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Nutritional Quality of the Walnut Male Inflorescences at Four Flowering Stages

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Abstract Nutrients, bioactive compounds and antioxidant activities of walnut male inflorescences at four flowering stages were analyzed in this study. The results showed that the male inflorescences were rich in carbohydrate, protein and mineral contents, and had antioxidant activities due to high contents of phenolic and flavonoid compounds. The nutritional compositions were varied at four stages. In the first half stage, including early flowering stage (EFS) and flowering stage (FS), the contents of protein, total amino acids, carbohydrates, antioxidants (phenolic, flavonoid and ascorbic acid) and some minerals (phosphorus, zinc and copper) were higher than that of the second half stage, including pollen-scattering stage (PSS) and later pollen-scattering stage (LPSS). Due to high contents of phenolic and flavonoid at EFS, the highest antioxidant activity was also observed at this stage. However, the higher amounts of crude fat, crude fiber and some mineral elements (potassium, iron, manganese, calcium and magnesium) were found in the second half stage. These results could contribute to using walnut male inflorescences as food, as well as developing new food products, and obtaining the bioactive substances from walnut male flowers.

Keywords: *male inflorescences of walnut, proximate composition, minerals, amino acids, bioactive compounds, antioxidant activity*

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1. Introduction

For centuries, flowers have been used for food in many parts of the world because they can increase and improve the appearance, taste and aesthetic value of foodstuffs [1]. Historically, for example the flowers of *Magnolia denudata* and *Chrysanthemum morifolium* have been used for more than two thousand years in China. In ancient Rome, flowers of roses were used for various kinds of purée and/or omelets. In medieval France, the flowers of calendula were used when preparing various salads. In Thailand, many flowers have been eaten since ancient time, and some have medicinal properties as well as nutritional value. Today, in addition to the aesthetic appearance, the specific taste and the smell of edible flowers, new data about their nutritive quality, especially about their high contents of bioactivity compounds, such as phenolic and flavonoid, have been a very important cause of an increasing interest for their consumption. Many studies indicated that edible flowers showed not only antioxidant and ROS scavenging activities but also had significant anti-inflammatory effects within the human body due to their bioactivity compounds. For instance, the flowers of ornamental roses were considered as a source of anti-inflammatory, anti-bacterial, anti-fungal and anti-viral substances [1,2]. The chrysanthemum flowers showed a marked antimicrobial, anti-inflammatory, and inhibiting effect on carcinogenesis in mice [1].

Juglans had high content of bioactivity compounds, such as melatonin, serotonin and total phenols in nuts [3]. In addition to nuts, the other organs or parts, including green husk, root and leaves, also had the anti-diarrheic, anti-helmintic, anti-septic and astringent properties, and are widely used for traditional medicine to cure skin inflammations and ulcers [4]. There were some studies indicated that the flowers of walnut were good source of phenol, flavonoid and mineral contents [5,6] and showed remarkable antihypoxic, anti-inflammatory, antioxidant, antidepressant and antihemolytic activities in safe dose. Moreover, the hydroalcoholic extract of walnut male flowers could increase serum levels of insulin, and decrease blood glucose levels in diabetic rats [7]. In China, the male inflorescence of walnut was also known as longevity food, and a traditional vegetable in Minority Ethnic Area of Guizhou province and Yunnan Province. The walnut plantation areas were about 2,000 000 hectares in 2010 in China and every mature walnut tree had about 2 to 4 kg male flowers. In production, 95% male inflorescences should thin in order to save the tree nutrients and improve the fruit yield, which caused a large waste of natural antioxidant food resources. Moreover, in

the pollen-scattering stage, a lot of pollen littered in the air and carried by wind, which could cause allergic reactions in people who were sensitive to it and they were manifested above all as sneezing and runny eyes even rash eczema. Therefore, using walnut male flower should not only benefit to save natural food resources, improve economic income, but also better for the environment. However, there was still lack of scientific information about the nutritional value of the male inflorescences of walnut, especial the species of *J.* According to Mlcek et al. [1], edible flowers were rich in common nutrients, bioactive compounds and had remarkable antioxidant activity, the objective of this study was to generate information about common nutritional components (proteins, fats, saccharides, minerals and vitamins ect.), the total phenolic and flavonoid contents and antioxidant properties of male flowers of *J. sigillaia* at four flower stages from southwest China and was expected to shed light on their potential health benefits that could be useful for consumers and public health workers.

