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BEE POLLEN ANTIOXIDANT ACTIVITY - A REVIEW: ACHIEVEMENTS AND FURTHER CHALLENGES

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Abstract- In the last decades, extensive literature has been published concerning bee pollen. The finding that pollen's flavonoid and phenolic profiles are species-specific, revolutionized bee pollen studies approach and increased the curiosity towards its potential antioxidant activity. In addition, the search for natural antioxidants had an amazing improvement over the years, justified by their safely character and effective but mild properties, as well as their recent popularity as alternative therapeutics in a rising and profitable market. Most studies suggest an intense correlation between bee pollen antioxidant activity and phenolic content, although a linear, direct relation is yet to be proved. The most used biochemical methodologies were described. Few published papers based their studies in *in vivo* systems, restraining therefore, the most important conclusions at this matter. These results are the only ones that can be transposed to living organisms, due to the antioxidant's role as potential pro-oxidants *in vivo*, at certain metabolic conditions and/or concentrations. The aim of the present work is to clarify and summarize all the progress that has been done in this matter and knowledge achieved, in order to direct further research and have a glimpse of what lies in the future and corresponding challenges.

Key Words: bee pollen, phenolic composition, antioxidant activity, pro-oxidant activity

INTRODUCTION

Pollen is collected by honeybees, as the most important source of proteins for the beehive [1-2]. Bee pollen, that is, floral pollen, collected, pelletizated and processed by the honey bees [3], is now recognized as a well balanced food [4-5] and its nutritional content may be partially released by digestive juices, although only a part of it is assimilated by humans [5]. Nevertheless, bee pollen is a product with added value, as it can also be used for several health benefits, recently reviewed [5], due to its main biologically active compounds, which include phenolic derivatives, such as flavonoids. These compounds are found in a species-specific profile [6], allowing bee pollen botanical origin identification and, thus, suggesting that it's therapeutic value and, further, its antioxidant capacity, are as variable as their phenolic content [4]. These findings have a major impact for the depth necessity of the establishment of bee pollen Quality International Standards, in order to homogenate the traded product and achieve, consequently, standard bioactivities [5]. The evaluation and quantification of bee pollen antioxidant capacity can be evaluated through several methodologies, according to the different existing antioxidant mechanisms. One of the most used techniques consists on the quantification of bee pollen radical scavenger capacity. Pollen, according to its role in plant reproduction, should have an efficient method to protect its DNA, against environmental conditions, especially UV exposure and, consequently, able to scavenge radicals [7]. In fact, polyphenolic UV-B screens such as phenolic

acids and flavonoids, are mostly found at the pollen wall surface, for protecting vulnerable pollen protoplasm against solar radiation and other environmental (microbial and chemical) damaging agents [8]. Previously published articles indicate that the bee pollen grains are only scarcely digestible for humans, due to the difficulty of enzyme penetration inside pollen [9]. Therefore and, despite its poor digestibility, bee pollen is still a rich source of these valuable antioxidant compounds. It is important to enhance that *in vivo* studies results are more reliable, providing a different picture from those *in vitro*, as they illustrate better the duality of antioxidant behavior in living organisms [10]. In certain conditions, they can act as oxidative substances, exerting a completely opposite effect from the intended one. According to this theme relevance and the efforts already made, it is worth doing a situation point, in order to systematize contents, rearranging and coordinating the available information and thereby improving their accessibility and objectivity, facilitating sustainable further studies.

BEE POLLEN - DEFINITION AND CHEMICAL COMPOSITION

Pollen is the male gametophyte of flowers [11]. Bee pollen is an apicultural product, made up of natural flower pollen mixed with nectar and bee secretions and it is rich in sugars, proteins, lipids, vitamins and flavonoids (3-5% dry weight) [12]. Commercially traded bee pollen is mainly collected by the honey bee (*Apis mellifera* L.) for the purpose of feeding its larvae in the early stages of development [11, 13]. Nevertheless, many other social bee species collect and use bee pollen, including the stingless bees (*Melipona subnitida* Ducke), native to Northeastern Brazil [12]. After touching flower's stamens and pollen dust covers their bodies, bees use their hind legs, compressing pollen into the pollen baskets. Then, use their mouth secretions, rich in different enzymes, to moisten the pollen and hold it together and nectar, about 10%, for packing. The collected pollen is accumulated as pellets in pollen baskets and it is the mixture of these pellets that comprises bee pollen [11]. The bees place the pollen in honeycombs and cover it with honey [7]. Whereas honey represents the major energy source for the beehive, pollen is used as the main source of other important nutrients, including proteins, minerals and lipids. In general and after intense research on this subject, recent reviews indicate that bee pollen (dried) is usually composed of 13-55% total carbohydrates, 0.3-20% dietary fiber, pectin, 1-13% lipids (with a good ratio of unsaturated/saturated fatty acids, including α-linolenic acid), 10-40% protein, 2-6% ash [11], accompanied by a variety of secondary plant products, such as flavonoids, carotenoids and terpens [6]. In addition, it should be enhanced that pollen contains important minerals as Zn, Cu and Fe [14], a desirable high K/Na ratio, essential aminoacids and significant quantities of several vitamins: provitamin A, Vitamin E, niacin, thiamine, folic acid and biotin [5, 15]. Vitamin C is also usually present [16]. The amount of these nutrition-relevant components, however, largely depends on the botanical origin of the pollen, as demonstrated by the variations presented, between the minimum and maximum values established [5, 15]. Its nutritional content led to worldwide consumption as a dietary supplement [16], with a special appeal to the elderly, in order to delay the aging process [6]. In addition, its valuable composition also led pollen to be considered as a human food and, fortunately, certain countries, including Brazil, Bulgaria, Poland and Switzerland, have already national pollen standards.

