



Determination of biologically active phenolic compounds in propolis by LC–MS/MS according to seasons and altitudes

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Abstract

The present study aimed to determine the effect of season and altitude to amount and prevalence of phenolic compounds in propolis. For the aim, 174 propolis samples were collected from 58 stationary apiaries from altitudes of 0–200 m, 200–500 m and above 500 m in spring, summer, and autumn distributed in the Marmara region of Turkey. Ethanol-extracted propolis samples were analyzed by LC–MS/MS to determine nine flavonoids and seven phenolic acids. Significant differences were found in average levels of galangin, naringenin, pinocembrin, quercetin, luteolin, caffeic acid, trans-cinnamic acid, p-coumaric acid, m-coumaric acid, and CAPE across the seasons and altitudes ($P < 0.05$). However, no differences were found levels of rutin, apigenin, kaempferol, ferulic acid and gallic acid ($P < 0.05$). Most of the significant differences were found between spring and autumn (10 differences). The prevalence of flavonoids was generally lower than phenolic acids. CAPE and pinocembrin had majority of the significant differences in phenolic compounds according to season and altitude. The prevalence of pinocembrin 100%, m-coumaric acid 99.4%, CAPE 99.4%, naringenin 99.4%, caffeic acid 98.9%, p-coumaric acid 98.3%, quercetin 96% and luteolin 95.4% were determined that dominant phenolic compound of Marmara Region of Turkey's propolis. Furthermore, *Salix* spp. 75.8%, *Quercus* spp. 74.4%, *Populus* spp. 72.4%, and *Pinus* spp. 53.5% were mainly determined source of propolis around the apiaries. These results show that different sampling of seasons and altitudes effect chemical composition of propolis even from the same area. The study indicated that seasons and altitudes are important factors for standardization of propolis.

Keywords Propolis · Standardization · Phenolic compounds · Season · Altitude · LC–MS/MS

Introduction

Propolis is an important natural product which is collected by honeybees from various plant's resin and bud exudates [1]. Propolis is used by the honey bees as a building and protection material, for instance, filling holes and cracks,

repairing of honeycombs in the hive, narrowing the hive entrance against intruders and cold weather [2]. Raw propolis is generally composed of 50% resin, 30% wax, 10% essential oil and aromatic compounds, 5% pollen, 5% terpenoids, steroids, amino acids and other compounds [3]. The chemical composition of propolis varies directly depend on the vegetation around apiaries [4]. Propolis has various biological activities owing to chemical composition that has been used for centuries, especially in traditional medicine [3]. Most of its biological activities are related to the presence of phenolic compounds in the resin [5–12].

Propolis standardization is important for global pricing and the determination of scientific effects but standardization of propolis could not be possible due to variables like regional vegetation, altitudes and seasons. Hence, it should require chemical analysis of different propolis constituents for determination of phenolic compounds [13–16]. Previous studies reported that seasonal variants could effect as a factor of different antioxidant, antibacterial and antitumor activities

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of propolis [17–27]. There are few numbers of reports proved effects of different altitude and season to chemical compositions and concentrations of propolis samples [22, 27, 28]. This study indicated influence both of seasons and altitudes on level, constituent, and prevalence of phenolic compounds in propolis. The objective of this study was to determine and quantitate the biologically active nine flavonoids and seven phenolic acids from 174 propolis specimens collected across the stationary apiaries distributed at different altitudes in three different seasons.

Materials and methods

Chemicals and reagents

Galangin (CAS-491-70-3) (CAS: Chemical Abstracts Service Number), quercetin hydrate (CAS: 849061-97-8), kaempferol (CAS: 520-18-3), gallic acid (CAS: 149-91-7) (\pm) naringenin (CAS: 67604-48-2), pinocembrin (CAS: 480-39-7), apigenin (CAS: 520-36-5), trans-cinnamic acid (CAS: 140-10-3), luteolin (CAS:491-70-3), p-coumaric acid (CAS: 501-98-4), m-coumaric acid (CAS: 14755-02-3), caffeic acid (CAS: 331-39-5), caffeic acid phenethyl ester (CAPE) (CAS: 104594-70-9), trans-ferulic acid (CAS: 537-98-4) and rutin (CAS: 250249-75-3) were purchased from Sigma-Aldrich® (Steinheim, Germany). HPLC grade acetonitrile, methanol, ethanol and analyzes quality formic acid were buy in Merck® (Darmstadt, Germany). Ultrapure water was obtained from ELGA® LabWater, Purelab flex.