2. Material and Methods

2.1. Samples Preparation

The experiment was performed at Guizhou Privincial Technology Research Center of Fruit Crop Engineering and Walnut Bureau of Hezhang from April of 2012 to September of 2013. Male inflorescences of walnut cultivar 'Qianhe-7' (*J. sigillata*) were harvested from 36 year-old walnut tree in four different flower stages (early flowering stage, EFS; flowering stage, FS; pollen-scattering stage, PSS; later pollen-scattering stage, LPSS) at April in 2012. The evaluation criterion for the flower stage was made according to the method of Zhang [8]. The sample trees located in a southwest region of China (Hezhang County, Guizhou Province, 27.13 N, 104.71 E, 1750 m altitude, 13°C mean annual temperature, 835 mm total annual precipitation). After harvesting, male inflorescences were placed into an ice box and carried to the laboratory within 3-4 hrs. Then the samples were divided into two parts. One fresh sample were used for determination of ascorbic acid contents, the other samples were oven-dried at 50°C until a constant weight was obtained. Then the dry matters were obtained according to the method of Association of the Official Analytical Chemists [9]. The dry samples were ground and passed through a mesh sieve (40, meshes) for use.

2.2. Reagents

High-performance liquid chromatography (HPLC)-grade methanol and acetonitrile, and analytically grade ethanol, octanol, perchloric acid, boric acid, anthrone, metaphosphate, metavanadate, dinitrophenol, oxalic acid, curcumin, strontium chloride and bromocresol green were all purchased from Sinopharm Chemical Reagent Co., Ltd (China). 2,4,6-tripiridyl-s-triazine (TPTZ), Folin–Ciocalteu reagent, amino acid standard and mineral standard were obtained from Sigma (St. Louis, MO, USA). Gallic acid and rutin were obtained from Fluka (Buchs, Switzerland). 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Wako (Japan). All chemicals were analytically grade, unless otherwise noted. All aqueous solutions were prepared with ultrapure water purified with the Labpure Water Purification System (AKSW-24, Bedford, Chengdu, China).

2.3. Methods

2.3.1. Proximate Analysis

The ash content was calculated by incineration of the samples at 525°C for 4 h to a constant weight [9]. The total content of protein was calculated by multiplying the total nitrogen (Kjeldahl method) by 6.25 [9]. The fat content was determined by acid digestion prior to continuous extraction using petroleum ether in Soxtec system [9]. The contents of total soluble sugars and starch were analyzed using the method of anthrone colorimetry. The content of total dietary fiber was quantified following the acid-base scrubbing method [10]. The nonstructural carbohydrates were obtained by difference (100-∑(protein + fat + ash + dietary fiber)), according to Sánchez-Machado et al. [11]. The available energy was calculated by multiplying the percent protein, fat and digestible carbohydrates by 4.0, 9.0 and 4.0, respectively and summing up the values. The values were reported as kilocalorie per 100 g on dry matter basis. All samples were analyzed three replicates.

2.3.2. Mineral Analysis

For determination the nitrogen (N), potassium (K) and phosphorous (P), 0.50 g oven dried power of sample was digested in flask with a 10 ml of sulphuric acid. After complete digestion and cooling, the solution was diluted to 50 ml with ultrapure water. The contents of N, K and P were analyzed by Kjeldahl method, flame photometry method and spectrophotometric method, respectively. The total N content was expressed as crude protein. The contents of boron (B), calcium (Ca), iron (Fe), zinc (Zn), manganese (Mn), copper (Cu) and magnesium (Mg) were analyzed according to the method of Association of the Official Analytical Chemists [9]. About 1.00 g of sample was incinerated in the muffle furnace (SX2-2.5-12, Boxun Industry & Commerce Co., Ltd, Shanghai, China) for 4 h. The ash was dissolved by hydrochloric acid (6 $mol \cdot L^{-1}$) and diluted to 50 ml with ultrapure water. The content of B was measured by the means of spectrophotometric method. The contents of Ca, Fe, Zn, Mn, Cu, and Mg were evaluated by atomic absorption spectrophotometer (TAS-990, PERSEE, Beijing, China). All samples were analyzed five replicates.

2.3.3. Amino Acids Analysis

To determine the total content of the amino acids, an acid hydrolysis was performed on the proteins (500 mg of the sample was added with 10 ml of HCl (6 M) and was heated at 110°C for 24 hrs). The hydrolyzed product was vacuumed and filtered, and then diluted to 50 ml in a volumetric flask [11]. 2 mL solution was dried in oven at 60°C; the residue was diluted with 2 ml of borate buffer and agitated for 15 seconds, then filtered with a 0.45 μm membrane. A 20 μL sample of this solution was injected into the column of the automatic amino acid analyzer (A-300, membrapure GmbH, Germany).

