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Preliminary Analyses of Phenolic Compounds and Antioxidant Activities in Tea Pollen Extracts

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ABSTRACT

Tea (*Camellia sinensis*) pollen is one of the main bee-collected pollen staples in Taiwan. We analyzed the contents of the phenolic compounds (total phenols, flavonoids, free aglycones of flavonoid and phenolic acids) in tea pollen, obtained by aqueous extraction (cold and hot water) or ethanolic extraction (50% EE and 95% EE). The antioxidant activity, capacity of scavenging H_2O_2, and chelating ability were measured. Nine phenolic compounds, including gallic acid, catechin, methyl gallate, caffeic acid, *p*-coumaric acid, ferulic acid, rutin, myricetin, and chlorogenic acid, were identified and quantified, while gallic acid (0.69 ± 0.01 to 2.55 ± 0.05 mg/g) was the most abundant. In the four tea pollen extracts, caffeic acid, *P*-coumaric acid, and ferulic acid presented high concentrations. Methyl gallate, rutin and myricetin were detected only in the ethanolic extracts (50% and 95% ethanolic extracts). Ethanolic extracts contained more phenolic compounds than in aqueous extracts. The total antioxidant activity, as measured by the ABTS (2, 2′-azino-bis-(3-ethylbenzthiazoline- 6-sulfonic acid) method, indicated that the ethanolic extracts had greater antioxidant activities than the aqueous extracts and the 50% ethanolic extract effectively chelated ferrous ions. The cold water extract had the greatest hydrogen peroxide scavenging ability. We believe this is the first report on the content of phenolic compounds, especially free flavonoid aglycones, and phenolic acids from tea pollen. Our findings indicated that tea pollen could be a good nutraceutical and dietary antioxidant supplement.

Key words: tea pollen, phenolic compounds, free flavonoid aglycones, phenolic acids, antioxidant activity

INTRODUCTION

The pollen collected by honeybees contains rich nutrients, such as proteins, lipids, minerals and vitamins, for brood rearing and bee growth and development[1,2]. Bee pollen is used as a folk medicine to alleviate or cure colds, flu, ulcers, premature aging, anemia, colitis, allergic reactions, enteritis, chronic prostatitis and other illnesses. Bee pollen have anti-inflammatory, antineoplastic, anti-oxidative, and anti-androgenic effects[2-4], and is a good nutritional supplement with the beneficial effect for health[2,3]. Pollen is recognized as a medicine in China and Germany[5,6]. In Taiwan, tea (*Camellia sinensis*) trees are commonly cultivated for manufacturing green tea, Oolong tea, and black tea. Tea (*C. sinensis*) pollen is, therefore, a major and popular bee-collected staple in the markets. Tea pollen is usually collected by honeybees from September to January of the following year, and its color is orange-red, with a sweet taste and pleasant floral aroma.

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Pollen possesses several valuable phytochemicals including carotenoids, steroids, terpenes, and flavonoids[2,3,7,8]. Many investigations of pollen have focused on the phytochemicals with high antioxidant capabilities of the phenolic substances[6]. The major phenolic compounds are flavonoids and phenolic acids[3,9] in plants. Flavonoids can be categorized as flavonols, flavanones, flavanols, flavones, anthocyanidins and isoflavones. The flavonol glycosides and phenolic acids also constitute the main classes of polyphenols in pollen[3]. Recently, many researchers have been focusing on flavonoids that are anticarcinogenic, anti-inflammatory, antibacterial, immune-modulatory, anti-allergic, antiviral, and estrogenic[4,7,10,11]. Almaraz-Abarca *et al*.[3] reported that phenolics and flavonoids played significant roles in the antioxidant capacity of pollen. Various flavonoid substances have been isolated from pollen. In a recent study, Almaraz-Abarca *et al*.[3] found flavonoids, such as flavones, flavonols, and derivatives of apigenin, luteolin, and cinnamic acid, in the pollen of the anthers of the mesquite tree *(Prosopis juliflora*, Leguminosae) in Mexico. Markham and Campos[12] reported

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that flavonoids, both aglycones and glycoside derivatives, presented extensively on the surface of natural pollen. The predominant form of flavonoids generally present is glycosides[13]. The flavonoid glycosides can be hydrolyzed into aglycones *in vivo* by the action of glucosidases[7]. Bee pollen is composed of natural pollen grains, nectar and bee saliva containing the hydrolytic enzymes α- and β-glucosidases which could hydrolyze the flavonoid derivatives (glycosides) to sugar and aglycone. As a result, the composition of the free flavonoid aglycones in pollen have been extensively studied and identified[7,14].

Serra Bonvehí *et al.*[7] identified and quantified 15 free flavonoid aglycones from 11 *Cistus ladaniferus* pollen samples using HPLC without prior hydrolysis. Almaraz-Abarca *et al.*[3] established a phenolics profile for *Prosopis juliflora* pollen, identifying 12 major phenolics without any chemical pretreatment. We chose to analyze the free aglycones and phenolic acids in honeybee-collected tea pollen directly by HPLC.

While several reports have shown pollen is rich in flavonoids and high in antioxidant activities[15,16], to our knowledge, no report is available for individual aglycones and the antioxidant activities of tea pollen. Therefore, we determined the total polyphenol and flavonoid contents of the tea pollen, and systematically investigated the free aglycone and phenolic acid composition of polyphenolic compounds, and their antioxidant properties characterized using several different assays. These include (1) using $ABTS^{\cdot+}$ to assess antioxidant activity, (2) adding horseradish peroxidase to determine the scavenging ability of H_2O_2, and (3) measuring the ferrozine-Fe^{2+} complex absorbance to establish the iron chelating abilities.

