm shift occurred about the turn of the 21st century. It became clear that unlike beeswax or bee venom, from chemical point of view there is no single product that is "just propolis". This resulted in a new approach to propolis research, and especially in studying the biological activity of propolis. It was understood that it was not enough to report that experiments were performed with propolis, but that it was necessary to chemically characterise the particular propolis used in the experiments [8].

Although of different chemical composition, propolis from different locations always demonstrates considerable biological activity [9, 10]. For this reason, the chemical diversity of different propolis samples has the potential to provide valuable leads to active components. The study of bee glue in unexplored regions offers possibilities to uncover new biologically active compounds with important pharmacological effects, especially antibacterial, antioxidant and anticancer substances. In this chapter, the recently discovered new propolis constituents (since 2009), including such coming from newly found source plants will be discussed.

2. GENERAL PHYSICO-CHEMICAL PROPERTIES OF PROPOLIS

Propolis is a sticky material; its colour is variable and depends on the plant source: brown, brown-yellow, yellow, green, brown-orange, red. Its smell also varies with the botanical origin but is in general pleasant, balmy or resinous; the taste is bitter and pungent. At temperatures lower than 15°C propolis is hard and brittle, while over 30°C it is soft, flexible and very sticky. Important characteristic of propolis is the amount of substances soluble in 70% ethanol, called “balsam”. Its content can be between 20 and 80%. The balsam contains mainly the plant derived bioactive compounds [5]. Besides the resins collected from plants, propolis contains also variable amounts of beeswax, pollen, mechanical impurities, up to 2% minerals.

The percentage of beeswax varies significantly: values between 5 and 49 % have been reported in the literature [11, 12]. Moreover, the wax amount depends on the harvesting method: the use of a propolis collector usually reduced the wax content below 20% [13].

Mechanical impurities consist of wood particles, remains of dead bees, moth cocoons, plant parts, etc. Their amount is variable too; differences in plant sources could explain the variations. In general, green Brazilian propolis is characterized by much higher percentage of mechanical impurities, up to 40%, compared to poplar type propolis (usually less than 10%). This could be due to the way bees collect resins from *Baccharis dracunculifolia* plants to produce green propolis:

CHAPTER 3

Chemical Composition of Bee Pollen

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Abstract: Bee pollen, usually used as an important source of nutrients and micronutrients for the young bees in the hive, is also an important food for humans. This product is very rich in proteins, lipids, free sugars, carbohydrates, and it contains trace amounts of minerals, phenolic acids, flavonoids and a good range of vitamins. A brief look at bee pollen composition, it is easily recognised that it is a balanced food that can be used as a stand-alone food or as a nutritional supplement or even as a medicinal product. Several bioactivities, due to some of these compounds, were studied in bee pollen samples from different floral sources and the results conduce to important properties. The amount and diversity of micronutrients could induce vast benefits if used for health purposes following a complete risk assessment. Nevertheless, the results pointing towards the encouraged use of bee pollen, the risk assessment of some floral species containing toxic compounds has not been fully studied to insure the safety of consumption for all the gathered flowers, so this will also be discussed in this chapter. Admiration for its goodness and medicinal properties, bee pollen has been consumed for centuries, however, currently the efficacy and safety for all consumed products, foods, supplements or medicines is an important tool to guarantee correct quality control and essential to add value to the product.

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To summarise, in this chapter we will put the situation of gaps in bee pollen research into some kind of perspective, outlining some important points and discussing in more depth the implications of collecting samples, chemical composition and risk assessment.

Keywords: *Apis mellifera*, Chemical composition, Collecting, Dietary product, Food, Gametophyte, Medicinal product, Micronutrients, Nutrients, Nutritional supplement, Pollen, Risk assessment.

1. INTRODUCTION

Bee pollen is flower pollen collected by the honey bee, *Apis mellifera,* for the purpose of feeding its larvae in the early stages of development. Collected flower pollen is accumulated as pellets (corbicular pollen) in pouches on the rear legs of the bee and it is the mixture of these pellets that comprises bee pollen.

Pollen itself is the male gametophyte in flowers. The female gametophyte produces nectar, a sweet liquid gathered and eaten by insects and other animals. This important substance, rich in sucrose and water, is the optimal environment necessary for the germination of the pollen tube and the release of its DNA in the female organ. For the fertilization of the flower minerals are also needed. We also suppose that polyphenolic compounds can be important in this step, perhaps in an allelopatic way, as they are species-specific [1].

Bees are very selective in the flora they choose to collect pollen. In fact some research has pointed out that for each *genera* they prefer only certain *species*, sometimes probably only one [2]. Spontaneous plants are the main floral resources selected. In any case they also pollinate some breeding plants such as the fructiferous trees. For example, bees can be "forced" to pollinate kiwis (*Actinidia deliciosa*), but once these insects have found a better source of pollen from a spontaneous plant, they leave the fields. In fact kiwis are genetically modified and they do not have any mate in nature to do the pollination.

Genetically modified (GM) plants are an important issue associated to bee pollens because their impact on bees, as on Humans is still unclear. Theoretically, bees do not visit breeding plants, but in practice, for example, they collect maize (*Zea mays* L.) pollen that is one of the major genetically modified plants. In these plants the introduction of *Bt* toxin from *Bacillus thuringiensis* is mainly in pollen and the impact on insects and humans is still controversial. *Bt* toxin is one of many microbial pesticides. Its formulated fermentation cultures can be sprayed on foliage to control selected insects because the ubiquitous bacterium synthesizes a toxic protein, known as the delta-endotoxin, every time it stops growing and produces a spore [3, 4]. The insecticidal gene that molecular biologists moved into corn, cotton, or potatoes is actually a truncated version of the natural gene. For the gene to function in plant cells, small snippets of DNA are attached that allow the code to be read. To track the location of the gene and to help select plant cells that have successfully incorporated the gene into their chromosome, marker genes encoding for either antibiotic or herbicide resistance are also spliced onto the toxic protein gene. We need to know how much protein we might be exposed to when eating food made from transgenic corn [4]. This is also true for bee pollen from maize that is commercialized for human consumption. Fifteen years ago researchers studied the sensibility of *Apis mellifera* and in the protocol they concluded that *Bt* toxin did not affect bees [5]. Nevertheless up until now, we have considered all the results in this field as preliminary and a further wait is necessary to see what will happen. Those toxins are the compounds that can be found in pollen, so reporting on these aspects is the new point that needs to be highlight. In the meantime humans continue to use the product in simultaneous to these evaluations.

2. BEE POLLEN COLLECTING FOR HUMAN INTAKE

The definition of "Bee pollen" is the result of the agglutination of flower pollens, made by the worker bees, with nectar (and/or honey) and salivary substances, which is collected at the entrance to the hive [6].

The concept of honeybees is usually associated to honeybees collecting nectar, however there are many other import products collected from the hive. Pollen is a crucial part of the honeybees´ diet, providing a wide range of nutrients namely protein, lipids, carbohydrates, vitamins, and minerals, *e.g.* pollen is the major source of amino acids in their diet, although it does have many other constituents. Before discussing some of them in detail we will give an overview on pollen

Section II

In this section, emphasis will be given to the analytical techniques of chromatography, nuclear magnetic resonance, electrochemical sensors and infrared spectroscopy as tools for the improvement of knowledge of the chemical composition of honeybee plant-derived products, or even to discriminate samples from different botanical or geographical origins or eventually for targeting adulterations.