Propolis samples

In the current study, 58 selected stationary apiaries located which 28 of them at 0–200 m altitudes, 17 of them between 200 and 500 m altitudes and 13 of them above 500 m altitudes. In total, 174 propolis samples were collected in spring (March, April, and May), summer (June, July, and August) and autumn (September, October, and November) from the apiaries in Marmara region of Turkey. Propolis specimens could not be collected during the winter season because of cold weather of sampling region. Coordinates of apiaries are shown in Fig. 1. Vegetation around apiaries was recorded in order to estimate the source of propolis (Supplementary Table 1). Seasonal propolis samples were collected by using plastic propolis traps shown in Supplementary Fig. 1 (Civan Incorporation, Turkey). The propolis samples were stored at +4 °C until extraction procedure (Supplementary Fig. 2).

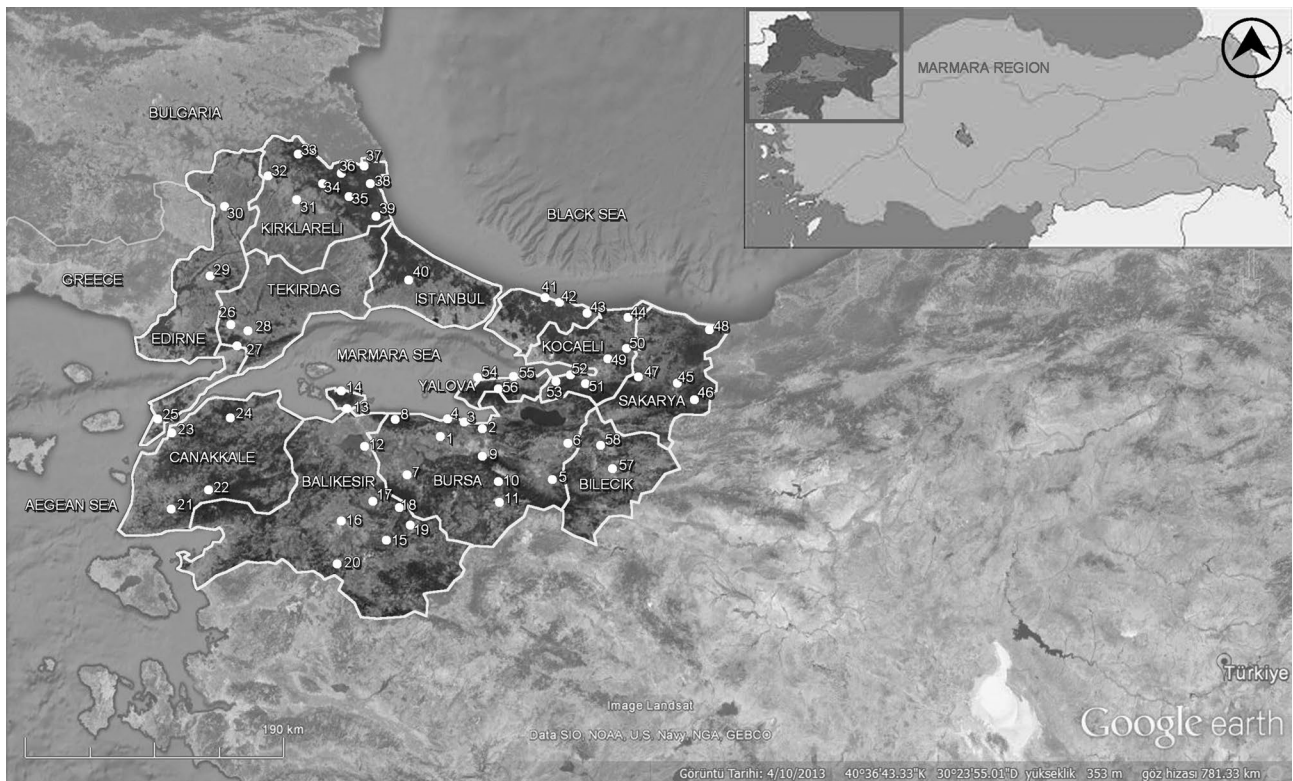
Extraction

The extraction method was carry out as defined by Erdogan et al. [29] and Trusheva et al. [30]. Propolis traps