MATERIALS AND METHODS

I. *Chemicals and Reagents*

Peroxidase, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-carboxylic acid), trifluoroacetic acid, phenol red, gallic acid, methyl gallate, caffeic acid, *p*-coumaric acid, ferulic acid, rutin, myricetin, quercetin, ferrozine (3-(2-pyridyl)-5,6-diphenyl -1,2,4- triazine-4′,-4″-disulfonic acid sodium salt), chlorogenic acid, catechin, and ABTS (2, 2'-azino-bis (3-ethylbenzthiazoline - 6-sulfonic acid)) were purchased from Sigma -Aldrich (St. Louis, MO, USA). Sodium carbonate, aluminum trichloride and Folin-Ciocalteu reagent were from Merck (Darmstadt, Germany). Ferrous chloride and methanol were obtained from Mallinckrodt (Phillipsburg, NJ, USA). All reagents were of analytical grade.

II. *Pollen Sources*

The tea (*C. sinensis*) pollen samples were harvested by local beekeepers from western regions of Taiwan in 2008. The monospecificity of the samples was confirmed by examining the color uniformity and observing the shape and size of pollen grains under microscope. All samples were stored at -18°C until analysis.

III. *Preparation of the Pollen Extracts*

The appropriate amount of distilled water was used to make 95% or 50% ethanol solutions for 10 g of tea pollen to make a final volume of 100 mL. Four types of pollen extracts were obtained by the following two steps: sonication (disruption) and agitation (extraction). First, a 60 mL sample was homogenized using a sonicator (Sonicator XL2020, Misonix Inc., Farmingdale, NY, USA) with the pulsar set at 35% of output power for 30 min, followed by stirring with a magnetic stirrer at room temperature (designated as the cold water extract, CWE) or incubated at 95°C in water bath (designated as the hot water extract, HWE) for 4 h. The other two samples were prepared at room temperature by 50% or 95% ethanolic extractions, designated as 50% EE and 95% EE, respectively. Finally, all samples were centrifuged at 9167 g at 4°C for 20 min to obtain the supernatant (extract I). To the precipitate, aliquots of 40 mL solvent were added, and the above extraction steps were repeated to obtain the supernatant extract II. Combining extracts I and II, four sets of samples were prepared. Samples of the CWE and HWE were dehydrated by lyophilization, while the 50% EE and 95% EE samples were concentrated by vacuum evaporation at 40°C.

IV. *The Phenolic Compounds Analysis*

(I) *The Total Polyphenolic Compound and Flavonoid Contents*

The analysis of total phenolic content was based on the Folin-Ciocalteu method modified by Gorinstein *et al.*[17] Briefly, tea pollen extract solution (0.1 mL) was mixed with 2 mL of 20 g/L sodium carbonate, then 0.1 mL of Folin-Ciocalteu aqueous reagent (50 mL in 100 mL water) was added and the solution was let stand for 30 min. The samples were measured spectrophotometrically at 760 nm, and the total phenolic content was expressed as gallic acid equivalents in mg per gram of pollen extract. The flavonoid content was determined using crystalline aluminum chloride assay according to the method described by Maksimović *et al.*[18] Pollen extract solution (1.5 mL) was added to an equal volume of 20 g/L aluminum trichloride and let stand for 10 min to allow formation of a flavonoid-aluminum complex then the absorbance at 430 nm was read. The flavonoid amount was calculated as a quercetin equivalent and expressed as mg of quercetin per gram of pollen extract.

(II) *Identification of Free Aglycone of Flavonoid Compounds*

Pollen extracts were separated by a reverse phase column (Mightysil RP-18 GP 250 × 4.6 mm, 5 μm, Kanto Corporation, Portland, OR, USA) according to the method of Nuutila *et al.*[19] The HPLC system (Hitachi, Tokyo, Japan)

was equipped with an autosampler (Hitachi, L-2200) and a photodiode-array detector (Hitachi, L-2455). The injection volume was 10 μL, and the pump (Hitachi, L-2130) applied a gradient of solvent A (20% methanol with 300 μL/L of trifluoroacetic acid) and solvent B (100% methanol with 300 μL/L of trifluoroacetic acid) at a flow-rate of 1.0 mL/min. The gradient elution was used starting with 0% A (100% of solvent B), by a linear increase of solvent A to 50% in 25 min, and then brought mobile phase composition back to the initial condition at 45 min (100% of solvent B). Finally, 100% A was obtained at 55 min for a cycle. It was re-equilibrated for the next analysis. The analyses were monitored at a wavelength of 280 nm, and the calibration standards used for quantification of the pollen extracts were gallic acid, catechin, methyl gallate, caffeic acid, *p*-coumaric acid, ferulic acid, rutin, myricetin, and chlorogenic acid.

V. *Antioxidant Activity*

The antioxidant effects of the tea pollen extracts were evaluated by several methods. All measurements were performed in triplicate using a spectrophotometer (Hitachi U-2000, Tokyo, Japan).