CHAPTER 4

Chromatography as a Tool for Identification of Bioactive Compounds in Honeybee Products of Botanical Origin

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Abstract: Honey, propolis, and pollen are three important components of the beehive produced by honeybees mixing different plant parts (nectar, resin and pollen) with their own secretions, for further usage with different purposes in the hive. The fact that these natural products have been associated with numerous health benefits has attracted the attention of researchers resulting in a significant raise of scientific studies attesting their biological properties. Among the various constituents of honey, propolis and pollen, the phenolic compounds are the ones most frequently related to the beneficial properties of these products and hence, one of the main investigated groups. Their characterization is important to understand individual contribution(s) and synergistic effects of each compound for the overall biological effects of the bee product. To pursuit this goal, spectrophotometric techniques including HPLC, GC and TLC, alongside with the respective detection methods such as DAD, FLD and MS, have been developed and improved in order to offer better and more accurate separative performances.

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The aim of this review is to give an approach on the course that the chromatographic techniques have taken until the most recent trends on this field applied to the separation and characterization of the phenolic constituents of honey, propolis and bee pollen as well as an overall perspective of variability in terms of phenolic composition that can be found in the three bee products mentioned.

Keywords: Bee pollen, Benzoic acids, Bioactive compounds, Caffeic acid derivatives, Chromatography, Cinnamic acids, Coumaric acids, DAD, Flavonoids, FLD, GC, Honey, Honeybee-derived products, HPLC, MS, Phenolic compounds, Propolis, TLC.

1. INTRODUCTION

Honey, propolis, and bee pollen are produced from nectar, resin and floral pollen, respectively, mixed with different bee secretions and further used in the beehive for distinct purposes [1, 2]. Notably, these three bee products have also been used for centuries by Men, for food and medicinal purposes [3 - 5].

More recently, Men´s interest for these natural products have significantly raised, since scientific studies have attested their abundance on nutrients and bioactive compounds, together with their association with beneficial properties, including those of cardio-, neuro-, hepato- and chemo-protective, as well as chemo-preventive, antiseptic, antimicrobial, anti-allergic, antioxidant, anticancer, anti-radiation, anti-inflammatory and wound-healing activities, among many others [6 - 9]. Hence, overall, honey, propolis and pollen are now envised as very tempting and useful for a large spectrum of applications in different industries including foods, cosmetics, perfumes and pharmaceutics [7].

Among the numerous compounds from honey, propolis or bee pollen, the phenolic compounds are undoubtedly more frequently associated with the beneficial properties of these products [4, 5, 10, 11]. These compounds are a class of metabolites that are ubiquitously distributed through plant kingdom and plant-derived products [12], where they are important players in growth and reproduction, providing protection against pathogens and predators, besides contributing towards the color and sensory features of fruits, vegetables and their derived products [13].

Chemically, all the phenolic compounds possess at least one phenyl ring in its structure, and most of them arise from a common origin: the amino acids phenylalanine or tyrosine. These amino acids are deaminated to give cinnamic acids, further entering the phenylpropanoid pathway where one or more hydroxyl groups are added to the aromatic ring(s), ranging from simple phenols to complex compounds generally known as polyphenols or phenolic compounds [14]. The most common examples of phenolic compounds that can be found in foods include the phenolic acids (C_6-C_1), cinnamic acids (C_6-C_3) and flavonoids (C_6-C_3-C_6) [13] (Fig. **1**) and hence, in general these are also important groups of phenolic constituents of honey, propolis and bee pollen.

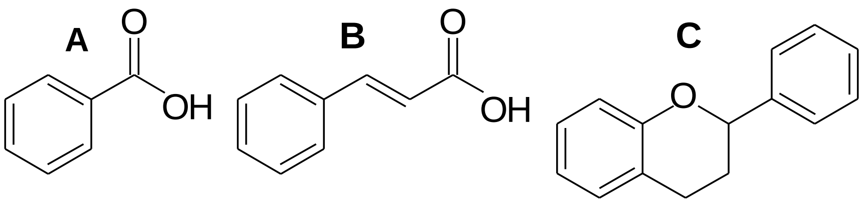

Fig. (1). Basic structure of phenolic acids (**A**), cinnamic acids (**B**) and flavonoids (**C**).

2. CHROMATOGRAPHIC METHODS

The close association between the beneficial properties of honey, propolis and pollen with their phenolic constituents boosted the need for characterizing them so that individual contribution(s) and synergistic effects on their biological activities can be elucidated.

Spectrophotometric assays, including Folin-Ciocalteu and Folin-Denis, for determination of the total phenolic content in plant samples, or reaction with $AlCl_3$ for total flavonoids measurement, are simple and economical, and can be useful for rapid and relatively inexpensive screening of numerous samples. However these techniques only give an estimation of the concentrations of the phenolic compounds over a certain minimum level and do not quantify phenolics individually. Besides, these reagents do not react specifically with phenols, since cross reactions commonly occur in complex samples and hence, unreliable data can be generated [15].

CHAPTER 5

Valuable Analytical Tools in Analysis of Honeybee Plant-Derived Compounds: Nuclear Magnetic Resonance Spectroscopy

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Abstract: Over the past fifty years, Nuclear Magnetic Resonance (NMR) spectroscopy proved to be a powerful tool in the structural characterisation of bioactive compounds from natural sources. In this chapter we cover the basic theory behind each NMR technique used to determine the structure of several families of compounds (*e.g.* carbohydrates, phenolics and sesquiterpenoids) present in honey and propolis. We also provide basic information how 1D and 2D NMR techniques can help in the structure establishment of honeybee constituents. The ^{1}H and ^{13}C NMR data of several of these constituents are compiled and described, being some of them used as botanical and geographical markers. In the case of propolis, a list of compounds identified by NMR is presented. A basic overview in quantitative NMR determinations and in NMR coupled to chemometric methodologies highlights their use to detect honey adulteration and assign their authenticity.

Keywords: Adulteration, Authenticity, Botanical marker, ^{13}C NMR, COSY, DEPT, DOSY, Geographical marker, HMBC, HMQC, ^{1}H NMR, Honeybee, HSQC, Natural products, NMR spectroscopy, NOESY, Propolis, ROESY, Structure elucidation, TOCSY.

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1. INTRODUCTION

The most important tools used by chemists for the structural elucidation of natural and synthetic compounds are based on spectroscopic techniques, being NMR spectroscopy a useful resource on a routine basis. The data provided by 1D NMR techniques, such as ^{1}H and ^{13}C NMR experiments, is the first step to achieve this goal. The complexity of molecular structures implies the use of more advanced 1D and 2D NMR techniques for the complete assignment of proton, carbon or other nuclei resonances. It is a non-destructive technique and with the greatest advances over the past years, excellent results can be obtained from samples weighing less than a milligram.

Thus, a brief discussion on the basic concepts of the commonly used NMR experiments from the point of view of structural elucidation of organic compounds is required to highlights the usefulness of these spectroscopic techniques.

The ^{1}H nuclei is the most commonly observed nuclei in NMR spectroscopy and is the starting point for most structure determinations. Valuable information can be obtained from a ^{1}H NMR spectrum: the chemical shift (δ, ppm; using TMS as standard) that is correlated with its chemical environment; the coupling constant (J, Hz) that are determined by the interactions between individual nuclei and promoted by electrons in a chemical bond and finally, under suitable conditions, the area of a resonance that is related to the number of nuclei giving rise to the ^{1}H NMR signal [1, 2].