The automatic amino acid analytic system was equipped with an eluent unit, an autosampler, a main unit

with a double piston pump, a rack for 2 glass bottles containing reagent and reactor washing solution, 2 photometers, a damping unit, a reactor, a separating column, a precolumn and a 2/3 way valve, an online solvent degasser, a chromatography data handling system. The amino acid analytic conditions were as follows: separation of the amino acid derivatives was achieved by a flow of 200 µL per min at a column temperature of 40°C. The reactor temperature was 115°C. The presses of buffer and reagent were 60 bar and 7 bar, respectively. Amino acid samples were separated by ion exchange chromatography and determined by reaction with ninhydrin. The detection was by photometric detector using the wavelengths of emission at 570 nm (except proline at 440 nm). All samples were analyzed three replicates.

The automatic amino acid analytic system allowed simultaneous analysis of 17 kinds of amino acids, and the different amino acids were identified by comparison with retention times for amino acid stock solutions. For determination of retention times, the reference standards were injected individually. The concentration of each amino acid was obtained by direct interpolation of the peak area in the correspondent linear calibration curve (peak area vs. concentration). These calibration curves were obtained over a wide concentration range in concordance with the level of each amino acid found in the samples that were analyzed. Three replications were done for amino acid determination.

2.3.4. Bioactive Compounds Analysis

Determination of ascorbic acid (AsA)

A high-performance liquid chromatographic (HPLC) method with ultraviolet detection was developed for determination of AsA [9,12]. 2.0 g of fresh sample was grounded with 7 ml of metaphosphoric acid (6%, w/v) in a mortar. The homogenate was centrifuged at 10000 rpm for 10 mins at 4°C, the supernatant was collected and diluted to 50 ml with ultrapure water. The solution was filtered with a membrane 0.45 µm (Millipore) and then 20 µL sample of it was injected into the column of the HLPC system.

The HLPC system (LC-15C, Shimadzu, Kyoto, Japan) was equipped with an autosampler (SIL-10AF), an online solvent degasser, a system controller with for chromatography data analysis, a pump (2LC-15C), a column oven (CTO-15C), and an UV detector (SPD-15C). Chromatographic analysis was performed using an analytical scale (150 × 4.6 mm i. d.) and Alltima C18 column with a particle size of 5 µm (Supelco, Bellefonte, PA). The HPLC conditions were as follows: The mobile phase was 0.2% (w/v) metaphosphoric acid solution. The flow rate was constant at 1 ml per min and the column was maintained at 30°C. The detection was used the UV detector at the wavelengths of emission at 254 nm.

Determination of total phenolic content (TPC)

For extraction of TPC, dried flower powder (1.5 g, d=0.0001g) was extracted with 60 ml of methanol (40%, v/v) for 50 min at 50°C in ultrasonic cleaner and then filter. The TPC was estimated by a colorimetric assay described by Conde-Hernández et al. [13] with some modifications. The reaction mixture which consisted of 0.5 ml of the extracted solution, 5.0 ml of Folin-Ciocalteau reagent and 15 ml Na_2CO_3 solution (20%, w/v) was incubated at room temperature for 2 h in the dark and then diluted to 50 ml with ultrapure water. The content of TPC was determined by measuring absorbance at 765 nm, usinga UV-Vis spectro-photometer (UV-2550, Shimadzu, Kyoto, Japan). The TPC was expressed as mg gallic acid equivalent (GAE) per 100g dry sample.

Determination of total flavonoid content (TFC)

The extraction of TFC was performed according to the method as described by Feng et al. [14] with minor modifications, dried flower powder (0.5 g, d=0.0001 g) was added with 30 ml ethanol (30%, v/v) and incubated for 1 h at 70°C in a water bath. The extracted production was filtered and the filtrate was used for TFC assay. The TFC was assayed by measuring the absorption at 510 nm in 3 ml reaction mixture containing 0.8 ml extract, 1.2 ml ethanol (30%, v/v), 0.5 ml $NaNO_2$ solution (5%, w/v) and 0.5 ml $Al(NO_3)_3$ solution (10%, w/v). The TFC was expressed as mg rutin equivalents (RE) per 100 g dry sample.

2.3.5. Antioxidant Activity Analysis

DPPH radical-scavenging activity

Dried flower powder (1.5 g, d=0.0001g) was extracted with 30 ml of ethanol (50%, v/v) for 50 min at 50°C in ultrasonic cleaner and then filtered. The filter was used for assay the antioxidant activity. The scavenging activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical was determined according to the method described by Motamed et al. [15] with a slight modification. Briefly, 2 ml DPPH ethanol solution (0.2 mM) was prepared and mixed with 2 ml sample ethanol solution. The mixture was incubated for 30 min at room temperature and measured the absorbance at 517 nm. The DPPH solution without sample and AsA ethanol solution were used as control and standard, respectively. The activity of radical-scavenging was given as % DPPH radical scavenging that was calculated by the equation: % DPPH radical scavenging = (control absorbance – sample absorbance)/control absorbance *100.