(I) *Total Antioxidant Activity*

Total antioxidant activity was obtained by measuring the scavenging activity of radical $ABTS^{\cdot+}$ according to the method described by Villaño *et al.*[20] with modifications. The color of the $ABTS^{\cdot+}$ was blue-green and generated by the interaction of ABTS, H_2O_2, and peroxidase. $ABTS^{\cdot+}$ has a characteristic absorption long wavelength at 734 nm, measured after a reaction time of 10 min. The ABTS, peroxidase, and H_2O_2 were freshly prepared in a 5 mM phosphate buffer solution (pH 7.6). First, 0.3 mL of ABTS (333 μM) was added to a peroxidase solution (20 units/mL), then 0.4 mL of H_2O_2 (125 μM) solution was added and vortexed vigorously for 1 min. The mixture was then kept in the dark for 1 h before adding 0.3 mL of pollen extract (0.05 - 0.5 mg/mL) to the reaction solution. The antioxidant potency of trolox, a water-soluble analogue of vitamin E, was used as the positive control. The final total antioxidant activity of the pollen extract was based on the decolorization of $ABTS^{\cdot+}$ at 734 nm as compared to the control. The total antioxidant activity was calculated as follows:

Total antioxidant activity (TAA %) =

$[1- (A_{sample} - A_{blank}) / (A_{control} - A_{blank})] \times 100$

(II) *Scavenging Capacity of Hydrogen Peroxide*

Determination of the hydrogen peroxide scavenging activities of the pollen extracts was made using the method of Cui, Kim and Park[21], with modifications. An aliquot of pollen extract (0.5 mL) was mixed with 0.2 mL of freshly prepared 4 mM hydrogen peroxide and allowed to react for 20 min. Next, 0.3 mL of peroxidase-phenol red (a mixture of 800 units/mL of peroxidase and 7.5 mM of phenol red) was added and allowed to stand at room temperature for 15 min. The reaction was stopped by placing the tubes in an ice bath, and the absorbance of the reaction mixtures was measured at 610 nm. The H_2O_2 scavenging capacity was calculated as follows:

Scavenging effect % =

$[1- (A_{sample} - A_{blank}) / (A_{control} - A_{blank})] \times 100$

(III) *Metal Ion Chelation*

The ability of the pollen extracts to chelate Fe^{2+} was measured using the method of Gülçin[22], with modifications. EDTA (ethylenediaminetetraacetic acid) was used as the positive control. The extract sample (200 μL) was mixed with 20 μL of 2 mM $FeCl_2$ and 640 μL of methanol for 30 sec. The reaction was then initiated by adding 40 μL of 5 mM ferrozine, and the reaction mixture was incubated at room temperature for 10 min. The absorbance at 562 nm was then read. The inhibition of ferrozine- Fe^{2+} complex formation was calculated as follows:

Chelating effects (%) =

$[1- (A_{sample} - A_{blank}) / (A_{control} - A_{blank})] \times 100$

VI. *Statistical Analysis*

Results were evaluated using one-way ANOVA and Duncan's multiple range test. The level of significance was set at $p < 0.05$. SPSS software (SPSS for Windows, version 10.0; SPSS, Inc., Chicago, IL, USA) was used for the analyses. Data are presented as means ± standard deviations (SD) and all analyses were done in triplicate.

RESULTS AND DISCUSSION

I. *Identification of Pollen*

Samples of honeybee-collected tea (*C. sinensis*) pollen were checked on the flavor for organoleptic quality, the mouth-feel, and the color (orange), and by performing microscopic analyses. Each pellet grain was largely homogeneous and predominantly from one floral source. The microscopic examination of the pollen shape indicated the pollen particle was a solid equilateral triangle with a 40 - 50 μm side length (Figure 1).

II. *The Phenolic Compounds*

(I) *The Total Polyphenolic Compounds and Flavonoids Contents*

The phenolic constituents, flavonoid glycosides, flavonoid aglycons, and phenolic acid derivatives have been isolated and identified from some specific species of bee pollen[23-25]. Kroyer and Hegedus[4] have been analyzed the total polyphenol contents from mixture of bee collected pollen, which was extracted individually using ethanol,

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Table 1. The extract yield, total phenolic content (TPC) and flavonoid content (FC) for four extracts of *Camellia sinensis* pollen

Pollen[a] extract	Yield (% w/w)	TPC[bc] (mg/g)	FC[bc] (mg/g)
CWE	58.5	30.80 ± 0.24^{A}	2.04 ± 0.38^{A}
HWE	54.4	30.20 ± 0.14^{A}	1.94 ± 0.27^{A}
50% EE	53.1	56.33 ± 0.30^{B}	2.50 ± 0.39^{A}
95% EE	21.3	61.27 ± 0.28^{C}	8.12 ± 0.32^{B}

[a]CWE (cold water extract); HWE (hot water extract); 50% EE (50% ethanol extract); 95% EE (95% ethanol extract).
[b]The total phenolic acid (TPC) and flavonoid contents (FC) are expressed as gallic acid equivalents and quercetin equivalents (mg/g of dry weights of the extracts), respectively.
[c]The values are expressed in means ± SD (n=3). Values with different upper-case letter in each column are significantly different at the $p < 0.05$ level.

