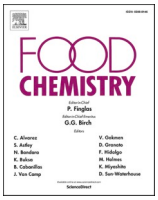
Food Chemistry 405 (2023) 134800

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Phenolamide and flavonoid glycoside profiles of 20 types of monofloral bee pollen

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ARTICLE INFO

Keywords:
Bee pollen
Phenolamides
Flavonoid glycosides
DNA barcoding
MS/MS cleavage

ABSTRACT

This study aimed at investigating phenolamides and flavonoid glycosides in 20 types of monofloral bee pollen. The plant origins of pollen samples were determined by DNA barcoding, with the purities to over 70 %. The 31 phenolamides and their 33 *cis/trans* isomers, and 25 flavonoid glycosides were identified; moreover, 19 phenolamides and 14 flavonoid glycosides as new-found compounds in bee pollen. All phenolics and flavonoids are present in the amidation or glycosylation form. The MS/MS cleavage modes of phenolamides and flavonoid glycosides were summarized. Isorhamnetin-3-*O*-gentiobioside presented the highest levels 23.61 mg/g in apricot pollen. Phenolamides in 11 types of pollen constituted over 1 % of the total weight, especially 3.9 % in rose and 2.8 % in pear pollen. Tri-*p*-coumaroyl spermidine and di-*p*-coumaroyl-caffeoyl spermidine respectively accounted for over 2.6 % of the total weight in pear and rose pollen. The richness in phenolamides and flavonoid glycosides can offer bee pollen more bioactivities as functional foods.

1. Introduction

Pollen is the male reproductive cells from the anthers of flowering plants (Bogdanov, 2011). Pollen is usually transmitted to the stigma of receptive female flowers by pollinators such as honey bees, butterflies, moths, and hummingbirds (Bogdanov, 2011). Honey bees are the most important pollinator for fruits, vegetables, and crops; specially, more than one-third of the world's crop production depends on bee pollination (Wu et al., 2021). Through pollination, honey bee collect pollen to supply almost all nutrients for brood rearing as well as for adult growth and development (Aylanc, Falcão, Ertosun, & Vilas-Boas, 2021; Dong, Yang, Wang, & Zhang, 2015). Bee pollen also benefits human health, such as helping recovery from chronic illness, slowing the aging process, lowering cholesterol levels, regulating intestinal functions, and promoting cardiovascular system (Aylanc et al., 2021; Thakur & Nanda, 2020). These functional properties of bee pollen are mainly attributed to its various bioactive compounds, such as proteins, carbohydrates, crude fibers, amino acids, vitamins, minerals, and fatty acids, as well as polyamines and flavonoids (Wu et al., 2021).

The previous works have documented that polyamines may be an "anti-aging vitamin" for humans (Madeo, Bauer, Carmona-Gutierrez, & Kroemer, 2019; Madeo, Eisenberg, Büttner, Ruckenstuhl, & Kroemer, 2010). For example, an epidemiological study firstly reported that the tissue levels of spermidine decline with aging in humans; in comparison, the concentrations of spermidine in healthy nonagenarians and centenarians are retained like younger individuals (Madeo, Eisenberg, Pietrocola, & Kroemer, 2018). Notably, Swedish Nutrition Recommendations estimated the recommended daily amount of spermidine approximately 30 mg for males and 25 mg for females (Madeo et al., 2020). Bee pollen contains numerous polyamines, such as putrescine, spermine and spermidine, usually conjugated with hydroxycinnamic acids (for example, *p*-coumaric, ferulic, and caffeic acid) (Gardana, Del Bo', Quicazán, Corrrea, & Simonetti, 2018; Kim et al., 2018; Thakur & Nanda, 2020; Wang, Snooks, & Sang, 2020; Zhang, Liu, & Lu, 2020; Zhang, Yu, Zhu, Liu, & Lu, 2022). These conjugated components are generally named as hydroxycinnamic acid amide (HCAA) derivatives or phenolamides. Recently studies have shown that bee pollen is rich in phenolamides, for example, 18 phenolamides in *Quercus*

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https://doi.org/10.1016/j.foodchem.2022.134800
Received 22 June 2022; Received in revised form 19 October 2022; Accepted 26 October 2022
Available online 1 November 2022

mongolica bee pollen (Kim et al., 2018); 13 phenolamides in *Mimosa pudica L.* bee pollen (Pereira Gomes et al., 2022); 5 phenolamides in rapeseed bee pollen (H. Zhang et al., 2020), and 18 phenolamides in bee pollen from Colombia, Italy and Spain (Gardana et al., 2018). Additionally, the previous research demonstrated that bee pollen contains various flavonoids (Giampieri et al., 2022; Thakur & Nanda, 2020), which attribute to a variety of functional activities, such as antioxidant, antidiabetic and anti-inflammatory properties (Giampieri et al., 2022). The flavonoids predominantly include kaempferol, quercetin, isorhamnetin, luteolin, and apigenin, and their glycosylated forms (Giampieri et al., 2022; Kieliszek, Piwowarek, Kot, Błażejak, Chlebowska-Śmigiel, & Wolska, 2018). Such variation mirrors the differences of bee pollen species and geographical origins. Rapeseed bee pollen from China contained isorhamnetin, kaempferol and its 3-*O*-glucosides, rutin, and quercetin and its 3-*O*-glucosides (Zhang, Yang, Jamali, & Peng, 2016); except rutin, the similar compounds in *Cistus* sp. bee pollen from Spain (Thakur & Nanda, 2020). Bee pollen from Egypt possessed apigenin, catechin, kaempferol, naringenin, rutin, and quercetin (Thakur & Nanda, 2020). The glucosides of anthocyanins (delphinidin, petunidinm, and malvidin) were detected in *Echium plantagineum* bee pollen from Spain (Thakur & Nanda, 2020). According to the previous reports, the flavonoids constituted 1.4 % of pollen (Kieliszek et al., 2018), and the higher concentration of quercetin-3-O-β-D-glucosyl-(2 → l)-β-glucoside range from 0.65 to 5108 μg/g of pollen (Giampieri et al., 2022). Overall, the wide variations remain a need to provide deep insights regarding phenolamide and flavonoid profiles of bee pollen as an underutilized source of functional foods.

The current study aimed to detect phenolamides and flavonoids in 20 types of monofloral bee pollen collected from China. The plant origins and purities of bee pollen samples were determined using DNA barcoding. Phenolamides and flavonoids were identified using HPLC, LC-MS/MS, or NMR, and their contents were quantified with external standards.

2. Materials and methods

2.1. Bee pollen samples

Twenty types of monofloral bee pollen were collected from eighteen Chinese apiaries in the Modern Agro-industry Research System during the flowering season of 2020, and then stored in a refrigerator at −18℃ until use. To ensure pollen purities, the locations of apiaries and the bee forage area were selected around with monofloral plant sources. The plant origins of pollen samples were recorded according to the locations of their hives and the floral sources available in flowering periods.

2.2. Chemicals and materials

Kaempferol-3-*O*-rutinoside-7-*O*-glucoside, quercetin-3-*O*-rutinoside, kaempferol-3-*O*-sophoroside, isovitexin, syringetin-3-*O*-rutinoside, kaempferol-3-*O*-rutinoside, syringetin-3-*O*-glucoside, and isorhamnetin-3-*O*-glucoside were purchased from Shanghai Yuanye Biotechnology Co., ltd. (Shanghai, China). Isorhamnetin-3-*O*-gentiobioside was from Sichuan Victory Biological Technology Co., ltd. (Chengdu, Sichuan, China). Isorhamnetin-3-*O*-neohesperidoside, quercetin-3-*O*-sophoroside and quercetin-3-*O*-glucoside were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Standards of some phenolamides (purities ≥97 %) were prepared by ourselves using preparative HPLC, including N(E), N'(E)-di-*p*-coumaroyl putrescine, N1(E), N5(E), N10(E)-tri-*p*-coumaroyl spermidine, N1(E), N10(E)-di-hydroxyferuloyl-N5(E)-feruloyl spermidine, N1(E), N5(E), N10(E), N14(E) tetra-*p*-coumaroyl spermine, N1(E), N5(E), N10(E), N14(E)-tetra-caffeoyl spermine, N1(E), N10(E)-di-*p*-coumaroyl spermidine, N(E)-*p*-coumaroyl-N'(E)-caffeoyl putrescin, N1(E), N14(E)-di-*p*-coumaroyl-N5, N10-di-acetyl spermine, N1(E), N5(E), N10(E) tri- caffeoyl spermidine, N1(E), N5(E)-di-*p*-coumaroyl-N10-feruloyl spermidine.

Methanol (HPLC grade) was purchased from Thermo Fisher Scientific Inc. (Fair Lawn, NJ, USA). Dimethyl sulfoxide-d6 (DMSO-d_6) and methanol-D4 were from Adamas (Shanghai, China). Ultrapure water was purified using a Milli-Q-Integral System (Millipore, Billerica, MA, USA). Acetic acid (HPLC and MS grade) was purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA). DNeasy Plant Mini Kit was from Qiagen (Qiagen, CA, USA). Ethyl alcohol (analytical grade) and all other reagents were from Solarbio Inc. (Beijing, China).

2.3. Plant origins and purity detections using DNA barcoding

Bee pollen was powdered by a pulverizer (LS-FSJ, Taisite, Tianjin, China). Bee pollen powder (100 mg) was used to extract DNA according to the protocol described in DNeasy Plant Mini Kit (Qiagen, CA, USA). The concentration of DNA was analyzed by a NanoDrop ND-1000 (Thermo Scientific, Massachusetts, USA), and checked by running the extracted DNA samples on 1 % agarose gel. The rbcL2 barcoding region was amplified using primers (rbcL2 F: 5′-CTTACCAGYCTTGATCGTTACAAAGG-3′ and R: 5′-GTAAAATCAAGTCCACCRCG-3′). PCR procedure was set as initial denaturation at 95 °C for 5 min, 28 cycles at 94 °C denaturations for 45 s, annealing at 55 °C for 50 s and extension at 72 °C for 45 s, and a final extension step at 72 °C for 10 min. Sequencing of the library was performed on Illumina® MiSeq PE300 platform (Illumina, Inc., CA, USA).

2.4. Compound identification of bee pollen extracts

Extraction of Bee pollen: One gram of pollen powder was extracted using 30 ml 75 wt% ethanol/water solvent, and then sonicated at 100 W for 30 mins at 40 °C. The pollen solution was centrifuged at 5000 × *g* for 10 min to remove the solid particles. The supernatants were filtered through MILLEX-GA 0.22 μm filter prior to HPLC injection.

HPLC-PDA and HPLC-ESI -*Q-TOF analysis*: The profiles of bee pollen extracts were analyzed at a wavelength of 280 nm using HPLC (PDA-20A diode array detector, SIL auto-injection valve, CTO-10A thermostat, and pump LC-6AD, Shimadzu, Tokyo, Japan). The 20 μL solution was loaded, and eluted through a 150 mm × 4.6 mm 3 μm Gemini C18 reversed-phase column (Phenomenex, Inc., CA, USA) with a gradient elution. The mobile phase consisted of water (Phase A) and methanol (Phase B), containing 1 % acetic acid, with a flow rate of 0.7 ml/min. The temperature of the column oven was set at 35 °C. A 150 min linear gradient was performed as follows: 0–10 min, 5 %-20 % B; 10–25 min, 20–24 % B; 25–45 min, 24–30 % B; 45–50 min, 30–38 % B; 50–60 min, 38–40 % B; 60–70 min, 40–42 % B; 70–80 min, 42–44 % B; 80–90 min, 44–46 % B; 90–100 min, 46–48 % B; 100–110 min, 48–50 % B; 110–120 min, 50–52 % B; 120–130 min, 52–54 % B; 130–140 min, 54–56 % B; 140–150 min, 56–90 % B.

To identify compounds in bee pollen extracts, HPLC-ESI -Q-TOF (Agilent 6520, Agilent Technologies, Palo Alto, CA, USA) was used, with the operating parameters: source voltage, 4 kV; capillary voltage, 130 V; capillary temperature, 350℃, full-scan spectra were obtained over a mass range of 50–800 Da; ESI-MS/MS spectra were acquired by a collision energy of 20 eV. Compounds were identified by their retention times (RT), UV spectra, and ESI-MS/MS information with either authentic standards or published reports.