Ferric reducing ability of plasma (FRAP)

FRAP assay is a simple, reproducible, rapid, and inexpensive method that measures reductive ability of antiradical and it is evaluated by the transformation of Fe^{3+}-TPTZ to a blue color Fe^{2+}-TPTZ, as a measure of total antioxidant capacity [16]. The FRAP reagent contained of 2.5 ml of TPTZ (10 $mmolL^{-1}$) dissolved in 40 $mmolL^{-1}$ HCl, 2.5 ml of $FeCl_3 \cdot 6H_2O$ (20 $mmolL^{-1}$) and 25 ml of acetate buffer (0.3 $mmolL^{-1}$, pH 3.6). The antioxidant potential of the extract was determined against standard curve of $FeSO_4$ (0, 25, 50, 100, 200, 400, and 800 $\mu molL^{-1}$) in 0.1% (v/v) HCl. Briefly, 2.7 ml of FRAP reagent, prepared freshly and warmed at 37°C, mixed with 0.3 ml of sample solution was used to measure the absorbance at 593 nm, methanol as the reagent blank.. The FRAP was expressed as mmol $FeSO_4$ per 100g DW.

2.6. Statistical Analysis

Data was processed with the statistics package SPSS 12.0 for Windows (SPSS Inc., Chicago, IL, USA) which

analyzed the average and the standard deviation of repetitions.

3. Results and Discussion

3.1. Proximate Composition

The proximate compositions of walnut male inflorescences in four different stages were shown in Table 1. The dry matter of the male inflorescences in LPSS and FS were the highest (20.11 g $100g^{-1}$) and lowest (14.56 g $100g^{-1}$), respectively. There were no significant differences in the dry matter of the first two stages. Dry matter content of walnut male flowers was relatively high as a kind of vegetable, and was mainly composed of carbohydrates, proteins, fiber and fats (Table 1). From EFS to LPSS, the ash content showed an increase trend, the peak value (9.85 g $100g^{-1}$) was observed at LPSS, and these values also indicated that the walnut male flower contained some nutritionally important minerals required by the body. A high crude protein content was found in the walnut male inflorescences and it was in the range of 20.34 g $100g^{-1}$ (EFS) to 22.31 g $100g^{-1}$ (FS). These values were similar to that presented by Chen et al. [17] of 21.23 g $100g^{-1}$ and higher than that determined in the walnut (15 g $100g^{-1}$). Moreover, the protein contents of walnut male flowers also could be comparable with peanut kernels (25-29 g $100g^{-1}$) [18], which indicated that male inflorescence could be considered as a good source of protein. With the extension of flower time, the protein content decreased at PSS and LPSS, which might be due to the high protein content in pollen [19]. The highest crude fiber content (15.47 g $100g^{-1}$) was found in the later pollen-scattering stage, and there were no significant differences among EFS, FS and PSS. These data showed that the crude fiber contents of walnut male flower (2.03-3.11 g $100g^{-1}$, converted into fresh weight) were higher than those determined in some kinds of vegetable species, such as fresh celery stalks (1.0-1.8 g $100g^{-1}$) [20] and fresh chive flowers (1.22 g $100g^{-1}$) [21]. Crude fiber was a group of indigestible carbohydrates. For human, it could improve the function of the alimentary tract and also lower blood glucose and cholesterol levels [22]. The content of crude fat and total sugars all increased firstly and then decreased from EFS to LPSS and the highest contents were noted at PSS (16.44 g $100g^{-1}$ and 12.04 g $100g^{-1}$, respectively). And fat was involved in the insulation of body organs and in the maintenance of body temperature and cell function. Additionally, fats were sources of omega-3 and omega-6 fatty acids and were required for the digestion, absorption, and transport of vitamins A, D, E, and K. While the peak value of starch (0.98 g $100g^{-1}$) was found at FS. An increase in sugars from EFS to PSS might be due to accumulation of more sugars due to hydrolysis of starch and slight decline at LPSS was due to utilization of sugars respiration process.

The content of carbohydrates was more than 40% in the dry flower sample and that of EFS was the highest (47.54 g $100g^{-1}$). High carbohydrates percent in food could provide energy for human, which enabled cells to do all of their functions, including building protein's and others substances needed by the body [23]. According to the relationship of coefficient conversion, the available energy was ranging from 469.18 (LPSS) to 484.79 kcal (FS) in 100 g dry flower sample. The consumption of 100 g of this sample would provide energy intake from 23.46% to 24.24% in a standard daily diet of 2000 kcal, which indicated that male inflorescence was a good source of available energy.