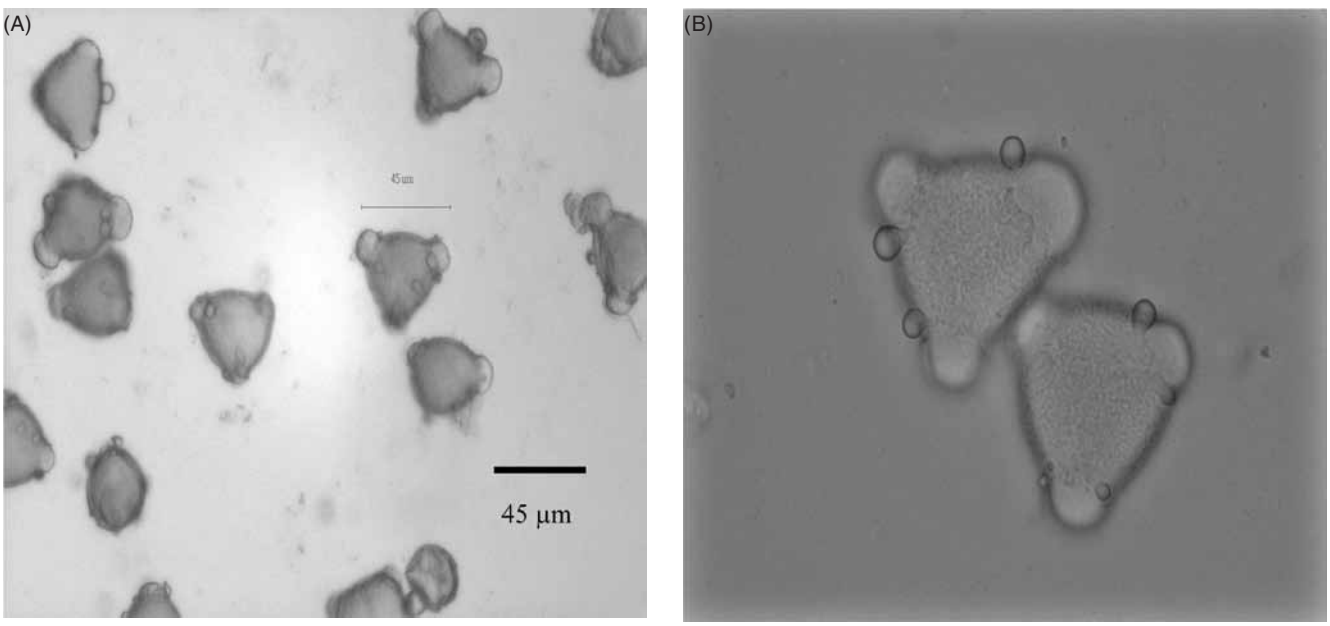

Figure 1. Biomorphic shape and size of tea (*Camellia sinensis*) pollen under microscopic examination. (Scale bar = 45 μm; (A) 10 × 20 and (B) 10 × 40).

methanol/water (1 : 1) and water, the ethanolic extract has the highest content of total polyphenol than others. In this study, the ethanolic tea pollen extracts contained more total phenolic compounds (TPC) than water extracts (Table 1) are comparable to the results of Kroyer and Hegedus[4]. In Table 1, both the phenolic compounds and flavonoid levels were found to be highest in the 95% EE, followed by the 50% EE, CWE, and HWE. High concentrations of pollen flavonoids correlate with high antioxidant activity and, therefore, high phytotherapeutic value[7]. The flavonoid content in tea pollen extracts ranged from 1.94 ± 0.27 to 8.12 ± 0.32 mg/g, expressed as quercetin equivalents (Table 1). Sarmento Silva *et al.*[26] and Campos *et al.*[2] reported that flavonoid/phenolics contents are associated with floral pollen type, and can therefore be used to determine the floral source because the profiles are unaffected by geography.

(II) *Identification and Quantification of the Free Flavonoid Aglycones*

Natural bee pollen contains 3 - 5% flavonoids by dry weight, and the flavonoids are principally found as glycosides and free aglycones[3,7,12,13]. To determine individual flavonoid glycosides is difficult, as most standards are not commercially available. However, the glycosides are hydrolyzed to aglycone (the sugar-free moiety) by the hydrolytic enzymes α- and β-glucosidase, making this process a good choice for quantifying pollen flavonoids[7]. Most honeybee-collected pollen possesses α- and β-glucosidase, which hydrolyze the pollen flavonoid derivatives (glycosides) because the pollen grains are coherent with nectar and bee secretions[7]. Therefore, we analyzed the free aglycones of tea pollen directly by HPLC using the method of Nuutila *et al.*[19]. As Figure 2 shows, the nine phenolic standards assumed in the tea pollen were well separated by HPLC within 35 min. These compounds were chosen because they had been previously reported [7,23,27] and the standards were available. Identification of the chromatographic peaks for the samples was made by comparing the retention times to

those of the standards (Figure 2). A representative HPLC chromatogram for the 50% ethanol extract (50% EE) of tea pollen was presented in Figure 3.

Flavonoid glycosides are important phenolic compositions of pollen[3,12]. Our study (Table 2) indicated that tea pollen extracts contain a variety of phenolic compounds. Gallic acid (0.69 ± 0.01 to 2.55 ± 0.05 mg/g) was abundant in the pollen extracts. Furthermore, the concentration of gallic acid (2.55 ± 0.05 mg/g) in the HWE was much greater than that of other extracts. In addition, rutin was found only in the ethanolic extracts of tea pollen, and the amount extracted increased significantly as the ethanol concentration increased. Other phenolic components of tea pollen present in high concentrations, such as caffeic acid, *p*-coumaric acid, and ferulic acid. The amount of chlorogenic acid in CWE and 50% EE, and catechin in HWE and 50% EE were much higher than that in other two extracts. Methyl gallate, rutin, and myricetin, were detected only in the ethanolic extracts.