HPLC purification of phenolamides: To further elucidate the chemical structure, some phenolamides were purified using a preparative HPLC-DAD system equipped with a fraction collector (NP7005-10C, Hanbang, Jiangsu, China). The separation was achieved with a 250 × 50 mm × 10 μm C18 column (Hanbang, Jiangsu, China) at 30 °C. Mobile phase A: 0.1 % (v/v) aqueous acetic acid and B: methanol were used with a flow rate of 70 ml/min.

NMR detection: N1(E), N10(E)-di-*p*-coumaroyl spermidine and N1(E), N14(E)-di-*p*-coumaroyl-N5, N10-di-acetyl spermine were further elucidated using a 600 MHz NMR spectrometer (Bruker AVANCE III 600 MHz, Rheinstetten, Germany) with a cryoprobe at room temperature.

Chemical shifts (δ) were quoted relative to tetramethylsilane (TMS). The 2D NMR data of heteronuclear single quantum coherence (HSQC), heteronuclear multiple-bond correlation (HMBC), and correlation spectroscopy (COSY) were used for analysis.

2.5. Quantification of bee pollen compounds

Compound quantification was performed by the peak areas at a wavelength of 280 nm using external calibration curves with pure standards (mentioned in 2.2) (Wang, Hu, Luo, Liu, & Zhang, 2018).

The contents of N1, N5, N10-tri-*p*-coumaroyl spermine, N1, N10-di-*p*-coumaroyl-N14-hydroxyferuloyl spermine, N1, N10-di-*p*-coumaroyl-N14-caffeoyl spermine, N1-hydroxyferuloyl-N5-*p*-coumaroyl-N14-caffeoyl spermine, N1-hydroxyferuloyl-N5-*p*-coumaroyl-N14-feruloyl spermine, N1-feruloyl-N5, N14-di-*p*-coumaroyl spermine, N1, N14-di-p-coumaroyl-N5-hydroxyavenalumoyl spermine, tetra-*p*-coumaroyl spermine, N1-caffeoyl-N5, N10-di-*p*-coumaroyl-N14-hydroxyferuloyl spermine, and N1, N5, N10-tri-*p*-coumaroyl-N14-hydroxyferuloyl spermine were quantified by N1(E), N5(E), N10(E), N14(E)-tetra-*p*-coumaroyl spermine; the contents of N, N' -di-*p*-coumaroyl putrescine, N, N'-dicaffeoyl putrescine, *N*-feruloyl-N'-caffeoyl putrescine, and *N*-*p*-coumaroyl-N'-feruloyl putrescine by N(E), N'(E)-di-*p*-coumaroyl putrescine; the contents of N1, N10-di-*p*-coumaroyl-N5-caffeoyl spermidine, tri-*p*-coumaroyl spermidine, N1-*p*-coumaroyl-N5, N10-di-caffeoyl spermidine, tri-feruloyl-spermidine, and N1, N5-di-*p*-coumaroyl-N10-hydroxyferuloyl spermidine by N1(E), N5(E), N10(E)-tri-*p*-coumaroyl spermidine; the contents of N1, N10-di-hydroxyferuloyl-N5-feruloyl spermidine and N1, N10-di-feruloyl-N5-hydroxyferuloyl spermidine by N1(E), N10(E)-di-hydroxyferuloyl-N5(E)-feruloyl spermidine; the contents of tetra-caffeoyl spermine, N1, N5, N10-tri-caffeoyl-N14-hydroxy feruloyl spermine, N1, N14-di-hydroxyferuloyl-N5, N10-di-caffeoyl spermine, N1, N5, N10-tri-caffeoyl-N14-feruloyl spermine, and N1-feruloyl-N5, N10-dicaffeoyl-N14-hydroxyferuloyl spermine by N1(E), N5(E), N10(E), N14(E)-tetra-caffeoyl spermine; the contents N1, N10-di-*p*-coumaroyl spermidine, *N*-*p*-coumaroyl-N'-caffeoyl putrescin, N1, N14-di-*p*-coumaroyl-N5, N10-di-acetyl spermine, tri-caffeoyl spermidine, and N1, N5-di-*p*-coumaroyl-N10-feruloyl spermidine were quantified by their prepared standards.

The contents of quercetin-3-*O*-sophoroside, quercetin-3,7-*O*-diglucoside and quercetin-3,4′-*O*-diglucoside were quantified by quercetin-3-*O*-sophoroside; kaempferol-3-*O*-rutinosyl-7-*O*-glucoside and kaempferol-3-*O*-(2″-*O*-glucosyl)-rutinoside by kaempferol-3-*O*-rutinoside-7-*O*-glucoside; isorhamnetin-3-*O*-(6″-*O*-malonyl)-glucosyl-7-*O*-glucoside, and isorhamnetin-3-*O*-(2″-*O*-glucosyl)-rutinoside, isorhamnetin-7-*O*-vicianoside, isorhamnetin-3-*O*-gentiobioside, and isorhamnetin-3-*O*-vicianoside by isorhamnetin-3-*O*-gentiobioside; quercetin-3-*O*-arabinoglucoside and quercetin-3-*O*-rutinoside by quercetin-3-*O*-rutinoside; kaempferol-3-*O*-sambubioside, kaempferol-3-*O*-sophoroside, and kaempferol-3-*O*-(6″-*O*-malonyl)-glucoside by kaempferol-3-*O*-sophoroside; syringetin-3-*O*-rutinoside and syringetin-3-*O*-(6″-*O*-malonyl)-glucoside by syringetin-3-*O*-rutinoside; isorhamnetin-3-*O*-neohesperidoside and isorhamnetin-3-*O*-(6″-O-malonyl)-glucoside by isorhamnetin-3-*O*-neohesperidoside; quercetin-3-*O*-(6″-*O*-malonyl)-glucoside and quercetin-3-*O*-glucoside by quercetin-3-*O*-glucoside. Isovitexin, kaempferol-3-*O*-rutinoside, isorhamnetin-3-*O*-glucoside, and syringetin-3-*O*-glucoside were respectively quantified by their standards.

2.6. Statistics

The quantification was performed in triplicate. The values were presented as means ± standard deviation and analyzed with SPSS software (version 16.0, SPSS GmbH Software, Munich, Germany).

3. Results

3.1. Plant origins and purities of 20 types of bee pollen

To identify the plant source of bee pollen, the traditional approaches focused on palynological analysis (Bell et al., 2016). These techniques are time-consuming, and rely on a high level of expertise in pollen morphological characters (Bell et al., 2016). However, these characters lack a high and specific resolution in species-level taxonomy for many plant groups (Bell et al., 2016). Over the past decade, DNA barcoding has gained popularity in taxonomic, ecological, and evolutionary research, and provides high efficiencies in species identification (Bell et al., 2016; Picron et al., 2021). For plants, the three regions of the chloroplast genome (rbcL, matK, and trnH-psbA) and the ITS region of nuclear ribosomal genome have been widely used as DNA barcodes, either together or separately.

We employed DNA barcoding to identify the plant origins and purities of the collected 20 types of bee pollen (Fig. 1). As shown in Fig. 1, the purities of 16 bee pollens reach more than 80 %. Generally, 80 % is recognized as lower limit for monofloral pollen (Campos et al., 2008). Five pure monofloral bee pollens were rapeseed (*Brassica rapa,* with a purity of 95.1 %), corn poppy (*Papaver rhoeas,* with a purity of 97 %), tea (*Camellia sinensis,* with a purity of 90.4 %), pear (*Pyrus bretschneideri,* with a purity of 91.5 %), Lotus (*Nelumbo nucifera,* with a purity of 97.9 %) (Fig. 1). The main botanical origins by DNA barcoding analysis (Fig. 1) were consistent with our records of the available floral sources near bee hives, when pollen samples were collected (as mentioned in Materials and Methods 2.1).

3.2. Identification of phenolamides

Through the HPLC profiles of 20 types of bee pollen (Fig. 2), 31 phenolamides and their 33 *cis/trans* isomers were identified and listed in Table 1. The MS^2 profiles and cleavage patterns of these phenolamides (A1 to A31) are shown in Supplementary Fig. 1.

Compounds A1-1, A1-2, A1-3, and A1-4 exhibited the same quasi-molecular ion at *m/z* 438 $[M + H]^+$ ($C_{25}H_{31}N_3O_4$) (Table 1), and yielded three MS/MS fragments at *m/z* 292, 204 and 147 (Fig. 3A), indicating isomers. Quasi-molecular ion at *m/z* 438 generated fragment ion at *m/z* 420 $[M + H - H_2O]^+$ and *m/z* 292 $[M + H - C_9H_6O_2]^+$, indicating the loss of 18 amu (water) and 146 amu (*p*-coumaroyl residue). The *m/z* 147 $[C_9H_6O_2]^+$ fragment from *i-cleavage* further validated the presence of *p*-coumaroyl residue. The *m/z* 204, 218 and 275 fragments were the characteristic fragment ions of *p*-coumaroyl spermidine (Fig. 3A). The fragment ion *m/z* 204 resulted from the cleavage of C4 and N5 by *H* rearrangement, indicating one *p*-coumaroyl residue substituted at N1. The fragment ion at *m/z* 218 was generated by the break of N5 and C6, indicating another *p*-coumaroyl conjugated at N10. The fragment ion at *m/z* 275 represented the empty of the N5 position. Thus, compound A1 and isomers were N1, N10-di-*p*-coumaroyl spermidine. According to the retention time and UV maximum absorption wavelength, compounds A1-1 to A1-4 are *cis/trans* isomers (Fig. 3A and Table 1). Compounds A1-1 to A1-4 displayed the redshift of UV maximum absorption wavelength and the decrease of polarity. The maximum UV absorption wavelength of *p*-coumaric acid *cis*-isomer was actually lower than that of *trans*-isomer. Because *trans-p*-coumaric acid is easy to form a π-π conjugate bond through steric hindrance, the UV spectrum of *trans-p*-coumaric acid will be redshift (Fuks-Janczarek, Kityk, Miedziński, Gondek, Danel, & Zagorska, 2006). In addition, *cis*-isomer presents a shorter retention time than *trans*-isomer, probably because the dipole moment of *cis*-isomers is usually higher than *trans*-isomers (Putschögl, Zirak, & Penzkofer, 2008), leading to *cis-p*-coumaric acid performing slightly stronger polarity (hydrophilic) than *trans-p*-coumaric acid. Based on the redshift of their UV λ max at 274, 284, 292, and 306 nm, compounds A1-1, A1-2, A1-3, A1-4 were identified respectively as N1(Z), N10(Z)-di-*p*-coumaroyl spermidine, N1(Z), N10

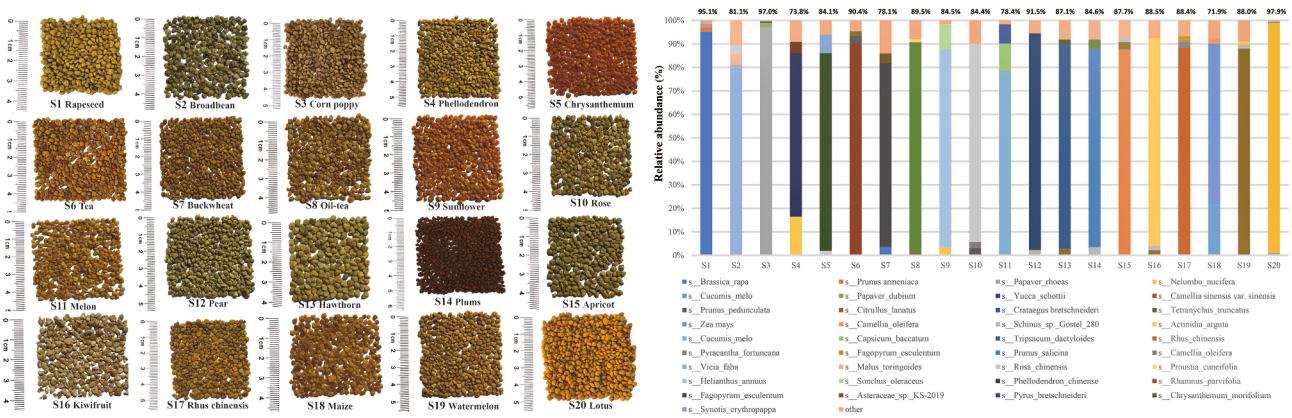

Fig. 1. Plant origins and purities of 20 types of monofloral bee pollen. Note: The plant origins were identified as rapeseed (*Brassica rapa*), broad bean (*Vicia faba*), corn poppy (*Papaver rhoeas*), phellodendron (*Phellodendron chinense*), chrysanthemum (*Chrysanthemum morifolium*), tea (*Camellia sinensis* var. *sinensis*), buckwheat (*Fagopyrum esculentum*), oil-tea (*Camellia oleifera*), sunflower (*Helianthus annuus*), rose (*Rosa chinensis*), melon (*Cucumis melo*), pear (*Pyrus bretschneideri*), hawthorn (*Crataegus bretschneideri*), plums (*Prunus salicina*), apricot (*prunus armeniaca*), kiwifruit (*Actinidia arguta*), rhus chinensis (*rhus chinensis*), watermelon (*Citrullus lanatus*), louts (*Nelumbo nucifera*) pollen.

