



Characterization of chemical compounds of fractions from four different bee pollens with chemometric classification

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Abstract

Bee pollen continues to increase its popularity as a ‘complete food’ among natural resources with its high health value and therapeutic chemical compounds. This study focused on HPLC-DAD (high-performance liquid chromatography-diode array detection) based on the identification of chemical compounds of *Cistus creticus*, *Helianthus annuus*, *Papaver somniferum* and *Salix* sp. bee pollens. Also, water extracts and the fractions (hexane, dichloromethane, ethyl acetate, butanol, water) obtained from the methanol extracts of all bee pollens were investigated for chemical compounds. A total of 29 chemical compounds were screened and kaempferol was detected in all studied bee pollen samples at concentration of 0.4 and 331.6 µg/g. Luteolin (68.7–694.8 µg/g), *trans*-aconitic acid (12.2–479.1 µg/g), myricetin (160.4–1534.2 µg/g), quercetin (162.1–608.1 µg/g), rosmarinic acid (273.8–435.6 µg/g), rutin (3.6–202.8 µg/g), and *trans*-cinnamic acid (5.1–1854.6 µg/g) were found as the most abundant chemical compounds. Additionally, nonpolar and polar fractions of the bee pollens were chemometrically clustered for similarities and differences of chemical compounds via principal component analysis (PCA) and hierarchical cluster analysis (HCA). In nonpolar fractions, *C. creticus*, *H. annuus*, *P. somniferum*, and *Salix* sp. ethyl acetate fractions (CEA, HEA, PEA, SEA) were separated from other fractions, while in polar fractions, *C. creticus*, *H. annuus*, *P. somniferum*, and *Salix* sp. butanol fractions (CB, HB, PB, SB) were separated from other fractions. This study provides additional data on the characterization of valuable chemical compounds in bee pollens and constitutes a beginning in the chemometric classification of chemical compounds.

Keywords Phenolic compounds · Chemometric analysis · *Salix* sp. · *Cistus creticus* · *Helianthus annuus* · *Papaver somniferum*

Introduction

Natural resources (plants, microorganisms, bee products, algae, mushrooms and some animal species) represent sources of biomolecules with various activities in different structural and vital forms [1]. The World Health Organization (WHO) announced that 80% of the global population prefers natural source medicines (especially medicinal plant-based medicines) to alleviate or cure diseases [2]. In addition, it has been reported that currently 50% of the pharmaceutical market originates from natural products. New molecules with potential bioactivity are identified from natural sources every day, and their evaluations continue for their suitability for use as drugs. Identifying biologically activated target or lead compounds is the first step in drug discovery. In this regard, the choice of appropriate method for the characterization of bioactive compounds to evaluate

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of both activities against disease and potency is an important goal [3].

Bee products constitute an important class of natural products with both nutritional and medicinal characteristics. Honeybees (*Apis mellifera* L.) can produce many by-products, such as the bee pollen, bee bread, royal jelly, propolis, and beeswax, in addition to honey. The medicinal properties of these by-products have been acknowledged by ancient civilizations for thousands of years, and they have an increasing use in the modern era, mainly as nutritional supplements or health products [4]. Bee pollen is produced by collecting pollen grains by bees to form a granule that is agglutinated using salivary secretions, nectar or honey and stored in the honeycomb cell. The main role of bee pollen is to supply a source of nutrients that guarantee the survival of the colony, consequently ensuring its development and maintenance [5]. Today, bee pollen has an important use in humans as nutritional supplements and some alternative medical treatments [6, 7]. Additionally, bee pollen is a concentrated, nutrient-dense food that is high in energy and vitamins. It is one of nature's most complete and nutritious foods, as it contains almost all the nutrients vital to humans. Bee pollen is called a 'complete food' or 'life-giving powder' because it contains many components not found in foods of animal origin and is richer in protein [8]. Bee pollen is a natural product rich in bioactive compounds. More than 250 bioactive substances have been identified in bee pollen of various plant species. This group of basic chemical substances includes lipids, amino acids, proteins, carbohydrates, fatty acids, phenolic compounds, carotenoids, flavonoids, vitamins, and bio-elements [9]. It has been informed that the bee pollen has antioxidant, antimicrobial, antidiabetic, anti-hyperglycemic, anti-inflammatory, hepatoprotective, nephroprotective, and anti-cancer properties with the synergism of this versatile and diverse mixture in its content [10, 11].

Phenolic compounds are a valuable member of secondary metabolites found in fruits, vegetables, coffee, fruit juice, mushrooms, and bee products. To date, the isolation or characterization of around 8000 phenolic compounds from natural sources has been reported. In terms of their structural differences, they are basically categorized into five sub-classes: flavonoids, phenolic acids, lignans, tannins and stilbenes [12]. Consumption of phenolic compounds is critically important for health as they are responsible for the removal of reactive oxygen species *in vivo* [13]. In this way, phenolic compounds support the prevention or alleviation of oxidative damage-based diseases such as inflammation, cardiovascular, diabetes diseases and cancer. Various studies have been managed on the mechanisms of action of phenolics, and it is accepted that the main bioactive property

underlying these effects is related to their antioxidant activities [14].

Bee pollen is a significant apitherapeutic product highly admired by natural medicine for its potential medicinal and nutritional applications. In recent years, it has become the focus of both the consumer and the scientific community. In this study, chemical compounds of *Cistus creticus*, *Helianthus annuus*, *Papaver somniferum* and *Salix* sp. bee pollens, water extracts and the fractions (hexane, dichloromethane, ethyl acetate, butanol, water) obtained from the methanol extracts of all bee pollens were characterized by HPLC-DAD. Also, chemical compounds found in the non-polar and polar fractions obtained from the bee pollens were chemometrically clustered via principal component analysis (PCA) and hierarchical cluster analysis (HCA) methods.

Materials and methods

Bee pollen materials

Cistus creticus bee pollen was collected from Antalya (36°55'N-32°01'E)-Türkiye in April, 2023; *Helianthus annuus* bee pollen from Konya (37°57'N-32°77'E)-Türkiye, in July, 2023; *Papaver somniferum* bee pollen in May, 2023 and *Salix* sp. bee pollen in April, 2023 from Afyon (38°45'N-30°26'E)-Türkiye with the contribution of expert staff members of the provincial organizations of the Central Union of Turkish Beekeepers. All bee pollen samples were transported to the laboratory with a cold chain and stored at −20 °C. Detailed information about the palynological analysis of all bee pollens is given in Supplementary information and the images of the bee pollens are shown in Figs. S1-S4.

Chemicals

Fumaric, vanillic, 2-hydroxy cinnamic, *trans*-aconitic, *p*-hydroxy benzoic, gallic, *p*-coumaric, protocatechuic, levulinic, chlorogenic, caffeic, *p*-hydroxy phenyl acetic, ferulic, 3-hydroxy benzoic, *trans*-cinnamic, and rosmarinic acids, and 6,7-dihydroxy coumarin, quercetin, guaiacol, rutin, naringenin, chrysin, methanol, kaempferol, *n*-hexane, apigenin, *n*-butanol, vanillin, coumarin, ethyl acetate, myricetin, catechin, dichloromethane, luteolin, and acetic acid were purchased from E. Merck (Darmstadt, Germany) and Sigma Chemical Co. (Sigma-Aldrich GmbH, Steinheim, Germany) as analytical grade.

Extraction and fractionation of the bee pollens

Initially, each bee pollen sample was dried in a freeze dryer and then ground. Ground bee pollens (999 g for *C. creticus*, 986 g for *H. annuus*, 1015 g for *P. somniferum*, 1019 g for *Salix* sp.) were separately extracted with 2 L of methanol at least 3 times at 24 h intervals under room conditions until no chemical compounds were extracted (3 times for *P. somniferum*, 4 times for *Salix* sp., 5 times for *C. creticus* and *H. annuus*). After the extraction was completed, the mixture was filtrated using a filter paper, then methanol was removed under vacuum in a rotary evaporator (Wigens, STRIKE 380 M3/M6, Beijing) at +40 °C to dryness to obtain the methanol extract. The residue of bee pollen was extracted with hot water at +80 °C for one day, the water was removed with a freeze dryer (Christ Alpha 1–4 LD, Christ, Germany) to dryness to obtain the water extract [15].

The methanol extract was dissolved in pure water and the water suspension was obtained. The water suspension was extracted with fresh solvents by liquid-liquid extraction method to give *n*-hexane, dichloromethane, ethyl acetate, *n*-butanol and water fractions, respectively. The solvents of *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol were removed separately under vacuum in a rotary evaporator to dryness to obtain the relevant fractions. Water solvent was removed with a freeze dryer to dryness to obtain the relevant fractions [16]. All the water extracts of bee pollens and fractions of bee pollen methanol extracts were maintained at +4 °C for a maximum of 10 days for further testing.

HPLC-DAD identification of chemical compounds

Chemical compounds of bee pollens, water extracts and fractions were identified using high performance liquid chromatography (HPLC) (LC-20AT, Shimadzu, Japan) following a procedure previously explained in our study [17, 18]. Bee pollens, water extracts and fractions were dissolved in water:methanol (80:20) and filtered by using a 0.20 µm disposable LC filter disk for HPLC-DAD. The separation was performed with the reverse phase C₁₈ column (Inertsil ODS-3, Inertsil, USA) (5 µm, 250 mm × 4.6 mm i.d) at 40 °C. An injection volume of 20 µL was used with mobile phase flow rate of 1 mL/min. The mobile phase used was: (A) 0.5% acetic acid in water, (B) methanol. The elution gradient was as follows: 0–10% B in 0.01 min, 10–20% B in 2 min, 20–30% B in 15 min, 30–100% B in 30 min, 100–0.01% B in 33 min, 0.01% B in 35 min, 10% B in 35.01 min. Detection was carried out photodiode array detector (PDA) using 280 nm as the preferred wavelength. In total, 29 different

standards were separated by HPLC, and the identification of the compounds was carried out with a photodiode array detector (PDA) at a wavelength of 280 nm. Comparison of retention times and UV data of standards was used in the characterization of the chemical compounds detected in the bee pollens, extracts and fractions. The amounts of identified chemical compounds were calculated by drawing the calibration graph obtained by injecting known concentrations (0.0–1.0 ppm) of different standards. All analysis was operated in triplicate. The results were given as µg/g dry weight (dw).