Serra Bonvehí *et al.*[7] stated that rutin is present in dried

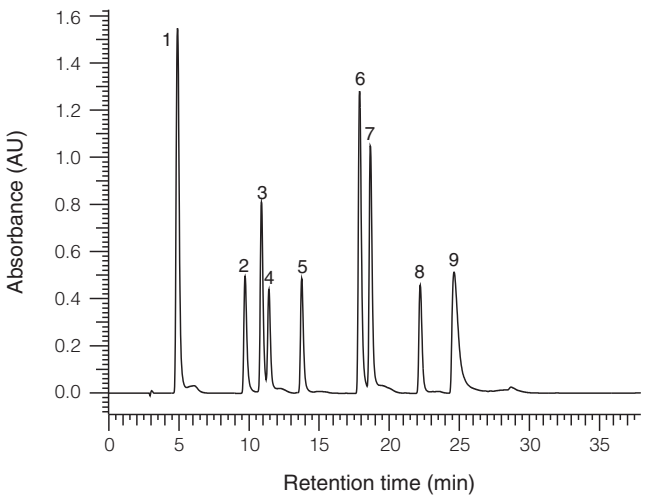

Figure 2. Chromatogram of the phenolic standards detected by reversed phase - high performance liquid chromatography (RP - HPLC) at 280 nm. peaks: 1, gallic acid; 2, catechin; 3, methyl gallate; 4, chlorogenic acid; 5. caffeic acid; 6, *p*-coumaric acid; 7, ferulic acid; 8, rutin; and, 9, myricetin. For analysis conditions, see Section IV of the Materials and Methods.

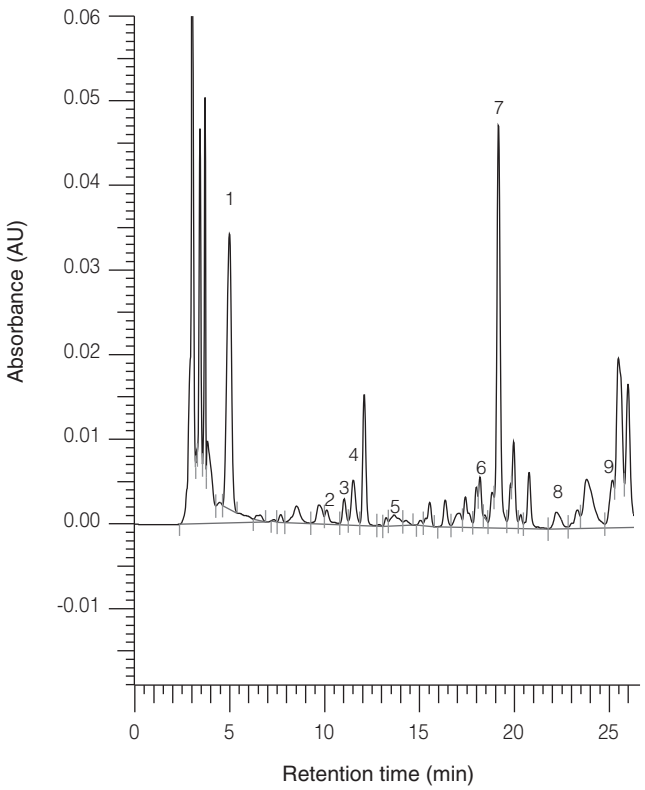

Figure 3. HPLC chromatogram of the phenolic compounds for 50% ethanol extract (50% EE) of tea (*Camellia sinensis*) pollen. Numbers 1 - 9 indicate the phenolic compounds as shown in Figure 2. For analysis conditions, see Section IV of the Materials and Methods.

Table 2. The contents of free flavonoid aglycones and phenolic acids (mean ± SD) in four pollen extracts

Component [a]	CWE[b] (mg/g)	HWE (mg/g)	50% EE (mg/g)	95% EE (mg/g)
Gallic acid	0.69 ± 0.01^{A}	2.55 ± 0.05^{C}	1.46 ± 0.08^{B}	1.44 ± 0.06^{B}
Catechin	0.13 ± 0.01^{A}	0.53 ± 0.03^{C}	0.52 ± 0.03^{C}	0.20 ± 0.01^{B}
Methyl gallate	—[c]	—[c]	0.007 ±0.002^{A}	0.006 ±0.001^{A}
Chlorogenic acid	0.42 ± 0.02^{C}	0.28 ± 0.01^{B}	0.48 ± 0.02^{D}	0.12 ± 0.01^{A}
Caffeic acid	0.41 ± 0.01^{A}	0.41 ± 0.02^{A}	0.45 ± 0.03^{B}	0.44 ± 0.01^{B}
p-Coumaric acid	0.41 ± 0.04^{C}	0.34 ± 0.01^{A}	0.38 ± 0.01^{B}	0.40 ± 0.04^{C}
Ferulic acid	0.25 ± 0.01^{A}	0.25 ± 0.01^{A}	0.35 ± 0.02^{B}	0.45 ± 0.03^{C}
Rutin	—[c]	—[c]	0.68 ± 0.03^{A}	1.18 ± 0.10 ^{B}
Myricetin	—[c]	—[c]	0.26 ± 0.01^{A}	0.55 ± 0.04^{B}

[a]The values are expressed in means ± SD (n = 3).
[b]CWE (cold water extract), HWE (hot water extract), 50% EE (50% ethanol extract), 95% EE (95% ethanol extract). Values (expressed as mg/g of dry weights of the extracts) with different upper-case letter in each row are significantly different at $p < 0.05$ level.
[c] Not detectable.

