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In vitro and *in vivo* antioxidant properties of water and methanol extracts of linden bee pollen

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ABSTRACT

Bee pollen is an important source of the herbal medicine with active bio-compounds, but there are no reports on its antioxidant properties. Herein, we report for the first time the antioxidant properties of two different (water and methanol) extracts of Linden bee pollen through analysis various *in vitro* and animal experimental (*in vivo*) antioxidant assays including DPPH free radical scavenging, superoxide radical scavenging, reducing power, superoxide dismutase (SOD) activity, lipid per-oxidant and total antioxidant capacity. The results demonstrated that the methanol extract of bee pollen exhibited the higher *in vitro* antioxidant activity than water extract. Besides, the animal experiments showed the dose dependent increase of superoxide dismutase and total antioxidant capacity in water and methanol extracts. MDA content was significantly lowered in medium and high treated mice group and there was no significant difference between two extracts. These results indicated that methanol extract of Linden bee pollen can be considered as a natural antioxidant source for the potential utilization in future herbal medicine.

1. Introduction

Linden tree (*Tilia Tuan Szyszyl*) belongs to the *Titiaceae*, *Tilia* plant genus and its average height is about 20 m, and widespread distributed across the north temperate and subtropical zone of the North parts of China. This plant species is significantly utilized by migratory beekeepers and reported as important source of bee honey which is the most common beehive product. All the bee natural products such as pollen, royal jelly and porpoise are gained the increased attention in recent years with valuable nutritional supplements. Nevertheless, among the bee products, bee pollens are commonly referred as the "life giving dust" containing high nutrients and essential amino acids needed by humans. Several reports revealed the biochemical and microbiological properties of bee pollen (Almeida-Muradian et al., 2005).

Bee pollen is agglutination of flower pollen, which is collected by honey bees with nectar and salivary substance (Feás et al., 2012). The collection of this natural products is relatively recent development, depend on the basic concept of scraping pollen off of the bees' legs as they enter the hive (Pascoal et al., 2014). Bee pollen is an important apicultural product as a source of proteins, amino acids, minerals and vitamins are providing the beneficial effects to the human immune defense system, anti-aging, anti-oxidant, and antimicrobial activities (Silva et al., 2006; Wu and Lou, 2007). Antioxidant and biochemical composition including flavonoids, anthocyanins, tannins in the bee pollen are significantly varied based their source of habitat and their (Kim et al., 2015). Polyphenols are known to trigger the antioxidant and radical scavenging activities and it might reduce the risk of chronic disease such as cancer, cardiovascular and neuro-degenerative diseases (Mărghitaş et al., 2009). Therefore, the recent studies give the more attention on study the antioxidant activity of different foods and the plant origin derived products (Li et al., 2016). Antioxidant activities of bee pollen extracts are extensively studied through the *in vitro* methods and results are indicated that phenolic compounds derived from the pollen are significantly enhance the antioxidant activity (Damir et al., 2014; Wu et al., 2014). The extensive in vivo studies are needed to ensure the antioxidant properties of biological samples because in some cases the in vitro potent antioxidants not active in vivo condition (Zhang and Shen, 1997). Thus, this study investigated the antioxidant properties of water and methanol extracts of Linden bee pollen by *in vitro* and *in vivo* antioxidant assays

2. Materials and methods

2.1. Materials

Bee pollen pellets were collected from beehives located in the

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Changbai Montain, North of Jilin province, China, during month on July 2016. Bee pollen were placed in pre-cleaned polypropylene bags and transported to laboratory within two hours and frozen at − 18 °C for biological compounds extractions.

2.2. Bee pollen wall break treatment

In this study, the bee pollen wall was broken by mechanical breaking method (Han et al., 2013). Briefly, 2 kg of fresh bee pollen was dried to 7–8% moisture content at room temperature, then it was disintegrated into bee pollen powder using a high-speed disintegrator (SB-10A, Puheng Lt. Co, Shanghai, China) and pass through 60 mesh. In order to improve the softness of the bee pollen the moisture content of bee pollen powder was increased to 30–35% through the water intake, then it was kept at 35 °C for 12 h, followed by dried in a hot air drying at 45 °C. During this processing steps, the bee pollen out wall structure would become wobbly, dry and easily breakable. These rapid dehydration process results the high extraction yield. After the rapid dried, the bee pollen was disintegrated again and pass through 60 mesh. Final bee pollen powder was kept in dried and dark condition for further use.

2.3. Preparation of the extracts

Each samples of bee pollen (5 g) were extracted with 50 mL of water and/or methanol for 1 h, respectively, in a mechanical shaker at room temperature. The residues were re-extracted by the same solvent and extract conditions. All extracts were pooled together and filtered with Whatman No. 1 filter paper. The water and methanol filtrate were concentrated under vacuum at 60 °C in a rotary evaporation, and the concentrates were dried to achieve constant weight. The dried samples were weighed to determine the yield of soluble constituents and stored at 4 °C until analysis.

