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Age-Induced Diminution of Free Radical Scavenging Capacity in Bee Pollens and the Contribution of Constituent Flavonoids

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Bee-collected pollen ("bee pollen") is promoted as a health food with a wide range of nutritional and therapeutic properties. The objective of the current study is to evaluate the contribution made through the free radical scavenging capability of bee-collected floval pollens by their flavonoid/phenolics constituents, and to determine whether this capability is affected by aging. The free radical scavenging effectiveness of a bee pollen (EC_{50}) as measured by the DPPH method is shown to be determined by the nature and levels of the constituent floral pollens, which can be assayed via their phenolics profiles by HPLC. Each pure floral pollen has been found to possess a consistent EC_{50} value, irrespective of its geographic origin or date of collection, and the EC_{50} value is determined to a large extent (ca. 50%) by the nature and the levels of the pollen's flavonoids and phenolic acids. Non-phenolic antioxidants, possibly proteins, account for the balance of the activity. Pollen aging over 3 years is demonstrated to reduce the free radical scavenging activity by up to 50% in the most active floral pollens, which tend to contain the highest levels of flavonoids/phenolic acids. It is suggested that the freshness of a bee pollen may be determined from its free radical scavenging capacity relative to that of fresh bee pollen containing the same floral pollen mix.

KEYWORDS: Bee pollen; free radical scavenging activity; α,α-diphenyl-β-picrylhydrazyl; DPPH; quality control; aging

INTRODUCTION

Active oxygen free radicals have been implicated as causative agents in conditions such as cancer, atherosclerosis, cerebral and cardiac ischemia, Parkinson's disease, gastrointestinal disturbances, and aging (*1*). These species can be produced both by normal metabolism and by "external" influences, e.g., UV light and carcinogens. When produced in quantities that overload the body's natural antioxidant and repair defense system, they can bring about breakdown of vital cellular components such as coenzymes, neurotransmitters, and macromolecules e.g., nucleic acids, proteins, lipids, and carbohydrates. Living cells have a limited capacity to nullify the activity of these oxidative free radicals, but it is believed that the ingestion of exogenous antioxidants can improve the protection of vital cellular components and thus their physiological function. Such exogenous antioxidants are commonly obtained from food and include vitamins C and E, β-carotene, and a variety of phenolic compounds, including flavonoids. The effectiveness of flavonoids in this respect is dependent on the presence of certain structural features (*2*).

Bee pollen, that is, floral pollen collected by the honey bee for its protein content, has been used as a nutrient-rich health food for many centuries (*3*), and its benefits have been widely lauded (*3*−*6*). The German Federal Board of Health has recently officially recognized pollen as a medicine (*3*). More specifically, ingestion of bee pollen by rats has been shown to decrease the level of the lipid oxidation products, malondialdehyde and conjugated dienes, in the erythrocytes (*7*), thus suggesting an antioxidant role for bee pollen. The same workers also demonstrated immunostimulation activity on primary and secondary levels of IgM and IgE in rabbits fed on a bee pollen-containing diet for 1 month (*8*).

Key components providing such activity are likely to be the known dietary antioxidants, flavonoids and other phenolic components. These components have been investigated extensively (*9*−*11*), and it has been demonstrated that each constituent floral pollen possesses its own distinct flavonoid/phenolic HPLC profile (*10*, *11*). Individual bee pollen pellets are comprised of just one floral pollen type, and this can be identified by HPLC comparison with reference profiles from authentic floral pollens. Differences in the nature and levels of the flavonoids and other phenolics would suggest that the effectiveness of various floral pollens (and therefore of the bee pollen mixes) as antioxidants/free radical scavengers may vary widely.

The objective of the current study is to evaluate the contribution made through the free radical scavenging capability of bee-collected floral pollens by their flavonoid/phenolics constituents, and to determine whether this capability is affected by aging.