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bee-collected pollen and could be an indicator of the quality of bee pollen, which might reflect long periods of storage or excessive heating during the drying process. An investigation into the average amount of rutin in 11 pollen samples extracted by ethyl acetate, ammonium sulfate, and metaphosphoric acid showed a mean of 29 ± 7.98 mg/100 g from western Spain (Extremadura and Salamanca) in 1997 and 1998[8]. In our study, rutin was only found in the ethanolic extracts of tea (*C. sinensis*) pollen, especially in the 95% EE (Table 2). Our study shows that the phenolic compounds (free flavonoid aglycones and phenolic acids) in ethanolic extracts are richer than that in aqueous extracts (Table 2).

Overall, the free flavonoid aglycones and phenolic acids obtained in the ethanolic extracts from tea pollen were plentiful. Consistent with previously published literature, a mixture of bee-collected pollen from Austria was extracted using ethanol, water, and methanol individually, with the highest content (24.6 mg/g) of total polyphenols being extracted using ethanol as the extraction solvent[4]. In our study, both gallic acid and rutin amounts (in 95% EE) were higher than others. While none of phenolics such as methyl gallate, rutin, and myricetin were present in the aqueous extracts.

III. *Antioxidant Activity*

Fruits and vegetables are the best sources of antioxidant compounds because of their abundance of flavonoids[11,28]. Free flavonoid aglycones and phenolic acids isolated from four extracts of tea pollen may be responsible for their high antioxidant and free radical scavenging activities.

Some discrepancies were observed among the different antioxidant-activity analysis systems for the tea pollen extracts. The ethanol-soluble pollen extracts (50% EE and 95% EE) had the strongest total antioxidant activities (Figure 4). The total antioxidant activity, as measured by the scavenging activity of the $ABTS^{+}$ radical, was also expressed as IC_{50}, and the values of IC_{50} for 50% EE and 95% EE were 0.08 mg/mL and 0.06 mg/mL, respectively. The IC_{50} using trolox as reference standard was 6.87 μg/mL ($K = 0.26$; $R^2 = 0.97$). The antioxidant abilities of the HWE were moderately higher than those of the CWE. High antioxidant activities are mainly dependent on the concentration of pollen extract with the greatest level of phenolic constituents[4,14,28]. The pollen ethanolic extracts contained higher amounts of the total polyphenols and flavonoids than the aqueous extracts (Table 1), and were consistent with the higher total antioxidant activity obtained in the study (Figure 4).

The CWE of the tea pollen had the greatest hydrogen peroxide scavenging activity, followed by 50% EE, 95% EE, and HWE (Figure 5). The results revealed that the scavenging capacity of H_2O_2 increased with the concentration of pollen extract. The percentages of H_2O_2 scavenging capacity for the CWE, HWE, 50% EE and 95% EE at the concentration of 1.2 mg/mL were 65.68%, 39.33%, 51.59%, and 49.22%, respectively (Figure 5). These results support the fact that pollen extracts have potential as H_2O_2 scavengers.

Most flavonoids bind available iron[29]. These chelating abilities are important for preventing hydroxyl radical formation from the iron-catalyzed reactions of superoxide and hydrogen peroxide. Four kinds of extracts were used to examine the ability of pollen extracts to chelate ferrous ions (Figure 6). A dose-dependent relationship was observed for the chelating effects of both 50% EE and HWE. In particular, a lower chelating ability in the 95% EE, nearly 11.27%, was observed at the concentration of 15 mg/mL. For 50% EE, the chelation was almost 18.7 times more efficient than that of 95% EE at the concentration of 3 mg/mL (chelating ability proportion, 54.2/2.9) and 6.5 times higher at the concentration of 15 mg/mL (chelating ability proportion, 73.5/11.3). It is possible that some components in the 95% EE could have

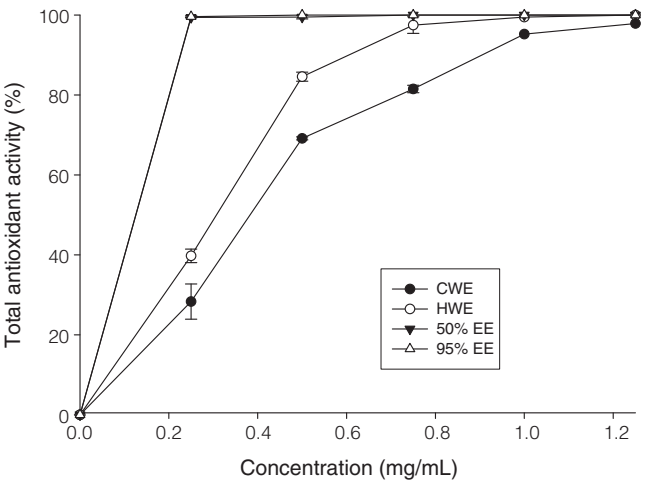

Figure 4. Total antioxidant activities of the four tea pollen extracts. (●) cold water extract (CWE), (○) hot water extract (HWE), (▼) 50% ethanol extract (50% EE), and (Δ) 95% ethanol extract (95% EE).