The ^{13}C NMR spectrum offers further characterization of a molecule and is directly related to the carbon skeleton. Typically, this spectrum is recorded with broadband decoupling of all protons, appearing each carbon resonance as a single line (δ, ppm; using TMS as standard) [1, 2]. The chemical shift of each resonance is once more indicative of their environment, being possible to identify certain functional groups that are not detected in a ^{1}H spectrum (*e.g.* carbonyls).

The ^{15}N NMR spectrum although having great importance for structural NMR analysis, since *N*-containing functional groups and N atoms are present in several molecular skeletons, the low natural abundance of ^{15}N (about 0.4%) and its extremely low relative sensitivity make difficult these measurements [1, 2]. The typical range of ^{15}N chemical shift is about 600 ppm and the recommend reference is nitromethane (δ = 0 ppm), although these values can also be quoted with respect to saturated aqueous solution of ammonium chloride (δ = - 359.5 ppm) or ammonium nitrate (δ = - 3.9 ppm). Unfortunately, the analysis and comparison of the reported data is many times hampered due to the lack of information of the reference standard used.

The ^1H-^1H COSY (COrrelation SpectroscopY) spectrum provides a means of identifying mutually coupled protons (typically geminal and vicinal couplings), allowing the assignment of all proton resonances [1, 3]. In this technique, 1D spectrum is displayed along each axis with a contour projection of this spectrum along the diagonal axis. Off-diagonal peaks represent proton shift correlations (or proton couplings). A related COSY technique, DQF-COSY (Double Quantum Filtered-COrrelated SpectroscopY), is sometimes used to simplify the diagonal of the COSY spectrum where the peaks are greatly reduced in intensity with consequent clarification of this region. A further advantage of DQF-COSY is that in the phase sensitive mode, both diagonal and cross peaks can be adjusted to have a pure absorption line shape. A similar spectrum to ^1H-^1H COSY can be obtained by a TOCSY (TOtal Correlated SpectroscopY) experiment. The correlations observed between all the protons within a given spin system is irrespective of whether they are directly coupled or not. This technique is quite useful in severe resonance overlapping, in which ^1H-^1H COSY spectra can leave to ambiguous assignments.

NOE (Nuclear Overhauser Effect) difference experiment provides information about the spatial proximity of two protons within a molecule [1, 3]. It involves the application of a radio-frequency field to a single resonance in which the corresponding protons become saturated (it means that the difference of population between their high and low energy levels are forced to zero). Recording of the proton spectrum after the period of saturation may, therefore, show changes in signal intensities for the protons in the vicinity of the saturated proton. Thus, 1D NOE studies generally presents the differences between the original proton spectrum and the irradiated proton spectra. In the 2D NOESY (Nuclear Overhauser Effect SpectroscopY) experiments, only one spectrum is

CHAPTER 6

Electrochemical Sensors for Assessing Antioxidant Capacity of Bee Products

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Abstract: This chapter is focused on the application of electrochemical techniques (*e.g.*, sensors and biosensors), as the predominant methodology, to the quantification of individual or total phenolic compounds, either in standard solutions or in real matrices (*e.g.*, plants, fruits and beverages) and their capability for assessing antioxidant activity/capacity. Specially, the potential application to evaluate antioxidant capacity of bee-hives products (*e.g.*, propolis, honey) is addressed. Finally, the voltammetric behavior of Portuguese monofloral honeys is discussed for the first time, taking into account the expected effects of honey color and floral origin.

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Also, a possible relation with the expected antioxidant capacity of honeys is discussed, considering their floral origin. Works describing the use of electrochemical detection imbibed on liquid chromatographic or capillary electrophoretic configurations among other analytical methods will not be focused in this review, although their undoubtedly potentials and proved applications.

Keywords: Antioxidant activity, Bee products, Cyclic voltammetry, Differential pulse voltammetry, Electrochemical techniques, Honey, Phenolic compounds, Pollen, Propolis, Square wave voltammetry.

1. INTRODUCTION

Honey, propolis and pollen are plant-derived products and a source of polyphenolic compounds. Honey has a high content of sugars and small amounts of minerals, proteins and other constituents namely flavonoids, phenolic acids, enzymes, amino acids and vitamins [1, 2]. In the case of honey, the total phenolic content appears to be strongly correlated with the antioxidant activity, being the highest values found in darker honeys [1, 3 - 6]. Propolis is a resinous material, which pharmaceutical properties are well known and attributed to the high contents in polyphenols [7] (flavonoids, phenolic acids and their esters), as well as terpenoids, steroids and amino acids [8]. Bee pollen also contains considerable amounts of phytochemicals and nutrients, being rich in carotenoids, flavonoids and phytosterols [9]. The floral and geographical origins affect greatly the quantity and composition of polyphenol compounds in these bee's products, which reflects on the antioxidant capacity of each product [1, 8].

Electroanalytical methods are well-known tools used to study chemical and biological systems, allowing evaluating the antioxidant capacity of compounds that act as reducing agents, like phenolic compounds, which are easily oxidized on the surface of electrodes. These methods have several advantages when compared to other more traditional techniques (for example, spectrophotometric methods) since, they are simple methodologies, have low detection limits, good selectivity, reduced time of analysis, low consumption of reagents, having an overall lower environmental impact. Since, the electrochemical signals are due to the presence of analytes with electrical properties (antioxidants), it is not necessary to generate or use oxidized species. Among the electroanalytical techniques, three voltammetric methods have been particularly reported as fast screening tools for assessing the composition quality and bioactivity of samples: cyclic voltammetry (CV), differential pulse voltammetry (DPV) and square-wave voltammetry (SWV). These techniques use electrolysis conditions to study the phenomena occurring between the electrode surface and the thin solution layer in contact, differing in the way that potential is applied [10 - 13]. They allow to obtain quantitative and qualitative information of chemical species during the electrolysis using the current-potential curve generated (*i.e.*, the voltammograms).

In the literature, some works report the direct application of electrochemical techniques for bioactivity assessment and/or quality evaluation of bee-hive products, being mainly focused in honey and propolis analysis. Indeed, to the best of the authors' knowledge there is no study concerning pollen evaluation. Despite not being widespread used [14], these analytical techniques are an attractive approach to characterize compounds that act as reducing agents in natural products, being envisaged an increase of their application in a near future for bee products analysis.

2. VOLTAMMETRIC TECHNIQUES: GENERAL CONCEPTS

The voltammetric techniques that use electrochemical cells are based on two or three electrodes, immersed in a solution, which allows the movement of ions by charge transfer (electrolytes). The electrodes (metals or semiconductors, solid or liquid) allow the charge transfer through the electrons movement.

2.1. Electrochemical Cells

The electrochemical cells can be galvanic or electrolytic cells. In the galvanic cells, the reactions occur spontaneously in the electrodes converting the energy generated in a chemical reaction into electrical energy. Applying a potential to the cell beyond the potential of the reversible reaction, the reaction direction is changed, being possible the conversion of electrical energy into chemical energy. In these conditions, it is an electrolytic cell. The electrolytic cells allow studying the reduction (electron capture) and oxidation (release of electrons) phenomena, in general, under the action of an external controlled potential, enabling to control the reaction's direction and their intensity. So, accurate information about the

CHAPTER 7

Infrared Spectroscopy as a Valuable Tool for the Analysis of Honey Bee Plant-Derived Products

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Abstract: The importance of honey has been recently promoted due to its nutritional, pharmaceutical and therapeutical characteristics. In recent years, the combination of novel and rapid instrumental techniques based on infrared spectroscopy [mid infrared (MIR), near infrared (NIR)] combined with multivariate data analysis has resulted in the development of both qualitative and quantitative methods for the analysis of honey and bee products. The most important applications of these technologies in honey have been associated with authenticity, discrimination or traceability issues. However, few reports can be found on the use of both NIR and MIR to quantitatively analyse honey composition and less information is available for the analysis of other bee products. This chapter aims to describe and discuss different applications on the use of NIR and MIR spectroscopies to analyse honey, pollen and bee derived products. A brief description of some qualitative applications will be also discussed.