BEE POLLEN HARVEST AND STORAGE

In order to collect bee pollen for human consume, a trap must be installed in front of the beehive entrance, so that the worker-bees, when coming home, lose their pollen pellets, which are withdrawn into a container [1]. The percentage of bee pollen retained in a trap may be quite variable, as well as the trap designs, but will always be considerably less than 100%. Due to its high water content and, in order to avoid microorganism proliferation and for preservation of a maximum quality, fresh bee pollen must be immediately placed in a freezer, after its daily harvest. Fresh, purified bee pollen can be frozen and stored under nitrogen, until consumption, thereby maintaining its optimal biological and nutritive properties. Nowadays, most of the commercialized bee pollen is produced by oven drying. This technology, however, should be improved, for example, by freeze-drying, since at the maximum oven recommended temperature of 40ºC, the vitamins' content decreases significantly. Finally, bee pollen should be stored in a cool, dry place, in well closed glasses or plastic recipients [5]. Water content, a fundamental parameter, is demanded by some regulations of certain countries, particularly in Brazil, which established a maximum of 4% for dried bee pollen, although there is not an official method for its determination yet [15].

BEE POLLEN ANALYSIS: IDENTIFICATION AND CHARACTERIZATION

Besides pollen grains, the pollen pellets contain lipid dyes from flower anthers. Several colors of pollen pellets can occur. From white and cream to dark brown and, with yellow, orange, red, greenish and gray degrees, their colors depend on pollen botanical *taxa* (referent to specie) and, consequently, chemical composition. However, in pollen pellet samples, a single pollen color of a specimen does not necessarily indicate a monofloral source, even though it has a greater probability to be one, when compared with samples

that show several colors. Bee pollen is considered as monofloral, if the major *taxon* is present at a minimum of 80% and different *taxa* will be used for specific nutritional and therapeutic purposes. On the other hand, multifloral bee pollen includes several *taxa* [11]. Concerning bee pollen pellets, worker-bees, in order to collect pollen, interact with certain plant species but, whenever there is not sufficient offering, the bees may visit flowers of other plant species and, sometimes, mix the pollen together in the same pollen pellet [1]. Bees, however, tend to be very selective when gathering bee pollen, as demonstrated by several studies, showing that each pellet of the honeybee-collected pollen is largely homogeneous [6], and, therefore, predominantly consist of pollen grains from one species [4]. In addition, bee pollens comprise pollen from only a few of the available species [3, 5-6, 11, 17-20]. Each kind of pollen has its own specificities, depending on its botanical origin [16]. In fact, this is the major factor for bee pollen variability, contributing to significant differences in chemical composition, both qualitatively and quantitatively [3, 5-6, 11, 19]. Therefore, pollen identification and characterization is of extremely importance. Botanical evaluation of pollen pellets should comprise a representative sample for pollen analysis. The pellets are first screened according to their colors and grouped into subsamples, then weighted. For microscopic identification, microscope slides should be prepared, in order to establish further comparisons with the existing literature database (palynological collections) [1]. Microscopic analysis, however, can usually only determine mostly plant family or genera, rather than species. Nevertheless, this approach is generally carried out routinely in pollen and bee pollen quality control [5]. Meanwhile, there is already another approach, by which the exact plant species can be determined. This identification technique was established previously [6] and resides on the analysis of pollen's flavonoid and phenolic profiles. Taking advantage of their properties as useful taxonomic markers and, through high performance liquid chromatography (HPLC/DAD) [6], this method shows that these compounds are species-specific and, for each species studied there is a unique phenolics' fingerprint [19, 21]. Therefore, bees collection and pelletization procedures, do not affect bee pollen flavonoid constituents, thus providing a worthwhile source of species-specific flower pollen [21-22]. The phenolic composition of pollen principally consists of flavonol glycosides and of hydroxycinnamic acids [21]. Bee pollen can, thus, be identified and characterized routinely, by creating a library of HPLC/DAD profiles, through data accumulation of all major pollen types available in the relevant collective area. Furthermore, flavonoids and other phenolic derivatives are also recognized as valuable antioxidants, improving bee pollen phytotherapeutic value [6].

BEE POLLEN ANTIOXIDANT ACTIVITY

In the last decades, pollen research has focused essentially on its botanical origin, chemical composition and nutritional value [5, 11, 13, 16, 23]. The results obtained led to continued studies concerning their phenolic profiles [6, 19, 22, 24-25] and, recently, to a proposal for the establishment of International Quality Standards [11]. These are of fundamental importance to assure bee pollen safety and efficacy, with a reliable scientific background supporting its commercialization worldwide and justifying its use as a valid phytotherapeutic alternative and/or dietary supplement [11]. In the mean time and, according to its composition, it became clear that bee pollen could have very promising bioactivities. Many studies have been published, ever since, concerning this matter, which, also, has been recently reviewed [5], further reinforcing bee pollen potential for nutritional and therapeutic uses. Although some bioactivities seem to have a brighter future over others, in terms of medical practice, antioxidant activity stands for almost all of them. Through its different mechanisms of action, already referred, this activity is somehow related to many others, as it is associated with several pathologies. In fact, anti-atherosclerotic, anti-inflammatory, anti-carcinogenic and even anti-anemic and antiallergenic activities have deep bases in oxidant-antioxidant equilibrium [3, 26-27]. On the other hand, antioxidant substances are essential in food, cosmetic and pharmaceutical industries. Furthermore, some of the widely used synthetic antioxidants such as BHT (butylated hydroxytoluene) and BHA (butylated hydroxyanisole) are now suspected to have toxic and mutagenic effects. BHT is under very careful monitoring and evaluation by FDA (Food and Drug Administration) [28]. All these factors and, in addition, the increasing interest for natural therapies, their major popularity and their profitable market demands, contribute to emphasize the importance of researching bee pollen antioxidant activity and justify and encourage further studies. Meanwhile, biological reactivity of free radicals and other reactive species, along with their role in oxidative stress, have been under considerable attention and controversy [29]. These species can be produced both by normal metabolism and exogenous stimuli, as UV light and air pollution [3, 27]. They have been implicated with various cellular injuries [30], including membrane lipid peroxidation [31-32], DNA alteration and protein damage [33], also with enzyme inactivation [29]. Therefore, they are associated with pathological, chronic conditions, including cancer [34-36], atherosclerosis [37-38], neurodegenerative and auto-immune disorders [39] and aging [3, 40]. Nevertheless, they also have a