were frozen at –20 °C in order to remove the propolis from traps. Each sample was classified by color, odor, and amount. A grater was used to break the propolis samples into small pieces and finally, a coffee bean grinder was used for homogenizing propolis (DeLonghi Kg 49, UK). Two grams of the propolis were weighed and added to 20 mL 70% Ethanol/water (v/v), shook with using a shaker (Nüve SL-35, Turkey) for an hour, then followed by ultrasonication (Bandelin Sonorex RK100, Germany) for 30 min. The mixture was filtered using filter papers (Watman No: 1, UK) in order to remove wax and bee parts. Finally, the filtrate was poured into glass tubes (tared tube) and evaporated using a vacuum centrifuge (Jouan, RC 10-10) (Supplementary Fig. 3). The obtained dried propolis ethanoic extract (DPEE) was weighed and stored at –20 °C until LC–MS/MS analyses (Supplementary Fig. 3).

Validation

The main stock solution of each phenolic standard prepared as 1 mg/mL by dissolving in methanol (0.1 formic acid). In order to determine phenolic compound's precursor ion (MS), fragment ion (MS/MS), retention time, collision energy and capillary voltage that each phenolic compound was performed by injection to the LC–MS/MS. Mass spectrometry was performed using SIM under a negative ion mode (Fig. 2). Mix phenolic compounds (MFC) solution was prepared from main stock solutions. MFC diluted with the methanol to prepare for 50, 25, 12.5, 6.75, 3.37 $\mu\text{g/mL}$ concentration in order to draw calibration curves. Each phenolic compound's calibration was curve plotted by linear regression (R^2) that shown in Table 1. The limit of detection (LOD) and limit of quantitation (LOQ) of the method realized by MFC serial dilution and by using the equations 3 S/N (signal to noise ratio) and 10 S/N , respectively. Phenolic compound's LOD ranged from 0.21 to 0.84 $\mu\text{g/mL}$ and LOQ ranged from 0.63 to 2.52 $\mu\text{g/mL}$ in the method. Four different kind of propolis samples were randomly selected for the identification of recovery. This propolis samples spiked which three different levels with MFC (20, 10, 5 $\mu\text{g/g}$). Recovery of the phenolic compounds was found out between 78 and 118% which differences of spike and blank propolis samples by analyzing (Table 1). MFC is analyzed five different days as five times for accuracy and repeatability of the method. The coefficient of variation (relative standard deviation: RSD) in result defined less than 4.3% and in retention time RSD less than 2%. The precision of the method was evaluated by repeat the measurements of MFC. Good precision was determined and the results were implemented to the uncertainty budget.