Keywords: Adulteration, Artificial neural networks, Authenticity, Chemometrics, Composition, Fourier, Glucose, Honey, Infrared, Mid infrared, Multivariate data, Near infrared, PLS, Pollen, Principal component, Propolis.

1. INTRODUCTION

The importance of honey has been recently promoted due to its nutritional, pharmaceutical and therapeutical characteristics [1]. In order to maintain high quality standards in the honey industry analytical methods are needed.

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Therefore, effective quality control methods like high performance liquid chromatography (HPLC), gas chromatography (GC), mass spectrometry (MS), thin layer chromatography (TLC), enzymatic tests and physical tests, nuclear magnetic resonance (NMR), mid infrared (MIR), and near infrared (NIR) have enriched the diversity of analytical tools available to monitor and analyse honey and derived products [3 - 6].

In recent years, the combination of novel rapid instrumental techniques with multivariate data methods (MVA) has resulted in the development of rapid and inexpensive methods of analysis. Techniques such as near infrared and mid infrared have been the most widely used due to their intrinsic characteristics (*e.g.* low cost, non-destructive) in order to qualitatively and quantitatively analyse several food matrices, including honey [4 - 8]. This chapter aims to describe and discuss different applications on the use of NIR and MIR spectroscopy to analyse honey, pollen and bee derived products. A brief description of some qualitative applications will be also discussed.

2. INFRARED SPECTROSCOPY AND MULTIVARIATE ANALYSIS

Infrared radiation (IR) lays between the visible (VIS) and the microwave wavelengths regions of the electromagnetic spectrum. The nominal range of wavelengths for NIR range is between 750 and 2,500 nm (13,400 to 4,000 cm^{-1}), while for the MIR, the spectral range is from 2,500 to 25,000 nm (4,000 to 400 cm^{-1}) [4 - 8]. In this wavelength range, solid, liquid or gaseous samples can absorb some of the incoming infrared radiation at specific wavelengths or frequencies resulting in a ‘fingerprint’ or spectrum of the sample [5 - 8]. Infrared spectroscopy is the absorption measurement of different IR frequencies by a sample positioned in the path of an IR beam (*i.e.* NIR and MIR beams); when the frequency of a specific vibration is equal to the frequency of the IR radiation directed at the molecule, this molecule absorbs the radiation [5 - 8].

Mid-infrared spectroscopy allows structural elucidation and compound identification; functional groups absorb photons at characteristic frequencies of MIR radiation and include mainly bands that come from stretching and bending fundamental vibrations. Stretching vibrations are those where the distance between atoms decreases or increases while atoms remain in the same bond axis [5 - 8]. However, in order to evaluate the composition of a complex food sample like honey and honey products, spectral interpretations should not be limited to one or two bands where the whole spectrum needs to be taken into consideration. In NIR the overlapping of many different overtone and combination vibrations results in broad bands with low structural selectivity in NIR spectra compared with MIR spectra where fundamentals are more resolved, allowing the structure of a sample to be better elucidated [4 - 8]. NIR spectroscopy is widely used to determine organic matter constituents and it is based on the absorption of electromagnetic radiation by a sample at wavelengths in the 800-2500 nm range. NIR spectra are composed of broad bands arising from overlapping absorption corresponding mainly to overtones and combinations of vibrational mode C-H, N-H, and O-H chemical bonds [4 - 8]. Overtones correspond to energy transitions that are higher than those for fundamentals and the frequencies of first and second overtones correspond to about two or three times that of the fundamentals [4 - 8]. Combination bands result from transitions involving two or more different vibrational modes of one functional group occurring simultaneously; the frequency of a combination band is the sum or the multiples of the relevant frequencies [4 - 8]. In addition the existence of combination bands (*e.g.* C-O stretch and N-H bend in protein), gives rise to a crowded NIR spectrum with strongly overlapping bands. A major disadvantage of this characteristic overlap and complexity feature of the NIR spectra has been the difficulty of quantification and interpretation of the spectra without the use of MVA methods. On the other hand, the broad overlapping bands can diminish the need for using a large number of wavelengths in calibration and analysis routines [4 - 8].

The combination of MVA with analytical instruments such as NIR or MIR spectroscopy provide with the ability to determine more than one component at a time [1 - 8]. The use of MVA also provides with the ability to detect patterns in a given data set as well as with different algorithms that can be used to develop mathematical models to predict or monitor composition or other quality characteristics (*e.g.* origin, traceability) [4 - 8]. Multivariate data or chemometrics covers quite a broad range of methods such as exploratory data analysis, pattern recognition (PR), and statistical experimental design (DoE) [1 - 6]. The most

Section III

The following chapters are focused on relevant bioactive properties presently attributed to bee products of botanical origin *i.e.,* antioxidant, anti-inflammatory, anti-tumoral and antimicrobial. Naturally, because of the variable chemical composition of honey, propolis and pollen from distinct geographic regions, biological properties also show variations, overall hampering the understanding of their potencialities. This highlights the need of standardization of beeproducts, so that conditions under which such natural products may promote health can be established.

CHAPTER 8(I)

Antioxidant Properties of Bee Products of Plant-Origin. Part 1. Honey

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Abstract: The study of antioxidant-antiradical activity of food products has received a rising interest since last decades, parallel to the boom of functional foods and healthy consumption trends, and to the increasing number of scientific evidence linking this physicochemical property to prevention of several degenerative diseases, in particular of cancer. Honey belongs to the category of natural foods showing high antioxidant activity, which depends largely on its botanical/geographical origin. Different studies have been conducted to describe the antioxidant activity of honey by both *in vitro* and *in vivo* techniques, which are reported in a vast extension of articles. However, the lack of a standard protocol for measuring the antioxidant activity has been one of the main drawbacks found. Techniques such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), Trolox Equivalent Antioxidant Capacity (TEAC), Ferric Reducing Antioxidant Power (FRAP) and Oxygen Radical Absorbance Capacity (ORAC) are the most common *in vitro* methods. It has been suggested to use at least two techniques for measuring antioxidant activity, since these are only an approximation to what occurs in the body. It is known that biologically active ingredients which may contribute to the antioxidant effect of honey include vitamins, minerals, organic acids, flavonoids, phenolic compounds and even products derived from Maillard reaction.

Keywords: Antiradical, Bioactive compounds, Biological activity, DPPH, Flavonoids, FRAP, Free radical, Honey enzymes, Honeybee products, Maillard reaction, Minerals, Natural foods, ORAC, Origin, Oxidative stress, Phenolic compounds, Scavenging capacity, TEAC, Vitamins.