Table 1. Proximate compositions of walnut male inflorescence at four flower stages

Nutrients	EFS	FS	PSS	LPSS
Dry matter (g $100g^{-1}$ FW)	14.56±0.68b	14.56±0.20b	18.65±5.24ab	20.11±1.6a
Protein (g $100g^{-1}$ DW)	20.34±0.20d	22.31±0.44a	21.01±0.09c	21.72±0.25b
Ash (g $100g^{-1}$ DW)	5.99±0.48d	6.66±0.29c	6.61±0.09b	9.85±0.08a
Fat (g $100g^{-1}$ DW)	12.19±0.19c	14.92±0.73b	16.44±1.23a	8.05±0.28d
Carbohydrates (g $100g^{-1}$ DW)	47.54±0.36a	41.98±0.68c	42.20±1.01c	44.92±0.57b
Total soluble sugar (g $100g^{-1}$ DW)	11.19±0.16c	11.71±0.05b	12.04±0.10a	11.28±0.16c
Crude fiber (g $100g^{-1}$ DW)	13.94±0.09b	14.14±0.14b	13.74±0.28b	15.47±0.19a
Starch (g $100g^{-1}$ DW)	0.72±0.04b	0.98±0.09a	0.48±0.06c	0.46±0.02c
Available energy (Kcal $100g^{-1}$ DW)	477.74±2.85b	484.79±3.43a	478.61±0.76b	469.18±1.52c

Mean value ± standard deviation (n=3, by duplicate). Different low letters in each line indicate the significant difference among four flower stages at $p<0.05$.

3.2. Mineral Composition

As shown in Table 2 and Table 3, among all mineral elements, the contents of potassium (from 27.16 to 38.38 g kg^{-1} DW) were the highest, followed by phosphorus (3.48 to 4.89 g kg^{-1} DW), calcium (2.73 to 7.05 g kg^{-1} DW) and magnesium (2.29 to 2.95 g kg^{-1} DW). In all micronutrients, the highest contents were found in iron elements (315.63 to 688.21 mg kg^{-1} DW), followed by manganese (113.45 to 214.85 mg kg^{-1} DW), zinc (49.76 to 74.86 mg kg^{-1} DW) and boron (22.77 to 28.61 mg kg^{-1} DW). The content of copper (19.80 to 23.95 mg kg^{-1} DW) was the lowest. Among four flowering stages, the highest contents of phosphorus, zinc, copper and boron were found at FS, where as the highest potassium, calcium, magnesium, iron and manganese at LPSS. These results showed that the mineral contents of walnut male flower, especially the micronutrients, were similar to or above those determined in some kinds of vegetable species including carrot and cauliflower that the contents of manganese, copper and zinc were 18 and 56 mg kg^{-1} DW, 8 and 15 mg kg^{-1} DW, 32 and 47 mg kg^{-1} DW, respectively [24]. Minerals accounted for 4.7% of the weight of the human organism. Most of the minerals were salts containing calcium and phosphorus, as the building blocks of the human skeleton [25]. According to Shajib et al. [26], the daily iron, zinc and copper requirements of an adult man were 10 to 15 mg per day, 12 to 15 mg per day and 2 to 3 mg per day. Our results indicated that the male inflorescences of walnut, to some extent, can meet the daily requirement of these minerals. Furthermore, many of minerals were the fundamental part of enzyme systems and served as the prevention of many diseases and strengthened the human immune system [1,27]. The present study suggested that there were higher mineral

contents in male inflorescences of walnut, which might be one of the causes of anti-inflammatory effect of walnut flowers [5].

Table 2. Contents of macronutrients of male inflorescence of *J. sigillaia* at four flower stages

Stages	Phosphorus / g kg^{-1}	Potassium / g kg^{-1}	Calcium / g kg^{-1}	Magnesium / g kg^{-1}
EFS	3.95±0.26b	27.16±0.17d	2.95±0.05bc	2.39±0.07bc
FS	4.89±0.24a	28.76±0.16b	2.73±0.167c	2.46±0.08b
PSS	4.13±0.10b	27.75±0.62c	3.04±0.04b	2.29±0.07c
LPSS	3.48±0.14c	38.38±0.44a	7.05±0.18a	2.95±0.03a

Mean value ± standard deviation (n=5, by duplicate). Different low letters in each row indicate the significant difference among four flower stages at $p<0.05$.

Table 3. Contents of micronutrients of walnut male inflorescence of *J. sigillaia* at four flower stages

Stages	Iron /mg kg^{-1}	Manganese /mg kg^{-1}	Copper /mg kg^{-1}	Zinc /mg kg^{-1}	Boron /mg kg^{-1}
EFS	318.67±30.68b	113.45±2.28c	20.77±0.30b	49.76±2.24c	22.77±1.04c
FS	351.62±3.47b	128.06±4.37b	23.95±0.38a	74.86±1.60a	28.61±0.55a
PSS	315.63±20.35b	115.46±5.39c	19.80±0.35c	53.17±0.50b	25.89±0.11b
LPSS	688.21±24.16a	214.85±0.45a	23.51±0.18a	53.71±1.70b	23.70±1.03c

Mean value ± standard deviation (n=5, by duplicate). Different low letters in each row indicate the significant difference among four flower stages at $p<0.05$.