1-N40°13.513"E28°40.300" İkizce	21-N39°35.685"E26°28.963" Ayrıcalık	41-N41°08.994"E29°38.028" Çayırbaşı
2-N40°16.442"E29°08.115" Dedeçavuş	22-N39°47.352"E26°46.245" Evciler	42-N41°08.977"E29°38.028" Yeniköy
3-N40°20.703"E28°56.419" Güzelyalı	23-N40°10.105"E26°17.730" Sarıcaali	43-N41°07.986"E29°49.617" Kurfalı
4-N40°23.827"E28°46.169" Triliye	24-N40°15.309"E26°55.675" Beyçayır	44-N41°03.810"E30°11.942" Kandıra
5-N39°56.965"E29°39.119" Tahtaköprü	25-N40°10.105"E26°17.730" Ecebat	45-N40°38.384"E30°39.966" Akyazı-1
6-N40°11.927"E29°46.314" Gökçesu	26-N40°52.192"E26°51.251" Malkara	46-N40°39.341"E30°37.887" Akyazı-2
7-N39°59.336"E28°25.221" Orhaniye	27-N40°46.563"E26°57.240" Elmalı-1	47-N40°41.988"E30°12.538" Sapanca
8-N40°18.199"E26°17.625" Örencik	28-N40°48.056"E26°57.572" Elmalı-2	48-N40°56.045"E30°51.628" Sungüt
9-N40°05.433"E29°04.390" Kirazlı	29-N41°10.892"E26°38.988" Kavacık	49-N40°47.563"E30°12.564" Ortaburun
10-N39°50.197"E29°12.116" Kemaliye-1	30-N41°25.283"E26°45.854" Aslıhan	50-N40°47.925"E30°10.983" Kurtdere
11-N39°49.861"E29°13.278" Kemaliye-2	31-N41°44.056"E27°20.231" Yündalan	51-N40°42.155"E29°47.342" Gölçük
12-N40°07.772"E28°03.583" Ergilli	32-N41°52.563"E27°04.310" Çayırli	52-N40°42.254"E29°41.560" Ulaşlı
13-N40°21.790"E27°55.249" Bandırma	33-N42°00.235"E27°17.694" Kula	53-N40°38.767"E29°12.858" Tepeköy
14-N40°29.919"E27°47.114" Kapıdağ	34-N41°52.588"E27°30.466" Armutveren	54-N40°37.965"E29°00.577" Şenköy
15-N39°32.459"E28°16.301" Mezitler	35-N41°49.823"E27°49.360" Demirköy	55-N40°38.767"E29°12.857" Samanlı
16-N39°37.198"E28°14.063" Sarfaklar	36-N41°52.588"E27°30.466" Avcılar	56-N40°36.806"E29°09.996" Termal
17-N39°34.618"E28°18.823" Tuzak	37-N41°52.472"E27°55.855" Yğneada	57-N40°04.559"E30°00.259" Bekdemir
18-N39°44.736"E28°20.350" Gökköy	38-N41°58.343"E27°54.910" Sisioba	58-N40°13.023"E29°52.144" İlyasbey
19-N39°47.005"E28°27.679" Yaylıbaşı	39-N41°38.530"E28°05.232" Kırıyköy	
20-N39°26.541"E27°56.889" Akçaköy	40-N41°15.696"E28°21.332" İhsaniye	

Fig. 1 The locations and global positioning system (GPS) coordinates in the Marmara region of Turkey where the propolis samples were collected

LC–MS/MS analyses

The analyses were basically conducted as described by Pelati et al. [31]. DPEE was dissolved as a 1/60 ratio in methanol, filtered with PVDF (polyvinyl difluoride) syringe filter (Millipore Millex-HV, 0.45 μm), and finally 5 μL injected into the LC–MS/MS ($n=3$). Analyses were performed using a Zivak HPLC and Zivak Tandem Gold Triple quadruple mass spectrometry equipped with a 460 autosampler, 212 pump and separation with *C18* column (150 \times 2.1 mm ID, 3 μm , Phenomenex, Germany). The column was eluted using a linear gradient. Mobile phases with a flow rate of 1.2 mL/min which are mobile phase A (0.1% formic acid in water)

and mobile phase B (0.1% formic acid in acetonitrile). The gradient elution was applied; 20–30% B at 0–5 min, 30–45% B at 5–6.18 min, 45–50% B at 6.18–10 min, 50–83% B at 10–11.59 min, 83–100% B at 11.59–17.29 min, 100–20% B at 17.29–19 min and 20% B at 19–25 min. The column temperature was set at +30 $^{\circ}\text{C}$. Mass spectrometry was performed using SIM under a negative ion mode by the Tandem Gold Quan 42 (Thermo Scientific). Helium at 34 psi was used as a drying gas, and nitrogen was used as the nebulizer gas at 55 psi. Details of the, precursor (main) ion, product (fragment) ion and method parameters (collision energy, capillary voltage) were also presented in Table 1, the spectra of phenolic compounds presented with Supplementary

Fig. 2 Fragment ions spectra of phenolic compounds. *CAPE* caffeic acid phenethyl ester, *GA* gallic acid, *RT* rutin, *CA* caffeic acid, *t-FA* trans-ferulic acid, *m-COU* m-coumaric acid, *p-COU* p-coumaric acid, *LT* luteolin, *KF* kaempferol, *QE* quercetin, *NR* naringenin, *AP* apigenin, *GL* galangin, *t-CA* trans-cinnamic acid, *PN* pinocembrin