(E)-di-*p*-coumaroyl spermidine, N1(E), N10(Z)-di-*p*-coumaroyl spermidine and N1(E), N10(E)-di-*p*-coumaroyl spermidine. Purified compound A1-4 was then confirmed as N1(E), N10(E)-di-*p*-coumaroyl spermidine by NMR data: ^{1}H NMR (600 MHz, Methanol-d_4) δ 7.65–7.45 (m, 2H, 13, 23), 7.45–7.36 (m, 4H, 19, 15, 29, 25), 6.81 (d, J = 8.0 Hz, 4H, 16, 18, 28, 26), 6.44 (d, J = 15.6 Hz, 2H, 12, 22), 3.47–3.41 (m, 2H), 3.40–3.35 (m, 1H), 3.11–2.99 (m, 4H), 1.83 – 1.75 (m, 2H), 1.74–1.65 (m, 2H). ^{13}C NMR (126 MHz, Methanol-d_4) δ 172.73 (11), 169.36 (21), 160.77 (17), 160.63 (27), 142.57 (13), 142.00 (23), 130.68 (19, 15), 130.56 (29, 25), 127.58 (14), 127.45 (24), 118.23 (12), 117.66 (22), 116.78 (16, 18), 116.75 (28, 26), 48.52 (6), 46.27 (4), 39.51 (9), 37.01 (2), 27.86 (8), 27.62 (7), 24.65 (3).

Compound A15 and its isomers displayed the quasi-molecular ion $[M + H]^+$ at *m/z* 584 (Table 1 and Supplementary Fig. 1 A15). These compounds were 146 amu higher than compounds A1, suggesting the third *p*-coumaroyl residue linked in the N5 position of spermidine, tri-*p*-coumaroyl spermidine (Kim et al., 2018). Compounds A13-1, A13-2, A13-3, and A13-4 were also *cis/trans*-isomers with quasi-molecular ions at *m/z* 600 $[M + H]^+$ (Fig. 3 B), and were 16 amu higher than compounds A15. Fragment ion at *m/z* 163 in MS/MS spectrum indicated the replacement of *p*-coumaroyl by caffeoyl (Fig. 3 B). Fragment ion at *m/z* 204 and 218 indicates two *p*-coumaroyl residues bound in N1 and N10; thus, those compounds were identified as N1, N10-di-*p*-coumaroyl-N5-caffeoyl spermidine. For compounds A14-1, A14-2, and A14-3, fragment ion at *m/z* 177 originated from feruloyl, and fragment ion at *m/z* 193 from hydroxy feruloyl (Table 1 and Supplementary Fig. 1 A14). Fragment ions at *m/z* 250 and 264 indicated the two hydroxy feruloyl substituted at N1 and N10, respectively; thus, those compounds were identified as N1, N10-di-hydroxyferuloyl-N5-feruloyl spermidine. Based on the above trisubstituted spermidine MS/MS cleavage rules, compounds A17-1, A17-2, A18-1, A18-2, A19-1, A19-2, A19-3, A21, A25 and A27 were identified and listed in Table 1, and the MS/MS fragment cleavage patterns in Supplementary Fig. 1 A17, A18, A19, A21, A25, and A27.

Compounds A6-1, A6-2, and A6-3 displayed the same quasi-molecular ion at *m/z* 381 $[M + H]^+$ and fragmented ions (Fig. 3 C and Table 1), indicating they are isomers. Quasi-molecular ion yielded fragment ion at *m/z* 235 $[M + H - C_9H_6O_2]^+$ via losing a *p*-coumaroyl residue (146 amu), and then generated fragment ion at *m/z* 89 $[M + H - C_9H_6O_2 - C_9H_6O_2]^+$ via losing another *p*-coumaroyl residue (146 amu), suggesting di-*p*-coumaroyl putrescine (Kim et al., 2018). According to the UV spectrum and polarity, compounds A6-1, A6-2, and A6-3 were identified as N(Z), N' (Z)-di-*p*-coumaroyl putrescine, N(Z), N' (E)-di-*p*-coumaroyl putrescine and N(E), N' (E)-di-*p*-coumaroyl putrescine. Similarly, compounds A9-1, A9-2, A10, A12, and A16 were then identified as putrescine conjugated phenolics (Table 1 and Supplementary Fig. 1 A9, A10, A12, A16).

Compound A7 displays quasi-molecular ion at *m/z* 717 $[M + H]^+$ ($C_{39}H_{48}N_4O_9$). As seen in MS/MS spectrum (Fig. 3 D), *m/z* 717 $[M + H]^+$ lost 146 amu (*p*-coumaroyl residue) to yield fragment ion at *m/z* 571 $[M + H - C_9H_6O_2]^+$, or losing 176 amu (feruloyl residue) to generate fragment ion 541 $[M + H - C_9H_6O_2 - C_{10}H_8O_3]^+$, or losing 192 amu (hydroxy feruloyl residue) to produce fragment ion 525 $[M + H - C_9H_6O_2 - C_{10}H_8O_3 - C_{10}H_8O_4]^+$. Thus, compound A7 was identified as *p*-coumaroyl-feruloyl-hydroxy feruloyl spermine. The fragment ion at *m/z* 234 suggested feruloyl residue substituted at N1 or N14 of spermine. The fragment ion at *m/z* 250 indicated hydroxy feruloyl residue also linked in N1 or N14 of spermine. Moreover, the fragment ion at *m/z* 467 indicated hydroxy feruloyl residue and *p*-coumaroyl residue substituted at two adjacent N positions (Fig. 3 D). Therefore, compound A7 was identified as N1-hydroxyferuloyl-N5-*p*-coumaroyl-N14-feruloyl spermine.

Compounds A2-1, A2-2, and A2-3 showed quasi-molecular ion at *m/z* 641 $[M + H]^+$ and the same fragments (Table 1). Quasi-molecular *m/z* 641 ion yielded fragment ion at *m/z* 495 $[M + H - C_9H_6O_2]^+$ via losing 146 amu (*p*-coumaroyl residue), and then lost another *p*-coumaroyl residue to generate fragment ion at *m/z* 349 $[M + H - C_9H_6O_2 - C_9H_6O_2]^+$ (Fig. 3 E). They were identified as trisubstituted *p*-coumaroyl spermine. The fragment ion at *m/z* 204 and 421 indicated two *p*-coumaroyl residues substituted at N1 and N5, or N10 and N14. Quasi-molecular ion (*m/z* 641) lost a $-NH_3$ group through *H* rearrangement to form fragment ion at *m/z* 624, demonstrating the empty of N1 or N14 (Fig. 3E). Thus, three *p*-coumaroyl residues were respectively attached to the N5, N10, and N14 positions of spermine (Fig. 3 E). According to the UV absorption and polarity, compounds A2-1, A2-2, and A2-3 were respectively identified as N5(Z), N10(E), N14(Z)-tri-*p*-coumaroyl spermine, N5(E), N10(E), N14(Z)-tri-*p*-coumaroyl spermine and N5(E), N10(E), N14(E)-tri-*p*-coumaroyl spermine. Based on the above trisubstituted spermine MS/MS cleavage rules, compounds A3-1, A3-2, A4-1, A4-2 A5, A8, A20-1, A20-2 and A20-3 were identified and listed in Table 1 and MS/MS fragment cleavage patterns in Supplementary Fig. 1 A3, A4, A5, A8, and A20.

As seen in the MS spectra of compounds A24-1 and A24-2 (Fig. 3 F and Table 1), they seemed to exist the two protonated ions *m/z* 441.1833 and *m/z* 881.3603 $[M + H]^+$. However, the isotopic ion of *m/z* 441.1833 additionally existed at *m/z* 441.6849, an extra 0.5 Da; in contrast, the isotopic ion of 881.3603 displayed at 882.3629, an extra 1 Da. This situation indicated that ion 441.1833 can carry two charges in