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1. INTRODUCTION

Since recent decades, there has been an increase in degenerative or chronic diseases such as diabetes mellitus, hypertension, cancer, Alzheimer's disease, atherosclerosis and heart disease as a consequence of oxidative stress [1 - 3]. Sies and Jones [4] stated that oxidative stress could be defined as "an imbalance between oxidants and anti-oxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage". It is caused by a concentration of reactive oxygen species (ROS) or reactive nitrogen species (RNS) higher than antioxidants. ROS include superoxide ($O2^{\bullet-}$), hydroxyl ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2), while RNS comprise nitric oxide ($NO^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet-}$) and peroxynitrite ($OONO^-$) [4, 5]. Several authors [5 - 7] commented the ability of cells to scavenge excess reactive species is largely dependent on the efficiency of the overall antioxidant defense system.

Halliwell and Gutteridge defined an antioxidant as "any substance that delays, prevents or removes oxidative damage to a target molecule" [5]. The overall antioxidant defense network consists of endogenous and exogenous antioxidants. The endogenous antioxidants comprise the enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) and non-enzymatic antioxidants including glutathione (GSH), vitamins C and E, and small molecules [4, 5]. The exogenous antioxidants comprise the micronutrients present in foodstuff [7].

Although there are a wide variety of natural antioxidant substances, the best sources of antioxidant compounds seems to be those from plant origin [8]. Some studies [9, 10] demonstrated that medicinal aromatic herbs, fruits and leaves of some berry plants, can biosynthesize phytochemicals possessing antioxidant activity and they may be used as a natural source of free radical scavenging compounds. Since the majority of these plants are used by the bees to collect honey nectar, bioactive components can be also found in bee products of plant-origin. For this reason, these products are recognized as a rich and natural source of bioactive compounds with potential antioxidant activity, similar to some fruits and vegetables [11].

Several methods for antioxidant activity evaluation have been carried out in order to assess potential biological effects of honey, propolis or pollen extracts. Often, as a first approach, the antioxidant potential of extracts is directly evaluated using chemical models, which in the case of hive products (as in general) include 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, ferric reducing antioxidant power (FRAP) assay, β-carotene bleaching assay, inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS) [12], allowing to cover the different mechanisms of action of antioxidants, either by transfer of hydrogen atoms or electron transfer. However, as many of these methods are based on radicals not found in biological systems (that hinders their transposition to *in vivo* systems), many studies also focused on absorption and on the impacts at biological level, in particular oxidative stress through biological markers, by using cell lines and *in vivo* models. In these tests, the antioxidant activity of extracts is measured through assessing the activity of antioxidant enzymes such as GPx, SOD or CAT at the cellular level, often accompanied by monitoring lipid oxidation (*e.g.* malonaldehyde, isoprostanes, *etc*), protein oxidation (protein carbonyls) and DNA damages (8-oxo-2-deoxyguanosine) [13].

Nevertheless, the lack of a validated assay to measure the antioxidant capacity difficults the comparison of antioxidant activity data when bee products of plant-origin are analyzed, and even when investigators use the same method, different modifications to the technique are often introduced. Thus the results among samples from different origins, even if they are of the same botanical species, are hard to compare [14, 15]. Different reviews have been published discussing the chemistry of antioxidant assays or the advantages/disadvantages of each method [16 - 18]. Dezmirean *et al.* mentioned that "choosing the correct method depends on the food matrices due to the nature of biological antioxidants present in the sample to be analyzed (enzymatic, nonenzymatic, biological or just dietary)" [19].

2. ANTIOXIDANT PROPERTIES OF HONEY

Honey, as a source of antioxidants, has been proven to be effective against deteriorative oxidation reactions in food, such as enzymatic browning of fruit and vegetables [20, 21], lipid oxidation in meat [22, 23], and to inhibit the growth of

CHAPTER 8(II)

Antioxidant Properties of Bee Products of Plant-Origin Part 2. Propolis and Pollen

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Abstract: Over the last years, the hive products such as propolis and pollen have been highlighted due to their potential health benefits, including antioxidant abilities that have been correlated with their content in phenolic compounds. Regardless of the several factors that may affect propolis and pollen antioxidant activity, these products have been shown to possess, either through the use of *in vitro* or *in vivo* models, important features concerning the modulation of cellular oxidative stress caused by environmental factors (*e.g.* UV-light), metals, pesticides and other xenobiotics. This modulatory effect focus not only on the capture of radicals that these elements might eventually generate, but also by the activation of cellular antioxidant mechanisms such as enzymatic antioxidants or by modifying gene expression patterns. Although the mechanisms behind these responses are not fully known, it has been showed that caffeic acid phenethyl ester, pinocembrin and chrisin are some of the compounds responsible for some of these responses. Taking into account the gathered results, propolis and pollen can be viewed as potential agents in the re-stabilization of cellular oxidative imbalance and in the prevention of oxidative stress related diseases.

Keywords: Antioxidant activity, Antioxidant defenses, Antioxidant enzymes,

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CAPE catalase, Chrysin, DPPH, Glutathione, Glutathione peroxidase, Hive, Lipid oxidation, Oxidative stress, Phenolic compounds, Pinocembrin, Pollen, Propolis, ROS.

1. ANTIOXIDANT PROPERTIES OF PROPOLIS

Propolis *i.e.*, the sticky dark-colored substance that bees produce from the collected resins of plants, has been the focus of many studies over the past decades with regard to biological activities, in particular that of antioxidant. In general, authors have associated this biological property to phenolic compounds present in propolis samples [1 - 7]. Obviously, since the phenolic content and profile of propolis is dependent on several factors (*e.g.* botanical origin [1, 8] and geographical location [2]), variations in antioxidant abilities of samples are also expected.

Following, relevant work on antioxidant properties of worldwide propolis is presented. Overall, this capacity has been mainly assessed in chemical models that, as known, do not mimic an *in vivo* environment and should only be used as a first approach. Still, over the last years, there has been also a considerable number of works dealing with the evaluation of antioxidant properties of propolis in biological models, both in cellular and *in vivo*. Due to the numerous literature data on this theme, we will mainly focus on recently reported data.

1.1. Assessment using Chemical Models

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) [1, 2, 4 - 18] assay, along with the 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) [1, 2, 12, 13, 17] and ferric reducing antioxidant power (FRAP) assays [4, 5, 7 - 8, 12, 14, 15, 17], are undoubtedly the most frequently used methods for assessing the antioxidant activities of propolis extracts (see Table **1**), as they are reliable and fast-executing methods [19]. This last also facilitates the obtaining of experimental data from more than one assay, as recommended, in a short time period [20].

There is a wide variation among the reported values of EC_{50} (most of the times defined as the concentration of extract needed to reduce half of the radical or the ferric ion concentration) when evaluated by DPPH, ABTS and FRAP assays and often, the antioxidant abilities are expressed in variable units, which clearly hamper the comparison of results.

Fluctuations in EC_{50} values (or antioxidant ability) of propolis samples have been closely correlated with their total phenolic content (TPC) [1, 2, 5 - 7, 13, 15, 17]. Still, there are exceptions to this *e.g.*, not always the sample with the highest TPC shows the highest scavenging activity or reducing power [8, 22]. Cases like that can be due to the distinct antioxidant ability of individual phenolics in the extracts and/or to the presence of other antioxidants besides phenolic compounds.

Table 1. Selected studies (2009-2015) of antioxidant activities of worldwide propolis extracts, as measured using chemical models.