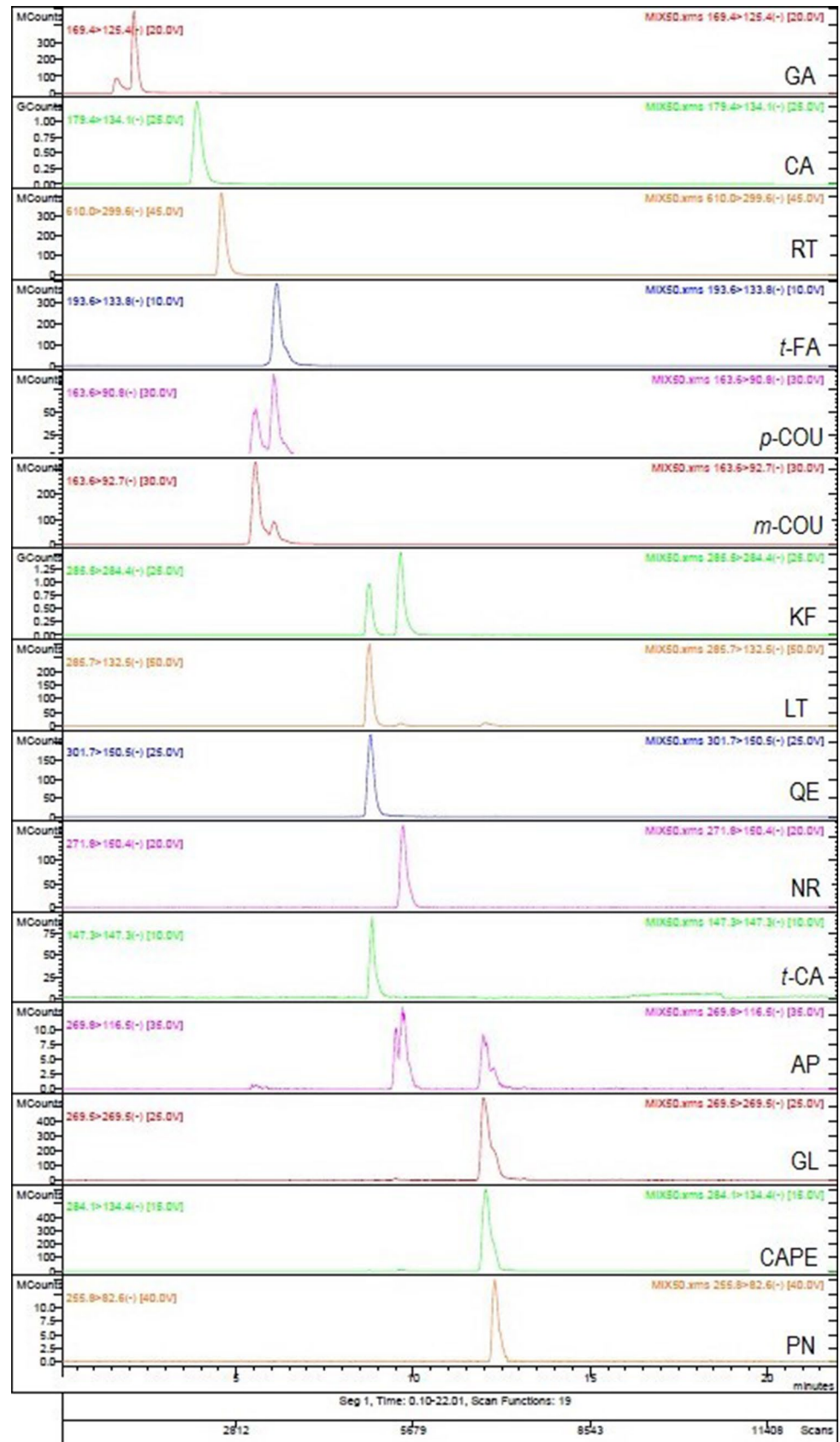


Table 1 The precursor (main) ion, fragment (daughter) ion MS/MS and method parameters of phenolic compounds

