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Antioxidant activity of Sonoran Desert bee pollen

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ABSTRACT

Bee pollen (pollen collected by honey bees) was collected in the high intensity ultraviolet (UV) Sonoran Desert and analyzed by the DPPH (radical 2,2-diphenyl-1-picryhydrazyl) assay and the FRAP (ferric reducing-antioxidant power) assay on six different pollen samples and in eight different water miscible solvents at 50 mg/ml. The bee pollen taxa were characterized for each pollen type by acetylization of the pollen extracts followed by microscopy and comparison with a library of samples native to the Sonoran Desert. The standards (R-(+)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), known as TROLOX, gallic acid and α-tocopherol (vitamin E) were analysed as standards to determine the potency of each pollen sample in the most efficient solvent. The Mimosa pollen sample displayed the highest antioxidant activity. Total polyphenolics, flavanols, flavones were determined, and the results are reported in milligrams of gallic acid, quercetin and naringenin per gram of pollen, respectively. There was good correlation between antioxidant activity and total phenolics. The order of effectiveness of the pollen samples in regard to antioxidant activity was determined and the most effective extraction solvents are discussed. Finally, solid phase micro-extraction, coupled with gas chromatography–mass spectroscopy was utilized to identify and quantify polyphenolic compounds known to have free radical scavenging activity in the pollen samples.

1. Introduction

Pollen is the reproductive cells of plants. Bees, other insects, wind and water pollinate plants by transferring pollen from the stamen to the stigma of another plant. Honey bees collect pollen by adding sugars from nectar to hold the grains together and then transfer them back to the colony by packing them into hairs on the corbiculae (hind legs) of bees (Snodgrass, 1975). The role of pollen to the sustenance of the bee colony cannot be overestimated. The bees consume pollen in their own diets and use it to feed larvae. The bees place the pollen in honeycombs with their legs and cover this pollen with honey. This pollen store is referred to by beekeepers as "bee bread" (Stanley & Linskens, 1974, p. 98). It was determined that an average value of 145 mg of pollen is required to rear just one worker bee. Results from survival studies conducted, on caged bees, where bees were fed pollen samples collected from the Sonoran Desert revealed that Mesquite pollen was most desirable to bees (Schmidt & Johnson, 1984), while Palm pollen was much less desirable. Bee pollen is also used as a dietary supplement for humans and reports have appeared in the literature regarding the antioxidant activity of bee collected pollen and the total phenolics (Campos, Webby, Markham, Mitchell, & Da Cunha, 2003; Leja, Mareczek, Wyzgolik, Klepacz-Baniak, & Czekonska, 2007; Rozema et al., 2001). Propolis, which is a resinous, high phenolic containing apiculture product has been well characterized for its health benefits related to its anti-microbial and antioxidant activities (Kosalec, Bakmaz, & Pepeljnjak, 2003; Russo et al., 2004).

Pollen serves several purposes in plant reproduction. An open reproductive system where the DNA of one plant is transferred to another plant is likely to have an efficient method of protection against environmental conditions, especially UV exposure. Evidence of this is that mountain plants have correspondingly higher levels of epoxy-carotenoids and xanthophylls with an increase in altitude (Asbek, 1958; Stanley & Linskens, 1974, pp. 228–229). The DNA in pollen does not have a metabolic repair mechanism found in other diploid cellular DNA. Therefore, it is likely that antioxidative compounds in pollen could offer protection against solar radiation. Previously it was reported that pine pollen could be harmed during 3–4 h exposure to UV radiation from light of the sun. Pine needles and pollen have been found to have a relatively high percentage of ascorbic acid. It was also shown that the flavonoid content and concentrations of outer pollen walls differ compared to the intracellular cytoplasmic concentrations, cell wall fractions, and extraction residues and that many of the phenolics are organic acids such as: p-hydroxybenzoic, p-coumaric, vanillic, gallic and ferulic acid (Rosema et al., 2001; Standifer, 1966; Strohl & Siekl, 1965). Many of the pigments are phenolic components and

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have been isolated as flavonoid glycosides (Heslop-Harrison, 1973; Tapi & Menziani, 1955).

There are very few reports characterizing the antioxidant activities of bee pollen. We are not aware of any previous reports on the antioxidant assays of Sonoran Desert bee pollen except those listed on the world wide web site (http://www.ccpollen.com/ORAC.shtml) that compares the oxygen radical absorbing capacity assay (ORAC) results on high desert bee pollens and reports that the bee pollen is more effective as an antioxidant than pomegranate extract or black raspberry extracts. Thus, the aim of this work was to evaluate the antioxidant activity of six pollen samples collected in the Sonoran Desert that are exposed to high levels of UV radiation from the sun. Also, since very little research has been reported on pollen extraction, we report our optimized extraction procedure. For the extraction, eight different solvents were used to determine the most effective solvents. Once the most facile solvent(s) were determined, the active antioxidant compounds were quantified by spectrophotometric assays and identified by solid phase micro-extraction and gas chromatography mass spectroscopy. Furthermore, this study evaluates the hypothesis that an early fall blooming taxa, such as the Yucca plant, does not have the reactive oxygen species (ROS) quenching ability as a taxa that blooms during a UV-intense period such as the Mesquite, Yucca, or especially the Chenopod and Mimosa pollen samples, which were collected during July through August.