key role in normal signaling processes [41-42]. Therefore, potential prejudice, instead of being related to their presence, which is inherent to all aerobic organisms, is associated with deficient antioxidant defenses, leading to their accumulation and consequent damage. The cellular balance between oxidant challenge and antioxidant response is, indeed, of great complexity. Knowledge of the antioxidant capacity of specific chemical scavengers and their activities against different oxidants is fundamental to understand and predict the susceptibility to oxidative stress of biological tissues [29]. Despite the existence of a masterful endogenous strategy antioxidant defense mechanism [43], the production of oxidant reactive species may overcome these defenses and, therefore, it is believed that exogenous antioxidants, in certain conditions, may have a key role in additional protection of vital cellular components and their physiological function. Such antioxidants are commonly obtained from food and include vitamins and a wide variety of phenolic compounds [3]. Interestingly, vitamins C and E, which play a vital role in the scavenger defense strategy of humans and other primates, are not synthesized by them. Consequently, there are mechanisms to monitor and regenerate these vitamins, promoting their recycle and, thus, allowing a self-sustaining activity. In fact, regeneration of α-tocopherol at membrane's surface by ascorbate is well documented and, strategically, it is a masterful mechanism [43-44]. That lypophilic vitamin, although inhibiting the peroxidative chain propagation, leads to the formation of lipidic hydroperoxides and thus becoming, in a certain way, a pro-oxidant substance. This concept constitutes the "antioxidant paradox", which claims that antioxidant species, in certain conditions and/or concentrations, can have a pro-oxidant activity, meaning an oxidant behavior. In this way, it is absolutely fundamental that these antioxidants exert their functions in a concerted and synchronized way. For example, in the case described above, the presence of vitamin C is crucial, not only because of vitamin E regeneration, but also to prevent the respective radical from further reacting in a harmful way. In addition and, to avoid the hydroperoxides produced from initiating new peroxidative chains, the glutathione-peroxidase (Gpx) activity is fundamental. In fact, many studies had already showed these effects, demonstrating that ascorbate presence restrains vitamin E pro-oxidant activity [45-46]. This paradox is also clearly illustrated by the role of vitamin C. Despite being a terminal reducer in the radicals' scavenging chain, at certain concentrations it may also reduce Fe^{3+} to Fe^{2+}, this way increasing or sustaining Fe^{2+} concentrations, which, in turn, may catalyze the reduction of hydrogen peroxide (H_2O_2), producing the hydroxyl radical ($OH^{\bullet}$), with the harmful consequences inherent to its formation [47-48]. Therefore, it's of extreme importance that the eventual additional intake of dietary antioxidant supplements respects the physiological concentrations and needs of the particular organism, which represents a difficult aim to achieve, as the boundaries between antioxidant and pro-oxidant activities are not still very clear and, obviously, because of the wide variety shown on people's metabolism and their different lifestyles [43, 45, 49]. Vitamin E also has a fundamental role in cell proliferation inhibition, monocytes adhesion and protection of LDL oxidation. These properties transform this vitamin into a major defensive antioxidant at a cardiovascular level [37-38, 45]. This system also illustrates the importance of the antioxidant synergistic effect. In fact, under oxidative pressure, vitamin E is rapidly consumed in order to protect LDL from being oxidized. The presence of vitamin C and/or dietary phenolic compounds, as caffeic acid, delays this lipophilic vitamin consumption, thereby increasing LDL resistance to oxidation [50-51]. Therefore, diet plays a vital role as a fundamental source of antioxidants, such as vitamin E, C and β-carotene, plant phenolic derivatives and essential minerals, as zinc and selenium, important co-factors of some antioxidant enzymes [27]. In order to preserve food and cosmetic formulations, synthetic antioxidants have been developed, which act by mechanisms similar to biological antioxidants. Phenolic antioxidants have been used as radical scavengers in inhibition propagation chains. Probucol, a lipophilic phenol, has been used in LDL's protection [52]. Metal chelators, such as desferoxiamin, were designed as preventive antioxidants. Other compounds "mimic" enzyme activities, such as Ebselen. Trolox, a water soluble analogue of vitamin E, is used as a reference antioxidant [43]. Antioxidant activity importance and impact along with the advantages of the utilization of natural occurrence products, led to intense research on this matter and phenolic compounds, with a particular enhance to flavonoids, which are now worldwide established and recognized for their antioxidant properties. Those, are well documented by innumerous studies [21, 43, 51, 53-55] and dependent of the presence of certain structural features [3, 56], such as the conjugated double bonds and the number of hydroxyl groups in the aromatic ring, mostly attributed to flavonoids and cinnamic acid derivatives [57, 58]. Corroborating these findings, many studies have suggested that flavonols and catechins have the most powerful antioxidant activity [59]. Flavonoids are able to exert their antioxidant properties by several mechanisms of action, which include direct radical scavenger, metals' chelating, synergistic interaction with endogenous antioxidants, xanthine oxidase inhibition and induction of antioxidant enzymes' activity [60]. These phenolic compounds have a major and determinant role, both in bee

pollen composition and bioactivity properties, with a greater emphasis to antioxidant bee pollen capacity [3, 4, 6, 19, 61-62].