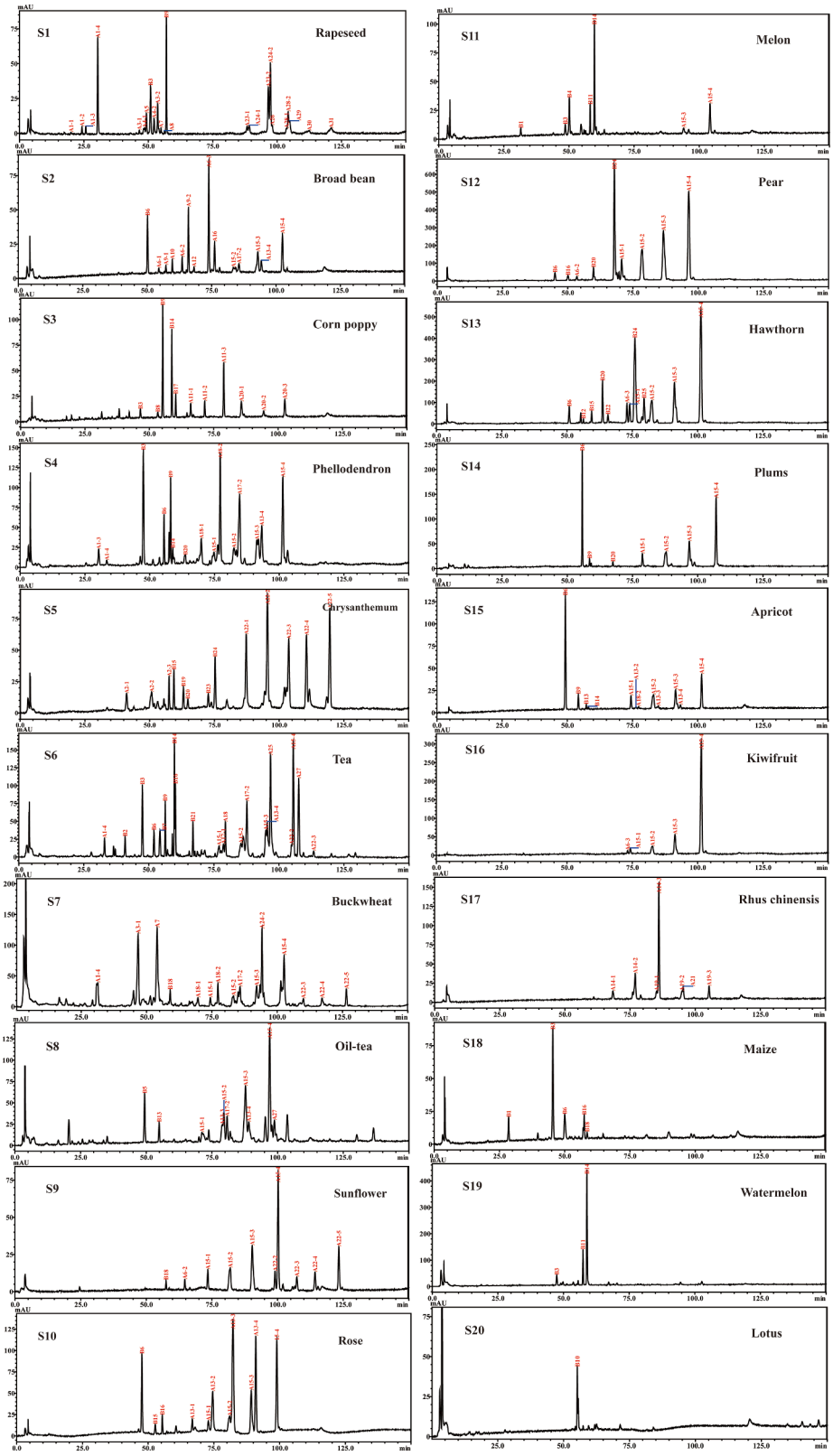

Fig. 2. HPLC chromatograms of bee pollen extracts. (S1) rapeseed; (S2) broad bean; (S3) corn poppy; (S4) phellodendron; (S5) chrysanthemum; (S6) tea; (S7) buckwheat; (S8) oil-tea; (S9) sunflower; (S10) rose; (S11) melon; (S12) pear; (S13) hawthorn; (S14) plums; (S15) apricot; (S16) kiwifruit; (S17) rhus chinensis; (S18) maize; (S19) watermelon; (S20) lotus pollen.Note: A1: N1, N10-di-*p*-coumaroyl spermidine; A2: N1, N5, N10-tri-*p*-coumaroyl spermine; A3: N1, N10-di-*p*-coumaroyl-N14-hydroxyferuloyl spermine; A4: N1, N10-di-*p*-coumaroyl-N14-caffeoyl spermine; A5: N1-hydroxyferuloyl-N5-*p*-coumaroyl-N14-caffeoyl spermine; A6: N, N'-di-*p*-coumaroyl putrescine; A7: N1- hydroxyferuloyl-N5-*p*-coumaroyl-N14- feruloyl spermine; A8: N1- feruloyl-N5, N14-di-*p*-coumaroyl spermine; A9: *N*-*p*-coumaroyl-N'-caffeoyl putrescine; A10: N, N'-di-caffeoyl putrescine; A11: N1, N14-di-*p*-coumaroyl-N5, N10-di-acetyl spermine; A12: *N*-feruloyl-N'-caffeoyl putrescine; A13: N1, N10-di-*p*-coumaroyl-N5-caffeoyl spermidine; A14: N1, N10-di-hydroxyferuloyl-N5-feruloyl spermidine; A15: Tri-*p*-coumaroyl spermidine; A16: *N*-*p*-coumaroyl-N'-feruloyl putrescine; A17: N1-*p*-coumaroyl-N5, N10-di-caffeoyl spermidine; A18: Tri-caffeoyl spermidine; A19: Tri-feruloyl spermidine; A20: N1, N14-di-*p*-coumaroyl-N5-hydroxyavenalumoyl spermine; A21: N1, N10-di-feruloyl-N5-hydroxyferuloyl spermidine; A22: Tetra-*p*-coumaroyl spermine; A23: Tetra-caffeoyl spermine; A24: N1, N5, N10-tri-caffeoyl-N14-hydroxy feruloyl spermine; A25: N1, N5-di-*p*-coumaroyl-N10-hydroxyferuloyl spermidine; A26: N1, N14-di-hydroxyferuloyl-N5, N10-di-caffeoyl spermine; A27: N1, N5-di-*p*-coumaroyl- N10-feruloyl spermidine; A28: N1, N5, N10-tri-caffeoyl-N14-feruloyl spermine; A29: N1-feruloyl-N5, N10-dicaffeoyl-N14-hydroxyferuloyl spermine; A30: N1-caffeoyl-N5, N10-di-*p*-coumaroyl-N14-hydroxyferuloyl spermine; A31: N1, N5, N10-tri-*p*-coumaroyl-N14-hydroxyferuloyl spermine. B1: quercetin-3,7-*O*-diglocuside; B2: kaempferol-3-*O*-rutinoside-7-*O*-glucoside; B3: quercetin-3-*O*-sophoroside; B4: quercetin-3,4′-*O*-diglucoside; B5: isorhamnetin-3-*O*-(2″-glucosyl)-rutinoside; B6: isorhamnetin-3-*O*-gentiobioside; B7: quercetin-3-*O*-rutinoside; B8: quercetin-3-*O*-arabinoglucoside; B9: kaempferol-3-*O*-sophoroside; B10: isorhamnetin-3-*O*-vicianoside; B11: isovitexin; B12: syringetin-3-*O*-rutinoside; B13: kaempferol-3-*O*-(2″-glucosyl)-rutinoside; B14: kaempferol-3-*O*-rutinoside; B15: isorhamnetin-3-*O*-(6″-*O*-malonyl)-glucosyl-7-*O*-glucoside; B16: isorhamnetin-3-*O*-neohesperidoside; B17: kaempferol-3-*O*-sambubioside; B18: quercetin-3-*O*-glocuside; B19: quercetin 3-*O*-(6″-*O*-malonyl)-glucoside; B20: isorhamnetin-3-*O*-glocuside; B21: isorhamnetin-7-vicianoside; B22: syringetin-3-*O*-glucoside; B23: kaempferol 3-*O*-(6″-*O*-malonyl)-glucoside; B24: isorhamnetin-3-*O*-(6″-*O*-malonyl)-glucoside; B25: syringetin-3-*O*-(6″-*O*-malonyl)-glucoside.

the *m/2z* form (Fig. 3 F). Thus, *m/z* 881.3603 $[M + H]^+$ was the real quasi-molecular ion of compounds A24-1 and A24-2 ($C_{47}H_{52}N_4O_{13}$). As seen in MS/MS spectrum (Fig. 3 F), *m/z* 881 $[M + H]^+$ lost 162 amu (caffeoyl residue) to yield fragment ion at *m/z* 719 $[M + H - C_9H_6O_3]^+$; then *m/z* 719 lost 162 amu (caffeoyl residue) and 192 amu (hydroxy feruloyl residue) to generate fragment ion at *m/z* 557 $[M + H - C_9H_6O_3 - C_9H_6O_3]^+$ and *m/z* 527 $[M + H - C_9H_6O_3 - C_{10}H_8O_4]^+$, respectively. The fragment ion at *m/z* 250 and *m/z* 483 indicated that hydroxy feruloyl residue substituted at N1 or N14 of spermine. Therefore, compound A24 was identified as N1, N5, N10-tri-caffeoyl-N14-hydroxyferuloyl spermine (Fig. 3 F). MS spectra of compounds A22, A23, A24, A26, A28, A29, A30, and A31 similarly presented *m/2z* protonated ions (Table 1

Table 1
Phenolamides identified in bee pollen and their UV and MS characteristics.

Peak	Mw	UV λ max (nm)	Formula	*m/z* of main fragments, MS^2	Compounds identified	Pollen
A1-1	437	274	$C_{25}H_{31}N_3O_4$	72, 119, 147, 204, 292	N1(Z), N10(Z)-di-*p*-coumaroyl spermidine	Rapeseed
A1-2	437	284	$C_{25}H_{31}N_3O_4$	72., 119, 147, 204, 292	N1(Z), N10(E)-di-*p*-coumaroyl spermidine	Rapeseed
A1-3	437	292	$C_{25}H_{31}N_3O_4$	72., 119, 147, 204, 292	N1(E), N10(Z)-di-*p*-coumaroyl spermidine	Rapeseed, Phellodendron
A1-4	437	306	$C_{25}H_{31}N_3O_4$	72., 119, 147, 204, 292	N1(E), N10(E)-di-*p*-coumaroyl spermidine	Rapeseed, Phellodendron, Tea, Buckwheat
A2-1	640	281	$C_{37}H_{44}N_4O_6$	147, 204, 275, 349, 421, 495,621	N1, N5, N10-tri-*p*-coumaroyl spermine	Chrysanthemum
A2-2	640	289	$C_{37}H_{44}N_4O_6$	147, 204, 275, 349, 421, 495,621	N1, N5, N10-tri-*p*-coumaroyl spermine	Chrysanthemum.
A2-3	640	294	$C_{37}H_{44}N_4O_6$	147, 204, 275, 349, 421, 495,621	N1, N5, N10-tri-*p*-coumaroyl spermine	Chrysanthemum.
A3-1*	686	295	$C_{38}H_{46}N_4O_8$	147, 204, 250, 275, 321, 349, 395, 495, 541	N1, N10-di-*p*-coumaroyl-N14-hydroxyferuloyl spermine	Rapeseed, Buckwheat
A3-2	686	309	$C_{38}H_{46}N_4O_8$	147, 204, 250, 275, 321, 349, 395, 495, 541	N1, N10-di-*p*-coumaroyl-N14-hydroxyferuloyl spermine	Rapeseed
A4-1*	656	299	$C_{37}H_{44}N_4O_7$	147, 163, 204, 220, 275, 349, 437, 495, 511	N1, N10-di-*p*-coumaroyl-N14-caffeoyl spermine	Rapeseed
A4-2	656	309	$C_{37}H_{44}N_4O_7$	147, 163, 204, 220, 275, 349, 437, 495, 511	N1, N10-di-*p*-coumaroyl-N14-caffeoyl spermine	Rapeseed
A5*	702	314	$C_{38}H_{46}N_4O_9$	147, 193, 220, 250, 275, 291, 321, 349, 395, 467, 511, 541, 557	N1- hydroxyferuloyl-N5-*p*-coumaroyl-N14-caffeoyl spermine	Rapeseed
A6-1	380	276	$C_{23}H_{28}N_2O_4$	89, 119, 147, 218, 235	N(Z), N'(Z)-di-*p*-coumaroyl putrescine	Broad bean
A6-2	380	290	$C_{23}H_{28}N_2O_4$	89, 119, 147, 218, 235	N(Z), N'(E)-di-*p*-coumaroyl putrescine	Broad bean, sunflower, Pear
A6-3	380	298	$C_{23}H_{28}N_2O_4$	89, 119, 147, 218, 235	N(E), N'(E)-di-*p*-coumaroyl putrescine	Broad bean, Hawthorn, kiwifruit
A7*	716	313	$C_{39}H_{48}N_4O_9$	147, 177, 193, 234, 250, 275, 305, 321, 349, 395, 421, 467, 525	N1-hydroxyferuloyl-N5-*p*-coumaroyl-N14-feruloyl spermine	Rapeseed, Buckwheat
A8*	670	307	$C_{38}H_{46}N_4O_7$	147, 177, 204, 234, 275, 305, 349, 451, 495, 525	N1-feruloyl-N5, N14-di-*p*-coumaroyl spermine	Rapeseed
A9-1*	396	293	$C_{22}H_{24}N_2O_5$	89, 147, 163, 218, 235, 251	*N*-*p*-coumaroyl-N'-caffeoyl putrescine	Broad bean
A9-2	396	308	$C_{22}H_{24}N_2O_5$	89, 147, 163, 218, 235, 251	*N*-*p*-coumaroyl-N'-caffeoyl putrescine	Broad bean
A10	412	294/303/319	$C_{22}H_{24}N_2O_5$	89, 145, 163, 234, 251, 277	N, N'-di-caffeoyl putrescine	Broad bean
A11-1*	578	277	$C_{32}H_{42}N_4O_6$	147, 171, 204, 275, 287, 313, 391, 416, 519., 537	N1(Z), N14(Z)-di-*p*-coumaroyl-N5, N10-di-acetyl spermine	Corn poppy
A11-2	578	292	$C_{32}H_{42}N_4O_6$	147, 171, 204, 275, 287, 313, 391, 416, 519., 537	N1(Z), N14(E)-di-*p*-coumaroyl-N5, N10-di-acetyl spermine	Corn poppy
A11-3	578	299/306	$C_{32}H_{42}N_4O_6$	147, 171, 204, 275, 287, 313, 391, 416, 519., 537	N1(E), N14(E)-di-*p*-coumaroyl-N5, N10-di-acetyl spermine	Corn poppy
A12*	426	293/318	$C_{23}H_{26}N_2O_6$	234, 251, 265	*N*-feruloyl-N'-caffeoyl putrescine	Broad bean
A13-1	599	275	$C_{34}H_{37}N_3O_7$	147, 163, 204, 218, 275, 292, 318, 420, 438, 454	N1, N10-di-*p*-coumaroyl-N5-caffeoyl spermidine	Rose
A13-2	599	292	$C_{34}H_{37}N_3O_7$	147, 163, 204, 218, 275, 292, 318, 420, 438, 454	N1, N10-di-*p*-coumaroyl-N5-caffeoyl spermidine	Apricot, Rose
A13-3	599	298	$C_{34}H_{37}N_3O_7$	147, 163, 204, 218, 275, 292, 318, 420, 438, 454	N1, N10-di-*p*-coumaroyl-N5-caffeoyl spermidine	Apricot, Rose, Oil-tea
A13-4	599	307	$C_{34}H_{37}N_3O_7$	147, 163, 204, 218, 275, 292, 318, 420, 438, 454	N1, N10-di-*p*-coumaroyl-N5-caffeoyl spermidine	Apricot, Rose, Oil-tea, Broad bean, Tea, Phellodendron
A14-1	705	308	$C_{37}H_{43}N_3O_{11}$	145, 177, 193, 250, 264, 321, 338, 364, 497, 512, 530	N1, N10-di-hydroxyferuloyl-N5-feruloyl spermidine	Rhus chinensis
A14-2	705	316	$C_{37}H_{43}N_3O_{11}$	145, 177, 193, 250, 264, 321, 338, 364, 497, 512, 530	N1, N10-di-hydroxyferuloyl-N5-feruloyl spermidine	Rhus chinensis
A14-3	705	321	$C_{37}H_{43}N_3O_{11}$	145, 177, 193, 250, 264, 321, 338, 364, 497, 512, 530	N1, N10-di-hydroxyferuloyl-N5-feruloyl spermidine	Rhus chinensis
A15-1	583	270	$C_{34}H_{37}N_3O_6$	147, 204, 275, 292, 420, 438	N1(Z), N5(Z), N10(Z)-tri-*p*-coumaroyl spermidine	Sunflower, Plums, Pear, Oil-tea, Tea, Phellodendron, Apricot, Hawthorn, kiwifruit, Buckwheat, Rose
A15-2	583	280	$C_{34}H_{37}N_3O_6$	147, 204, 275, 292, 420, 438	N1(Z), N5(Z), N10(E)-tri-*p*-coumaroyl spermidine	Sunflower, Plums, Pear, Oil-tea, Tea, Phellodendron, Apricot, Hawthorn, kiwi fruit, Broad bean, Buckwheat, Rose
A15-3	583	290	$C_{34}H_{37}N_3O_6$	147, 204, 275, 292, 420, 438	N1(E), N5(Z), N10(E)-tri-*p*-coumaroyl spermidine	Sunflower, Plums, Pear, Oil-tea, Tea, Phellodendron, Apricot, Hawthorn, kiwifruit, Broad bean, Melon, Buckwheat, Rose
A15-4	583	298	$C_{34}H_{37}N_3O_6$	147, 204, 275, 292, 420, 438	N1(E), N5(E), N10(E)-tri-*p*-coumaroyl spermidine	Sunflower, Plums, Pear, Oil-tea, Tea, Phellodendron, Apricot, Hawthorn, kiwi fruit, Broad bean, Melon, Buckwheat, Rose
A16*	410	294/309	$C_{23}H_{26}N_2O_5$	89, 147, 177, 218, 235, 265	*N*-*p*-coumaroyl-N'-feruloyl putrescine	Broad bean