Phenolic compounds	RTm	R ²	RCV %	Precursor (main) ion (MS) m/z	Fragment (daughter) ion MS/MS	CV	CE (V)
GA	2.115	0.998	83	169.4	125.4	−40.0	20.0
RT	4.592	0.997	78	610	299.6	−80.0	45.0
CA	3.898	0.994	88	179.4	134.1	−30.0	25.0
<i>t</i> -FA	5.926	0.998	75	193.6	133.8	−40.0	10.0
<i>m</i> -COU	5.762	0.993	81	163.6	90.8	−40.0	30.0
<i>p</i> -COU	5.849	0.999	85	163.6	92.7	−40.0	30.0
KF	7.096	0.994	101	285.5	284.4	−60.0	25.0
LT	7.102	0.975	70	285.7	132.5	−70.0	50.0
QE	7.125	0.991	97	301.7	150.5	−40.0	25.0
NR	7.763	0.990	77	271.8	150.4	−20.0	20.0
<i>t</i> -CA	7.189	0.999	118	147.3	147.3	−60.0	10.0
AP	7.580	0.989	106	269.8	116.5	−80.0	35.0
GL	12.031	0.967	99	269.5	269.5	−70.0	25.0
CAPE	12.05	0.986	96	284.1	134.4	−80.0	15.0
PN	12.307	0.989	95	255.8	82.6	−80.0	40.0

GA gallic acid, RT rutin, CA caffeic acid, *t*-FA *trans*-ferulic acid, *m*-COU *m*-coumaric acid, *p*-COU *p*-coumaric acid, KF kaempferol, LT luteolin, QE quercetin, NR naringenin, *t*-CA *trans*-Cinnamic acid, AP apigenin, GL galangin, CAPE caffeic acid phenethyl ester, PN pinocembrin, RTm retention time, R² correlation coefficients, RCV recovery, CV capillary voltage, CE collision energy

Fig. 4 and propolis sample spectra were shown in Supplementary Fig. 5. Data acquisition was carried out using Varian MS Workstation Method Builder 6.9.3 (Walnut Creek, CA, USA).

Statistical analyzes

LC–MS/MS data were analyzed using Mixed Linear Model. Chi-square Test was used to compare prevalence of data. Differences were considered significant probability level of $P < 0.05$. All statistical analyses were carried out by using SPSS (Version 20.0) software for Windows (SPSS Inc., Chicago, IL, USA).

Results and discussion

Sampled propolis specimens had different natural colors such as yellow, green, brown, reddish, orange and blackish, and their appearances were bright and matt with sticky and not-sticky textures as shown Supplementary Fig. 6. The propolis amounts obtained from a propolis trap ranged from 15 to 285 g for each season. When the propolis sources insufficient, honey bees filled the sides of the traps with resin and filled the middle of the traps with wax (Supplementary Fig. 1). Totally, 15 phenolic compounds were determined in propolis samples and measured by using LC–MS/MS. Statistical results and mean of phenolic compounds concentrations of propolis samples across seasons and at different altitudes were presented in Table 2.

Fifteen phenolic compounds result of 174 samples and information of 58 apiaries were presented in Supplementary Table 2. Generally, the highest levels of phenolic compounds were seen the number of 3, 45 and 46 apiaries (Supplementary Table 1).

The prevalence of flavonoids were generally lower than of phenolic acids. Pinocembrin is a flavonoid which was the most common phenolic compound found in all location. The prevalence (as percentage) of phenolic compounds were defined as pinocembrin 100%, *m*-coumaric acid 99.4%, CAPE 99.4%, naringenin 99.4%, caffeic acid 98.9%, *p*-coumaric acid 98.3%, quercetin 96%, luteolin 95.4%, ferulic acid 94.8%, galangin 90.8%, *trans*-cinnamic acid 84.5%, kaempferol 81%, apigenin 57.5%, gallic acid 55.7%, and rutin 44.3% (Supplementary Table 3). The seasonal prevalence of kaempferol and *t*-cinnamic acid in spring compared to autumn were determined significantly higher ($P < 0.05$) (Supplementary Table 3). The seasonal prevalence of naringenin in spring and autumn compared to summer were determined significantly higher ($P < 0.05$) (Supplementary Table 3). The seasonal prevalence of rutin in autumn compared to spring were determined significantly higher ($P < 0.05$) (Supplementary Table 3). Inter-seasonal prevalence of altitudes; rutin in spring according to autumn in 0–200 m, apigenin in summer according to autumn in 500 m and above and gallic acid in spring according to summer and autumn in 500 m and above were determined significantly higher ($P < 0.05$) (Supplementary Table 3). However, there is no significant difference according to seasons in 200–500 m altitude