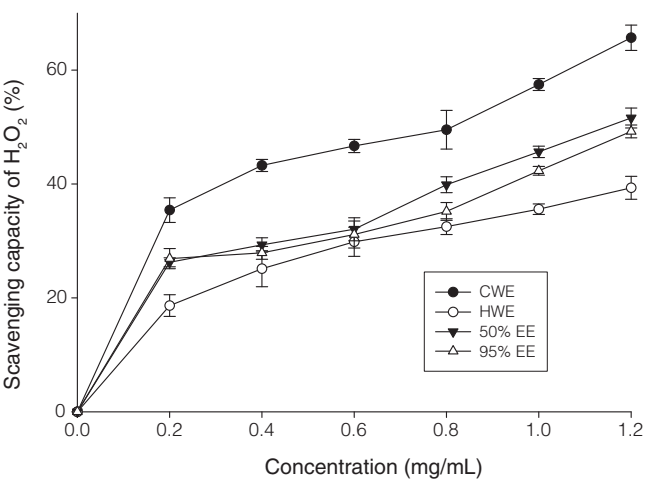

Figure 5. Hydrogen peroxide scavenging capacity of the four tea pollen extracts on ferrous ions. (●) cold water extract (CWE), (○) hot water extract (HWE), (▼) 50% ethanol extract (50% EE), and (Δ) 95% ethanol extract (95% EE).

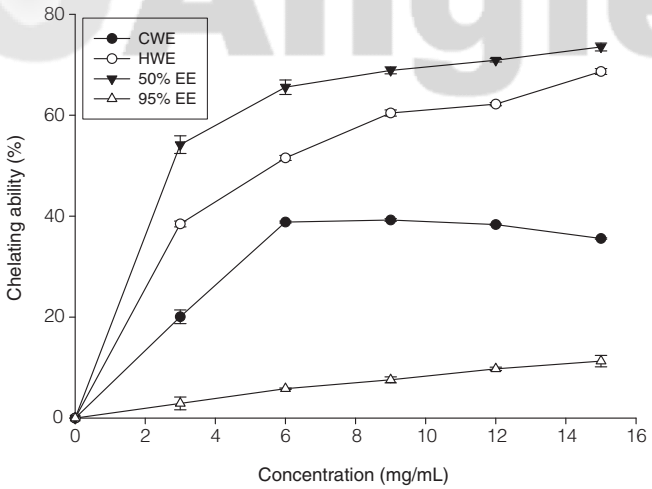

Figure 6. Chelating ability of the four tea pollen extracts to chelate ferrous ions. (●) cold water extract (CWE), (○) hot water extract (HWE), (▼) 50% ethanol extract (50% EE), and (Δ) 95% ethanol extract (95% EE).

interfered with the iron chelation activity. The intermediate chelating ability of the aqueous extracts (CWE and HWE) was less efficient than that of 50% EE, but still much higher than that of 95% EE. The chelation assay showed that the significant chelating activity of the 50% EE captured the ferrous ion before ferrozine.

CONCLUSIONS

To our knowledge, this is the first report to determine the content of the free aglycones of phenolic compounds obtained from tea pollen. The phenolic compounds (free flavonoid aglycones and phenolic acids) profile comprises gallic acid, catechin, methyl gallate, caffeic acid, *p*-coumaric acid, ferulic acid, rutin, myricetin, and chlorogenic acid. The various polyphenolic compounds may also be regarded as bioactive dietary supplements. The different types of tea pollen extracts (aqueous and ethanolic) prepared in this study showed diverse antioxidant activities. In conclusion, the results indicated that tea (*C. sinensis*) pollen could be a good source of dietary antioxidants.

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REFERENCES

1. Human, H. and Nicolson, S. W. 2006. Nutritional content of fresh, bee-collected and stored pollen of *Aloe grestheadii* var. *davyana* (Asphodelaceae). Phytochem. 67: 1486-1492.
2. Campos, M. G., Markham, K. R., Mitchell, K. A. and Cunha, A. P. 1997. An approach to the characterization of bee pollens via their flavonoid/phenolic profiles. Phytochem. Anal. 8: 181-185.
3. Almaraz-Abarca, N., Graça Campos, M., Ávila-Reyes, J. A., Naranjo-Jiménez, N., Corral, J. H. and González-Valdez, L. S. 2007. Antioxidant activity of polyphenolic extract of monofloral honeybee-collected pollen from mesquite (*Prosopis juliflora*, Leguminosae). J. Food Compost. Anal. 20: 119-124.
4. Kroyer, G. and Hegedus, N. 2001. Evaluation of bioactive properties of pollen extracts as functional dietary food supplement. Innov. Food Sci. Emerg. Technol. 2: 171-174.
5. Chu, Q., Tian, X., Jiang, L. and Ye, J. 2007. Application of capillary electrophoresis to study phenolic profiles of honeybee-collected pollen. J. Agric. Food Chem. 55: 8864-8869.
6. Medeiros, K. C. P., Figueiredo, C. A. V., Figueredo, T. B., Freire, K. R. L., Santos, F. A. R., Alcantara-Neves, N. M., Silva, T. M. S. and Piuvezam, M. R. 2008. Anti-allergic effect of bee pollen phenolic extract and myricetin in ovalbumin-sensitized mice. J. Ethnopharmacol. 119: 41-46.
7. Serra Bonvehí, J., Soliva Torrentó, M. and Centelles Lorente, E. 2001. Evaluation of polyphenolic and flavonoid compounds in honeybee-collected pollen produced in Spain. J. Agric. Food Chem. 49: 1848-1853.
8. Almeida-Muradian, L. B., Pamplona, L. C., Coimbra, S. and Barth, O. M. 2005. Chemical composition and botanical evaluation of dried bee pollen pellets. J. Food Compost. Anal. 18: 105-111.
9. Pyrzynska, K. and Biesaga M. 2009. Analysis of phenolic acids and flavonoids in honey. Trends Anal. Chem. 28: 893-902.
10. Zhang, Y., Chen, J., Ma, X. M. and Shi, Y. P. 2007. Simultaneous determination of flavonoids in *Ixeridium gracile* by micellar electrokinetic chromatography. J. Pharm. Biomed. Anal. 45: 742-746.
11. Dini, I. 2011. Flavonoid glycosides from *Pouteria obovata* (R. Br.) fruit flour. Food Chem. 124: 884-888.
12. Markham, K. R. and Campos, M. 1996. 7-and 8-*O*-methylherbacetin-3-*O*- sophorosides from bee pollens and some structure/activity observations. Phytochem. 43: 763-767.
13. March, R. E., Lewars, E. G., Stadey, C. J., Miao, X. S., Zhao, X., Metcalfe, C. D. 2006. A comparison of flavonoid glycosides by electrospray tandem mass spectrometry. Int. J. Mass Spectrom. 248: 61-85.
14. Silva, T. M. S., Camara, C. A., Silva Lins, A. C., Barbosa-Filho, J. M., Silva, E. M. S., Freitas, B. M. and Santos, F. D. A. R. 2006. Chemical composition and free radical scavenging activity of pollen loads from stingless bee *Melipona subnitida* Ducke. J Food Compost. Anal. 19: 507-511.