(*continued on next page*)

Table 1 (*continued*)

Peak	Mw	UV λ max (nm)	Formula	*m/z* of main fragments, MS^2	Compounds identified	Pollen
A17-1	615	296	$C_{34}H_{37}N_3O_8$	147, 163, 204, 220, 436, 454, 470	N1-*p*-coumaroyl-N5, N10-di-caffeoyl spermidine	Tea
A17-2	615	309	$C_{34}H_{37}N_3O_8$	147, 163, 204, 220, 436, 454, 470	N1-*p*-coumaroyl-N5, N10-di-caffeoyl spermidine	Broad bean, Oil-tea, Tea, Phellodendron, Buckwheat
A18-1	631	315	$C_{34}H_{37}N_3O_9$	163, 220, 291, 308, 452, 470	Tri-caffeoyl spermidine	Phellodendron, Buckwheat
A18-2	631	321	$C_{34}H_{37}N_3O_9$	163, 220, 291, 308, 452, 470	Tri-caffeoyl spermidine	Apricot, Tea, Phellodendron, Buckwheat
A19-1	673	317	$C_{37}H_{43}N_3O_9$	145, 177, 234, 305, 348, 480, 498	Tri-feruloyl spermidine	Rhus chinensis
A19-2	673	317	$C_{37}H_{43}N_3O_9$	145, 177, 234, 305, 348, 480, 498	Tri-feruloyl spermidine	Rhus chinensis
A19-2	673	317	$C_{37}H_{43}N_3O_9$	145, 177, 234, 305, 348, 480, 498	Tri-feruloyl-spermidine	Rhus chinensis
A20-1*	682	282	$C_{39}H_{46}N_4O_7$	147, 204, 275, 317, 374, 391, 417, 519, 537	N1, N14-di-*p*-coumaroyl-N5-hydroxyavenalumoyl spermine	Corn poppy
A20-2	682	292	$C_{39}H_{46}N_4O_7$	147, 204, 275, 317, 374, 391, 417, 519, 537	N1, N14-di-*p*-coumaroyl-N5-hydroxyavenalumoyl spermine	Corn poppy
A20-3	682	299	$C_{39}H_{46}N_4O_7$	147, 204, 275, 317, 374, 391, 417, 519, 537	N1, N14-di-*p*-coumaroyl-N5-hydroxyavenalumoyl spermine	Corn poppy
A21*	689	320	$C_{37}H_{43}N_3O_{10}$	145, 177, 234, 305, 496, 514	N1, N10-di-feruloyl-N5-hydroxyferuloyl spermidine	Rhus chinensis
A22-1	786	271	$C_{46}H_{50}N_4O_8$	147, 204, 275, 421, 495, 623, 641	N1(Z), N5(Z), N10(Z), N14(Z) tetra-*p*-coumaroyl spermine	Chrysanthemum
A22-2	786	275	$C_{46}H_{50}N_4O_8$	147, 204, 275, 421, 495, 623, 641	N1(E), N5(Z), N10(Z), N14(Z) tetra-*p*-coumaroyl spermine	Chrysanthemum, Tea, Sunflower
A22-3	786	288	$C_{46}H_{50}N_4O_8$	147, 204, 275, 421, 495, 623, 641	N1(E), N5(Z), N10(Z), N14(E) tetra-*p*-coumaroyl spermine	Chrysanthemum, Tea, Sunflower, Buckwheat
A22-4	786	294	$C_{46}H_{50}N_4O_8$	147, 204, 275, 421, 495, 623, 641	N1(E), N5(E), N10(Z), N14(E) tetra-*p*-coumaroyl spermine	Chrysanthemum, Sunflower, Buckwheat
A22-5	786	309	$C_{46}H_{50}N_4O_8$	147, 204, 275, 421, 495, 623, 641	N1(E), N5(E), N10(E), N14(E) tetra-*p*-coumaroyl spermine	Chrysanthemum, Sunflower, Buckwheat
A23-1	850	318	$C_{46}H_{50}N_4O_{12}$	163, 220, 291, 365, 453, 527, 689	Tetra-caffeoyl spermine	Rapeseed
A23-2	850	322	$C_{46}H_{50}N_4O_{12}$	163, 220, 291, 365, 453, 527, 689	Tetra-caffeoyl spermine	Rapeseed
A24-1*	880	315	$C_{47}H_{52}N_4O_{13}$	163, 193, 220, 250, 291, 321, 453, 483, 527, 557, 701, 719	N1, N5, N10-tri-caffeoyl-N14-hydroxy feruloyl spermine	Rapeseed
A24-2	880	320	$C_{47}H_{52}N_4O_{13}$	163, 193, 220, 250, 291, 321, 453, 483, 527, 557, 701, 719	N1, N5, N10-tri-caffeoyl-N14-hydroxy feruloyl spermine	Rapeseed, Buckwheat
A25*	629	308	$C_{35}H_{39}N_3O_8$	147, 193, 204, 264, 275, 292, 338, 438, 466, 484	N1, N5-di-*p*-coumaroyl-N10-hydroxyferuloyl spermidine	Tea
A26*	910	321	$C_{48}H_{54}N_4O_{14}$	163, 193, 220, 250, 291, 321, 483, 557, 587, 719, 748	N1, N14-di-hydroxyferuloyl-N5, N10-di-caffeoyl spermine	Rapeseed
A27*	613	310	$C_{35}H_{39}N_3O_7$	147, 177, 204, 248, 275, 292, 322, 348, 438, 450, 468	N1, N5-di-*p*-coumaroyl- N10-feruloyl spermidine	Oil-tea, Tea
A28-1*	864	315	$C_{47}H_{52}N_4O_{12}$	147, 163, 193, 220, 250, 321, 467, 527, 557, 689, 703, 719	N1, N5, N10-tri-caffeoyl-N14-feruloyl spermine	Rapeseed
A28-2	864	317	$C_{47}H_{52}N_4O_{12}$	147, 163, 193, 220, 250, 321, 467, 527, 557, 689, 703, 719	N1, N5, N10-tri-caffeoyl-N14-feruloyl spermine	Rapeseed
A29*	894	320	$C_{48}H_{54}N_4O_{13}$	163, 177, 193, 220, 250, 291, 321, 467, 510, 539, 57, 571, 701, 719, 733	N1-feruloyl-N5, N10-dicaffeoyl-N14-hydroxyferuloyl spermine	Rapeseed
A30*	848	312	$C_{47}H_{52}N_4O_{11}$	147, 163, 220, 250, 275, 291, 321, 437, 467, 494, 511, 557, 657, 685, 703	N1-caffeoyl-N5, N10-di-*p*-coumaroyl-N14-hydroxyferuloyl spermine	Rapeseed
A31*	832	310	$C_{47}H_{52}N_4O_{10}$	147, 204, 250, 275, 291, 321, 495, 541, 641, 669, 687	N1, N5, N10-tri-*p*-coumaroyl-N14-hydroxyferuloyl spermine	Rapeseed

*: first identified from bee pollen.

and Supplementary Fig. 2 A22, A23, A24, A26, A28, A29, A30 and A31).

Compounds A11-1, A11-2, and A11-3 display the same quasi-molecular ion at *m/z* 579 $[M + H]^+$ (Table 1 and Fig. 3 G) as isomers ($C_{32}H_{42}N_4O_6$). The protonated molecular ion at *m/z* 579 $[M + H]^+$ lost 146 amu (*p*-coumaroyl residue) to yield fragment ion at *m/z* 433 $[M + H - C_9H_6O_2]+$, and then lost 42 amu (acetyl) to generate fragment ion at *m/z* 391 $[M + H - C_9H_6O_2 - C_2H_2O]^+$; fragment ion at *m/z* 391 lost another 146 amu (*p*-coumaroyl residue) to produce fragment ion at *m/z* 287 $[M + H - C_9H_6O_2 - C_2H_2O - C_9H_6O_2]^+$ (Fig. 3 G). The fragment ion at *m/z* 204 indicates that two *p*-coumaroyl residues substituted at the N1 and N14 positions of spermine. The fragment ion at *m/z* 317 suggested that acetyl residue was substituted at the N5 and N10 positions of spermine. According to the retention time and UV maximum absorption wavelength, compounds A11-1, A11-2, and A11-3 were identified as N1(Z), N14(Z)-di-*p*-coumaroyl-N5, N10-di-acetylspermine, N1(Z), N14(E)-di-*p*-coumaroyl-N5, N10-di-acetyl spermine and N1(E), N14(E)-di-*p*-coumaroyl-N5, N10-di-acetyl spermine. Compound A11-3 was then confirmed by NMR data: ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.82 (s, 4H, 10a, 10b, 10c, 10d), 8.08 –7.89 (m, 4H, 1, 14, 1′, 14′), 7.48 – 7.36 (m, 8H, 9a, 5a, 9b, 5b, 9c, 5c, 9d, 5d), 7.35 – 7.24 (m, 4H, 3b, 3a, 3d, 3c), 6.82 – 6.72 (m, 8H, 8a, 6a, 8b, 6b, 8c, 6c, 8d, 6d), 6.44 – 6.34 (m, 4H, 2a, 2b, 2c, 2d), 3.30 – 3.08 (m, 24H, 2, 4, 6, 8, 11, 13, 2′, 4′, 6′, 9′, 11′, 13′),