Statistical analysis

All HPLC-DAD analysis were operated in triplicate and the results were given as the average of triplicate. The results were registered as the mean ± S.E. (standard error). Student's *t* test was used to determine the significant differences among the average values and *p* < 0.05 was considered to indicate statistical significance. Chemometric analysis of the chemical compounds of the fractions by principal component analysis (PCA) and hierarchical cluster analysis (HCA) was completed by MINITAB 16.0 software. PCA was used to observe the similarities and differences between fractions and extracts in terms of their chemical compounds. HCA was used to separate fractions and extracts into clusters and groups according to the proximity or diversity of their chemical compounds. Euclidean distance and Ward Link method were used to define hierarchical relationships in cluster analysis [19].

Results and discussion

Chemical compounds

Chemical compounds of bee pollens of *C. creticus*, *H. annuus*, *P. somniferum* and *Salix* sp., the fractions (hexane, dichloromethane, ethyl acetate, butanol, water) and water extracts of all four pollens were characterized by HPLC-DAD. The analytical parameters of chemical compounds for HPLC-DAD analysis are described in Table S1. In total, 29 different chemical compounds consisting of phenolic acids, organic acids, flavonoids, and phenolics are screened and the chromatograms of the standards, the pollens, fractions and water extracts are given in Figs. S5–33. The screened and detected chemical compounds in *C. creticus* bee pollen, fractions and water extract are given in Table 1, *H. annuus* bee pollen, fractions and water extract in Table 2, *P. somniferum* bee pollen, fractions and water extract in Table 3, *Salix* sp. bee pollen, fractions and water extract in Table 4.

Table 1 Chemical compounds ($\mu\text{g/g}$) of *C. creticus* bee pollen, water extract and fractions

Peak number	Compounds	Retention time (min)	C	CH	CD	CEA	CB	CW1	CW2
1	Fumaric acid	3.061	nd	nd	nd	nd	nd	nd	nd
2	<i>trans</i> -Aconitic acid	3.303	15.8 $\pm$ 0.01	nd	34.0 $\pm$ 0.01	239.4 $\pm$ 0.02	222.4 $\pm$ 0.01	12.2 $\pm$ 0.01	479.1 $\pm$ 0.02
3	Gallic acid	3.585	nd	nd	nd	nd	nd	nd	nd
4	Levulinic acid	3.809	nd	nd	nd	nd	nd	nd	nd
5	Protocatechuic acid	5.345	nd	nd	nd	nd	nd	nd	nd
6	<i>p</i> -Hydroxy phenyl acetic acid	6.326	nd	nd	nd	nd	nd	nd	nd
7	Catechin	6.402	nd	nd	nd	nd	nd	nd	nd
8	Chlorogenic acid	7.749	nd	nd	nd	nd	nd	nd	nd
9	<i>p</i> -Hydroxy benzoic acid	7.950	nd	nd	3.5 $\pm$ 0.01	3.2 $\pm$ 0.01	nd	nd	nd
10	6,7-Dihydroxy coumarin	8.921	nd	nd	nd	nd	nd	nd	nd
11	Vanillic acid	9.635	nd	nd	11.0 $\pm$ 0.01	nd	nd	nd	nd
12	Caffeic acid	9.722	nd	nd	nd	4.3 $\pm$ 0.01	nd	nd	nd
13	3-Hydroxy benzoic acid	10.214	nd	nd	nd	nd	nd	nd	nd
14	Vanillin	11.714	nd	nd	0.1 $\pm$ 0.01	nd	nd	nd	nd
15	<i>p</i> -Coumaric acid	14.556	nd	nd	nd	nd	nd	nd	nd
16	Guaiaicol	14.822	nd	nd	nd	nd	nd	nd	nd
17	Ferulic acid	16.642	nd	nd	5.9 $\pm$ 0.01	nd	nd	nd	nd
18	Coumarin	18.701	nd	nd	nd	nd	nd	nd	nd
19	Rutin	20.459	4.1 $\pm$ 0.01	nd	5.3 $\pm$ 0.01	nd	202.8 $\pm$ 0.01	nd	nd
20	2-Hydroxy cinnamic acid	20.509	nd	3.8 $\pm$ 0.01	nd	287.9 $\pm$ 0.02	nd	nd	0.5 $\pm$ 0.01
21	Rosmarinic acid	21.095	281.3 $\pm$ 0.02	nd	nd	435.6 $\pm$ 0.02	309.9 $\pm$ 0.01	nd	277.5 $\pm$ 0.02
22	Myricetin	21.517	160.4 $\pm$ 0.01	163.4 $\pm$ 0.02	211.6 $\pm$ 0.01	270.2 $\pm$ 0.01	nd	nd	nd
23	Quercetin	23.351	nd	nd	586.9 $\pm$ 0.02	608.1 $\pm$ 0.01	162.2 $\pm$ 0.01	nd	nd
24	<i>trans</i> -Cinnamic acid	23.443	nd	nd	nd	nd	nd	46.4 $\pm$ 0.01	43.6 $\pm$ 0.01
25	Naringenin	23.568	nd	nd	nd	nd	nd	nd	nd
26	Luteolin	23.900	nd	73.3 $\pm$ 0.01	694.8 $\pm$ 0.01	558.4 $\pm$ 0.01	69.8 $\pm$ 0.01	nd	nd
27	Kaempferol	24.765	32.4 $\pm$ 0.02	30.4 $\pm$ 0.02	8.8 $\pm$ 0.01	6.1 $\pm$ 0.01	30.4 $\pm$ 0.02	32.8 $\pm$ 0.02	32.2 $\pm$ 0.01
28	Apigenin	25.015	nd	nd	nd	nd	nd	nd	nd
29	Chrysin	27.172	nd	0.4 $\pm$ 0.01	nd	nd	nd	nd	nd
	Total		494.0	271.3	1561.9	2413.2	997.5	91.4	832.9

Values represent the means $\pm$ S.E. of three parallel measurements ($p<0.05$). nd: Not detected. C: *C. creticus* bee pollen, CH: Hexane fraction, CD: Dichloromethane fraction, CEA: Ethyl acetate fraction, CB: Butanol fraction, CW1: Water fraction, CW2: Water extract

Kaempferol is a powerful bioactive flavonol found in fruits and vegetables. It has been described to prevent various diseases such as diabetes, obesity, arthritis, glaucoma, osteoporosis and skin diseases. Additionally, it has been revealed that kaempferol has hepatoprotective, kidney protective, cardioprotective, neuroprotective and gastroprotective properties through its antioxidant and anti-inflammatory effects [20]. Moreover, it has been determined that kaempferol, in combination with other drugs or natural compounds, synergistically increases the effectiveness of drugs [21]. Kaempferol was the only compound identified in all bee pollens, fractions and water extracts in the concentration range of 0.4 and 331.6 $\mu\text{g/g}$. PEA (*P. somniferum* ethyl acetate fraction), HEA (*H. annuus* ethyl acetate fraction) and HB (*H. annuus* butanol fraction) were documented

as the richest fractions in kaempferol among all bee pollens, fractions and water extracts.

Luteolin is a flavone found in many foods commonly consumed by humans such as vegetables, fruits, and herbs. In addition to its wide range of bioactivities, its anti-convulsion, antitumor, anti-inflammatory, antidiabetic, neuroprotection, anti-cardiovascular, and antioxidant properties have been documented in *in vitro* or animal experiments [22]. Luteolin was the second most identified compound. The very high concentration of luteolin was found in CD (*C. creticus* dichloromethane fraction), CEA (*C. creticus* ethyl acetate fraction), SB (*Salix* sp. butanol fraction), PEA, HEA, HB, PD (*P. somniferum* dichloromethane fraction), SEA (*Salix* sp. ethyl acetate fraction), PB (*P. somniferum* butanol fraction), SD (*Salix* sp. dichloromethane fraction), CH (*C. creticus* hexane fraction), S (*Salix* sp. bee pollen),

Table 2 Chemical compounds ($\mu\text{g/g}$) of *H. annuus* bee pollen, water extract and fractions