Methodologies

The methods used in assessing the antioxidant activity take advantage of the various defense mechanisms against oxidative processes already identified in aerobic organisms. It can thus be said that the wide variety of antioxidant strategies requires a great diversity of methodologies. Therefore, the several existent approaches can be classified into the following major groups, concerning: lipid peroxidation inhibition capacity; free radical scavenger inhibition capacity and ferric-reducing capacity. Furthermore, these methodologies can be performed at *in vitro* biological and/or chemical systems, or *in vivo* systems. It is known that antioxidant evaluation capacity in the latter is, obviously, more reliable, when compared to *in vitro* systems, since those provide results that cannot be directly transposed to real biological organisms [10]. Since there are many documents published concerning this matter and, being impossible and inadequate to expose and summarize all of them, accompanying the detailed methodologies, some significant studies that, respectively, illustrate them, were here analyzed.

Evaluation of the ability to inhibit lipid peroxidation

This method is based on the absorbance's measurement of the resulting products of lipid peroxidation, including hydroperoxides and degradation products, mainly malondialdehyde (MDA). Reactions are typically carried out in triplicate, for each sample and, also, require a blank control (without the substances studied) and a positive control (reference substances) [10].

Thiobarbituric Acid Reactive Species (TBARS)

The measurement of Thiobarbituric Acid Reactive Substances (TBARS) is a well-established method for screening and monitoring lipid peroxidation. Even though there remains a controversy regarding the specificity of TBARS toward compound other than MDA- naturally occurring product of lipid peroxidation, it still remains the most widely employed assay to determine lipid peroxidation [64]. Modifications of the TBARS assay by many researchers have been used to evaluate human and animal tissues and fluids, drugs and foods, including bee pollen. The reaction between malondialdehyde (MDA) and thiobarbituric acid (TBA), results in the formation of a colored adduct that absorbs radiation at a wavelength λ_{max} 535 nm. Thus, by measuring the colour ingtensity of MDA-TBA complex it is possible to quantify the formation of MDA and assess the extent of lipid peroxidation.

(1) In vitro studies

TBARS assay has been used to evaluate lipid peroxidation inhibition capabilities of the total extract of a bee pollen mixture and its six constituent pollens [4] and of monospecific bee pollen extract, from the mesquite tree (*Prosopis juliflora*), from Mexico [10] (Table 1). The antioxidant capacity was evaluated in relation to the phenolic composition of bee pollen extracts, by using quercetin, quercitrin and caffeic acid as reference substances. The authors utilized mouse hepatic microsomal preparations, in an *in vitro*-biological system. Lipid inhibition activities were expressed in terms of the concentration of antioxidant required to inhibit MDA formation by 50% values, (IC_{50} in μg mL^{-1}), after induced oxidative stress. These values were calculated from MDA concentrations against the sample flavonol concentration curves by linear regression, using MDA extinction coefficient ($1{,}56x10^5 M^{-1}cm^{-1}$). Butanol was the solvent chosen for quantification, but for the extracts preparation and further determination of flavonol content, was used an ethanol-water (50% v/v) solution. Some samples (*Amaranthus hybdridus* pollen and the mixture of bee pollen) are proved to be more effective lipid oxidation inhibitors than caffeic acid and quercetin standards, this last one considered as a powerful antioxidant [4]. The results showed that the antioxidant activity of the extracts were higher than those shown by the references.

(2) In vivo studies

In an *in vivo* system, using homogenized livers of bromobenzene-intoxicated mice, the antioxidant capacity of bee pollen samples was evaluated and compared with the *in vitro* results obtained in the same study [10]. Eighty mice were divided into eight groups: a control group, provided with cooking oil; a second group provided with the mesquite bee pollen extract at a determined volume and flavonol concentration; a third group provided with the mesquite bee pollen extract at the same volume but with more than twice of the flavonol concentration than the previous group; a group provided with the same volume of vitamin E (reference positive control); a group intoxicated with bromobenzene in cooking oil; the three last groups were all intoxicated with bromobenzene in cooking oil at the same volume and concentration than the previous but after the administration of, respectively, vitamin E, the second group extract and, finally, the third group extract. After mice's sacrifice, the inhibition of lipid peroxidation was assessed in liver homogenates by the same procedures of those in the *in vitro* system. The results, expressed in TBARS levels (μg mg^{-1} liver), showed that neither of the mesquite bee pollen extracts reached vitamin's E levels to inhibit *in vivo* lipid oxidation. In the presence of bromobenzene, both extracts of mesquite pollen showed antioxidant activity. The most interesting results in this experience, reside on the antioxidant capacity showed by the mesquite

bee pollen extract with less than a half of the flavonoid concentration, compared with the pro-oxidant capacity, showed by the other extract, more concentrated in flavonoids, as toxic as bromobenzene itself (Table 1). In order to characterize *in vivo* antioxidant activity of the *Cystus incanus* L. bee pollen, other method was developed [63]. After palynological analysis, botanical origin of the used bee pollen was determined, showing *C. incanus* L. as the major predominant species. Qualitative and quantitative chromatographic analysis of phenolics was also performed through an HPLC/DAD system. The mice were sacrificed and in a portion of liver and brain tissue the lipid peroxidation was estimated, according to TBARS method. In a lysate of erythrocytes, the authors determined the antioxidant enzymes activity, including SOD, CAT and Gpx, as for the liver and brain samples. Before being killed, the mice were divided into two groups, an experimental and a control one. Mice were fed fourteen days before testing, either with commercial food pellets (for control group), or with those mixed with bee pollen, with a correspondent dose of that of humans, after correction adjustments for mouse metabolism. The results showed that bee pollen significantly decreased TBARS concentration in the liver, but not in the brain (Table 1).