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Table 1. Phenolic Content and EC_{50} Values for Individual Bee-Collected Floral Pollens[a]

	EC_{50} (μg/mL)					
pollen (taxon)	extract with 50% ethanol	phenolic fraction	total phenolic glycosides content (mg rutin equiv/ g pollen)	total phenolic aglycon content (mg quercetin equiv/ g pollen)	total phenolic acids content (mg caffeic acid equiv/ g pollen)	total phenolic content Σ (mg equiv/ g pollen)
Eucalyptus globulus Labill.	40 ± 0.3	8 ± 0.1	3.93 ± 0.1	24 ± 0.2	4.62 ± 0.18	32.58 ± 0.18
Metrosideros umbellata	82 ± 0.5	*b*	5.85 ± 0.1	2.5 ± 0.05	1.5 ± 0.07	9.85 ± 0.1
Raphanus raphanistrum L.	130 ± 2.9	26 ± 0.1	4.67 ± 0.2		7.91 ± 0.3	12.58 ± 0.3
Salix atrocinera Brot.	150 ± 4.7	30 ± 0.09	10.35 ± 0.2		5.55 ± 0.17	15.9 ± 0.2
Ranunculus sardous Crantz	157 ± 7.2	47.7 ± 0.5	8.45 ± 0.12			16.6 ± 0.12
Ulex europeus L	335 ± 10	15 ± 0.05	1.01 ± 0.1		4.30 ± 0.08	5.31 ± 0.09
Cistus ladanifer L.	>500		5.3 ± 0.08			5.3± 0.08
Echium plantagineum L.	over 500		5.87 ± 0.17		0.02 ± 0.002	5.89 ± 0.17
Erica australis L.	over 500		11.77 ± 0.2			11.77 ± 0.2
Ixerba brexioides	over 500		4.3	1.7		6 ± 0.2
Knigthia excelsa	over 500		0.8		0.285	1.085 ± 0.1

[a] $\bar{x}$ ± SME; $\bar{x}$, arithmetic mean; SEM, standard error of mean, calculated at 95% ($P = 0.05$). [b] Insufficient pollen available for phenolics fractionation.

Aging, and in particular the effect this has on the therapeutic (and nutritional) properties of bee pollen, is of importance both to the consumer and to the producer, and a convenient method for the determination of freshness would be of value.

MATERIALS AND METHODS

Pollen Sources and Identification. The floral pollens used in the present study were obtained from selected bee pollen "pellets" containing pure *Salix atrocinera* Brot., *Ranunculus sardous* Crantz, and *Ulex europeus* L. [collected in Portugal and in New Zealand (PT/NZ)]; *Eucalyptus globulus* Labill., *Cistus ladanifer* L., *Echium plantagineum* L., and *Erica australis* L. [collected in Portugal (PT)]; and *Metrosideros umbellata*, *Ixerba brexioides*, and *Knightia excelsa* [found only in New Zealand (NZ) bee pollen].

The identification of each taxon was carried out by HPLC, and the phenolics profiles were compared with our database of floral pollen profiles. Individual components were isolated by paper chromatography, followed by cleanup using RP18 methanol, and structure determination was based on UV absorption spectra, hydrolysis of the glycosides, and NMR spectroscopy. Data relating to this work have been published previously (*9*, *10*, *12*, *13*).

Free Radical Scavenging Effectiveness. Each constituent floral pollen "pellet" was extracted with ethanol:water (1:1), aided by ultrasound, and centrifuged. The extract was then analyzed by HPLC to determine its taxon and the level of phenolics, prior to the measurement of its radical scavenging activity. The concentrations of the compounds in pollen samples were determined using the following curve equations (A = HPLC peak area):

$$\text{flavonoid glycosides} \rightarrow \mu\text{g standard rutin} = 3 \times 10^{-7}A + 5.33 \times 10^{-2} \qquad R^2 = 0.9982$$

$$\text{flavonoid aglycons} \rightarrow \mu\text{g standard quercetin} = 6 \times 10^{-7}A + 6 \times 10^{-5} \qquad R^2 = 0.9999$$

$$\text{caffeic acid derivatives} \rightarrow \mu\text{g standard caffeic acid} = 1 \times 10^{-7}A + 5.62 \times 10^{-2} \qquad R^2 = 0.9993$$