Peak number	Compounds	Retention time (min)	H	HH	HD	HEA	HB	HW1	HW2
1	Fumaric acid	3.061	nd	nd	nd	nd	nd	nd	nd
2	<i>trans</i> -Aconitic acid	3.303	nd	nd	nd	363.4 $\pm$ 0.03	273.6 $\pm$ 0.01	15.8 $\pm$ 0.01	32.9 $\pm$ 0.02
3	Gallic acid	3.585	nd	nd	nd	nd	nd	nd	nd
4	Levulinic acid	3.809	nd	nd	nd	nd	nd	nd	nd
5	Protocatechuic acid	5.345	nd	nd	nd	nd	nd	nd	nd
6	<i>p</i> -Hydroxy phenyl acetic acid	6.326	nd	nd	nd	nd	nd	nd	nd
7	Catechin	6.402	nd	nd	nd	nd	nd	nd	nd
8	Chlorogenic acid	7.749	nd	nd	nd	nd	nd	nd	nd
9	<i>p</i> -Hydroxy benzoic acid	7.950	nd	nd	20.4 $\pm$ 0.02	17.6 $\pm$ 0.01	nd	nd	nd
10	6,7-Dihydroxy coumarin	8.921	nd	nd	nd	nd	nd	nd	nd
11	Vanillic acid	9.635	nd	nd	8.7 $\pm$ 0.01	nd	nd	nd	nd
12	Caffeic acid	9.722	nd	nd	nd	4.1 $\pm$ 0.02	nd	nd	nd
13	3-Hydroxy benzoic acid	10.214	nd	nd	nd	nd	nd	nd	nd
14	Vanillin	11.714	nd	nd	nd	nd	nd	nd	nd
15	<i>p</i> -Coumaric acid	14.556	nd	nd	0.1 $\pm$ 0.01	nd	nd	nd	nd
16	Guaiaicol	14.822	nd	nd	nd	nd	nd	nd	nd
17	Ferulic acid	16.642	nd	nd	nd	nd	nd	nd	nd
18	Coumarin	18.701	nd	nd	nd	nd	nd	nd	nd
19	Rutin	20.459	nd	3.6 $\pm$ 0.01	3.9 $\pm$ 0.02	nd	132.6 $\pm$ 0.01	nd	nd
20	2-Hydroxy cinnamic acid	20.509	4.6 $\pm$ 0.01	nd	nd	86.1 $\pm$ 0.01	nd	nd	nd
21	Rosmarinic acid	21.095	nd	nd	nd	319.8 $\pm$ 0.01	280.2 $\pm$ 0.02	nd	nd
22	Myricetin	21.517	169.1 $\pm$ 0.01	nd	194.8 $\pm$ 0.03	nd	nd	nd	nd
23	Quercetin	23.351	166.5 $\pm$ 0.02	165.7 $\pm$ 0.02	nd	nd	nd	nd	nd
24	<i>trans</i> -Cinnamic acid	23.443	nd	nd	48.4 $\pm$ 0.01	1854.6 $\pm$ 0.02	1474.6 $\pm$ 0.02	nd	nd
25	Naringenin	23.568	nd	nd	nd	nd	nd	nd	nd
26	Luteolin	23.900	69.9 $\pm$ 0.02	71.5 $\pm$ 0.01	nd	345.9 $\pm$ 0.02	247.1 $\pm$ 0.02	nd	nd
27	Kaempferol	24.765	30.5 $\pm$ 0.01	34.1 $\pm$ 0.02	20.8 $\pm$ 0.01	221.8 $\pm$ 0.01	193.4 $\pm$ 0.01	31.2 $\pm$ 0.01	32.5 $\pm$ 0.01
28	Apigenin	25.015	nd	nd	nd	nd	nd	nd	nd
29	Chrysin	27.172	nd	0.7 $\pm$ 0.01	2.6 $\pm$ 0.01	nd	0.5 $\pm$ 0.01	nd	nd
	Total		440.6	275.6	299.7	3213.3	2602	47	65.4

Values represent the means $\pm$ S.E. of three parallel measurements ($p < 0.05$). nd: Not detected. H: *H. annuus* bee pollen, HH: Hexane fraction, HD: Dichloromethane fraction, HEA: Ethyl acetate fraction, HB: Butanol fraction, HW1: Water fraction, HW2: Water extract

SH (*Salix* sp. hexane fraction), HH (*H. annuus* hexane fraction), H (*H. annuus* bee pollen), CB (*C. creticus* butanol fraction), P (*P. somniferum* bee pollen), and PW1 (*P. somniferum* water fraction).

trans-Aconitic acid was recognized as one of 30 chemicals with the most added value by the Department of Energy due to its industrial use as a chemical building block and precursor of other important chemicals and polymers. The reported biological activities of *trans*-aconitic acid could be listed as nematocidal, anti-leishmanial, antifungal, antioxidant, antifeedant, anti-inflammatory, and anti-quorum sensing [23]. *trans*-Aconitic was the third most identified compound. The presence of *trans*-aconitic was detected in C (*C. creticus* bee pollen), CD, CEA CB, CW1 (*C. creticus* water fraction), CW2 (*C. creticus* water extract), HEA, HB,

HW1 (*H. annuus* water fraction), HW2 (*H. annuus* water extract), PW2 (*P. somniferum* water extract), S, SD, SEA, SB, SW1 (*Salix* sp. water fraction), and SW2 (*Salix* sp. water extract).

Myricetin is a flavonol that has an important position in protecting health. The explored biopotentials of myricetin were reported as antidiabetic, anti-cancer, anti-obesity, osteoporosis protection, cardiovascular protection, hepatoprotective, and anti-inflammatory activities [24]. The very high concentration of myricetin was found in C, CH, CD, CEA, H, HD (*H. annuus* dichloromethane fraction), P, PD, PEA, S, SH, SD, SEA, SB, SW1, and SW2.

Rosmarinic acid is an important polyphenolic acid that has uses in various fields such as food preservative, cosmetic and pharmaceutical industries. Bioactive properties of

Table 3 Chemical compounds ($\mu\text{g/g}$) of *P. somniferum* bee pollen, water extract and fractions

Peak number	Compounds	Retention time (min)	P	PH	PD	PEA	PB	PW1	PW2
1	Fumaric acid	3.061	nd	nd	nd	nd	nd	nd	118.4 $\pm$ 0.02
2	<i>trans</i> -Aconitic acid	3.303	nd	nd	nd	nd	nd	nd	31.7 $\pm$ 0.01
3	Gallic acid	3.585	nd	nd	nd	nd	nd	nd	nd
4	Levulinic acid	3.809	nd	nd	nd	nd	nd	nd	nd
5	Protocatechuic acid	5.345	nd	nd	nd	nd	nd	nd	nd
6	<i>p</i> -Hydroxy phenyl acetic acid	6.326	nd	nd	nd	nd	nd	nd	nd
7	Catechin	6.402	nd	nd	nd	nd	nd	nd	nd
8	Chlorogenic acid	7.749	nd	nd	nd	nd	nd	nd	nd
9	<i>p</i> -Hydroxy benzoic acid	7.950	nd	nd	3.6 $\pm$ 0.01	2.8 $\pm$ 0.01	nd	nd	nd
10	6,7-Dihydroxy coumarin	8.921	nd	nd	4.1 $\pm$ 0.01	nd	nd	nd	nd
11	Vanillic acid	9.635	nd	nd	8.4 $\pm$ 0.02	nd	nd	nd	nd
12	Caffeic acid	9.722	nd	nd	nd	4.6 $\pm$ 0.01	nd	nd	nd
13	3-Hydroxy benzoic acid	10.214	nd	nd	nd	nd	nd	nd	nd
14	Vanillin	11.714	0.1 $\pm$ 0.01	0.6 $\pm$ 0.01	116.3 $\pm$ 0.01	0.8 $\pm$ 0.02	1.2 $\pm$ 0.01	nd	nd
15	<i>p</i> -Coumaric acid	14.556	nd	nd	0.6 $\pm$ 0.01	0.4 $\pm$ 0.01	nd	nd	nd
16	Guaiaicol	14.822	nd	nd	nd	nd	1.8 $\pm$ 0.01	nd	nd
17	Ferulic acid	16.642	nd	nd	6.0 $\pm$ 0.02	nd	nd	nd	nd
18	Coumarin	18.701	nd	nd	nd	128.2 $\pm$ 0.01	nd	nd	nd
19	Rutin	20.459	4.0 $\pm$ 0.01	nd	nd	41.1 $\pm$ 0.01	11.9 $\pm$ 0.01	nd	3.6 $\pm$ 0.01
20	2-Hydroxy cinnamic acid	20.509	nd	nd	nd	nd	nd	nd	nd
21	Rosmarinic acid	21.095	nd	294.6 $\pm$ 0.01	nd	277.3 $\pm$ 0.02	nd	nd	nd
22	Myricetin	21.517	161.2 $\pm$ 0.02	nd	185.8 $\pm$ 0.01	196.2 $\pm$ 0.01	nd	nd	nd
23	Quercetin	23.351	168.8 $\pm$ 0.01	170.9 $\pm$ 0.02	nd	312.1 $\pm$ 0.01	195.8 $\pm$ 0.02	162.1 $\pm$ 0.01	nd
24	<i>trans</i> -Cinnamic acid	23.443	nd	17.9 $\pm$ 0.01	19.2 $\pm$ 0.03	nd	nd	nd	nd
25	Naringenin	23.568	nd	2.5 $\pm$ 0.01	nd	nd	nd	nd	nd
26	Luteolin	23.900	69.4 $\pm$ 0.01	nd	193.5 $\pm$ 0.02	359.5 $\pm$ 0.01	96.2 $\pm$ 0.01	68.7 $\pm$ 0.01	nd
27	Kaempferol	24.765	26.3 $\pm$ 0.01	17.1 $\pm$ 0.01	0.4 $\pm$ 0.01	331.6 $\pm$ 0.02	0.6 $\pm$ 0.01	29.8 $\pm$ 0.01	1.6 $\pm$ 0.01
28	Apigenin	25.015	nd	nd	nd	nd	nd	nd	nd
29	Chrysin	27.172	nd	1.3 $\pm$ 0.01	nd	12.8 $\pm$ 0.01	0.7 $\pm$ 0.01	nd	nd
	Total		429.8	504.9	537.9	1666.9	308.2	260.6	155.3

Values represent the means $\pm$ S.E. of three parallel measurements ($p < 0.05$). nd: Not detected. P: *P. somniferum* bee pollen, PH: Hexane fraction, PD: Dichloromethane fraction, PEA: Ethyl acetate fraction, PB: Butanol fraction, PW1: Water fraction, PW2: Water extract

rosmarinic acid such as antiviral, antibacterial, antiepileptic, anti-inflammatory, anti-nociceptive, anti-anxiolytic, antioxidant, anti-depressant, and neuroprotective have been highlighted in previous studies. Its potential use in preventing Alzheimer's disease, improving cognitive function, treating kidney diseases, and cancer has also been reported [25]. C, CEA, CB, CW2, HEA, HB, PH (*P. somniferum* hexane fraction), PEA, S, SH, SD, SEA, SB, and SW1 were identified as having very high contents of rosmarinic acid.