Quantification of the hidroperoxides formed

Another important methodology in the evaluation of lipid peroxidation is the measurement of lipid hydroperoxides formed, by measuring the absorbance at a wavelength of 232 nm. The mixture in question contains a polyunsaturated fatty acid, the bee pollen extract and the same amount of solvent in the control case [57]. The aim of their study was to determine the antioxidant ability of phenolic constituents (total phenols, phenylpropanoids, flavonols and anthocyanins) in bee pollen of 12 plant species (Table 1). Antioxidant capacity was measured as total antioxidant activity (TAA), radical-scavenging activity and activity against free hydroxyl radical. Total antioxidant activity, was determined according to this methodology. The unsaturated acid used was exogenous linoleic acid (LA) and its peroxidation products were measured spectrophotometrically at λ_{max} 232 nm, in the control and bee pollen solution. The peroxidation was initiated by the ferrous-EDTA system. TAA was expressed as a percentage inhibition of LA peroxidation by pollen extract in comparison to the oxidative level in the control. The bee pollen loads were collected in the Krakow area (Poland), referring the date and conservation process of fresh bee-collected pollen. Twelve species were identified and further analyzed. For extracts preparation the solvent chosen was methanol. The results showed that pollen antioxidant activity differed considerably according to the pollen species, but for most of them exceeded 60% (60-90%).

Evaluation of the radical scavenging activity

Several methods allow the assessment of the antioxidant activity in this perspective. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is the most used for this effect and illustrates the general capacity of antioxidants to inhibit or stop the radical chain propagation [3-4, 7, 28, 57, 61-62]. Hydroxyl radical is also frequently used to evaluate antioxidant activity. Due to the high hydroxyl radical reactivity, the scavenger capacities against this radical are considered separately.

DPPH assay

This method employs a solution of DPPH radical, at a certain concentration, in the respective solvent, mixed with the appropriately diluted extracts. The blank is made only by DPPH/solvent solution. The radical has a purple coloration, whereas in the reduced form (DPPH-H) shows a yellow one. Quantification of the remaining DPPH radicals is recorded from the absorption at λ_{max} 517 nm (or near wavelengths). The DPPH$^{\bullet}$ concentration in the reaction medium against, for example, the flavonoids concentration of samples are plotted to determine, by linear regression, the efficient concentration at 50%, meaning, the amount of the antioxidant needed to decrease by 50% the initial DPPH$^{\cdot}$ concentration (EC_{50} µg/mL) (Table 1) [4, 65]. The results may also be expressed as the equivalent Trolox concentration (mmol Trolox g^{-1} sample) [61].

TEAC assay- The ABTS$^{\bullet+}$ bleaching method

TEAC (Trolox Equivalent Antioxidant Capacity) assay is based on the ability of antioxidant molecules to quench the long-lived ABTS$^{\bullet+}$ (2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) cation, a blue-green chromophore with characteristic absorption at 734 nm, compared with that of Trolox, a water-soluble vitamin E analog. A stable stock solution of ABTS$^{\bullet+}$ is prepared 12-16 h before use, by reacting aqueous solution of ABTS (2,2'-azinobis-3-ethylbenzotiazoline-6-sulfonic acid) with potassium persulfate. The blenching of ABTS$^{\bullet+}$ is registered spectrophotometrically at 734 nm, being a measure of the antioxidant capacity of the sample added in the reaction mixture. The TEAC assay was carried out to measure the antioxidant activity of twelve uniflorral bee pollens harvested from Romania [61]. The results were calculated by standard curves prepared with known concentrations of Trolox, and expressed as "mmol Trolox g^{-1} sample" (Table 1).

β-carotene bleaching (BCB) assay

The method is based on the color change of β-carotene, an orange antioxidant, when exposed to

light and oxygen, meaning, to oxidative conditions. The β-carotene, in the radical, oxidized form, loses the orange color and becomes colorless. However, the presence of antioxidants gives it additional protection, keeping their reduced state and, consequently, its orange color, while this protection lasts. The test is carried out in a β-carotene/linoleic acid emulsion system. The β-carotene's bleaching mechanism is a free radical-mediated phenomenon, by means of neutralizing the linoleate free radical and other free radicals formed in the system from linoleic acid (formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups), which attack the highly unsaturated b-carotene models. In the model system used, b-carotene undergoes rapid discoloration, as the molecules lose their double bonds by oxidation, a fact that can be monitored spectrophotometrically. The presence of antioxidants can hinder the extent of b-carotene-bleaching by neutralizing the free radicals formed in the system. The absorbance is measured at 470 nm, against a blank, consisting of the extracts emulsion without β-carotene. The measurement is continued, at determined time intervals, until β-carotene color disappears. The β-carotene linoleate model system was used to assess the antioxidant activity of bee pollen extracts by means of the inhibition of the β-carotene bleaching [28, 65-66]. However, some studies were not analyzed in more detail, due to the absence of detailed species identification [28]. Lipid peroxidation (LPO) inhibition is calculated as a percentage by means of the b-carotene content after 2 h of assay/initial b-carotene content [28, 66], or by the extract concentration providing 50% antioxidant activity (EC_{50}), calculated by interpolation from the graph of antioxidant activity percentage against extract concentration [65].

Activity against free hydroxyl radical

In order to assess hydroxyl radical-scavenging activity (HRSA) of bee pollen extracts, a chemical system was used, consisted of a buffered reaction mixture composed of Fe(III)-EDTA, 2-deoxy-D-ribose, ascorbic acid, and H_2O_2 to induce the hydroxyl radical formatiom [57]. The antiradical activity was expressed as an inhibition of deoxyribose degradation [67]. The absorbance of the reaction mixture was read in a spectrophotometer at λ_{max} 532 nm against a solution prepared without ascorbic acid. The activity was expressed as the percentage of reaction inhibition. According to the results obtained, HRSA was above 60% (61-98%), for eight of the twelve identified species (Table 1).