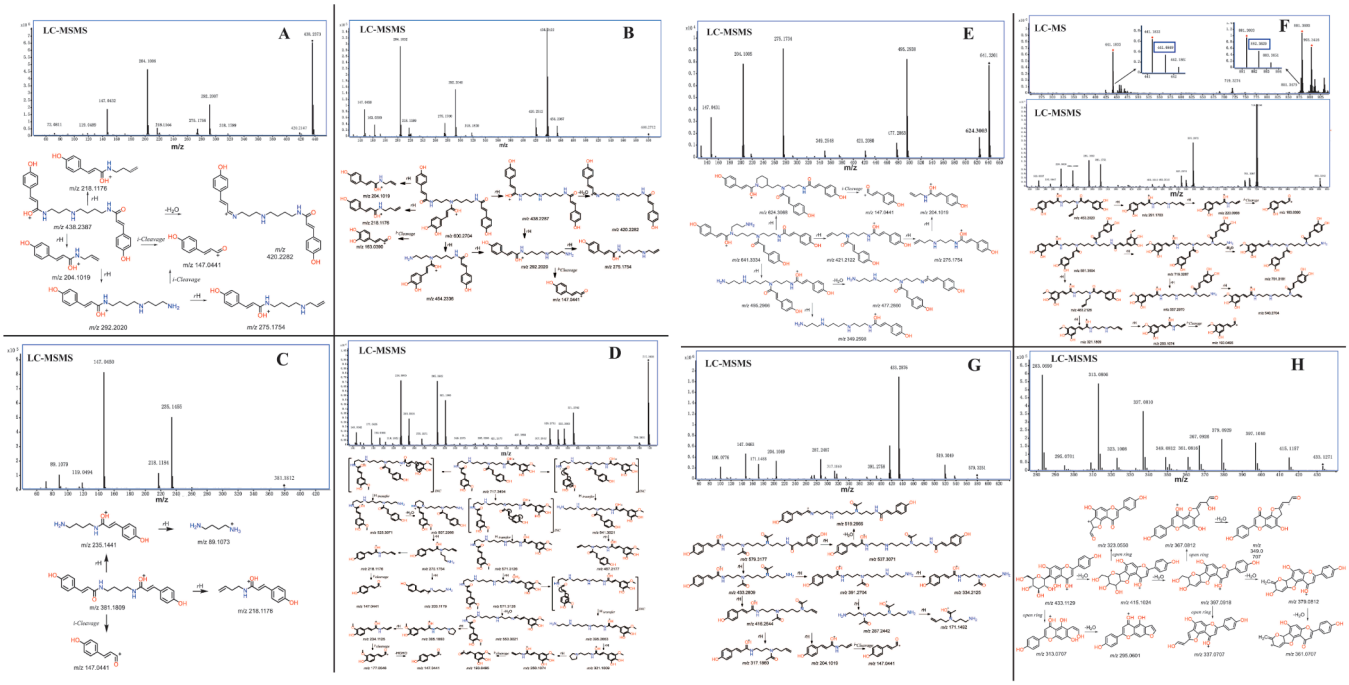

Fig. 3. Mass spectra (ESI) and fragment cleavage patterns of phenolamides and flavonoid glycosides Note: A: N1, N10-di-*p*-coumaroyl spermidine; B: N1, N10-di-*p*-coumaroyl-N5-caffeoyl spermidine; C: N, N'-di-*p*-coumaroyl putrescine; D: N1-hydroxyferuloyl-N5-*p*-coumaroyl-N14-feruloyl spermine; E: N1, N5, N10-tri-*p*-coumaroyl spermine; F: N1, N5, N10-tri-caffeoyl-N14-hydroxy feruloyl spermine; G: N1, N14-di-*p*-coumaroyl-N5, N10-di-acetyl spermine; H: isovitexin.

2.01 – 1.94 (m, 12H, 16, 18, 16′, 18′), 1.75 – 1.56 (m, 8H, 3, 7, 3′, 12′), 1.55 – 1.32 (m, 8H, 12, 7′, 8′). ^{13}C NMR (101 MHz, DMSO-d_6) δ 169.28 (15), 169.26 (15′), 169.23 (17), 169.10 (17′), 165.47 (1a, 1b), 165.27 (1c, 1d), 158.81 (7a, 7b), 158.77 (7c, 7d), 138.72 (2b, 3b, 3a), 138.58 (3d, 3c), 129.18 (9c, 5c, 9d, 5d), 129.15 (9a, 5a, 9b, 5b), 125.89 (4b, 4a), 125.85 (4d, 4c), 118.69 (2a), 118.49 (2c, 2d), 115.72 (8a, 6a, 8b, 6b, 8c, 6c, 8d, 6d), 47.80 (4, 4′), 45.72 (6), 45.59 (11′), 44.36 (8), 44.08 (13′), 42.74 (2, 2′), 36.53 (10, 9′), 36.23 (13, 6′), 28.64 (3, 3′), 27.76 (7), 27.73 (12′), 25.78 (11, 8′), 24.74 (12), 24.55 (7′), 21.32 (16), 21.28 (18), 21.23 (16′, 18′).

3.3. *Identification of flavonoid glycosides*

Twenty-five flavonoid glycosides were identified in 20 types of bee pollen (Table 2). The MS^2 profiles and cleavage patterns of compounds B1 to B25 are shown in Supplementary Fig. 3.

Compounds B1, B3, and B4 displayed the same quasi-molecular ion at *m/z* 627.1512 $[M + H]^+$ ($C_{27}H_{30}O_{17}$) (Supplementary Fig. 3 B1, B3, and B4) as isomers. Fragment ions at *m/z* 465 $[M + H\text{-}C_6H_{10}O_5]^+$ and *m/z* 303 $[M + H\text{-}C_6H_{10}O_5\text{-}C_6H_{10}O_5]^+$ respectively corresponded to losing 162 amu (glucosyl residue) and 324 amu (two glucosyl residues) (Supplementary Fig. 3 B1, B3, and B4). The fragment ion at *m/z* 303.0449 $[C_{15}H_{10}O_7 + H]^+$ presented quercetin. Thus, the compounds appeared to be quercetin-di-glucoside isomers. Notably, the relative abundances of *m/z* 465 in compound B1(98 %) and compound B4 (100 %) were much higher than in compound B3 (2 %) (Table 2 and Supplementary Fig. 3 B1, B3, and B4). This indicated that compounds B1 and B4 were glycosylated on the two different hydroxyls of aglycon, while B3 on the single hydroxyl of the aglycon. Compound B3 was validated as quercetin-3-*O*-sophoroside by the commercial standard. Compounds B1 and B4 were identified as quercetin-3,7-*O*-diglucoside, and quercetin-3,4′-*O*-diglucoside based on their chromatographic behaviors in HPLC.

Compounds B2 and B13 were isomers with molecular weight 756 (Supplementary Fig. 3 B2 and B13). Quasi-molecular ion at *m/z* 757 $[M + H]^+$ yielded fragment ion at *m/z* 611 $[M + H - 146]^+$ via losing a rhamnosyl residue (146 amu). Meanwhile, *m/z* 611 $[M + H - 146]^+$ lost one and two glucosyl residues to generate fragment ions at *m/z* 449 $[M + H - 146\text{–}162]^+$ and *m/z* 287 $[M + H - 146\text{–}162\text{-}162]^+$, indicating diglucosyl-rhamnosyl kaempferol (Supplementary Fig. 3 B2 and B13). The relative abundance of *m/z* 449 in compound B2 (100 %) was much higher than that of B13 (5 %), indicating compound B2 glycosylated on the two different hydroxyls of aglycon, while compound B13 on the single hydroxyl (Table 2 and Supplementary Fig. 3 B2 and B13). Compound B2 was validated as kaempferol-3-*O*-rutinoside-7-*O*-glucoside by the commercial standard. Compound B13 was identified as kaempferol-3-*O*-(2″-*O*-glucosyl)-rutinoside.

Compounds B7, B9, B10, and B21 exhibited the same quasi-molecular ion *m/z* 611 $[M + H]^+$ (Supplementary Fig. 3). The low relative abundances of *m/z* 465 (19 % in B7), *m/z* 449 (12 % in B9), and *m/z* 479 (19 % in B10 and 8 % in B21) indicated that they were glycosylated on the single hydroxyl of aglycon (Supplementary Fig. 3 B7, B9, B10, and B21). Compound B7 lost a rhamnosyl residue (l46 amu) to yield the fragment ion at *m/z* 465 $[M + H\text{-}146]^+$, and then lost a glycosyl residue (162 amu) to produce the fragment ion at *m/z* 303 $[M + H\text{-}146\text{–}162]^+$ (Table 2 and Supplementary Fig. 3 B7). Compound B7 was validated as quercetin-3-*O*-rutinoside (rutin) by the commercial standard. For B9, MS/MS fragment ions at *m/z* 449 $[M + H\text{-}162]^+$ and *m/z* 287 $[M + H - 162\text{–}162]^+$ corresponded to lose one and two glycosyl residues (Supplementary Fig. 3 B9). Compound B9 was validated as kaempferol-3-*O*-sophoroside by the commercial standard. Compounds B10 and B21 displayed the same fragment ions at *m/z* 479 $[M + H\text{-}132]^+$ and *m/z* 317 $[M + H\text{-}132\text{–}162]^+$, corresponding to the loss of a pentosyl residue (132 amu) and a glucosyl residue (162 amu) (Supplementary Fig. 3 B10 and B21). The quasi-molecular ion at *m/z* 611 firstly lost a pentosyl residue (132 amu) to yield fragment ions at *m/z* 479, indicating the linkage of pentose with glucose via (2 → 6) glycosylic bond. Generally, the glycosylated positions of aglycone hydroxyls prefer at the 3- and 7-hydroxyls (Cavaliere, Foglia, Pastorini, Samperi, & Laganà, 2005). The 7-position glycosylation is less polar than 3-position; thus, compounds B10 and B21 were respectively identified as isorhamnetin-3-*O*-vicianoside and isorhamnetin-7-*O*-vicianoside.

Compounds B15, B19, B23, B24, and B25 all displayed loss of the

Table 2
Flavonoid glycosides identified in bee pollen and their UV and MS characteristics.

Peak	Mw	UV λ max (nm)	Formula	*m/z* of main fragments (relative intensity, %), MS/MS	Compounds identified	Pollen
B1	626	353	$C_{27}H_{30}O_{17}$	145(1), 85(1.5), 303(100), 465 (98)	Quercetin-3,7-O-diglucoside	Melon, Maize
B2*	756	317/327/340	$C_{33}H_{40}O_{20}$	287(40), 449(100), 595(11), 611 (10)	Kaempferol-3-O-rutinoside-7-O-glucoside	Tea
B3	626	354	$C_{27}H_{30}O_{17}$	303(100), 465(6)	Quercetin-3-O-sophoroside	Watermelon, Corn poppy, Watermelon, Rapeseed, Dandelion, Maize, Phellodendron, Melon, Tea
B4*	626	355	$C_{27}H_{30}O_{17}$	303(100), 465(54)	Quercetin-3,4′-O-diglucoside	Melon
B5*	786	329,342,354	$C_{34}H_{42}O_{21}$	317(100), 479(3), 641(3)	Isorhamnetin-3-O-(2″-O-glucosyl)-rutinoside	Oil-Tea
B6	640	328,340,354	$C_{28}H_{32}O_{17}$	317(100), 479(12)	Isorhamnetin-3-O-gentiobioside (Astragaloside)	Broad bean, Plum, Apricot, Maize, Pear, Hawthorn, Rose, Phellodendron, Tea
B7	610	355	$C_{27}H_{30}O_{16}$	129(4), 303(100), 465(19)	Quercetin-3-O-rutinoside (Rutin)	Tea
B8*	596	355	$C_{26}H_{28}O_{16}$	115(1), 303(100), 465(16)	Quercetin-3-O-arabinoglucoside (Peltatoside)	Corn poppy
B9	610	348	$C_{27}H_{30}O_{16}$	145(3), 287(100), 445(12)	Kaempferol-3-O-sophoroside	Plum, Apricot, Corn poppy, Rapeseed, Tea, Phellodendron
B10*	610	355	$C_{27}H_{30}O_{16}$	145(1), 317(100), 479(19)	Isorhamnetin-3-O-vicianoside	Lotus
B11*	432	338	$C_{21}H_{20}O_{10}$	313(90), 337(62), 349(14), 361 (14), 367(26), 379(33)	Isovitexin	Watermelon, Melon
B12*	654	362	$C_{29}H_{34}O_{17}$	347(100), 509(19)	Syringetin-3-O-rutinoside	Hawthorn
B13*	756	348	$C_{33}H_{40}O_{20}$	129(4), 287(100), 449(5), 611(6)	Kaempferol-3-O-(2″-O-glucosyl)-rutinoside	Apricot, Oil-Tea
B14	594	348	$C_{27}H_{30}O_{15}$	287(100), 449(19)	Kaempferol-3-O-rutinoside	Apricot, Watermelon, Corn poppy, Melon, Tea, Phellodendron
B15*	726	327/354	$C_{31}H_{34}O_{20}$	317(80), 565(100)	Isorhamnetin-3-O-(6″-O-malonyl)-glucosyl-7-O-glucoside	Hawthorn, Chrysanthemum, Rose
B16	624	355	$C_{28}H_{32}O_{16}$	317(100), 479(18)	Isorhamnetin-3-O-neohesperidoside	Rose, Tea, Pear, Maize
B17	580	348	$C_{26}H_{28}O_{15}$	287(100), 449(13)	Kaempferol-3-O-sambubioside (Leucoside)	Corn poppy
B18	464	353	$C_{21}H_{20}O_{12}$	303(100)	Quercetin-3-O-glucoside	Buckwheat, Maize, Sunflower
B19*	550	352	$C_{24}H_{22}O_{15}$	303(100)	Quercetin 3-O-(6″-O-malonyl)-glucoside	Chrysanthemum
B20	478	355	$C_{22}H_{22}O_{12}$	317(100)	Isorhamnetin-3-O-glucoside	Plum, Pear, Hawthorn, Chrysanthemum, Phellodendron
B21*	610	355	$C_{27}H_{30}O_{16}$	317(100), 479(8)	Isorhamnetin-7-O-vicianoside	Tea
B22	508	358	$C_{23}H_{24}O_{13}$	347(100)	Syringetin-3-O-glucoside	Hawthorn
B23*	534	346	$C_{24}H_{22}O_{14}$	287(100)	Kaempferol 3-O-(6″-O-malonyl)-glucoside	Chrysanthemum
B24*	564	355	$C_{25}H_{24}O_{15}$	317(100)	Isorhamnetin-3-O-(6″-O-malonyl)-glucoside	Pear, Hawthorn, Chrysanthemum
B25*	594	358	$C_{26}H_{26}O_{16}$	347(100)	Syringetin-3-O-(6″-O-malonyl)-glucoside	Hawthorn