Quercetin is a flavonol and is found in a great variety of natural sources. Numerous *in vivo* and *in vitro* studies have supported that quercetin commands various medicinal values such as neurovascular protection, anti-inflammatory, antioxidant, antihypertensive, hypoglycemic, anti-cancer, anti-aging, and immune strengthening effects [26]. The high

quantity of quercetin was recorded in CD, CEA, CB, H, HH, P, PH, PEA, PB, PW1, SH, SEA, SB, and SW1.

Rutin, a flavonoid glycoside derivative, is known as vitamin P or rutoside. A lot of studies have confirmed that rutin has antimicrobial, antioxidant, anti-arthritis, hepatoprotective, cardiovascular, anti-allergic, anti-cancer, antidiabetic, wound healing, and anti-inflammatory effects [27]. The presence of rutin was specified in C, CD, CB, HH, HD, HB, P, PEA, PB, and PW2.

Cinnamic acid is known for its various health benefits and low cytotoxicity, and is also known as phenylacrylic acid. The five most well-known cinnamic acid members are: ferulic, caffeic, *p*-coumaric and sinapic acids. Among its wide range of bioactive properties neuroprotective, anti-tumor, anti-parasitic, antidiabetic, antimicrobial, antioxidant and anti-inflammatory activities came to the fore [28].

Table 4 Chemical compounds ($\mu\text{g/g}$) of *Salix* sp. bee pollen, water extract and fractions

Peak numbers	Compounds	Retention time (min)	S	SH	SD	SEA	SB	SW1	SW2
1	Fumaric acid	3.061	nd	nd	nd	nd	nd	nd	nd
2	<i>trans</i> -Aconitic acid	3.303	42.2 $\pm$ 0.01	nd	29.4 $\pm$ 0.01	130.6 $\pm$ 0.01	154.6 $\pm$ 0.01	20.2 $\pm$ 0.01	66.9 $\pm$ 0.01
3	Gallic acid	3.585	nd	nd	nd	nd	nd	nd	nd
4	Levulinic acid	3.809	nd	nd	nd	2.10 $\pm$ 0.01	nd	nd	nd
5	Protocatechuic acid	5.345	nd	nd	nd	nd	nd	nd	nd
6	<i>p</i> -Hydroxy phenyl acetic acid	6.326	nd	nd	nd	nd	nd	nd	nd
7	Catechin	6.402	nd	nd	nd	nd	nd	nd	nd
8	Chlorogenic acid	7.749	nd	nd	nd	nd	nd	nd	nd
9	<i>p</i> -Hydroxy benzoic acid	7.950	nd	nd	nd	0.8 $\pm$ 0.01	nd	nd	nd
10	6,7-Dihydroxy coumarin	8.921	nd	nd	nd	nd	nd	nd	nd
11	Vanillic acid	9.635	nd	nd	1.0 $\pm$ 0.01	nd	nd	nd	nd
12	Caffeic acid	9.722	nd	nd	nd	82.4 $\pm$ 0.02	nd	nd	nd
13	3-Hydroxy benzoic acid	10.214	nd	nd	nd	nd	nd	nd	nd
14	Vanillin	11.714	nd	nd	nd	nd	nd	nd	nd
15	<i>p</i> -Coumaric acid	14.556	nd	nd	nd	nd	nd	nd	nd
16	Guaiacol	14.822	nd	nd	nd	nd	nd	nd	nd
17	Ferulic acid	16.642	nd	nd	nd	nd	nd	nd	nd
18	Coumarin	18.701	nd	nd	nd	nd	nd	nd	nd
19	Rutin	20.459	nd	nd	nd	nd	nd	nd	nd
20	2-Hydroxy cinnamic acid	20.509	nd	nd	nd	nd	nd	nd	nd
21	Rosmarinic acid	21.095	347.9 $\pm$ 0.02	298.8 $\pm$ 0.01	309.0 $\pm$ 0.02	402.5 $\pm$ 0.02	390.9 $\pm$ 0.01	273.8 $\pm$ 0.01	nd
22	Myricetin	21.517	421.2 $\pm$ 0.01	270.0 $\pm$ 0.01	463.8 $\pm$ 0.01	1162.8 $\pm$ 0.03	1534.2 $\pm$ 0.01	168.8 $\pm$ 0.01	160.4 $\pm$ 0.02
23	Quercetin	23.351	nd	163.3 $\pm$ 0.03	nd	205.2 $\pm$ 0.01	412.7 $\pm$ 0.02	162.1 $\pm$ 0.01	nd
24	<i>trans</i> -Cinnamic acid	23.443	5.1 $\pm$ 0.01	nd	22.6 $\pm$ 0.01	nd	nd	nd	nd
25	Naringenin	23.568	nd	nd	nd	nd	nd	nd	nd
26	Luteolin	23.900	72.5 $\pm$ 0.01	72.2 $\pm$ 0.01	91.7 $\pm$ 0.01	131.9 $\pm$ 0.01	525.7 $\pm$ 0.01	nd	nd
27	Kaempferol	24.765	32.2 $\pm$ 0.02	35.6 $\pm$ 0.01	25.0 $\pm$ 0.02	31.8 $\pm$ 0.01	19.2 $\pm$ 0.01	33.6 $\pm$ 0.01	32.6 $\pm$ 0.01
28	Apigenin	25.015	nd	nd	15.1 $\pm$ 0.01	nd	nd	nd	nd
29	Chrysin	27.172	nd	nd	nd	nd	nd	nd	nd
	Total		921.1	839.9	957.6	2150.1	3037.3	658.5	259.9

Values represent the means $\pm$ S.E. of three parallel measurements ($p<0.05$). nd: Not detected. S: *Salix* sp. bee pollen, SH: Hexane fraction, SD: Dichloromethane fraction, SEA: Ethyl acetate fraction, SB: Butanol fraction, SW1: Water fraction, SW2: Water extract

The presence of *trans*-cinnamic acid was defined in CW1, CW2, HD, HEA, HB, PH, PD, S, and SD. The decreasing amounts of 2-hydroxy cinnamic acid were in the following order: CEA, HEA, H, CH, and CW2.

p-Coumaric acid is a cinnamic acid analogue found in a wide variety of natural sources. The huge bioactive potentials of *p*-coumaric acid have been revealed as an antioxidant, bone formation, antimicrobial, anti-ulcer, anti-cancer, antiarthritic, anti-inflammatory, cardioprotective, gout prevention, antidiabetic, anti-melanogenic, gastroprotective, anti-platelet, hepatoprotective, reno-protective, and anti-angiogenic [29]. The presence of *p*-coumaric acid was found in HD, PD, and PEA.

Ferulic acid is an important cinnamic acid derivative and is pronounced as boon. It has tremendous potential therapeutic properties in the therapy of cancer, diabetes, lung, and cardiovascular diseases, mainly with versatile bioactivities such as activation of transcriptional factors, antiallergic, antimicrobial, anti-cancer, antiviral, metal chelation, antioxidant, hepatoprotective, modulation of enzyme activity [30]. Ferulic acid was characterized in PD and CD.

Caffeic acid is a dihydroxy cinnamic acid derivative found primarily in honey, coffee, potatoes, fruits, herbs, chard, olives, and vegetables [31]. Caffeic acid was correlated with various pharmacologic activities, consisting of anti-obesity, antidiabetic immunomodulatory, hypolipidemic,

hypotensive, antiproliferative, antioxidant, antimicrobial, neuroprotective, and anti-inflammatory [32]. Caffeic acid was identified in all ethyl acetate fractions of the bee pollens. The highest concentration of caffeic acid was found in SEA and followed by PEA, CEA, and HEA.

Hydroxy benzoic acids are phenolic compounds with remarkable antioxidant activity that contain hydroxyl groups in different positions and numbers in their aromatic rings. *p*-Hydroxy benzoic acid, one of the most important members of this family, has been ranked among various value-added bioproducts with potential biotechnological applications in recent years, especially in food, cosmetics, pharmaceuticals, and fungicides [33]. As a result of the studies, it was revealed that the various bioactive properties of *p*-hydroxy benzoic acid include nematocidal, antialgal, antimutagenic, hypoglycemic, antimicrobial, antiestrogenic, anti-platelet aggregation, anti-inflammatory, antiviral, and antioxidant [34]. *p*-Hydroxy benzoic acid was found in CD, CEA, HD, HEA, PD, PEA, and SEA.

Chrysin is a flavone found in fruit, honey, herbs and propolis. Chrysin, whose anti-cancer, antioxidant, hepatoprotective, antiviral, neuroprotective and anti-anxiety properties have been emphasized, has also been reported to be an adjuvant to some drugs [35]. Chrysin was identified in CH, HH, HD, HB, PH, PEA, and PB.