Scavenging ROS capacities by employing the ROS-sensitive probe

This methodology measured the reactive oxygen species (ROS) scavenging capacities by using antioxidant capacity assays that employed the reactive oxygen species: (ROS)-sensitive probe, such as 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) or aminophenyl fluorescein (APF). The time-kinetics of ROS reactivity is monitored as fluorescence generation. The effects of the ethanol extract of bee pollen (95% ethanol) on the production of hydrogen peroxide (H_2O_2), superoxide anion ($O2^{\cdot-}$) and hydroxyl radical ($HO\cdot$), was investigated on *in vitro* cultured retinal ganglion cells (RGCs), specifically RGC-5, an E1A virus-transformed rat cell line [2]. Among other bee products tested (propolis and royal jelly), bee pollen exhibited weaker scavenging activity against the $HO\cdot$ than against H_2O_2 and $O2^{\cdot-}$ (Table 1). There are marked differences in antioxidant activities among bee products, the rank order being: propolis > bee pollen > royal jelly.

Ferric ion reducing antioxidant power assay (FRAP)

The ferric reducing/antioxidant power (FRAP) is a simple, direct test of antioxidant capacity. In contrast to other tests of total antioxidant power, is faster and more robust [68]. This method was initially developed to assay plasma antioxidant capacity [69], and further adapted [70]. This assay measures the change in the absorbance at 593 nm owing to the formation of the blue colored Fe^{II}-tripycridyltriazine compound from the colorless oxidized Fe^{III} form do to the action of electron donating antioxidants, at low pH. The working FRAP reagent is prepared *in situ*, mixing acetate buffer, 2,4,6-tripyridyl-s-triazine and ferric chloride solution. The method was used in order to assess antioxidant capacity of twelve honeybee-collected pollens of selected floral species [61]. The authors identified pollen pellets botanical origin, prepared bee pollen extracts with methanol and determined total phenolic and total flavonoid content, to further comparisons and conclusions (Table 1). By measuring the conversion of the Fe^{III} / ferricyanide complex to the ferrous form, in a separate study, the antioxidant activity of six pollen samples, characterized as Mesquite, Yucca, Palm, Terpentine Bush, Mimosa and Chenopod, was evaluated [7].

DISCUSSION

In several studies, close relationship between pollen antioxidant bioactivity and phenolic compounds has been reported [7, 11, 57]. The flavonoid/phenolics contribution to bee-collected floral pollen radical scavenger capacity (RSC) was evaluated (expressed as EC_{50} values) [3]. Pollen sources were identified by HPLC/DAD and each phenolic profile was compared with those of the existing floral pollen database. This is a fundamental procedure, due to bee pollen variability which consequently, results in

significant differences in its chemical composition [3, 5, 11, 19]. In order to assess RSC, the DPPH method was used. For each pollen species, the phenolic fraction was extracted and also tested for DPPH assay. Further, individual fractions and pure compounds were similarly analyzed. Pollen samples RSC was also tested at one year regular intervals, during four years of storage. The results show that C fraction (corresponding to phenolic constituents), representing 6% of the total components, was the most active one, although did not reflect extract's full activity. In the other hand, it was evident that if the storage conditions aren't ideal and monitored, pollen's free radical scavenging capability can decrease over 50%, during the first year [3]. Bee pollen collected from the high intensity ultraviolet Sonoran Desert (USA) was analyzed by the DPPH and the FRAP assays [7]. For six different pollen samples, eight different water miscible solvents were tested, in order to determine the most effective ones. The bee pollen taxa were characterized for each pollen type and total polyphenolics, flavonols and flavones, were determined. Combinations of pollen type, extraction solvent, phenolic content, DPPH and FRAP values were then screened and analyzed. The results showed that methanol and dimethylformamide had provided the best combinations regarding antioxidant activity percentages. DPPH and FRAP assays generally provided coincident values. The *Mimosa* pollen sample had the highest antioxidant activity, of the tested pollen samples and, also, the highest polyphenolic content [7]. The antioxidant properties of bee pollen from twelve plant species were measured as the RSC, (trough the DPPH assay); as the inhibition of lipid peroxidation (expressed in total antioxidant activity) and as the antiradical activity against free hydroxyl radical [57] (Table 1). All species were properly identified and the extracts were prepared in 80% methanol. The results illustrated great variability of the phenolic content, in the investigated species. The highest and lowest levels of total phenols were found in pollens from *P. communis* and *Z. mays*, respectively. However, *P. communis* did not show the best TAA activity (although it was one of the species that exceeded 60% values). Each method used revealed considerable differences among the several pollen species. This finding is consistent with phenolic content great variability. In the other hand, these three methods did not express a coincident antioxidant activity. For example, *Z. mays* had poor values of TAA and RSC but a good hydroxyl radical scavenger activity (HRSA). *P. communis*, in turn, with the greater levels of flavonols, phenylpropanoids and total phenols, had the best HRSA values, but poor RSA values. Nevertheless, some of the species showed good activities, in the three assays and correspondent high phenols levels [57]. TBARS and DPPH assays were used to assess the antioxidant activities of total extracts of a mixture of honeybee-collected pollen and its six constituent pollens [4]. The results showed that all the extracts were effective antioxidants as free radical scavengers, although with lower levels when compared to the standards tested. In this way, caffeic acid provided the best result. Also, a linear and dependent relation was found for all total extracts, between DPPH radical concentration reduction and increased flavonol concentration. The same happened when assessing lipid peroxidation inhibition. In all cases, the relation between MDA concentration reduction and increased flavonol concentration was linear. Concerning the six studied species, their capacity to inhibit lipid peroxidation showed greater differences, compared with the RSC values (Table 1). These studies, as well as the majority of published research, were performed *in vitro*. In contrast, *in vivo* studies are scarce, although fundamental, when it comes to transpose results to living organisms, for further analysis related to effective antioxidant activity and eventual establishment of therapeutic parameters, including doses related ones. The antioxidant activity of *Prosopis juliflora* bee pollen in both systems was compared, by TBARS quantification [10]. In an *in vitro* biological system, the extracts antioxidant activities (test performed in triplicate), were higher than those shown by the references, including quercetin. In the *in vivo* system, the extracts of Mesquite pollen (a flavonoid concentrated and a diluted one) demonstrate antioxidant activity, in the presence of bromobenzene. However, in its absence, the diluted extract showed antioxidant activity, although not as high as vitamin E, in opposition to the concentrated extract, which acted as harmful as bromobenzene itself. This experience illustrates the complexity that resides beneath the dietary intake of antioxidant products, in healthy individuals, which is now under extensive controversy [49].