Table 4. Contents of amino acids (g 100g^{-1} DW) at four different stages of walnut male inflorescences

Amino acids	EFS	FS	PSS	LPSS
Aspartic acid	1.34±0.02b	1.41±0.03a	1.38±0.01a	1.21±0.02c
Threonine^{z}	0.65±0.04a	0.69±0.01a	0.68±0.01a	0.57±0.01b
Serine	0.79±0.01a	0.81±0.03a	0.81±0.01a	0.66±0.03b
Glutamic acid	1.68±0.04c	1.95±0.05a	2.01±0.01a	1.87±0.02b
Glycine	0.70±0.03b	0.77±0.01a	0.72±0.01b	0.61±0.04c
Alanine	0.81±0.03b	0.86±0.01a	0.83±0.01b	0.75±0.02c
Cysteine	0.06±0.01a	0.04±0.00bc	0.03±0.00c	0.04±0.00b
Valine^{z}	0.67±0.03ab	0.71±0.02a	0.63±0.02b	0.59±0.01c
Methionine^{z}	0.21±0.01a	0.15±0.01b	0.20±0.00a	0.15±0.01b
Isoleucine^{z}	0.63±0.02a	0.60±0.01b	0.59±0.01b	0.49±0.01c
Leucine^{z}	1.10±0.02b	1.16±0.03a	1.16±0.03a	0.95±0.02c
Tyrosine	0.45±0.06a	0.41±0.01ab	0.38±0.01b	0.32±0.01c
Phenylalanine^{z}	0.84±0.04b	0.88±0.02ab	0.92±0.01a	0.73±0.01c
Histidine^{z}	0.45±0.02a	0.37±0.03b	0.30±0.00c	0.28±0.01c
Lysine^{z}	0.61±0.01b	0.65±0.01a	0.64±0.02a	0.48±0.01c
Arginine^{z}	1.24±0.02b	1.33±0.04a	1.20±0.02bc	1.18±0.01c
Proline	0.69±0.05a	0.64±0.01a	0.65±0.03a	0.27±0.02b
EAA	6.40±0.07ab	6.54±0.13a	6.32±0.01b	5.42±0.06c
TAA	12.92±0.05b	13.43±0.24a	13.13±0.01b	11.15±0.17c
EAA/TAA (%)	49.54	48.70	48.13	48.61

Mean value ± standard deviation (n=3, by duplicate). Different low letters in each line indicate the significant difference among four flower stages at $p<0.05$.

^{z}Essential amino acid, EAA: essential amino acids. TAA: total amino acids.

3.3. Amino Acid

As shown in Table 4, the male inflorescence of walnut was rich in 17 kinds of amino acids; the tryptophan was not detected because it was completely destroyed by acid hydrolysis. The contents of total amino acids ranged from 11.15 g 100g^{-1} (LPSS) to 13.43 g 100g^{-1} (FS), which contained 9 kinds of essential amino acids (including the semi-essential amino acids, arginine and histidine). The essential amino acid contents ranged from 5.42 g 100g^{-1} (LPSS) to 6.54 g 100g^{-1} (FS), accounting for 48.13%-49.54% of total amino acids. Both total amino acid and essential amino acid contents of FS were higher than those in the other stages. The amino acids which were concentrated in a higher proportion in the different stages were glutamic acid, aspartic acid, arginine and leucine, whose average contents were 1.88 g 100g^{-1}, 1.34 g 100g^{-1}, 1.24 g 100g^{-1} and 1.09 g 100g^{-1}, respectively, while the lowest concentration was methionine (0.18 g 100g^{-1}), followed by tyrosine (0.39 g 100g^{-1}). The results of this study showed that the amino acids contents in walnut male inflorescence were comparable to or higher than that in walnut kernels, whose contents of arginine, glutamic acid, aspartic acid and leucine were 1.32 g 100g^{-1}, 1.22 g 100g^{-1}, 0.99 g 100g^{-1} and 0.90 g 100g^{-1}, respectively [28]. According to WHO/FAO [29], the EAA/TAA of high quality protein was more than 36%, and the EAA/TAA of walnut male inflorescence were all above 48%, therefore, the protein in the walnut male inflorescences could be considered as a kind of high-quality protein.