The free radical scavenging effectiveness of the pollen extract and/or the purified phenolic fraction (from the more active taxa) on the α,α-diphenyl-β-picrylhydrazyl (DPPH) radical was measured by using a spectrophotometric system as previously described by Navarro et al. (*14*, *15*). Briefly, this involved the mixing of 2 mL of DPPH solution (6×10^{-5} M in ethanol) with an appropriate amount of extract, followed immediately by homogenization using a vortex. After 10 min, quantification of the remaining DPPH radicals is recorded from the absorption at λ 517 nm. The reference standard was ascorbic acid with a normal $EC_{50} = 1.5 \times 10^{-2}$ mM ($R\wedge = 0.9993$). Results are presented in **Table 1** in EC_{50} values, which represent the weight of sample required to scavenge 50% of the DPPH radicals available.

Separations of Constituent Types from *E. globulus* (and Other) Pollens. The sequence of extractions used to separate lipids, phenolics, proteins, and carbohydrates from *E. globulus* pollen is summarized as follows.

First, bee pollen was defatted with *n*-hexane (fraction A, lipid constituents) and afterward filtrated. The sample was then recovered from the filter and extracted with 50% ethanol (fraction B). The ethanol was evaporated under vacuum at 35 °C and the remaining water extracted with ethyl acetate (1:1) to separate the phenolic content of the organic fraction (fraction C). The aquous extract was washed with *n*-butanol to separate the protein constituents (fraction D). The carbohydrate content remained in the aqueous fraction (fraction E). TLC for each group of constituents followed each process.

From the phenolic fraction, quercetin-3-*O*-sophoroside, 3-methoxyquercetin, luteolin, tricetin, and myricetin were isolated and identified by UV and NMR methods (*10*).

Individual fractions and pure compounds were subsequently assessed for free radical scavenging effectiveness using the DPPH method described above. The results were compared with the luteolin, tricetin, and myricitin standards (from Sigma). Quercetrin and ascorbic acid represent the reference standard. 7- and 8-methoxyherbacetin and the 8-methoxyherbacetinsophoroside were also assayed because they have a methoxylated structure (they are not available on the market and were isolated by the authors in a previous work) (*12*).

RESULTS AND DISCUSSION

The structures of the phenolics found in *Salix atrocinera* Brot., *Ranunculus sardous* Crantz, *Ulex europeus* L., *Eucalyptus globulus* Labill., *Cistus ladanifer* L., *Echium plantagineum* L., *Erica australis* L., *Metrosideros umbellata*, *Ixerba brexioides*, and *Knigthia excelsa* were determined in earlier work, and details of their structure determination have been published previously (*9*, *10*, *12*). The flavonoid/phenolic profiles, both qualitatively and quantitatively, appear to be species-specific for the individual pollens that make up bee pollen. Thus, the major taxa represented in a bee pollen mix can be identified from the bee pollen's HPLC profile, which is a composite, reflecting its constituent pollens (*10*). All the pollens analyzed contained flavonol glycosides, usually quercetin, kaempferol, herbacetin, or isorhamnetin derivatives, and some also contained aglycon types which were generally not represented as glycosides, e.g., myricetin, tricetin, luteolin, and 3-*O*-methylquercetin. Significative levels of phenolic acid derivatives were also present in some pollens (*9*, *10*, *12*, *13*).

The species-specific flavonoid/phenolics profiles of individual floral pollens appear to be mirrored in the free radical scaveng-