3. *In vitro* antioxidant activity

3.1. DPPH radical-scavenging activity

A modified method described by Li and Jia. (2014) was used to measure the scavenging activity to the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). In brief, 1 mL of each extract in different concentrations (0–5 mg/mL) was added to 1 mL ethanol solution of DPPH (0.1 mM) and were mixed completely. The mixture was incubated at room temperature in the dark for 30 min, and the absorbance was then measured at 517 nm. The control was sample was 1 mL of 100 mM DPPH mixed with 1 mL of Methanol. Vitamin C was used as the positive control. The percentage scavenging effect was calculated as

$$\text{Scavenging rate} = [1 - \text{As/Ac}] \times 100\%$$

Where; As was the absorbance of the extract and Ac was the absorbance of the control

A dose response curve was plotted to determine the IC_{50}, which is define as the concentration required to obtain a scavenging capacity of 50%. A lower IC50 value corresponds to a greater antioxidant activity.

3.2. O_2 scavenger activity

It is known that the superoxide radical can generated by the auto-oxidation of pyrogallol acid (PA). The scavenging radical activity was determined by Marklund and Marklund (1974). PA is readily auto-oxidized at pH 8.2, and the intermediate product produced by auto-oxidized exhibit a strong absorbance at 320 nm. At the beginning of the auto-oxidization, the observed absorbance is linear with respect to time, while when antioxidant agent was applied to inhibit the superoxide radical, the generation of the intermediate product is slow down. Thus, the activity was calculated from the tempered slope of the resulting absorbance–time curve. Briefly, the reaction was performed in a tube with 2.8 mL Tris-HCl buffer (50 Mm, pH 8.2). The mixture was added with 0.1 mL of each extract solution in varying concentrations (0–5 mg/mL) and incubated at 25 °C for 10 min. The reaction was stated by added by the addition of 0.1 mL PA solution into the tube. The absorbance at 320 was recorded every 30 s during the first 4 min. The slope of the absorbance- time curve represented the value of the oxidation velocity (V). The control sample was made using 0.1 mL distilled water instead of extract solution. The percentage inhibitory effect was calculated as:

$$\text{Inhibitory rate} = (1 - \text{Vs/Vc}) \times 100\%$$

Where, Vs was the oxidation velocity in the sample containing extract, and Vc was oxidation velocity in the control sample.

3.3. Reducing power assay

The reducing power was determined based on the principle increase of the absorbance in reaction system. The increases absorbance indicates the increases antioxidant activity (Tsai et al., 2006). The reaction mixture contained 2.5 mL deionized water, 2.5 mL PBS (200 mM, pH 6.6), 2.5 mL $K_3Fe(CN)_6$ (1%) and varying concentration of each bee pollen extract. After incubated at 50 °C for 20 min, 2.5 mL of 10% trichloroacetic acid (w/v) were added and centrifuged at 3000 rpm for 10 min. Five mL supernatant was mixed with 5 mL of deionized water and 1 mL of 0.1% $FeCl_3$, and the absorbance was measured at 700 nm. Ascorbic acid was used as the positive control.

4. *In vivo* antioxidant activity

4.1. Test animals

Male Kunming mice (20 ± 2 g) were purchased from the Experiment Animal Center of Yanbian University, China. The mice were housed in standard rat cage with free access to food and kept in a regulated environment at 25 ± 1 °C with a 12 h each of dark and light cycle. After 1 weeks of acclimatization to the home cage, the mice were randomly divided as follows: Five mice in the control group (C) and five mice in each group treated with water or methanol extract at different doses of 100 (T1), 300 (T2) and 500 μL/day (T3). The control group received only 1 mL of distilled water, and the treated groups were given the different doses of methanol or water bee pollen extract in 1 mL of distilled water by gavage once daily for 15 days. On day of 16th, the mice were sacrificed by cervical dislocated, and the blood were collected and processed to obtain serum used for the *in vivo* antioxidant assay.

4.2. Superoxide dismutase (SOD) assay

The assay for total SOD was based on its ability to inhibit the oxidation of oxymine by the xanthine-xanthine oxidase system (Oyanagui, 1984). Test of total SOD activity was carried out by using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the operate instrument. The hydroxylamine nitrite produced by the oxidation of oxymine had an absorbance peak at 550 nm. SOD activity was expressed as Units per milliliter of serum.

4.3. Lipid peroxidation index assay

Test of MDA content was performed by using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the operate instrument. The absorbance at 532 nm was measured spectrophotometrically and the levels of lipid peroxides can be expressed as nanomoles per milliliter of serum.