CONCLUSIONS - ACHIEVEMENTS

According to the extensive research that has already been done, it is possible to establish that:

The majority of bee pollens already analyzed contained flavonol glycosides, usually quercetin and kaempferol derivatives, which are, in turn, associated with powerful antioxidant properties [3-4];

In general, flavonoid/phenolic content is closely related to bee pollen antioxidant activity [3, 7, 57];

The relationship between flavonoid/phenolic content and bee pollen antioxidant activity is far from absolute, but linearity has been found between DPPH radical and MDA reduced concentrations and flavonoid increased ones [3-4];

Within the several fractions that can be extracted from bee pollen, including the lipid, carbohydrates

and protein fractions, the phenolic fraction had the best antioxidant capacity;

DPPH screening for free radical scavenging capability, revealed that each pollen species possess a characteristic EC_{50} value, despite its origin, which, in turn, it's consistent with species-specific flavonoid/phenolic content;

After one year storage, bee pollen RSC may decrease up to 50%, for uncontrolled storage conditions. This decrease is higher for bee pollens with higher antioxidant activities [3];

The existing different methodologies used to assess bee pollen antioxidant capacity, illustrate the different antioxidant mechanisms of action. Therefore and according to bee pollen complex phenolic composition, the analysis should be performed by, at least, two assays. Since pollen composition is species-specific, the qualitative and quantitative differences, between the phenolic derivatives, along with possible interactions among them and/or antioxidant vitamins eventually present, several times these assays exhibited great inter and intra-variability [57, 61]. Other times, they have shown similar results [7];

The antioxidant capacity in an *in vitro* biological system does not represent the real activity *in vivo* [10, 63];

In an *in vivo* system, flavonoids concentration in the bee pollen extract, can determine its antioxidant or pro-oxidant behavior, in the absence of oxidative stress. Under this condition, bee pollen extract exerts protective antioxidant action [10];

Whenever there is a high level of oxidative stress, bee pollen intake has positive benefits, protecting the organism and restraining the eventual damage and further tissue injuries. Therefore, under these conditions, pollen always exhibits antioxidant properties. In most cases, the stress conditions are induced through mice poisoning. In all the studies, including paracetamol [71], bromobenzene [10], carbaryl [72] and protoxur administration [73], the mice also fed with bee pollen, showed an increase in their survival, by preventing hepatic lesions [71]. In addition, the oxidative stress markers were positively changed [72], with the normalization of the activity of several glutathione system enzymes [74]. In this way, if the conclusions about bee pollen's prophylactic role are not clear yet, its effective detoxicating activity is already established.

In vivo studies also showed that bee pollen consumed as a food supplement (with the appropriated dose), modulated antioxidant enzymes, in the mice liver, brain and erythrocytes' lysate, also reducing hepatic lipid peroxidation [63].

CONSIDERATIONS FOR FUTURE RESEARCH

Reactive oxidant species, oxidative stress and antioxidant defenses, were, in the last few years, matters of great popularity and were intensively studied and explored. Nowadays, the research focuses in applying this knowledge to assess, mostly, the bioactive properties of natural occurring products. For bee pollen, however, there are few reports that characterize its antioxidant activities. In addition, many of the published papers illustrate studies that were not properly performed, since many did not provide bee pollen identification, or established comparisons under different experimental conditions, impossible to transpose, as well as comparisons between bee pollen antioxidant activities and other honey derived products, from different botanical origin. Furthermore, these studies would improve with the definition and standardization of experimental conditions, for the establishment of more comparable and reproducible results. Also, several studies use only one method for bee pollens antioxidant activity evaluation, restraining, therefore, the conclusions and thus having a very reductive character. The pollen plant species reported are not many, yet. According to the importance of bee pollen phenolic profile, it would be worthwhile the study of the best solvents for its extraction [7]. In order to establish more accurate relations between bee pollen phenolic content and its antioxidant activity, the performance of cross studies between pollen flavonoid/phenolic composition and antioxidant vitamins content, could be beneficial. These substances interact synergistically in human organism and their eventual concerted action could, perhaps, explain some controversial results. For example, the loss of bee pollen antioxidant activity, after one year storage, although the flavonoid/phenolic constituents' levels remained unchanged [3]. The stability of antioxidant vitamins in bee pollen samples has already been evaluated [75], but this information is yet to correlate with the phenolic content. In order to establish the role of each flavonoid/phenolic derivative, the individual compounds should be separated in further investigations as some previous studies dose [6]. Finally and, as already explained, the impact of performing *in vivo* studies to evaluate this activity should be enhanced. In future researches, they must be used more often, as they provide more reliable results and a deeper understanding of the global mechanisms that are involved within, dependent of many variables that restrain the process. These include the phenolic concentration of the extract used, the living organism degree of oxidative stress and its health condition, previous to the referred administration, the treatment´s duration in time, the presence or absence of other antioxidants intake. Therefore, the detailed analysis of each variable individually and together, as one, holds the key for the future establishment of therapeutic parameters, fundamental for bee pollen consumption in a safe and effective way. It´s of vital importance that, besides the desirable standardized quality, commercialized bee pollen is assayed for

the respective phenolic content, so that the daily dosage could be adequately assured, also providing and predicting the corresponding limited period of treatment.