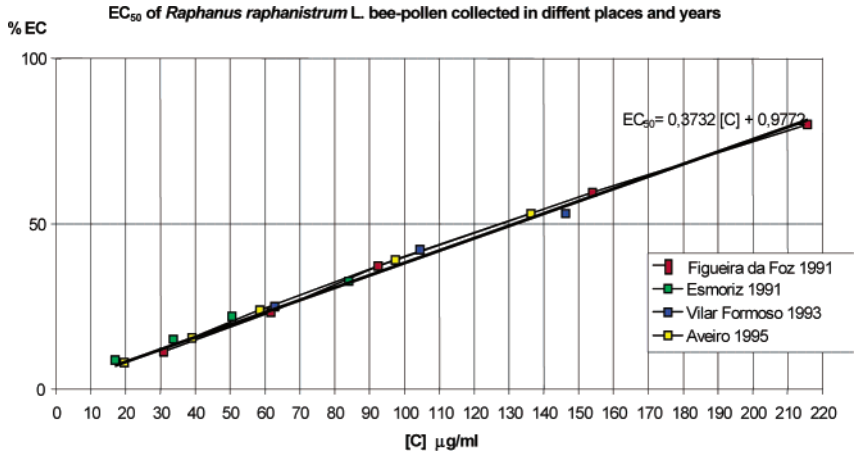

Figure 1. Free radical scavenging capacity of *Raphanus raphanistrum* L. bee pollen collected in four different places between 1991 and 1995.

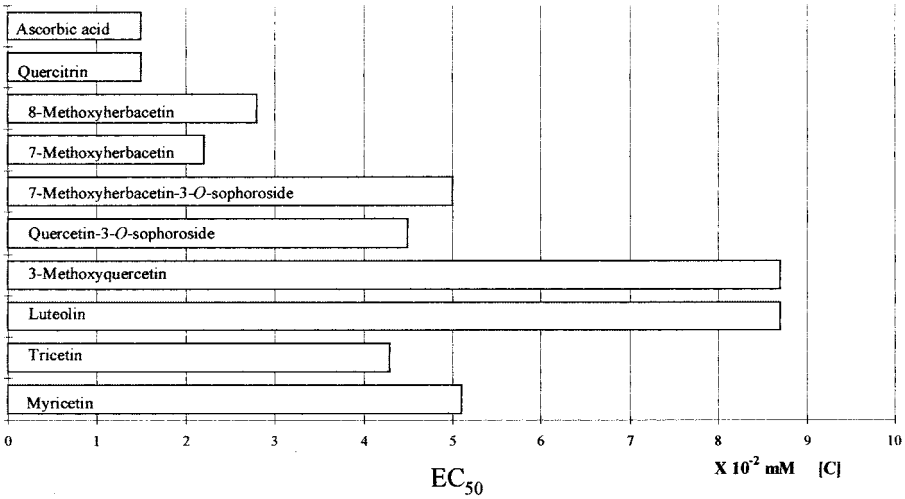

Figure 2. Comparison of EC_{50} values for a range of pollen flavonoids and ascorbic acid. Compounds were isolated from different pollens except for ascorbic acid. The same result can be obtained with standards when available, as for example quercetrin, luteolin, tricetin, and myricetin.

ing capability of floral pollens. DPPH screening for free radical scavenging capability revealed that each pollen species possesses a characteristic EC_{50} value, irrespective of its origin or when it was collected. A good example of this is presented in **Figure 1**, which illustrates the consistency of the free radical scavenging capacity of *Raphanus raphanistrum* L. pollen collected from different sites within Portugal at different times. There is some logic in relating these consistent EC_{50} values to the qualitatively and quantitatively consistent flavonoid/phenolics content associated with each floral pollen type, as each of these components possesses its own characteristic free radical scavenging capability (**Figure 2**).

The free radical scavenging activity, however, seems not to be due to the phenolics alone, as there is only an approximate correlation between total phenolic content (flavonoid glycosides, flavonoid aglycons, plus phenolic acid derivatives) and the EC_{50} values (**Table 1**).

In the current investigation it has become evident that upon aging, a pollen's free radical scavenging capability can decrease by up to 50% during the first year (for the more active pollens) if the storage conditions are not the ideal (e.g., if there is no control of temperature, moisture, etc.). A dry, cold, and darkened environment can be considered as the normal conditions to preserve bee pollen, as usual for this kind of product. The changes observed in the activity of *E. globulus* pollen over a period of 4 years are presented in **Figure 3**.

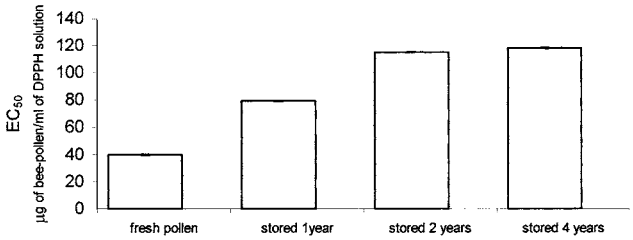

Figure 3. EC_{50} in *Eucalyptus globulus* pollen on aging.