Geographic location	Botanical Origin	Solvent	Assay	Results	Ref.
China (Shandong)	*Populus sp.*	Mix EtOH/H_2O	DPPH ABTS	DPPH: EC_{50} = 15.49±0.59 µg/ml Ext; ABTS: EC_{50} = 36.66±1.82 µg/ml Ext.	[1]
Turkey (Bengol, Rize, Tekirgard and Van)	N.D	Mix MeOH/H_2O/HCl	DPPH ABTS	DPPH: 409.6 to 503.7 mg trolox eq./g Ext; ABTS: 237.7 to 285.3 mg trolox eq./g Ext.	[2]
Italy (Venetia)	N.D	Mix H_2O/EtOH	DPPH LOPerox	DPPH: 100 to 150 mM catechin eq. for 150 µg/mL; LOPerox: 62% to 75% for 1.2 µg/mL Ext.	[3]
China (temperate, subtropical and tropical zones/26 regions)	N.D	HotH_2O	DPPH RP	DPPH: EC_{50} = 0.28 to 3.29 µg/mL Ext; RP: 0.20 to 3.47 %/µg/mL Ext.	[4]
Poland (Northern region)	N.D	EtOH	DPPH RP	DPPH: 60.78±10.12 % for 25 µL of liq. EtOH Ext (1 g propolis/10 mL EtOH); RP: 930.5±66.34 mmol Fe^{2+}/g Ext	[5]
Mexico (Sonora)	N.D	EtOH	DPPH	DPPH: 16 to 64.8% at 100 µg/mL Ext.	[6]

CHAPTER 9

Anti-Inflammatory Activity of the Honeybee Plant-Derived Products Honey, Pollen and Propolis

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Abstract: This chapter aims to discuss the effects of honeybee plant-derived products in inflammatory processes, with particular focus on honey, pollen and propolis. Honey is mainly composed by fructose and glucose, containing also minerals, proteins, free amino acids, vitamins and polyphenols and has long been used by humans not only for nutritional purposes but also as a medicine. The biological properties of honey can be ascribed to its polyphenolic content which, in turn, is usually associated to its anti-inflammatory activity, as well as antioxidant, antiproliferative and antimicrobial benefits. Bee pollen results from the agglutination of flower pollens with nectar and salivary substances of the honeybees. Due to its optimal nutritional balance, it has been considered as a perfect food all around the world and also used as a therapeutical agent. However, there is a lack of scientific support addressing the biological activities of bee pollen. Propolis is produced by bees from secretions of trees, trunks, buds, leaves and pollen, adding wax and substances secreted by bee glands.

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The large and diverse number of chemicals in propolis may justify their biological activities, namely anti-inflammatory properties. Herein we emphasize the anti-inflammatory potential of the honeybee plant-derived products propolis, honey and pollen. Whenever possible we also disclose the action mechanisms and the principal compounds responsible for the biological activity. The intracellular signaling targets of propolis, honey and pollen are highlighted and summarized in Fig. (**1**). Overall, the production of inflammatory mediators, *i.e.* nitric oxide (NO) and prostaglandins, are inhibited by the three products partially due to the inhibition of nuclear factor kappa B (NF-κB) and mitogen activated protein kinases (MAPKs) signaling pathways.

Keywords: Bee pollen, Chemokines, Cytokines, Flavonoids, Honey, Honeybee, Immune cells, Immune system, Inflammation, Intracellular signaling pathways, Lipopolysaccharide, Macrophages, Mechanism of action, Nitric oxide, Nuclear factor kappa B, Polyphenols, Propolis, Prostaglandins.

1. INFLAMMATORY PROCESS: AN INTRODUCTION

The innate immune system provides a first line of defense and consists of cells and proteins that are always present and ready to mobilize immediately or within hours of an antigen's appearance in the body. The main components of the innate immune system include physical epithelial barriers, dendritic cells, phagocytic leukocytes, natural killer (NK) cells and plasma proteins. Besides its crucial role in the clearance of the antigen, cells of the innate immune system also take part in the initiation and subsequent development of adaptive immune responses as well as in the elimination of antigens that have been targeted by an adaptive immune response. The adaptive immune system is mediated by lymphocytes that once activated, proliferate and generate potent mechanisms for neutralizing or eliminating the antigen, also providing increased protection against a subsequent attack by the same antigen. The two types of adaptive immune responses include: humoral immunity, mediated by antibodies produced by B lymphocytes, and cell-mediated immunity, mediated by T lymphocytes [1]. The activation of both types of immunity initiates inflammation.

Inflammation is part of the body's immune response that may be triggered by an infection, burn, or other injuries, as an attempt of self-protection. Two stages of inflammation exist, acute and chronic inflammation. Acute inflammation is an initial stage (innate immunity) that usually persists for a short period and is generally beneficial for the host. If inflammation lasts for a longer time period, the second stage of inflammation, or chronic inflammation, become self-perpetuating over time predisposing the host to various chronic illnesses, including rheumatoid arthritis [2], asthma [3], inflammatory bowel diseases [4], atherosclerosis [5] and cancer [6]. Several cell types and chemical mediators are involved in the inflammatory response. Briefly, immune cells present receptors on their surfaces named pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), which recognize molecules highly conserved and shared among pathogens, collectively referred to as pathogen-associated molecular patterns (PAMPs). Macrophages have a key role in providing an early and immediate defense against foreign agents. Upon recognition by TLR4 of the gram negative cell wall component, lipopolysaccharide (LPS), macrophages become activated and produce a variety of pro-inflammatory mediators, including prostaglandins, nitric oxide (NO), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and IL-10. These cytokines, which may be also produced by other immune cells, play a major role in the induction and regulation of inflammation, hematopoiesis, and immune response.

TNF-α is an endogenous pyrogen displaying systemic effects in the acute phase reaction and able to induce IL-1β and IL-6 production. IL-1β is a pleiotropic pro-inflammatory cytokine that stimulates systemic and local responses to infection. It induces the expression of adhesion molecules and chemokines on endothelial cells, leading to the traffic and infiltration of inflammatory and immunocompetent cells at the site of injury [7]. In addition, IL-1β causes vasodilatation and hypotension, fever and enhances pain sensitivity. IL-6 is a multifunctional regulator of immune response, hematopoiesis, and acute phase reactions [8]. In contrast, the anti-inflammatory cytokine IL-10 decreases the production of pro-inflammatory cytokines TNF-α and IL-1β [9].

Other important inflammatory mediators are prostaglandin E2 (PGE2), which is synthesized by the rate limiting enzyme cyclooxygenase (COX), and NO that is synthesized by nitric oxide synthase (NOS). The high-output of NO by the inducible form of NOS (iNOS) contributes to the pathogenesis of septic shock and inflammatory diseases. Therefore, the selective inhibition of COX-2 and iNOS in

CHAPTER 10

Antitumor Properties of Honeybee Plant-Derived Products: Honey, Propolis and Pollen

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Abstract: The majority of cancers have no curable treatment and the main available therapies have serious side effects, justifying the need for development of new antitumor agents. Several efforts have been made to identify natural products useful in the cancer setting. This area has emerged as an important research field, providing the possibility to both identify novel potentially useful agents and to study the mechanisms of antitumor action. Honeybee plant-derived products have shown anti-cancer activity in a series of experimental and clinical studies with cell lines, animals and humans. Honey, the viscous, golden and sweet liquid produced by bees from the nectar of flowering plants has proven to display antiproliferative and apoptotic effects, along with other activities that contribute for its antitumor properties. Propolis, a special substance made by honeybees through mixing tree saps with salivary secretions, is used to seal fissures and openings in the hive, strength combs, seal brood cells and protect the hive from infections. Propolis contains phytonutrients that may be useful in different pathological conditions, including cancer.