Vanillin is the primary ingredient that provides the characteristic taste and aroma of vanilla, which is a mixture of more than 200 compounds. Vanillin, which forms a multi-million-dollar market, is preferred as a fragrance ingredient in ice cream, confectionery, dairy products, perfume, pharmaceuticals, liqueurs and other beverage sectors [36]. The pharmacological characteristics of vanillin were reported as antiviral, neuroprotective, nephroprotective, antibiotic, antimicrobial, wound healing, anti-inflammatory, anti-cancer, antioxidant, and cardioprotective activities [37]. Vanillin was detected in CD, P, PH, PD, PEA, and PB.

Vanillic acid is a dihydroxybenzoic acid analogue used as an important flavoring agent. It attracts attention mostly due to its usage in the cosmetics, fruit, sweeteners, cigarettes, pharmaceuticals, alcohol, beverage and polymer sectors [38]. The well-identified bioeffects of vanillic acid were reported as: antimicrobial, antibacterial, sedative, antifungal, antimutagen, hepatoprotective, wound healing, antioxidant, cardioprotective, antidepressant, chemotherapy-preventative effects, and even herbal growth regulator [39]. Vanillic acid was identified in all dichloromethane fractions of the bee pollens. The presence of vanillic acid was determined in CD, HD, PD, and SD.

Coumarins are bioactive molecules with the most privileged structural skeleton of the heterocyclic benzopyrene family, found in a wide variety of natural sources [40]. It has attracted attention with its bioactive properties such as

anticonvulsant, antidiabetic, antitumor, anti-inflammatory, anti-cancer, antibacterial, antifungal, antiviral, neuroprotective, antihypertensive, antioxidant, anticoagulant, and antimicrobial. Today, many coumarin derivatives are approved for clinical use by the FDA (U.S. Food and Drug Administration) [41]. When coumarin was only found in PEA, 6,7-dihydroxy coumarin was detected in PD.

Guaiacol, also known as 2-methoxy phenol, is an important phenolic compound with antioxidant, analgesic and antimicrobial activities [42]. Guaiacol was only identified in PB.

Naringenin is a flavanone found in high concentrations mostly in citrus fruits. The positive effects of naringenin on health were revealed as antidiabetic, anti-cancer, gastroprotective, antimicrobial, anti-obesity, cardioprotective, neuroprotective, immunomodulator, and nephroprotective by *in vitro* and *in vivo* studied with related to antioxidant and anti-inflammatory activities [43]. Naringenin was only found in PH.

Apigenin is a flavone that attracts attention with its low toxicity. Apigenin is mainly found in fruits, nuts, vegetables, honey, herbs, and tea. Literature studies have reported that apigenin is a promising treatment candidate with its anti-inflammatory and antioxidant properties on a variety of diseases including insomnia, cancer, obesity, infection, depression, diabetes, respiratory, cardiovascular, neurodegenerative, and skin diseases [44]. SD was the only fraction containing apigenin.

Fumaric acid is a valuable dicarboxylic acid used in industrial products such as food, detergents, feed, and pharmaceutical products with antioxidant, anti-cancer and antimicrobial properties [45]. The only PW2 was found as rich in fumaric acid.

Levulinic acid is a value-added dicarboxylic acid that is in demand for its antimicrobial effect in the cosmetic industry, maintaining acid balance and protecting the skin from microbial infection and in food industry by improving food safety and quality without causing any negative effects [46]. Levulinic acid was only characterized in SEA.

Chemical compounds are extracted using organic solvents and their aqueous formulations in the basic context, and there are still uncertainties in the literature regarding the most suitable solvent and extraction technique, especially for the extraction of phenolic compounds. In particular, the extraction of phenolic compounds is carried out using acetone, ethyl acetate, water, methanol and butanol [47]. Serial exhaustive extraction is one of the most commonly used techniques for the extraction of chemical compounds from natural sources, involving fractionation of the crude extract from hexane to butanol. Although the extraction efficiency increases depending on the polarity of the solvent used, the extraction performance also depends on other factors such

as the chemical composition of the phenolic compounds to be extracted, the number and position of hydroxyl groups, molecular size, temperature, contact time, particle size and the effect of other compounds [48]. In general, ethyl acetate (1666.9–3213.3 µg/g) and butanol (308.2–3037.3 µg/g) fractions of bee pollens were determined to be significantly more chemically rich than other fractions in this study. The highest levels and yields of chemical compounds in these ethyl acetate and butanol fractions could be relevant with the increased solvent polarity and extraction efficiency.

It is noteworthy that previous studies on the chemical compositions of the bee pollens were limited. Consistent with the results here, luteolin (33.9 µg/g), kaempferol (31.93 µg/g), quercetin (31.4 µg/g), vanillic acid (10.89 µg/g), myricetin (9.09 µg/g), *p*-hydroxy benzoic acid (8.9 µg/g), isorhamnetin (7.76 µg/g), and rutin (4.02 µg/g) were identified as the most abundant phenolic acids and flavonoids in *Cistus* bee pollen (85%-monofloral) from Afyon-Türkiye [49]. The ethyl acetate and dichloromethane sub-extracts of *Cistus* sp. bee pollen (58%-monofloral) and *Salix* sp. bee pollen (64%-monofloral) from Samsun-Türkiye were investigated for phenolic compounds by Alimoglu et al. [50]. For *Cistus* sp. bee pollen when isoquercitrin (9030 µg/g), rutin (3390 µg/g) and caffeic acid (900 µg/g) were detected in the ethyl acetate sub-extract, no phenolic compounds were identified in the dichloromethane sub-extract. For *Salix* sp. bee pollen when only isoquercitrin (680 µg/g) was reported in the ethyl acetate sub-extract, galangin (40 µg/g) and chrysin (60 µg/g) were characterized in the dichloromethane sub-extract. The results of *H. annuus* bee pollen, water extract and fractions are in agreement with those reported in *H. annuus* bee pollen from North-East-Romania. Kaempferol (52.22 µg/g), myricetin (33.36 µg/g), *p*-coumaric acid (24.75 µg/g), vanillic acid (21.19 µg/g), quercetin (21.11 µg/g), luteolin (13.45 µg/g), rosmarinic acid (2.01 µg/g), and chlorogenic acid (0.78 µg/g) were reported as the main compounds in this *H. annuus* bee pollen [51]. In parallel with the findings obtained here, quercetin (38.96, 26.14 µg/g), caffeic acid (4.28, 2.16 µg/g), rutin (2.72, 8.64 µg/g), kaempferol (6.12, 2.42 µg/g), and luteolin (1.22, 1.14 µg/g) were reported in *H. annuus* bee pollen (from Vojvodina-Serbia) ethanol and methanol extracts [52]. The ethanol extracts of frozen, dried, freeze-dried of *H. annuus* bee pollen collected from Western-Slovakia were revealed to contain quercetin (14.30, 10.19, 12.04 µg/g), luteolin (66.39, 63.62, 46.96 µg/g), and apigenin (23.99, 32.01, 34.40 µg/g) [53]. Çobanoğlu [49] studied phenolic compounds of two different monofloral *P. somniferum* bee pollens and the presented results supported our findings. The main phenolic compounds of *P. somniferum* bee pollen (80.2%-monofloral) from Afyon Türkiye were reported as luteolin (450.68 µg/g), kaempferol (20.68 µg/g), rutin (12.3 µg/g),

p-coumaric acid (11.87 µg/g), genistein (7.03 µg/g), myricetin (8.37 µg/g), and quercetin (4.71 µg/g). *P. somniferum* bee pollen (81%-monofloral) from Afyon-Türkiye was described as rich in luteolin (399.97 µg/g), *p*-hydroxy benzoic acid (21.8 µg/g), *p*-coumaric acid (17.21 µg/g), kaempferol (14.12 µg/g), and rutin (10.27 µg/g). The amounts of quercetin (2.05, 3.99, 5.19 µg/g), luteolin (1105.80, 1190.67, 1340.58 µg/g), kaempferol (12.96, 22.40, 23.61 µg/g), and apigenin (4.60, 6.56, 15.65 µg/g) in the ethanol extract of frozen, dried and freeze-dried *P. somniferum* bee pollen from Western-Slovakia were reported [54]. Again, in the study of Çobanoğlu [49] the dominant phenolic compounds were recorded as luteolin (230.55 µg/g), *p*-coumaric acid (17.04 µg/g), genistein (10.37 µg/g), naringenin (13.13 µg/g), kaempferol (20 µg/g), quercetin (8.34 µg/g), isorhamnetin (15.14 µg/g), and rutin (8.66 µg/g) in *Salix* bee pollen (multifloral) from Kütahya-Türkiye. The ethanol extract of the multifloral bee pollen from Fez-Meknes-Morocco was detected as rich in caffeic acid (41.00 µg/g), chlorogenic acid (22.90 µg/g), isorhamnetin (11.0 µg/g), gallic acid (11.71 µg/g), and catechin (10.80 µg/g) by Aboulghazi et al. [55]. The methanol extract of the bee pollen (multifloral) from Rize-Türkiye was investigated for phenolic compounds and the most abundant compounds were reported as luteolin (52.52 µg/g), daidzein (16.18 µg/g), *p*-coumaric acid (13.10 µg/g), caffeic acid phenethyl ester (12.72 µg/g), and chrysin (4.46 µg/g) [56]. In a comprehensive study conducted by Mutlu and Erbas [57] in which 21 polyfloral and 29 monofloral bee pollens collected from different regions (Mediterranean, Anatolia, Aegean, Black Sea, Marmara) of Türkiye in two different seasons were chemically examined, methyl-3,4,5-trihydroxybenzoate (7.0–119.2 µg), *trans*-cinnamic acid (7.8–111.4 µg), naringenin (134.0–966.7 µg), *p*-coumaric acid (15.1–78.8 µg), 3,4-dimethoxy cinnamic acid (13.3–361.7 µg), rosmarinic acid (311.4–3933.8 µg), apigenin (128.7–758.7 µg), rutin (145.8–605.7 µg), myricetin (11.1–130.9 µg), quercetin (07.1–269.8 µg), luteolin (17.9–1068.9 µg), and kaempferol (14.2–72.5 µg) were identified in all bee pollens per one g. In a study on the phenolic compounds of the ethanol extracts of two monofloral (*Brassica* sp. and *Salix* sp.) and three multifloral bee pollens collected from Transylvania-Romania, quercetin-3-*O*-rutinoside (33.54–134.23 µg/g), quercetin (8.70–115.36 µg/g), kaempferol (0.26–234.78 µg/g), quercetin-3-*O*-galactoside (5.96–23.16 µg/g), ellagic acid (24.76–154.90 µg/g), and *p*-coumaric acid (0.43–26.85 µg/g) were described as the most abundant phenolic compounds [58]. The dominant phenolic compounds in nine different bee pollens (multifloral) (from Bayburt-Türkiye) were obtained as gallic acid (16.13–27.76 µg/g), sinapic acid (5.15–56.93 µg/g), ferulic acid (50.92–89.85 µg/g), isorhamnetin (44.67–82.80 µg/g), kaempferol (5.95–8.46 µg/g), naringenin (8.30–10.04 µg/g)