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LIST OF ABBREVIATIONS

ABTS - 2,2'-azinobis-3-ethylbenzotiazoline-6-sulfonic acid
ABTS$^{\bullet+}$ - 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) cation
APF - aminophenyl fluorescein
BCB - β-carotene bleaching assay
BHT- Butylated hydroxytoluene
BHA- Butylated hydroxyanisole
CAT - Catalase
CM-H2DCFDA - 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester
DNA - Deoxyribonucleic acid
DPPH - 2,2-diphenyl-1-picrylhydrazyl
EC_{50} - The efficient concentration at 50%
EDTA - Ethylenediaminetetraacetic acid
FDA - Food and Drug Administration
FRAP - Ferric Reducing/Antioxidant Power
Gpx – Glutathione peroxidase
HPLC/DAD -High Performance Liquid Chromatography
HRSA - Hydroxyl Radical Scavenging Activity
IC_{50} - The half maximal inhibitory concentration
LA - Linoleic acid
LDL- Low density lipoprotein
LPO - Lipid peroxidation
MDA - Malondialdehyde
RGCs - Retinal ganglion cells
ROS – Reactive Oxigen Species
RSC - Radical Scavenger Capacity
SOD – Superoxid dismutase
TAA - Total Antioxidant Activity
TBA - Thiobarbituric acid
TBARS -Thiobarbituric Acid Reactive Substances
TEAC - Trolox Equivalent Antioxidant Capacity
UV- Ultraviolet radiation
UV-B- Ultraviolet radiation (290–320 nm)

Table 1 - Antioxidant activity values of bee pollen with specific floral origins:

Pollen- Floral Origins	Methodologies										References
	Lipid peroxidation inhibition			Radical Scavenging Activity						Reducing antioxidant power	
	Thiobarbituric Acid Reactive Species (TBARS)		Hidroperoxides Quantification (%)	DPPH assay (*%; #EC_{50} µg ml^{-1}; †mmol Trolox g^{-1})	Activity against free hydroxyl radical (%)	ABTS assay (mmol Trolox g^{-1})	ROS-sensitive reagent tests (IC_{50} µg ml^{-1})			Ferric ion Reducing Antioxidant Power assay (FRAP) (mmol FeII g^{-1})	
	In vitro (*IC_{50} 10^{-1}µg ml^{-1}; †IC_{50} mg ml^{-1})	*In vivo* (*µg mg^{-1} liver; †nmol mg^{-1} protein)					H_2O_2	O^{-}	$HO^{\cdot}$		
Eucalyptus globulus Labill				40,0#							[3]
Metrosideros umbellata				82,0#							[3]
Raphanus raphanistrum L.				130,0#							[3]
Salix atrocinera				150,0#							[3]
Ranunculus sardous				157,0#							[3]
Ulex europeus L.				335,0#							[3]
Cistus ladanifer L.				>500,0#							[3]
							9,99	8,44	57,6		[2]
Echium plantagineum L.				>500,0#							[3]
Erica australis L.				>500,0#							[3]
Ixerba brexioides				>500,0#							[3]
Knigthia excelsa				>500,0#							[3]
Tagetes sp.	2,6*			6,8#							[4]
Amaranthus hybridus L.	0,7*			14,0#							[4]
Solanum rostratum	5,9*			8,4#							[4]
Bidens odorata	3,6*			9,3#							[4]
Ranunculus petiolaris	5,2*			9,9#							[4]
Zea mays	16,2*			10,3#							[4]
			6,8	23,5*	75,8						[57]
Sinapis alba			86,4	90,0*	61,0						[57]
Phacelia tanacetifolia			85,9	66,3*	73,5						[57]
Robinia pseudoacacia			84,4	91,0*	15,8						[57]
Aesculus hippocastamum			81,9	91,3*	10,5						[57]
Taraxacum officinale			77,3	15,2*	50,7						[57]
Chamerion angustifolium			27,2	23,7*	67,5						[57]

Lamium purpureum			51,1	8,6 *	76,1						[57]
Lupinus polyphyllus			38,5	61,7*	24,7						[57]
Malus domestica			76,5	16,0*	92,7						[57]
Pyrus communis			66,4	29,6*	98,0						[57]
Trifolium sp.			55,1	82,2 *	65,1						[57]
Prosopis juliflora	0,07†	0,1373*									[10]
Capsella bursa pastoris L.				1.342†		2.365				2.412	[61]
Helianthus annus L.				0.454†		1.860				1.491	[61]
Crataegus monogyna J.				1.313†		2.785				2.014	[61]
Pinus sp.				0.135†		0.546				0.697	[61]
Matricaria chamomilla L.				1.348†		4.466				5.355	[61]
Carduus sp.				1.432†		2.747				3.382	[61]
Taraxacum officinale Web.				0.348†		1.499				0.327	[61]
Onobrychis viciifolia				0.684†		1.883				1.040	[61]
Centaurea cyanus L.				2.615†		3.638				1.980	[61]
Knautia arvensis				0.274†		0.938				0.255	[61]
Salix sp.				2.814†		6.838				3.760	[61]
Carex sp.				1.533†		3.770				3.055	[61]
Cystus incanus L.		**0**,63†									[63]

2