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Susana M. Cardoso (Ed)

Bee pollen, the bees´ primary food source, is plant pollen collected from a variety of plants and processed by honeybees. Demand for this natural product is rising since it has effects on a variety of biological functions, which contribute to the fight and prevention of cancer. This review focuses on the antitumor properties of honey, propolis and bee pollen as well as on the potential use of these honeybee plant-derived products to develop new therapeutic approaches for patients with different types of tumors.

Keywords: Angiogenesis, Apoptosis, Autophagy, Bioactivities, Cancer, Flavonoids, Honey, Honeybees, Immortality, Invasion, Metastasis, Natural products, Oncogenes, Plants, Pollen, Polyphenols, Proliferation, Propolis, Standardization, Tumor suppressors.

1. CANCER

Cancer can be defined as a disease where cells suffered alterations and acquired an abnormal capacity of proliferation, invasion to adjacent tissues or metastasis to distant organs, through blood and lymphatic vessels. Two main types of genes are involved in cancer development: oncogenes and tumor suppressor genes. Oncogenes are genes that contribute to the development of cancer, while tumor suppressor genes have the opposite effect. The development of many cancers is often related to alterations in these genes, either by promoting the activity of oncogenes or decreasing the activity of tumor suppressors.

There are several types of cancer, with diverse characteristics and sensitivity to therapy, therefore with different mortality rates. The worldwide incidence and mortality of the main types of cancer are presented in Fig. (**1**) [1].

Despite advances of therapeutic regimens over the years, there are still some cancers which do not respond well to current therapy and consequently have high mortality rates. Thus, new therapeutic approaches are urgently needed.

1.1. Hallmarks of Cancer

In the publication “The hallmarks of cancer” [2], which was recently updated [3], Hanahan and Weinberg detail the main alterations - “hallmarks” - acquired by human cancer cells during the development of malignant tumors, which include the sustained capacity of proliferation, insensitivity to anti-growth signaling, resistance to cell death, replicative immortality, angiogenesis induction and the mechanisms of invasion and metastasis (Fig. **2**). In their most recent publication, the authors propose additional hallmarks, known as enabling characteristics and emerging hallmarks [3].

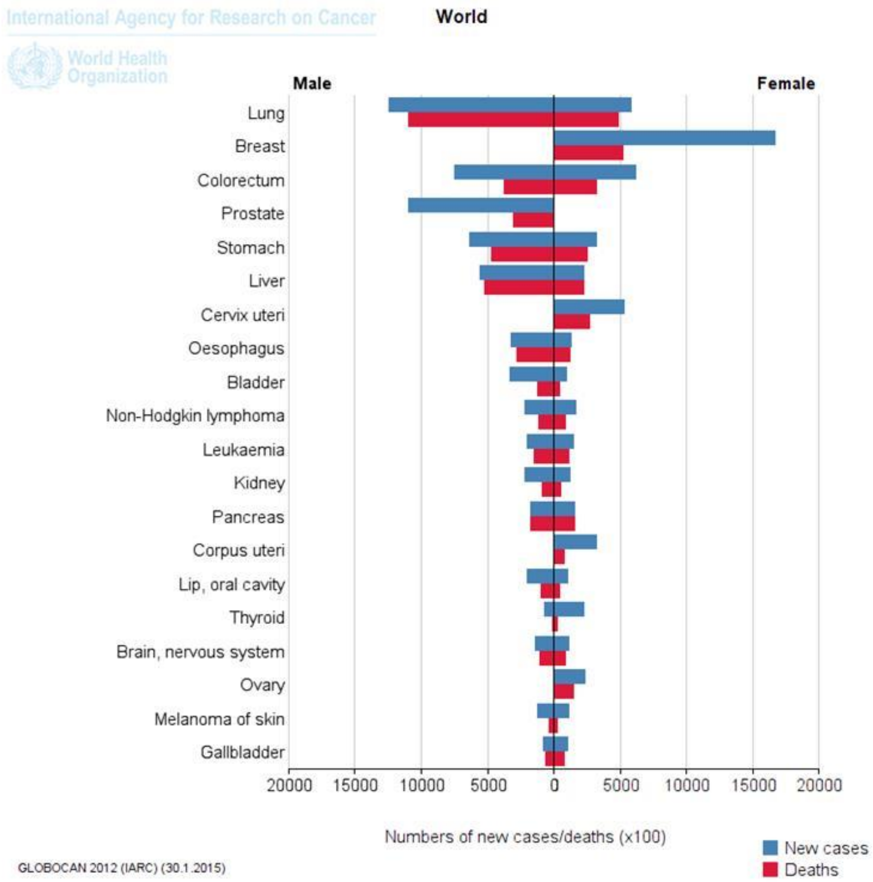

Fig. (1). Worldwide incidence and mortality of the main cancers [1].

1.1.1. Sustaining Proliferative Signaling

The capacity of chronic proliferation is probably the most important alteration of cancer cells. While the release of growth factors is tightly regulated in normal tissues, cancer cells present deregulation of this process, with consequent uncontrolled growth. Cell proliferation is mainly regulated by growth factors that

CHAPTER 11

Antimicrobial Activity of Honeybee Plant-Derived Products

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Abstract: Bee products have always been used as foods and/or as therapeutic agents against a number of diseases in alternative medicine since almost immemorial times. The millennial track record of the health and nutritional benefits of such natural bee products is being supported more recently by scientific research. The antimicrobial activity of bee products is considered one of its widespread and most important bioactivities, and highlights the potential of these natural products as promising antimicrobial agents for clinical and biotechnological applications. Honey and propolis fulfill all the criteria of ideal candidates for treatment of non-healing wounds and other diseases caused by microorganisms. These natural products find application against resistant pathogenic microorganisms without the risk of antimicrobial resistance acquisition and prevent the formation or distortion of biofilms. Honey is regarded as a pure natural and functional product of high nutritional value. Honey factors that contribute to its antimicrobial activity are diverse but researchers consider both the high sugar concentration and low pH as well as the presence of hydrogen peroxide, methylglyoxal and bee defensin-1 (an antimicrobial peptide) as the most relevant in different honeys.

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Propolis is a sticky resin produced by bees, especially from coniferous, having plant-derived and bee-released compounds, but with a chemical composition difficult to standardize due to its dependence on vegetation, season and environmental conditions at the collection site. The mechanism of propolis antimicrobial activity is complex, probably relying in a synergistic activity between different phenolic compounds such as flavonoids and phenolic acids along with other components. Pollen is a source of phytochemicals and nutrients, extremely rich in carotenoids, flavonoids and phytosterols. Although less studied for this bioactivity, bee pollen has also been proven to possess antimicrobial activity.

Keywords: Antibacterial activity, Antifungal activity, Antiviral activity, Bacteria, Bees, Biofilm, Concentration, Nectar, Pathogenic microorganisms, Peroxidase activity, Phenolic compounds, Pollen, Propolis, Resistance, Synergism, Yeasts.

1. INTRODUCTION

Antimicrobial agents are essential to ensure human/animal health and welfare, as well as food security. Overall, antimicrobials are vital in the reduction of the global burden of infectious diseases. However, the increasing number of immunocompromised individuals - patients suffering from AIDS (acquired immune deficiency syndrome), with organ transplantation and undergoing chemotherapy regimens for cancer treatment - increases the number of opportunistic infections and has been leading to the development and spread of resistant pathogens, diminishing the efficacy of antimicrobial drugs. The evolution of resistant strains is a natural phenomenon, occurring when microorganisms replicate erroneously or when strains exchange resistant traits, but the use and misuse of antimicrobial drugs speed up the appearance of such drug-resistant strains, further amplified by inadequate infection control practices, poor sanitary conditions and inappropriate food-handling [1].