2. Materials and methods

2.1. Materials

The pollen pellets were collected between March and November 2006 from beehives located in the Sonoran Desert, North of Tucson, AZ, USA and were purchased from Freddy T's Beeswax, Oracle, AZ, USA with the exception of the Palm pollen, which was collected at our facility. The six pollen samples analyzed in this study were characterized as: Mesquite, Yucca, Palm, Terpentine Bush, Mimosa and Chenopod. The pollen taxa and the occurrence of the pollen taxa in the samples are listed in Table 1. The pollen samples were dried at 50 °C and then kept at room temperature under a nitrogen atmosphere until the mass, measured on an analytical balance, was constant. The reagents and solvents were purchased from Sigma Aldrich and used as described.

2.2. Preparation of the extracts

2.2.1. Solvents comparisons

The pollen pellets (500 mg) were suspended in water miscible solvents (5 ml) (listed in Tables 2 and 3) in a screw cap centrifuge tube and the concentration maintained at 50 mg/ml volumetrically. The test tubes were vortexed and placed in an ultrasound bath at 41 °C for 90 min, with vortexing every 30 min. The samples were refrigerated at 3 °C overnight then centrifuged at 2500 RPM for 1 min. Any samples that remained turbid or opaque were refrigerated and centrifuged at 3200 RPM for 10 min and this step repeated until transparent. The supernatant was then pipetted into a 20 ml scintillation vial. Any samples that contained particulate were microfiltrated with a Pasteur pipet with a cotton filter. The samples were then capped and refrigerated at 3 °C. All antioxidant assays were performed within 7 days of extraction.

Methanolic extracts for ED_{50}, total polyphenolics, flavones, flavanols, flavonones and analysis by solid phase micro-extraction with gas chromatography–mass spectroscopy: The pollen pellets (2.000 g) were suspended in 7 ml methanol (MeOH) and placed in an ultrasound bath for 30 min followed by centrifugation at 3200 RPM for 5 min. The supernatant was transferred to a tared 100 ml round bottom flask. These steps were repeated 8 times. The MeOH was flash evaporated and dried under a gentle stream of nitrogen. To the residue was added 2 ml of water and the suspension was flash frozen and freeze dried overnight. The residue was dissolved in MeOH for a final concentration of 20 mg/ml.

2.3. Preparation for microscopic analysis

The centrifugants from the antioxidant assays were acetylated (9:1 solution of acetic anhydride and sulphuric acid), stained (safranin "O"), mounted in glycerin on microscope slides and the pollen grains counted (ca. 500 grains per sample) at 460× magnification.

2.4. Determination of antioxidant activity using the DPPH radical scavenging method

The 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay is a robust, facile antioxidant assay. We used a modification of the assay conditions reported by Hatano, Kagawa, Yasuhara, & Okuda (1988). The method was scaled to a 96 well plate format (330 μl scale). The DPPH molecule is a stable radical that accepts an electron from the analyte. Ethyl alcohol (280 μl) was mixed with the solvent extract (72 μl) in a scintillation vial and DPPH solution (242.5 μl of a 51 mg DPPH/100 ml MeOH) added and the vial was capped and vortexed. The more active samples were readily apparent, as the purple colour becomes a light yellow colour. Immediately following, 160 μl was transferred to each well and the samples run in triplicate. The micro-plate was sealed (Thermal Seal, Excel Scientific, Wrightwood, CA) and incubated in the dark for 1 h at room temperature. After a programmed 30 s shake cycle, the absorbance was recorded at 517 nm. Exaction solvents (72 μl) volumes was used as controls for each solvent extract. The percent activity calculated by the following equation by substituting the mean of the absorbance values, which were recorded in triplicate:

$$\%\text{scavenging activity} = 100 \times [\text{control-sample}]/\text{control}$$

ED_{50} values (concentration required to quench 50% of the radicals under the experimental conditions in this assay) were calculated for several pollen MeOH and DMF extracts showing high levels of scavenging activity.

2.5. Determination of antixoxidant activity using ferric reducing-antioxidant power

The ferric reducing-antioxidant power (FRAP) assay was performed according to conditions reported by Benzie and Strain (1999). Water (735 μl; 18 M-Ohm; Barnstead) was mixed with 450 μl of FRAP solution. The FRAP solution was freshly prepared by mixing: 10 mM 2,4,6-tripyridyl-S-triazine (TPZ) in 40 mM HCl, 20 mM $FeCl_3$ solution and 0.3 M NaOAc/HOAc buffer at pH 3.6 at (1:1:10) parts per volume respectively. To a 20 μl scintillation vial was added 450 μl of the FRAP solution and 20 μl of the pollen extract and 735 μl water and the mixture was capped and vortexed; the more active samples turned a dark blue color spontaneously. Next, 100 μl was transferred the well of a 96 well, flat bottom micro-plate (Costar, Cambridge, MA). The samples were run in triplicate and the micro-plate sealed (Thermal Seal) and incubated in the dark in an oven maintained at 37 °C for 30 min. The temperature was maintained at 37 °C, and absorbance was recorded at 593 nm, after a 30 s shake cycle. The mean values for the blanks were subtracted from the means of the absorbance values. Extraction solvent (20 μl) was used as a control. The reported values are expressed in terms of the amount of the ferrous form of [Fe^{2+}] produced from a standard curve plot