*: firstly identified from bee pollen.

same MS/MS fragment ions, 248 amu (Table 2 and Supplementary Fig. 3 B15, B19, B23, B24, and B25). According to the literature reports (Gardana et al., 2018; Song et al., 2013), 248 amu may be attributed to a malonyl residue connected to glucose [glc + malonyl]. Thus, compounds B19, B23, B24, and B25 were respectively identified as quercetin 3-*O*-(6″-*O*-malonyl)-glucoside, kaempferol 3-*O*-(6″-*O*-malonyl)-glucoside, isorhamnetin-3-*O*-(6″-*O*-malonyl)-glucoside, and syringetin 3-*O*-(6″-*O*-malonyl)-glucoside. For compound B15, quasi-molecular ion $[M + H]^+$ at *m/z* 727 $[C_{31}H_{34}O_{20} + H]^+$ was 162 amu (a glycosyl residue) higher than that of compound B24, indicating two glucosyl residues. Compound B15 was identified as isorhamnetin-3-*O*-(6″-*O*-malonyl)-glucosyl-7-*O*-glucoside. To our knowledge, the five malonyl flavonoid glycosides were firstly found in bee pollen.

Compound B11 showed the quasi-molecular ion *m/z* 433 $[M + H]^+$, and fragments at *m/z* 415 $[M + H\text{-}H_2O]^+$, *m/z* 397 $[M + H\text{-}H_2O\text{-}H_2O]^+$, *m/z* 379 $[M + H\text{-}H_2O\text{-}H_2O\text{-}H_2O]^+$ and *m/z* 361 $[M + H\text{-}H_2O\text{-}H_2O\text{-}H_2O\text{-}H_2O]^+$ (Fig. 3 H). This successive dehydration in MS/MS fragments is the typical C-glycoside characteristics (Xiang, Zhang, Apea-Bah, & Beta, 2019). The loss of 120 (*m/z* 313) and 150 amu (*m/z* 283) from quasi-molecular ion *m/z* 433 (Fig. 3 H) was the typical cleavage patterns of C-linked hexose. Thus, compound B11 was validated as isovitexin by the commercial standard.

Compounds B5, B6, B16, and B20 were identified as isorhamnetin glucosides based on the above MS/MS cleavage rules and listed in Table 2, and the MS/MS fragment cleavage patterns in Supplementary Fig. 3 B5, B6, B16 and B20.

3.4. MS/MS cleavage modes of phenolamides and flavonoid glycosides

Based on the MSMS spectra of 31 phenolamides and their 33 *cis/trans* isomers, we summarized the characteristics as follows: (1) + ESI-MS^2 spectra of phenolamides present some special fragment ions, usually losing 146 amu (*p*-coumaroyl residue), 162 amu (caffeoyl residue), 176 amu (feruloyl residue) and 192 amu (hydroxy feruloyl residue). (2) Typically, some fragment ions can help confirm the amidate position of hydroxycinnamic acids with spermidine and spermine, and the free N position in unsaturated substituted phenolamides, di- substituted spermidine and tri-substituted spermine (Supplementary Fig. 4). For example, the fragment ions at *m/z* 250, *m/z* 234, and *m/z* 467 in compound A7 indicate that N1, N5, and N14 positions were respectively substituted, and the N10 position is free. (3) MS spectra of some substituted spermine usually look confused by *m/z* or *m/2z* protonated ions, such as compounds A24, A26, A28, A20, A30, and A31 (Supplementary Fig. 2). This uncertainty can be solved by the presence of an extra 0.5 Da isotopic ion (Fig. 3F). For example, The MS spectra of compound A24 presented ion at *m/2z* 441.1833 with isotopic ion 441.6849, an extra 0.5 Da; and ion *m/z* 881.3603 with the isotopic ion at 882.3629, an extra 1 Da (Fig. 3F). Thus, *m/z* 881.3603 $[M + H]^+$ was the real quasi-molecular ion, not 441.1833. This finding can explain the unidentified compound with *m/z* 439 $[M - H]^-$ in previous research

(Zhang et al., 2020). (4) Maximum UV absorption wavelength of *cis*-isomer is lower than that of *trans*-isomer, presenting redshift from *cis*-isomer to *trans*-isomer. Additionally, *cis*-isomer presents the stronger polarity (hydrophilic) than *trans*- isomer. In this study, the *cis/trans* isomers of compounds A1 were respectively identified, based on maximum UV absorption wavelength and polarity (Table 1 & Fig. 2).

The MS spectrum characteristics of glycosylated flavonoids were summarized as follows: (1) + ESI-MS^2 spectra of glycosylated flavonoids present some specific fragment ions, usually losing 132 amu (pentosyl residue), 146 amu (rhamnosyl residue), 162 amu (glucosyl residue), and 248 (malonyl glucosyl residue). (2) Specific fragment ions in MS^2 spectrum are responsible for glycosyl or phenolic substitution. MS/MS spectra of glycosylated flavonoids and phenolamides all present the same loss of 146 amu and 162 amu. Rhamnosyl residue and glucosyl residue usually produce a neutral loss in MS/MS, while *p*-coumaroyl residue and caffeoyl residue are *H*-rearrangement cleavage. Thus, for *p*-coumaroyl or caffeoyl amidate, their fragment ions present at *m/z* 147 or *m/z* 163 in +ESI-MS^2 spectrum (Supplementary Fig. 1); in contrast, for rhamnosyl or glucosyl glycosylation, without fragment ions at *m/z* 147 or *m/z* 163 in +ESI-MS^2 spectrum (Supplementary Fig. 3). (3) The relative abundance of MS/MS fragment ions can mirror the glycosylated position in single or more hydroxyls. For example, the relative abundance of fragment ion at *m/z* 465 for compound B4 (100 %) is higher than compound B3 (2 %) (Supplementary Fig. 3 B3 and B4). This difference suggested that compound B4 was respectively glycosylated on two different hydroxyls, quercetin-3,4′-*O*-diglocuside; in comparison, compound B3 on the single hydroxyl, quercetin-3-*O*-sophoroside. (4) The 3-position glycosylation is more polar than the 7-position; thus, the