[59]. The ethanol extracts of 16 chestnut bee pollens from Black Sea and Marmara regions from Türkiye were screened for 29 phenolic compounds by Karkar et al. [60]. Rosmarinic acid (2042–13495 µg/g), vitexin (354–1720 µg/g), luteolin (288–907 µg/g), hyperoside (232–1126 µg/g), pinocembrin (60–232 µg/g), and *trans*-chalcone (8–58 µg/g) were stated as the most commonly identified compounds in investigated 16 chestnut bee pollens. Benzoic acid (8900.6 µg/g), apigenin (6477.2 µg/g), quercetin (3575.1 µg/g), luteolin (3930.2 µg/g), and kaempferol (3264.5 µg/g) were noted as the main compounds in the ethanol extract of the bee pollen collected from Muğla-Türkiye by Kahraman et al. [61]. The main chemical compounds of the methanol extract of the bee pollen from Kocaeli-Türkiye were reported as benzoic acid (26.493 µg/g), quercetin (10.254 µg/g), syringaldehyde (5.362 µg/g), protocatechuic acid (4.805 µg/g), rutin (2.445 µg/g), and catechin (2.071 µg/g) [62]. Sonmez et al. [63] characterized the main compounds in the methanol extract of Anatolian bee pollen (from Türkiye) as quercetin (169.793 µg/g), rutin (178.289 µg/g), *p*-coumaric acid (7.848 µg/g), and *p*-hydroxy benzoic acid (3.153 µg/g). In the findings of the above-mentioned literature studies, it is noticed that kaempferol, luteolin, myricetin, *p*-hydroxy benzoic acid, *p*-coumaric acid, rutin, and rosmarinic acid are among the most abundant compounds identified in the bee pollens. In sum, a total of 28 different samples (four different bee pollens, water extracts and fractions of these bee pollens) were screened for chemical compounds and kaempferol was detected in all samples, luteolin in 18 samples, myricetin in 16 samples, rosmarinic acid in 14 samples, rutin in 10 samples, *p*-hydroxy benzoic acid in 7 samples, *p*-coumaric acid in 3 samples. In various previous studies have been emphasized that these differences in the chemical compositions of the bee pollen arise from differences in climatic conditions such as temperature, genotype, water stress, light intensity; collection time and the botanical origin of the bee pollen [57].

The obtained findings revealed that *C. creticus*, *H. annuus*, *P. somniferum*, and *Salix* sp. bee pollens, fractions and extracts can be used as an alternative source of natural chemical compounds such as organic acids, phenolic acids, phenolics and flavonoids for food, cosmetic and pharmaceutical applications. Although more isolation and characterization studies are needed for the full chemical characterization of the pollens here, the obtained data have the basis to pave the way for future studies. Also, ethyl acetate and butanol fractions, especially thanks to their impressive contents of chemical compounds with previously proven bioactive properties, can serve as natural bioactive sources for researchers to conduct research on these species.

Chemometric analysis

Chemometric methods are a set of processes of extracting information by processing raw data. Chemometric methods include experimental design, classification and clustering studies, multivariate analysis, and multivariate calibration. Principal component analysis (PCA) is a versatile statistical analysis that reduces variables in a table of data to their essential properties, called principal components. Several linear combinations of the original variables are explained as principal components in which the variance of all variables is explained at the maximum level. With this method, an approximation of the original data table is obtained by using some main components [64]. Hierarchical cluster analysis (HCA) classifies similar units within the same cluster by calculating the values of the units observed in the research on all measured variables. The analysis focuses on the clusters and groups that will emerge, and the resulting clusters are expected to be homogeneous within themselves and heterogeneous among themselves [65]. In this study, nonpolar and polar fractions of the methanol extracts of four bee pollens were separately chemometrically classified for their identified chemical compounds by using PCA and HCA.

For nonpolar fractions of the methanol extracts of bee pollens, the score plot graphic is given in Fig. 1 and the loading plot graphic is given in Fig. 2. 1st component (PC1) was estimated for 21.8% and 2nd component (PC2) for 20.1% of total variance. By interpreting Figs. 1 and 2 together, it was determined that all nonpolar fractions differed according to the concentrations of their chemical compounds and there were five different groups in total. High concentrations of ferulic acid, vanillin, 6,7-dihydroxy coumarin and vanillic acid were effective in PD forming the 1st group. PEA was in the 2nd group with the highest contents of chrysin, rutin, coumarin and kaempferol. The 3rd group was consisted of CH, CD, HH, HD, PH, SH, SD related to *p*-hydroxy benzoic acid, quercetin, luteolin, apigenin and naringenin. The highest concentration of myricetin and levulinic acid caused SEA in the 4th group. CEA and HEA were grouped as the 5th group due to the highest amounts of rosmarinic, 2-hydroxy cinnamic, caffeic and *trans*-aconitic acids. Fig. 3 presents the dendrogram results of HCA for the nonpolar fractions of the methanol extracts of bee pollens. Two main clusters and four sub-clusters were attained according to the similarities and concentrations of the main characterized compounds in the nonpolar fractions of bee pollens. HEA was in the one main cluster whereas the other main cluster is other nonpolar fractions of bee pollens. The 1st sub-cluster was consisted of SEA with the higher concentration of myricetin than others. CD and CEA were in the 2nd sub-cluster due to similar concentration of luteolin and quercetin when

compared to other the nonpolar fractions. PEA was positioned in the 3rd sub-cluster with higher rutin and coumarin contents than the others. HH, HD, CH, PD, PH, SH, and SD were combined in the 4th sub-cluster due to the proximity of their kaempferol amounts.

For polar fractions of the methanol extracts of bee pollens, the score plot graphic is given in Fig. 4 and the loading plot graphic is given in Fig. 5. 1st component (PC1) was estimated for 27.6% and 2nd component (PC2) for 23.3% of total variance. By interpreting Figs. 4 and 5 together, it was determined that all polar fractions differed according to the concentrations of their chemical compounds and there were seven different groups in total. PB constituted the 1st group, as vanillin was identified only in this fraction. Since fumaric acid was identified only in PW2, this extract was called as the 2nd group. CW1, HW1, HW2, PW1, SW1, and SW2 were in the 3rd group because they contained similar chemical compounds. The identification of 2-hydroxycinnamic acid only in CW2 was effective in the formation of the 4th group. While the 5th group contained CB in connection with the highest concentration of rutin, the highest amounts of rosmarinic and *trans*-cinnamic acids provided HB in the 6th group. The presence of luteolin, myricetin and quercetin in SB led to the formation of the 7th group. Fig. 6 presents the dendrogram results of HCA for polar fractions from the methanol extracts of bee pollens. Two main clusters and six sub-clusters were attained according to similarities and concentrations of the main characterized compounds in the polar fractions of bee pollens. The 1st main cluster consists of SB and the 2nd main cluster consists of the other polar fractions of bee pollens. Additionally, 6 sub-clusters were given in the below:

1st sub-cluster: HB.

2nd sub-cluster: CW2.

3rd sub-cluster: CB.

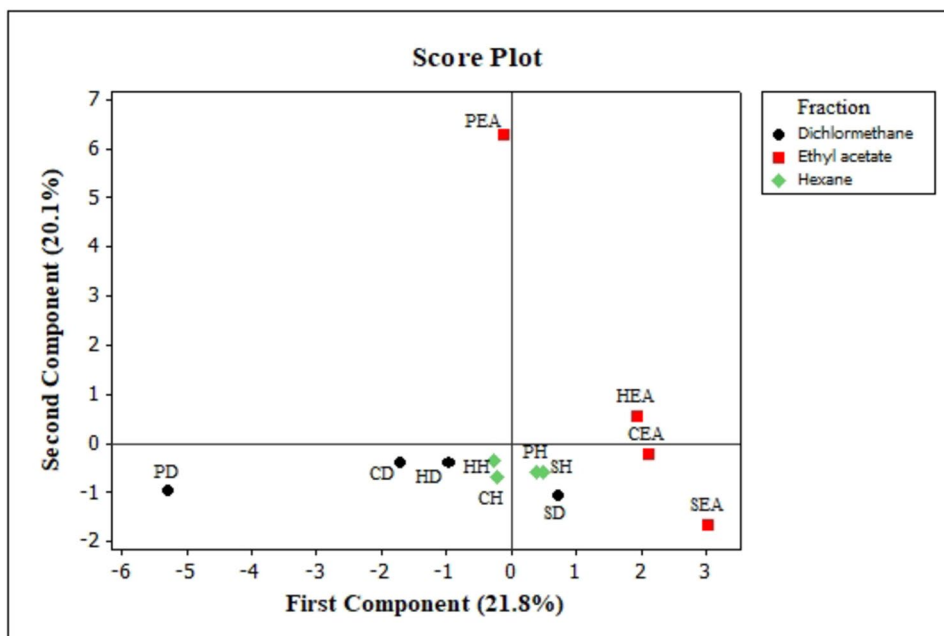
4th sub-cluster: SW1.