To determine the contribution that the phenolic fraction makes toward to the free radical scavenging capacity of a floral pollen extract, *Eucalyptus globulus* component types were separated into fractions, as outlined in Material and Methods. The exine (45 wt %) and the lipid fraction (3%) were found to be inactive and to constitute 48% of the original.

The EC_{50} for the crude extract (bee pollen ultrasonicated with 50% ethanol) is the same as that of fraction B (40μg/mL), corresponding to the fraction without exine and lipids. From this, it is evident that the activity of bee pollen will be found in the remaining 52% of the total compounds.

Fraction C (phenolic constituents), with $EC_{50} = 8$ μg/mL, represents 6% of the total components. This fraction was the most active one but did not reflect the full activity of the crude extract.

Table 2. Effect of 3-Year Aging on the EC_{50} of Selected Pollens

pollen (taxon)	EC_{50} (μg/mL) fresh	3 years old
Eucalyptus globulus Labill. (PT)	40 ± 0.3	120 ± 0.3
Metrosidros umbellate (NZ)	82 ± 0.5	152 ± 0.9
Raphanus raphanistrum L. (PT)	120 ± 2.9	120 ± 2.9
Salix atrocinera Brot. (PT/NZ)	150 ± 4.5	150 ± 4.5
Ranunculus sardous Crantz (PT/NZ)	157 ± 2	276 ± 2
Ulex europeus L. (PT/NZ)	332 ± 5.1	334 ± 6
Echium plantagineum L. (PT)	≫500	
Erica australis L. (PT)	≫500	
Cistus ladanifer L. (PT)	≫500	
Ixerba brexioides (NZ)	≫500	
Knigthia excelsa (NZ)	≫500	

[a] $\bar{x}$ ± SME; $\bar{x}$, arithmetic mean; SEM, standard error of mean, calculated at 95% ($P = 0.05$).

The other fractions which contain amino acids, proteins (fraction D, 17 wt %), and carbohydrates, mainly glucose and fructose (fraction E, 9 wt %), showed little activity and certainly was insufficient to make up the deficit. Earlier work involving aging studies on *E. globulus* pollen by one of us (*16*) demonstrated that the nature and the levels of phenolic/flavonoid constituents remained unchanged after 1 year of storage, yet the activity decreased by 50% (**Figure 3**). This indicates that the loss in the activity resulting from the separation of component types has occurred in the non-phenolic fractions during the workup, possibly as a result of protein denaturation.

That the flavonoid/phenolic components must play a significant role in the free radical scavenging capacity of a floral or bee pollen generally is indicated by the observation that the pollens which exhibit the highest activity *tend* to be those that contain the highest levels of flavonoids and phenolic acid derivatives (**Table 1**). This relationship is far from absolute, however, due to the varying activity of different flavonoid types (**Figure 2**) and to the presence of other antioxidant constituents (for example, vitamins).

The glycosidic forms of the polyphenols analyzed did not suffer hydrolysis during the workup. The aglycons found (3-*O*-methylquercetin, luteolin, tricetin, and myricetin) are free in the original samples and different from the aglycons of the glycosides identified (glycosides of quercetin, kaempferol, and herbacetin).

Another interesting finding of this work, although not entirely unexpected, is that the pollens with higher activity tend to suffer the greatest percentage losses on aging (**Table 2**).

On the basis of the above findings, it is concluded that the free radical scavenging effectiveness of a bee pollen is determined by its constituent pollens, and that the EC_{50} values for these constituent pollens are consistent (always the same) for each pollen species. The isolated compounds reveal the same result determined with the standards. The EC_{50} values are in large part determined by the free radical scavenging activity of the flavonoid/phenolic constituents, although other constituents, perhaps proteins, can contribute up to half the activity. This activity can decrease significantly on storage (aging), and it is therefore proposed that the freshness of a bee pollen may be determined from its free radical scavenging capacity relative to that of fresh bee pollen with the same floral pollen mix.

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