15. Tiziani, S., Schwartz, S. J. and Vodovotz, Y. 2008. Intermolecular interactions in phytochemical model systems studied by NMR diffusion measurements. Food Chem. 107: 962-969.
16. Yao, L., Jiang, Y., Singanusong, R., D'Arcy, B., Datta, N., Caffin, N. and Raymont, K. 2004. Flavonoids in Australian *Melaleuca, Guioa, Lophostemon, Banksia* and *Helianthus* honeys and their potential for floral authentication. Food Res. Int. 37: 166-174.
17. Gorinstein, S., Zachwieja, Z., Katrich, E., Pawelzik, E., Haruenkit, R., Trakhtenberg, S., and Martin-Belloso, O. 2004. Comparison of the contents of the main antioxidant compounds and the antioxidant activity of white grapefruit and his new hybrid. Lebensm. Wiss. Technol. 37: 337-343.
18. Maksimović, Z., Malenčić, D., and Kovačević, N. 2005. Polyphenol contents and antioxidant activity of *Maydis stigma* extracts. Bioresource Technol. 96: 873-877.
19. Nuutila, A. M., Kammiovirta, K. and Oksman-Caldentey, K. M. 2002. Comparison of methods for the hydrolysis of flavonoids and phenolic acid from onion and spinach for HPLC analysis. Food Chem. 76: 519-525.
20. Villaño, D., Fernández-Pachón, M. S., Troncoso, A. M. and García-Parrilla, M. C. 2004. The antioxidant activity of wines determined by the $ABTS^{\cdot+}$ method: influence of sample dilution and time. Talanta 64: 501-509.
21. Cui, Y., Kim, D. S. and Park, K. C. 2005. Antioxidant effect of *Inonotus obliquus*. J. Ethnopharmacol. 96: 79-85.
22. Gülçin, Í. 2006. Antioxidant activity of caffeic acid (3,4-dihydroxycinnamic acid). Toxicol. 217: 213-220.
23. Campos, M. G., Webby, R. F., Markham, K. R., Mitchell, K. A. and Cunha, A. P. 2003. Age-induced diminution of free radical scavenging capacity in bee pollens and the contribution of constituent flavonoids. J. Agric. Food Chem. 51: 742-745.
24. Ceska, O. and Styles, E. D. 1984. Flavonoids from *Zea mays* pollen. Phytochem. 23: 1822-1823.
25. Meurer, B., Wray, V., Grotjahn, L., Wiermann, R., and Strack, D. 1986. Hydroxycinnamic acid spermidine amides from pollen of *Corylus avellana* L.. Phytochem. 25: 433-435.
26. Sarmento Silva, T. M., Camara, C. A., da Silva Lins, A. C., Barbosa-Filho, J. M., Sarmento da Silva, E. M., Freitas, B. M., and Ribeiro dos Santos, F. D. A. 2006. Chemical composition and free radical scavenging activity of pollen loads from stingless bee *Melipona subnitida* Ducke. J. Food Compost. Anal. 19: 507-511.
27. Ferreres, F., Tomás-Barberán, F. A., Tomás-Lorente, F., Nieto, J. L., Rumbero, A. and Olías, J. M. 1989. 8-Methoxykaempferol 3-sophoroside, a yellow pigment from almond pollen. Phytochem. 28: 1901-1903.
28. Leja, M., Mareczek, A., Wyżgolik, G., Klepacz-Baniak, J. and Czekońska, K. 2007. Antioxidative properties of bee pollen in selected plant species. Food Chem. 100: 237-240.
29. Hynes, M. J. and Coinceanainn, M. Ó. 2001. The kinetics and mechanisms of the reaction of iron (III) with gallic acid, gallic acid methyl ester and catechin. J. Inorg. Biochem. 85: 131-142.