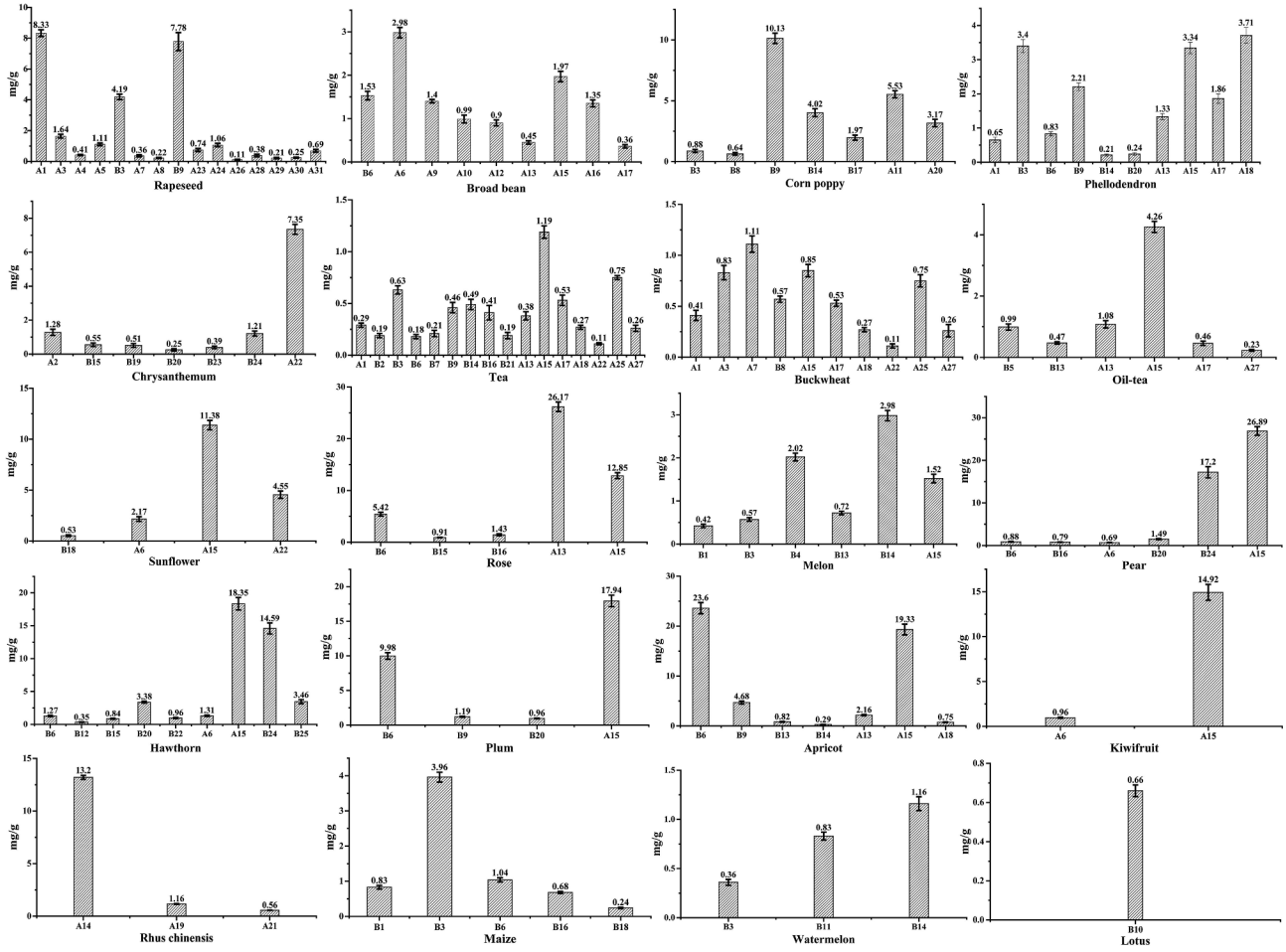

Fig. 4. Contents of phenolamides and flavonoid glycosides in 20 types of bee pollen. Note: A1: N1, N10-di-*p*-coumaroyl spermidine; A2: N1, N5, N10-tri-*p*-coumaroyl spermine; A3: N1, N10-di-*p*-coumaroyl-N14-hydroxyferuloyl spermine; A4: N1, N10-di-*p*-coumaroyl-N14-caffeoyl spermine; A5: N1-hydroxyferuloyl-N5-*p*-coumaroyl-N14-caffeoyl spermine; A6: N, N'-di-*p*-coumaroyl putrescine; A7: N1- hydroxyferuloyl-N5-*p*-coumaroyl-N14- feruloyl spermine; A8: N1- feruloyl-N5, N14-di-*p*-coumaroyl spermine; A9: *N*-*p*-coumaroyl-N'-caffeoyl putrescine; A10: N, N'-di-caffeoyl putrescine; A11: N1, N14-di-*p*-coumaroyl-N5, N10-di-acetyl spermine; A12: *N*-feruloyl-N'-caffeoyl putrescine; A13: N1, N10-di-*p*-coumaroyl-N5-caffeoyl spermidine; A14: N1, N10-di-hydroxyferuloyl-N5-feruloyl spermidine; A15: Tri-*p*-coumaroyl spermidine; A16: *N*-*p*-coumaroyl-N'-feruloyl putrescine; A17: N1-*p*-coumaroyl-N5, N10-di-caffeoyl spermidine; A18: Tri-caffeoyl spermidine; A19: Tri-feruloyl spermidine; A20: N1, N14-di-*p*-coumaroyl-N5-hydroxyavenalumoyl spermine; A21: N1, N10-di-feruloyl-N5-hydroxyferuloyl spermidine; A22: Tetra-*p*-coumaroyl spermine; A23: Tetra-caffeoyl spermine; A24: N1, N5, N10-tri-caffeoyl-N14-hydroxy feruloyl spermine; A25: N1, N5-di-*p*-coumaroyl-N10-hydroxyferuloyl spermidine; A26: N1, N14-di-hydroxyferuloyl-N5, N10-di-caffeoyl spermine; A27: N1, N5-di-*p*-coumaroyl- N10-feruloyl spermidine; A28: N1, N5, N10-tri-caffeoyl-N14-feruloyl spermine; A29: N1-feruloyl-N5, N10-dicaffeoyl-N14-hydroxyferuloyl spermine; A30: N1-caffeoyl-N5, N10-di-*p*-coumaroyl-N14-hydroxyferuloyl spermine; A31: N1, N5, N10-tri-*p*-coumaroyl-N14-hydroxyferuloyl spermine. B1: quercetin-3,7-*O*-diglocuside; B2: kaempferol-3-*O*-rutinoside-7-*O*-glucoside; B3: quercetin-3-*O*-sophoroside; B4: quercetin-3,4′-*O*-diglucoside; B5: isorhamnetin-3-*O*-(2″-glucosyl)-rutinoside; B6: isorhamnetin-3-*O*-gentiobioside; B7: quercetin-3-*O*-rutinoside; B8: quercetin-3-*O*-arabinoglucoside; B9: kaempferol-3-*O*-sophoroside; B10: isorhamnetin-3-*O*-vicianoside; B11: isovitexin; B12: syringetin-3-*O*-rutinoside; B13: kaempferol-3-*O*-(2″-glucosyl)-rutinoside; B14: kaempferol-3-*O*-rutinoside; B15: isorhamnetin-3-*O*-(6″-*O*-malonyl)-glucosyl-7-*O*-glucoside; B16: isorhamnetin-3-*O*-neohesperidoside; B17: kaempferol-3-*O*-sambubioside; B18: quercetin-3-*O*-glocuside; B19: quercetin 3-*O*-(6″-*O*-malonyl)-glucoside; B20: isorhamnetin-3-*O*-glocuside; B21: isorhamnetin-7-vicianoside; B22: syringetin-3-*O*-glucoside; B23: kaempferol 3-*O*-(6″-*O*-malonyl)-glucoside; B24: isorhamnetin-3-*O*-(6″-*O*-malonyl)-glucoside; B25: syringetin-3-*O*-(6″-*O*-malonyl)-glucoside.

chromatograph behaviors (polarity and retention time) are responsible for the glycosylated position of flavonoids. For example, compounds B10 is less retention time and more polar than B21; thus, compounds B10 glycosylated in 3-position, isorhamnetin-3-*O*-vicianoside; B21 in 7-position, isorhamnetin-7-*O*-vicianoside (Fig. 2 & Table 2).

3.5. Quantification of phenolamides and flavonoid glycosides

The contents of phenolamides and flavonoid glycosides in 20 types of monofloral bee pollen were quantified with external standards. As can be seen in Fig. 4, the compositions of phenolamides and flavonoid glycosides varied considerably in bee pollen samples. For 20 samples, 17 floral types of bee pollen contained 31 phenolamides; especially, kiwifruit and rhus chinensis bee pollens only possessed phenolamides. Those phenolamides can be subdivided into the following three groups: 5 phenolamides conjugated putrescine, 10 phenolamide conjugated spermidines and 16 phenolamides conjugated spermine. The levels of phenolamides ranged from 1.50 mg/g to 39.02 mg/g (Fig. 4); noteworthily, phenolamides in 11 types of bee pollen constituted more than 1 % of the total weight. The highest levels of phenolamides were presented in rose pollen 39.02 mg/g, followed by pear pollen at 27.58 mg/g and apricot pollen at 22.24 mg/g (Fig. 4). Among phenolamides, phenolamides conjugated spermidine presented the higher level than the other two types. Tri-*p*-coumaroyl spermidine existed in higher level than other phenolamides, accounting for more than 10 mg/g in 7 types of bee pollen; for example, pear pollen was with highest levels of 26.89 mg/g (Fig. 4). The contents of di-*p*-coumaroyl-caffeoyl spermidine and dihydroxy feruloyl-feruloyl spermidine reached to 26.17 mg/g in rose bee pollen and 13.2 mg/g in rhus chinensis bee pollen, respectively.

In addition, 18 types of bee pollen contained 25 flavonoid glycosides; especially, maize, watermelon, and lotus bee pollens only possessed flavonoid glycosides. The aglycones of these 25 flavonoid glycosides are uniquely identified as kaempferol, isorhamnetin, quercetin, and syringetin. The contents of flavonoid glycosides ranged from 0.53 mg/g to 29.39 mg/g in bee pollen (Fig. 4). Isorhamnetin-3-*O*-gentiobioside (astragaloside) and kaempferol-3-*O*-sophoroside respectively existed in nine and six types of bee pollen; furthermore, the highest value of isorhamnetin-3-*O*-gentiobioside was recorded as 23.61 mg/g in apricot pollen.

4. Discussions

Our results indicated that bee pollen is a treasure trove of phenolamides and flavonoid glycosides. The 31 phenolamides and their 33 *cis/trans* isomers were identified from 17 floral types of bee pollen. Compounds A1, A2, A13, A14, A15, A17, A18, A19, A22, and A23 were consistent with the previous work regarding phenolamides in bee pollen from Colombia, Italy and Spain using UHPLC - Orbitrap Mass (Gardana et al., 2018) and from *Mimosa pudica* L. using UPLC-QTOF-MS (Pereira Gomes et al., 2022). Recently, 21 phenolamides were identified in milk thistle, apricot, buckwheat, lotus, rose, camellia, rapeseed, and sunflower bee pollen using UPLC-QTOF-MS based metabolomics (Zhang et al., 2022), while lacking information about the substituent positions and *cis/trans* isomers of these phenolamides. Moreover, the previous research usually used amine moieties to unreliably quantify the amounts of phenolamides due to unavailable standards (W. Wang et al., 2020). In this study, we provided a reliable quantification using prepared phenolamides from bee pollen as standards. Our results showed high levels of phenolamides in bee pollen, especially constituting 3.9 % in rose and 2.8 % in pear pollen (Fig. 4). In addition, 25 flavonoid glycosides were found in 18 floral types of bee pollen. Compounds B1, B3, B6, B7, B9, B14, B16, B17, B18, B20, and B22 were similarly identified in previous studies (Giampieri et al., 2022; Mosić et al., 2019; Pinto et al., 2010; Thakur & Nanda, 2020; Truchado, Ferreres, & Tomas-Barberan, 2009; Turner, Nielsen, O'Connor, & Burton, 2006; Wu, Shao, Li, Zhang, & Wang, 2015). Compounds B2, B4, B5, B8, B10, B11, B12, B13, B15, B19, B21, B23, B24, and B25 seem to be firstly identified in bee pollen. We identified 8 isorhamnetin glycosides, 7 quercetin glycosides, and 6 kaempferol glycosides (Table 2), consistent with the previous reports that bee pollen mainly contained quercetin, isorhamnetin, and kaempferol glycosides (Thakur & Nanda, 2020). The contents of flavonoid glycosides in bee pollen ranged from 0.05 % to 3 % (Fig. 4), consistent with the previous research that flavonoids presented in pollen (1.4 %) (Kieliszek et al., 2018). So far, in edible plants, a total of 36 phenolamides were approximately found in barley, wheat, rice, maize, potato, tomato, wolfberry, foxtail millet, and their related food products (Wang et al., 2020). In comparison, containing 31 phenolamides and their 33 isomers, bee pollen seems to be the most abundant source of phenolamides among known natural products (Table 1). To the best of our knowledge, 19 phenolamides and 14 flavonoid glycosides were the first report in bee pollen (Tables 1 and 2). Thus, these new-found phenolamides and flavonoid glycosides can provide deep insights to develop bee pollen as functional foods.

Our results showed that phenolics and flavonoids in bee pollen are present in the amidation or glycosylation form, not in the free form. We speculate that the free phenolics and flavonoids previously reported in bee pollen (Hemmami, Ben Seghir, Ben Ali, Rebiai, Zeghoud, & Brahmia, 2020; Li et al., 2019) may be derived from cleavage fragments during ionization in mass spectrometry. The amidation of polyamine can reduce polarity and hydrophilicity, and facilitate their transport, stabilization, and organelle localization (Fujita et al., 2012). The glycosylation of flavonoids can render less reactive and more polar, and prevent cytoplasmic damage for the safe storage of flavonoids in the cell vacuole (Cavaliere et al., 2005). Therefore, derivatized polyamines and flavonoids may be more common form in plants. Phenolamides can be hydrolyzed *in vivo* to generate the phenolic moiety and the amine moiety by enzymes from the host and/or gut microbiota (Wang et al., 2020). The previous research showed that tri-*p*-coumaroyl spermidine can inhibit HIV-1 protease; noteworthily, spermidine and *p*-coumaric acid did not show the activity against HIV-1 protease (Wang et al., 2020). Thus, phenolamides seem to possess better bioactivities than free phenolic acids and amines.

The various functions of bee pollen may be considerably contributed to the richness of flavonoid glycosides and phenolamides. Numerous studies have demonstrated that flavonoid glycosides present a variety of functional properties, for example, antioxidant, anti-diabetic, and anti-inflammatory properties (Thakur & Nanda, 2020). In contrast, little research focused on phenolamides in the past. It is worth noting that phenolamides in bee pollen presented anti-tyrosinase activity (Khongkarat, Ramadhan, Phuwapraisirisan, & Chanchao, 2020; Kim et al., 2018; Su, Yang, Lu, & Liu, 2021; Zhang et al., 2022) and their inhibitory activity might differ depending on the numbers and types of phenolic moieties (Kim et al., 2018). The structures and orientations of phenolic moieties in the polyamine derivatives are very similar to tyrosine (Khongkarat et al., 2020). Therefore, phenolamides can bind to the active site of tyrosinase like a lock and key. As an amine moiety of phenolamide, spermidine has been reported as a universal longevity elixir and "anti-aging vitamin" in humans by stimulating apoptosis and autophagy (Madeo et al., 2019; Madeo et al., 2010). Our results revealed that bee pollen showed the highest level of conjugated spermidine, especially tri-*p*-coumaroyl spermidine with more than 1 % of the total weight in 7 types of bee pollen (Fig. 4). Bee pollen, therefore, seems to be a hopeful anti-aging food. Similarly, spermine, as a significant component of prostatic fluid, has been proved to alleviate prostatitis (Lynch & Nicholson, 1997). We speculate that the therapeutic properties of bee pollen in prostate diseases may be considerably attributed to 16 phenolamides conjugated spermine (Table 1 and Fig. 4). In the future research, 19 new-found phenolamides will be investigated regarding their anti-aging and prostate-caring benefits.

5. Conclusion

In 20 types of monofloral bee pollen collected from China, we identified 31 phenolamides, their 33 *cis/trans* isomers, and 25 flavonoid glycosides. Bee pollen can be the most abundant phenolamide sources among known natural products. Further studies may be necessary for investigating the bioactivities of new-found phenolamides and flavonoid glycosides. The current research seems to provide a better understanding on bee pollen for developing new functional foods directed at antioxidant, anti-inflammatory, and anti-aging properties.

CRediT authorship contribution statement

Jiangtao Qiao: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Visualization. **Zhouxu Feng:** Investigation, Data curation, Visualization. **Yong Zhang:** Investigation. **Xingying Xiao:** Investigation. **Jie Dong:** Data curation, Resources, Project administration. **Eric Haubruge:** Conceptualization, Methodology, Data curation. **Hongcheng Zhang:** Conceptualization, Validation, Resources, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This research was supported by the China Agriculture Research System of MOF and MARA (CARS-45-KXJ19), the Agricultural Science and Technology Innovation Program (CAAS-ASTIP-2019-IAR) from the Ministry of Agriculture of P.R. China, and 113 talent medicine research program of China medical city from Taizhou government.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.134800.

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