5th sub-cluster: PB and PW1.

6th sub-cluster: HW1, HW2, CW1, PW2 and SW2.

According to the chemometric analysis results, *C. creticus*, *H. annuus*, *P. somniferum*, and *Salix* sp. ethyl acetate fractions were separated from other fractions in nonpolar fractions. This may be due to the higher total amount of chemical compounds in the ethyl acetate fractions compared to the hexane and dichloromethane fractions. According to Alara et al. [48], provided that the existence of different effects is also considered, it is stated that increasing solvent polarity increases the solubility of chemical compounds and thus the extraction efficiency. *C. creticus*, *H. annuus*, *P. somniferum*, and *Salix* sp. butanol fractions were separated from other fractions in polar fractions in the chemometric analysis. Again, this may be due to the higher total amount of chemical compounds in the butanol fractions compared to the water fractions and extracts. Boeing et al. [66] reported that extraction with water was not as effective as extraction with organic solvent-water mixtures. Because the presence of water increases the permeability of the cell tissue, allowing better mass transfer by molecular diffusion and recovery of water-soluble chemical compounds. The differences in these bioactive compounds determined in polar and nonpolar fractions may provide an idea for predicting the biological effects of future studies aimed at these species.

Fig. 1 Score plot for PC1 and PC2 in the nonpolar fractions



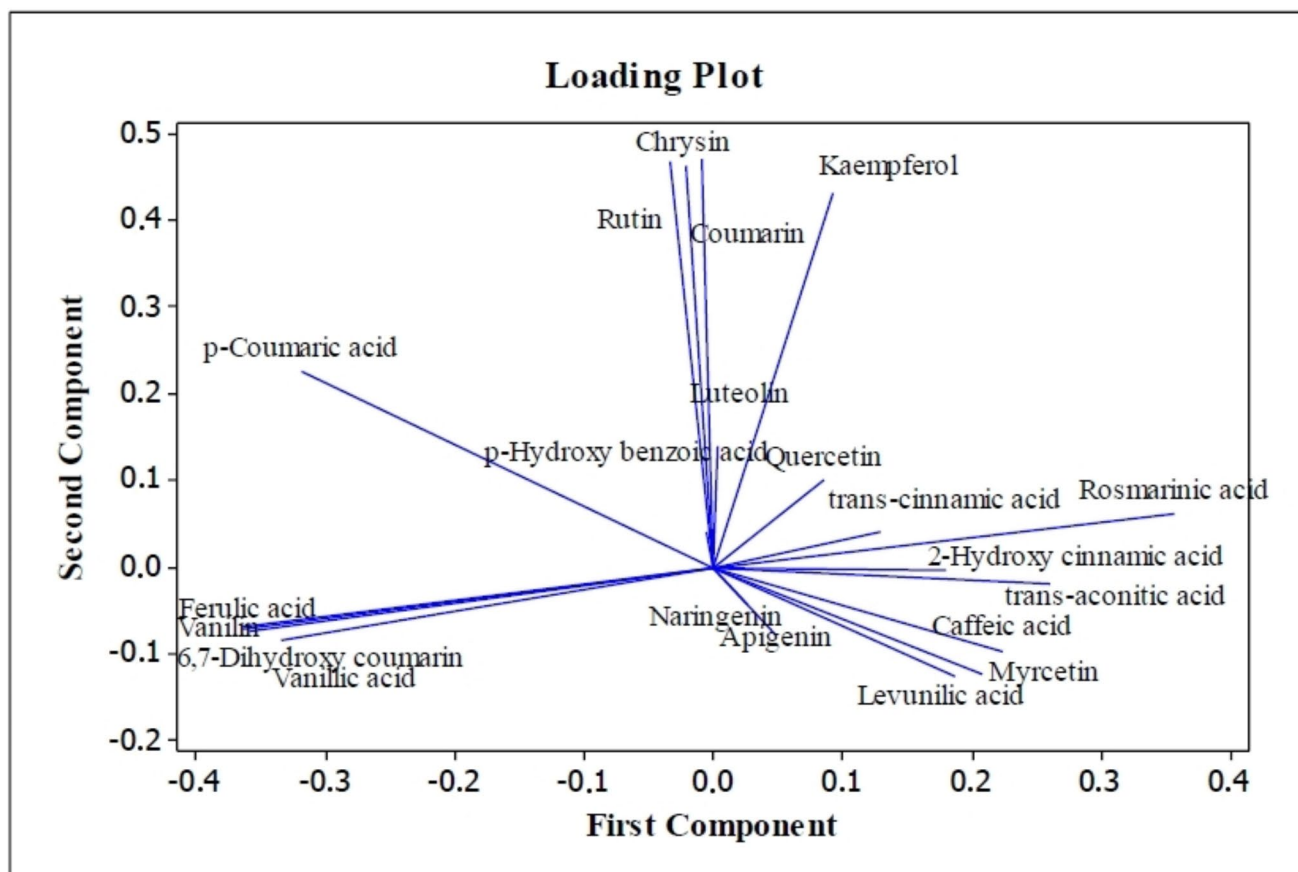


Fig. 2 Loading plot for PC1 and PC2 in the nonpolar fractions

Conclusion

Within the scope of this study, *C. creticus*, *H. annuus*, *P. somniferum* and *Salix* sp. bee pollens, water extracts and the fractions (hexane, dichloromethane, ethyl acetate, butanol, water) obtained from the methanol extracts of all bee pollens were investigated for chemical compounds by HPLC-DAD in detail. This study is the first to identify the chemical compounds of *C. creticus* bee pollen, water extract and fractions and, *H. annuus* and *P. somniferum* bee pollen water extracts and fractions. In these 28 different bee pollen samples studied in total, kaempferol, luteolin, *trans*-aconitic acid, myricetin, quercetin, rosmarinic acid, rutin, and *trans*-cinnamic acid were the most frequently identified compounds. All examined bee pollens, water extracts and fractions were found to be significantly rich in chemical compounds. Also, chemical compounds of nonpolar and polar bee pollen fractions were chemometrically clustered via PCA and HCA. Nonpolar and polar fractions were distinguished among themselves according to their chemical compounds, similarities and concentrations of these compounds. According to the chemometric analysis results, while *C. creticus*, *H. annuus*, *P. somniferum*, and *Salix* sp. ethyl acetate fractions

were separated from other fractions in nonpolar fractions, *C. creticus*, *H. annuus*, *P. somniferum*, and *Salix* sp. butanol fractions were separated from other fractions in polar fractions.

In conclusion, this study is the first to identify the chemical compounds of *C. creticus* bee pollen, water extract and fractions and, *H. annuus* and *P. somniferum* bee pollen water extracts and fractions. Since this study is one of the first chemical analyses of *C. creticus* pollen, further research on this species is necessary. The important chemical compounds characterized in the contents of *H. annuus*, *P. somniferum*, and *Salix* sp. bee pollens, fractions and extracts could provide a critical basis for future research on the biological potentials of these pollen species. Considering the chemical content of bee pollens, it can be considered that they can be used especially in the development of medicines and nutritional supplements.