3.4. Bioactive Compounds

3.4.1. Ascorbic Acid Content

In this study, the ascorbic acid contents (AsA) of male inflorescences in different stages were shown in Table 5. Among four flowering stages, the highest content of AsA was in the EFS (0.15 g 100g^{-1}) and the lowest in the LPSS (0.07 g 100g^{-1}). With the opening of the flower, the content of AsA showed a decline trend which may be due to reducing of synthesis and increasing of decomposition. These values showed that the AsA contents of walnut male flower were comparable to or higher than those determined in the some kinds of fruits, such as kiwifruit was about 0.07 g 100g^{-1} and guava about 0.07 g 100g^{-1} [30] or some vegetable species, such as red chilli (0.22 g 100g^{-1}) and green chilli (0.07 g 100g^{-1}) [31]. AsA was a potent water-soluble antioxidant in humans and its antioxidant effects had been demonstrated in many experiments in vitro [32]. Shajib et al. [26] reported that the minimum daily requirement of AsA for preventing clinical symptoms of the scurvy for an adult was about 10 mg or little less. In the present study, there was more than 10 mg AsA per 100g walnut male flower. Therefore, we could conclude that daily intake of 100g walnut male flower could prevent scurvy and the male inflorescences might be considered as a good source of AsA.

3.4.2. Total Phenolic Content (TPC)

The TPC were presented in Table 5. There were significant differences among different stages tested. The TPC of walnut male inflorescences ranged from 2.13 to 2.43 g GAE 100g^{-1} DW. The highest TPC was found in the EFS (2.43 g GAE 100g^{-1} DW), followed by FS (2.27 g GAE 100g^{-1} DW) and PSS (2.21 g GAE 100g^{-1} DW), while in the LPSS, the male inflorescence had the lowest TPC (2.13 g GAE 100g^{-1} DW). It was lower than the content obtained by Nabavi et al. [5] in *J. regia* flower (7.17 g GAE 100g^{-1} DW). It could be possible that the variations in the TPC of walnut flowers were influenced by the environmental conditions of growing fields and the species. However, the TPC of walnut male flower in this

study was higher than that of walnut kernels (1.25 g GAE $100g^{-1}$ DW) [33]. Furthermore, these values (0.33 to 0.43 g GAE $100g^{-1}$FW) were comparable to or slightly lower than those determined in the some kinds of edible flowers, such as garden nasturtium (0.33 g GAE $100g^{-1}$ FW), cornflower (0.48 g GAE $100g^{-1}$ FW) and begonias (0.49 g GAE $100g^{-1}$ FW) [2]. The TPC have been reported to be associated with antioxidant activity, which potentially had beneficial implications in human health [34]. Some reporters found that walnut flowers had remarkable pharmacological effects [5] and antihemolytic activity [6] both attributed to high phenol and flavonoid contents. Our study also proved that there were high TPC in the walnut male flower, which was accordance with the results of Nabavi et al [5].

3.4.3. Total Flavonoid Content (TFC)

The TFC of walnut male flowers had significant differences among four flowering stages (Table 5). In the FS, the male inflorescences of walnut had the highest TFC with a concentration of 2.15 g RE $100g^{-1}$ DW, followed by the EFS (2.06 g RE $100g^{-1}$ DW) and the PSS (1.70 g RE $100g^{-1}$ DW). The lowest TFC was observed in the LPSS, which was of 1.44 g RE $100g^{-1}$ DW. Our results suggested that the TFC in walnut male inflorescences (0.29 to 0.32 g RE $100g^{-1}$ FW) was higher than those determined in the some kinds of edible flowers, such as garden nasturtium (0.14 g RE $100g^{-1}$ FW), violet (0.20 g RE $100g^{-1}$ FW) and carnation (0.23 g RE $100g^{-1}$ FW) [2]. Previous works also indicated that yellow flowers had high the flavonoid content [34] and especially the pollen was rich in it, while the phenolic substances mainly existed in nectar [1]. According to results of TPC and TFC, it was found that the peaks of TPC and TFC were not appeared at the same flower opening stage, the TPC reached peak at the EFS, however, the peak of TFC was six days later than that of TPC and at FS, when the appearance of inflorescence was yellow. The reason might be associated with high content pollen at flowering stage. So we could deduce that the contents and types of antioxidant were varied with the opening of flower and the antioxidant effect might be different due to these causes. For humans, several health beneficial properties of dietary flavonoids were recognized for their antioxidant and antiproliferative effects which might protect the body from various diseases, such as cancers, cardiovascular disease and inflammatory [35]. Nabavi et al. [5] also found walnut flowers had antihypoxic activity and antidepressant activity effect due to the high content of flavonoids (6.17 g RE $100g^{-1}$ DW).

Table 5. Ascorbic acid (g $100g^{-1}$ FW), total phenols (g $100g^{-1}$ DW) and total flavonoid (g $100g^{-1}$ DW) content of male inflorescence of *J. sigillaia* at four flower stages

Bioactive compounds	EFS	FS	PSS	LPSS
Ascorbic acid (g $100g^{-1}$ FW)	0.15±0.01a	0.09±0.01b	0.09±0.01b	0.07±0.01c
Total phenols (g $100g^{-1}$ DW)	2.43±0.05a	2.26±0.02b	2.21±0.01c	2.13±0.02d
Total flavonoid (g $100g^{-1}$ DW)	2.06±0.02b	2.15±0.04a	1.70±0.04c	1.44±0.04d

Mean value ± standard deviation (n=3, by duplicate). Different low letters in each line indicate the significant difference among four flower stages at $p<0.05$.