Microbial resistance to antimicrobial agents is a global threat and is considered a serious public health problem by the World Health Organization (WHO), making the discovery of new antimicrobial agents an urgent need. In 2014, the WHO reported the results of a first global surveillance on antimicrobial resistance, with data from 114 countries. This WHO's report reveals that antibiotic resistance is not a vision but a frightening reality instead, which is happening here and now, threatening the capacity to treat common infections and minor injuries, considered treatable for decades [1]. Resistance to antimicrobial agents, in particular to antibiotics, including the major last-resort drugs, continues rising across the world. Even more worryingly is the fact that very few new antibiotics are under development and few new therapies are on the horizon. Alternative antimicrobial strategies and new antimicrobial agents are therefore urgently demanded and have been leading to reconsider the use of old traditional therapeutic resources [2]. In the actual trend towards the use of natural and renewable resources, natural compounds with antimicrobial properties and potential biomedical applications - particularly found in plants and plant products, including honey and other bee products - have been extensively researched recently [3 - 5] and are of highest interest. Such products and/or its compounds might be used either directly or as leads for the development of novel antimicrobials agents.

In this work we aim to report and discuss the potential of some honeybee plant-derived products for the development of new antimicrobial drugs, supported by the results of several studies regarding the assessment of antimicrobial properties in honeybee plant-derived products against diverse microorganisms. Likewise, the action mechanisms of the best known compounds responsible for the antimicrobial activity of honey, propolis and pollen will be described.

2. ANTIMICROBIAL PROPERTIES OF HONEY

Honey has long been used and prescribed as a remedy in traditional medicine of all civilizations, either for oral intake or topical application. Widely seen as a food source of high nutritional value, honey is being increasingly recognized by its healing properties. Since its first documented use, in Sumerian writings (2100 to 2000 BC), as medication and ointment, several scientific works have reported its use in the treatment of ulcers, wounds, bed sores and other skin infections resultant from burns and wounds [5 - 7]. This healing property of honey is mainly attributed to its antibacterial activity, further helped by the maintenance of a moist wound condition and the supply of a protective barrier by honey´s high viscosity, which helps preventing infection [2]. A number of medical grade honeys with standardized levels of antibacterial activity are currently marketed, and display an effective *in vitro* bactericidal activity against antibiotic-resistant bacteria,

Section IV

The latter section of the Book is dedicated to the applications of honey, propolis and pollen, in particular those that give add-value to those products. Potential applications are also focused, since we believe that for sure several of them will give rise to new products in a near future and overall fomenting the market of bee plant-derived products.

CHAPTER 12

Add Value Products of Honeybee Plant-Derived Origin

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Abstract: Although honey extraction is an ancient practice, its use as a source of income is more recent. Over the years several techniques have been developed to achieve greater amounts of honey and also to ensure the quality of the product. Furthermore, bees during their life cycle produce wax, royal jelly, pollen and propolis besides honey. Pollen and propolis are such as honey, plant derivatives, while the wax and royal jelly are products secreted by glands of the bees. This chapter aims to present ways to add value to bee products plant-derived, namely: honey, pollen and propolis. Uses and new applications are presented. Moreover, the production of honey-fermented products such as mead, honey beer and honey vinegar are also discussed.

Keywords: Antibacterial properties, Antioxidant properties, Applications, Chemical composition, Cosmetics, Drink, Fermentation, Food industry, Health, Honey, Honey beer, Honey vinegar, Mead, Medicine, Natural preservatives, Pollen, Propolis, Quality, Uses, Wort.

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1. INTRODUCTION

During their life cycle, bees produce honey, propolis, pollen, wax and royal jelly for their maintenance. Each of these products has an important role in the development and routine of the hive members. Since the beginning of beekeeping as an economic activity, research has been developed to understand the best way of management the bees' hives in order to obtain higher productivity.

Over the years many techniques have been developed to optimize the production of honey, pollen, wax and royal jelly that had contributed to these honey derivatives become a source of income for many rural families. Although honey production has already sufficient supply to put itself into a central position in agribusiness, more research is necessary in order to other products originating from beekeeping reach the market.

This chapter aims to show how to add value to products of honeybee plant-derived origin such as honey, propolis (bee glue) and pollen, as well as through the development of mead (honey fermented drink) and other honey fermented products.

2. ADDING VALUE TO PRODUCTS OF BEEKEEPING

2.1. Honey

Honey is the "natural sweet substance, produced by *Apis mellifera* bees from the nectar of plants or from secretions of living parts of plants, 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" [1]. After harvesting, honey does not necessarily need further treating. However, honey must always be handled hygienically and the cleaning status of all equipment is of great importance. Moreover, as honey is hygroscopic, all equipment and bottles used in its processing must be dry in order to reduce the chances of fermentation. Nevertheless, honey has a long shelf-life if harvested with care and stored in containers with appropriate lids.

Since ancient times honey has a high cultural value as a food. Besides that, this is also used for anointing in traditional birth, marriage and funeral ceremonies. This cultural connection is evident in the term “honeymoon”. In general terms and, as cited by Krell [1], honey has been used in many applications such as:

- For bees as a food supply for those times when the flowers are scarce;
- As food for humans. Honey can be eaten in its unprocessed state such as liquid or crystallized forms or in the comb;
- Food ingredient as natural sweetener for drinks or in other dishes. Honey has been used in several food products, for example: *i*) honey liqueurs; *ii*) spreads with dried fruits or milk, like “Dulce de Leche”, a popular Argentinean spread; *iii*) fruits and nuts (whole, chopped or pureed) in honey; *iv*) honey with pollen and propolis; *v*) fruit marmalade or jams where a portion of the sugar is replaced by honey; *vi*) honey jelly; *vii*) syrups; *viii*) rose honey, which is produced by mixing honey with red rose petals and boiling water; *ix*) caramels and candies; *x*) honey gums; *xi*) gingerbread; *xii*) marzipan; *xiii*) bakery products; *xiv*) non-alcoholic beverages like strengthening or replenishing isotonic drinks; and *xv*) sauces and some distilled alcoholic beverages. Some of these have honey as a flavoring agent such as Drambuie in Scotland, Benedictine in France, Irish Mist in Ireland, Krupnik in Poland, Grappa al Miele in Italy and Barenfang in Germany. Moreover, in the breakfast cereal industry, mixtures of honey (liquid or dried form) with cereal flakes and dried fruits such as roasted peanuts are used, improving oxidative stability [2];
- Medicine or tonic due to its medicinal properties. Indeed, hot milk, tea or other infusions with honey are a very usual home remedy to treat colds and throat infections. Recently, Raeessi *et al.* [3] found that “honey with coffee” is an effective treatment for post-infectious cough. As reviewed by Ediriweera and Premarathna [4], fresh bee’s honey may be used in the treatment of several diseases such as bronchial asthma, throat infections, hiccups and ulcers, while old bee’s honey is more used to treat diarrhea, vomiting, rheumatoid arthritis, diabetes mellitus and obesity. Nowadays, several products with honey in their formulation have been produced such as wound dressings [5 - 8] and gels for topical treatments for reepithelialization [9]. Furthermore, carbon nanoparticles from honey have given good results in real-time photoacoustic imaging [10] and

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