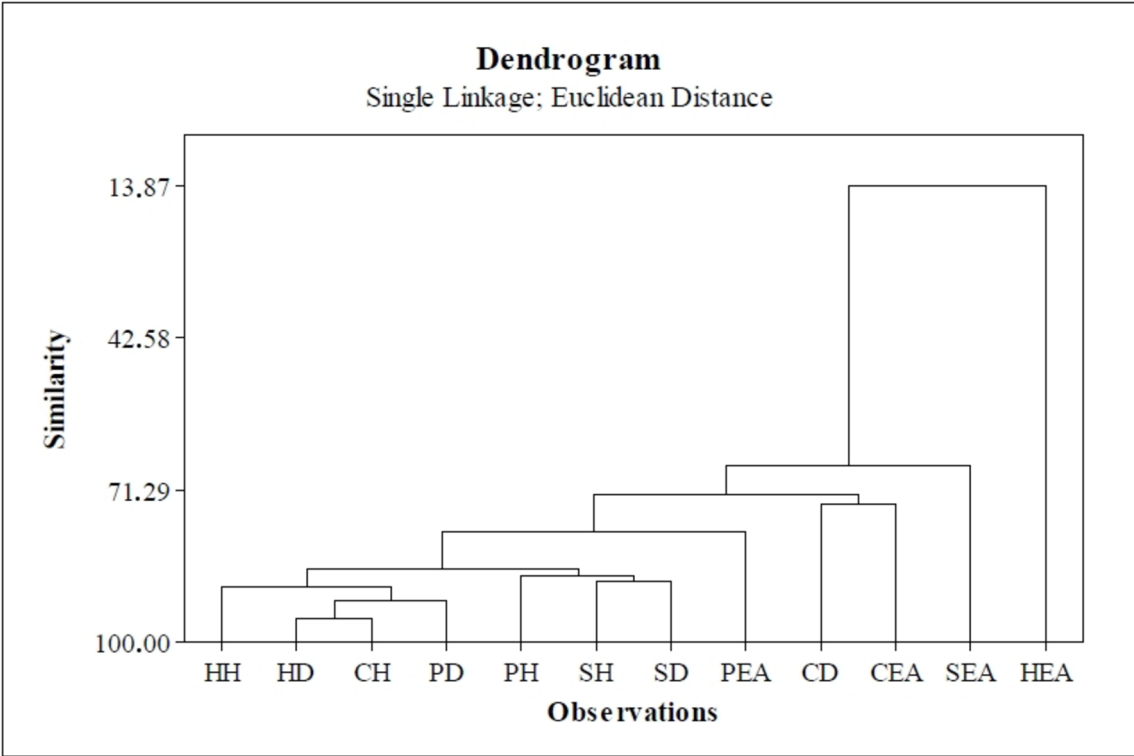


Fig. 3 Dendrogram results of the nonpolar fractions

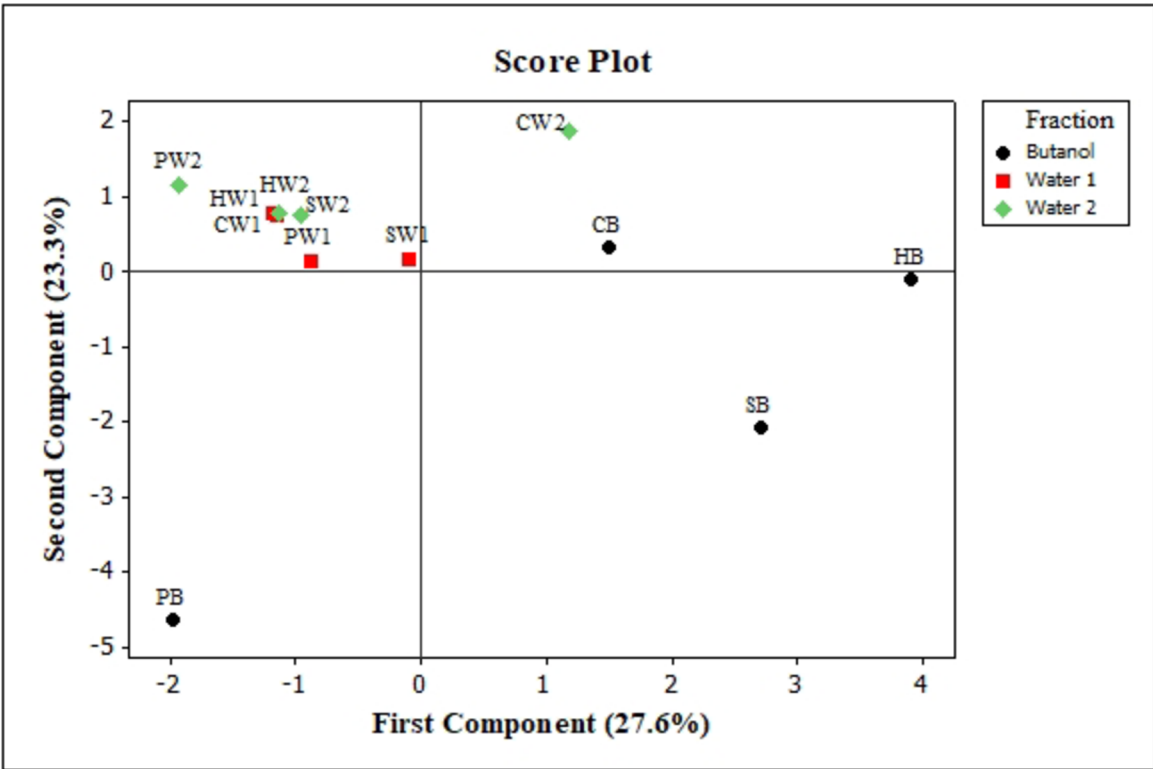


Fig. 4 Score plot for PC1 and PC2 in the polar fractions

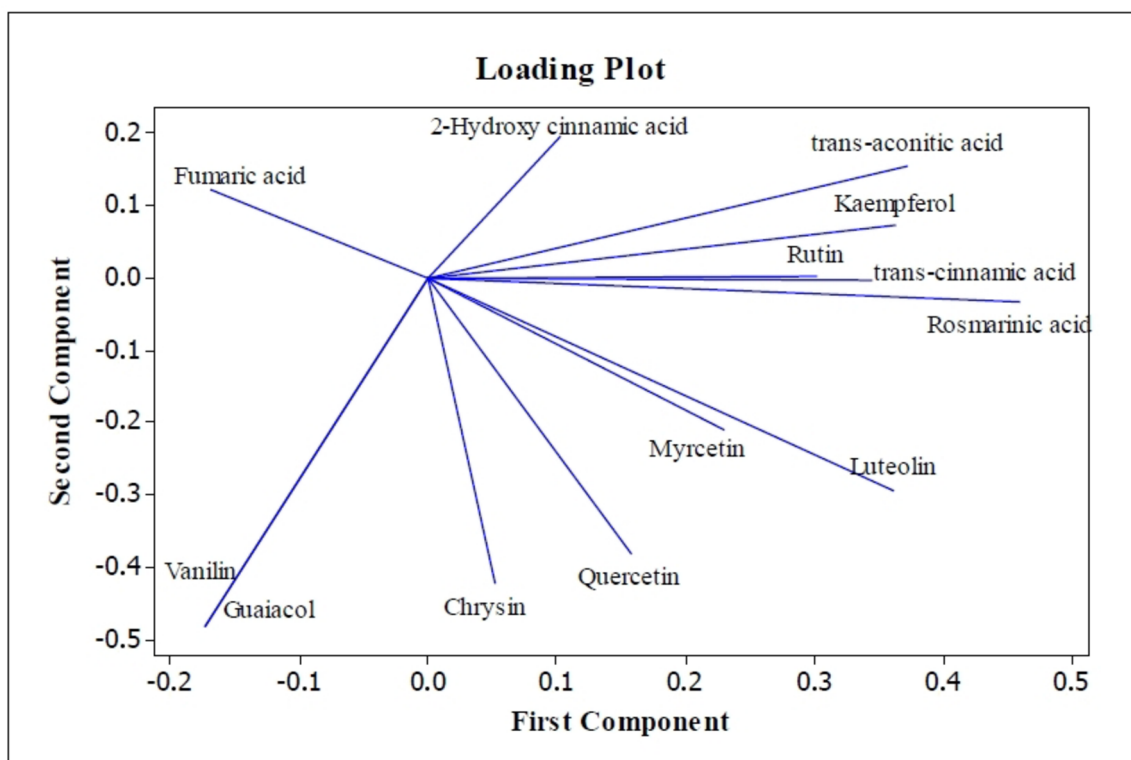


Fig. 5 Loading plot for PC1 and PC2 in the polar fractions

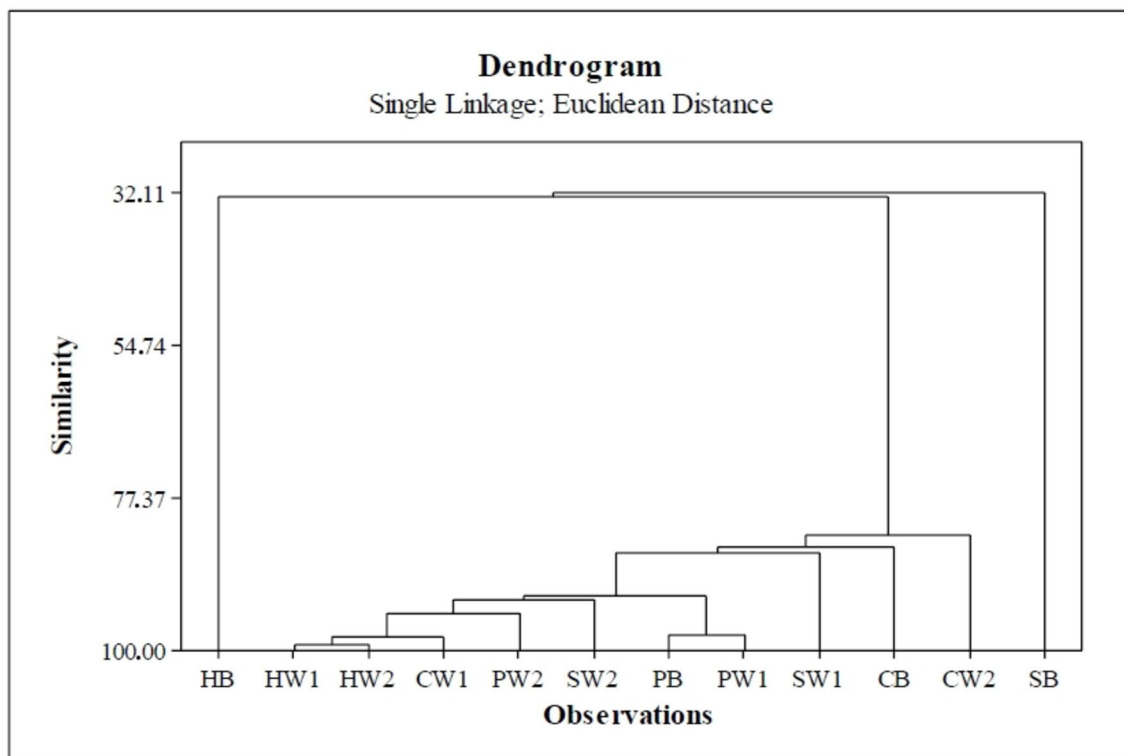


Fig. 6 Dendrogram results of the polar fractions

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors report no declarations of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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