3.5. Antioxidant Activity

3.5.1. DPPH Radical-Scavenging Activity

The DPPH radical-scavenging activity (percentage inhibition) of the male inflorescences extracts was given in Table 6. DPPH radical scavenging capacity of extracts ranged from 84.33% (EFS) to 79.26% (LPSS) and showed a trend of decline with the opening of flower. Significant differences were detected among four flower stages. The DPPH assay was a preliminary test to investigate the antioxidant potential of extracts [34]. Results showed that the DPPH radical-scavenging activity of walnut male flowers was higher than those determined in some kinds of edible flowers, such as Queen of tropic flower (31.39%), West Indian jasmine (62.52%) and pagoda tree (69.65%) [34]. The results were in agreement with previous works that the walnut male flowers had remarkable antioxidant activity reported by Nabavi et al. [5].

3.5.2. Ferric Reducing Ability of Plasma (FRAP) Assay

Among four flowering stages, the highest FRAP activity was observed at the EFS (2.06 mmol $FeSO_4$ $100g^{-1}$ DW) and then declined with the opening of flower (Table 6). Moreover, the trends of ferric ion-reducing activities in four flowering stages were similar to the DPPH radical-scavenging activities and the highest antioxidant activity at the first half stage may be attributed to higher total phenolic and flavonoid content at these stages. The average value of FRAP of four stages was 1.53 mmol $FeSO_4$ $100g^{-1}$ DW. This value was higher than those determined in some kinds of vegetable and fruit species, such as spinach (0.98 mmol $FeSO_4$ $100g^{-1}$), tomato (0.31 mmol $FeSO_4$ $100g^{-1}$), grape (0.83 mmol $FeSO_4$ $100g^{-1}$), and apple (0.29 mmol $FeSO_4$ $100g^{-1}$) [36], which indicated that the male inflorescences of walnut had high ferric reducing ability. Furthermore, the present study also indicated that walnut male flowers showed significant Fe^{2+} chelating ability, which might reduce iron-related complications in human and thereby improved quality of life [5].

Table 6. Antioxidant activity of male inflorescence of *J. sigillaia* at four flower stages

Antioxidant activity	EFS	FS	PSS	LPSS
DPPH (% inhibition)	84.33±0.05a	82.18±0.20b	82.60±0.38b	79.26±0.29c
FRAP (mmol $100g^{-1}$ DW)	2.06±0.08a	1.55±0.02b	1.30±0.01c	1.20±0.02d

Mean value ± standard deviation (n=3, by duplicate). Different low letters in each line indicate the significant difference among four flower stages at $p<0.05$.

4. Conclusions

In present study, the contents of carbohydrate, protein, amino acids, mineral elements, AsA, phenolic and flavonoid compounds and antioxidant activities of walnut male inflorescences were investigated in four flowering stages. This study confirmed that walnut male inflorescences were a good source of protein, fiber, mineral, amino acids, phenolic and flavonoid compounds, it could be utilized as food for human consumption.

Our results suggested that flower opening degree significantly affected the contents of proximate

composition, mineral elements, amino acids, phenolic and flavonoid, as well as the antioxidant activity. In the EFS and FS, the contents of crude protein, total amino acids, carbohydrates, antioxidants (phenolic, flavonoid and ascorbic acid) and some mineral elements, such as phosphorus, zinc and copper, were higher than the second half stage. Due to high contents of phenolic and flavonoid at EFS, the highest antioxidant activity was also observed at this stage. With the opening of flower, the higher amounts of crude fat, crude fiber and some mineral elements, such as potassium, iron, manganese, calcium and magnesium appeared. The results also showed that the antioxidant activity was strictly related to the antioxidants, including ascorbic acid, phenolic and flavonoid, and significantly decreased with the opening of flower.

To our knowledge, the present study is the first nutrimental composition research on the walnut male inflorescences, which could generate useful information for consumers and may encourage researchers to utilize walnut male flower as sources of phytochemicals. However, there are rich of bioactive compound and has high level antioxidant in walnut flower, so the toxicity should be tested to confirm their safety for use as food additives. The antioxidant mechanisms and the anti-proliferative of the extracts also should be further studied to gain more application for use as natural antioxidants. In addition, more complete and effective storage methods and culinary treatments are needed due to high water content in walnut male flowers, which lead the products to deteriorate and spoil easily by microorganism and chemical reactions.

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Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this article.

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