Table 2 Statistical analyses of the average levels of phenolic compounds across seasons and altitudes

Phenolic Compounds	Altitudes	Between 0 and 200 m (n=84)		Between 200 and 500 m (n=51)		Between 500 m and above (n=39)		Inter altitude P
		Seasons (n = 58)	Mean + S.E.	Inter-season P	Mean + S.E.	Inter-season P	Mean + S.E.	
AP	Spring	2629.37 ± 600.45	0.500	2736.23 ± 595.60	0.474	2981.96 ± 686.84	0.101	0.934
	Summer	2281.11 ± 518.19		1759.27 ± 516.40		1704.49 ± 486.21		0.433
	Autumn	3413.36 ± 870.70		2422.13 ± 874.92		1267.42 ± 474.32		0.382
CAPE	Spring	2933.25 ^{ab} ± 263.01	0.049	2769.28 ^{ab} ± 349.29	0.033	2699.53 ^a ± 340.60	0.022	0.857
	Summer	2640.52 ^a ± 240.79 ^B		2394.05 ^a ± 408.99 ^B		3939.36 ^b ± 259.35 ^A		0.02
	Autumn	3281.93 ^b ± 421.83		3479.50 ^b ± 384.72		3857.05 ^b ± 424.01		0.14
t-FA	Spring	2554.43 ± 377.06	0.471	2506.46 ± 499.93	0.09	2406.46 ± 424.67	0.875	0.974
	Summer	2588.52 ± 390.18		2103.45 ± 489.72		2536.59 ± 328.93		0.726
	Autumn	2209.17 ± 335.27		1520.64 ± 393.06		2357.63 ± 465.36		0.421
GA	Spring	56.03 ± 21.43	0.08	48.90 ± 19.70	0.929	148.97 ± 120.95	0.345	0.404
	Summer	117.40 ± 41.18		55.67 ± 15.79		23.28 ± 8.26		0.15
	Autumn	117.89 ± 36.82		53.06 ± 16.01		19.13 ± 7.76		0.321
GL	Spring	4457.80 ^a ± 664.97	0.008	3419.49 ± 562.09	0.943	3327.32 ± 502.80	0.666	0.37
	Summer	5259.56 ^{ab} ± 856.56		3245.98 ± 439.37		3776.15 ± 507.76		0.15
	Autumn	5872.01 ^b ± 1042.05 ^A		3190.17 ± 726.65 ^B		3912.31 ± 512.06 ^B		0.025
CA	Spring	4715.16 ± 475.28	0.317	4551.07 ± 666.09	0.349	3999.01 ^a ± 506.80	0.004	0.685
	Summer	4725.54 ± 556.67		4616.27 ± 738.08		4790.78 ^{ab} ± 448.55		0.64
	Autumn	4310.26 ± 517.26		3870.84 ± 520.74		5491.63 ^b ± 460.03		0.725
KF	Spring	212.11 ± 61.73	0.736	255.07 ± 62.54	0.680	223.75 ± 43.54	0.599	0.88
	Summer	280.24 ± 88.30		214.82 ± 64.55		283.88 ± 63.46		0.794
	Autumn	206.44 ± 60.70		187.33 ± 46.97		303.09 ± 82.03		0.73
QE	Spring	3509.21 ^a ± 479.18	0.040	3163.17 ± 484.27	0.806	2802.12 ± 585.741	0.072	0.649
	Summer	4537.36 ^b ± 698.71		2874.18 ± 434.61		3775.80 ± 418.90		0.217
	Autumn	4225.19 ^{ab} ± 600.81 ^A		2854.47 ± 582.73 ^B		4189.05 ± 819.81 ^{AB}		0.035
LT	Spring	332.01 ^a ± 70.64	0.011	300.78 ± 64.38	0.511	233.04 ± 36.26	0.065	0.628
	Summer	427.75 ^{ab} ± 85.63		357.34 ± 69.819		401.38 ± 38.70		0.747
	Autumn	464.55 ^b ± 78.72		315.52 ± 48.09		461.71 ± 98.53		0.378
m-COU	Spring	743.86 ± 108.83 ^A	0.394	696.21 ± 126.84 ^{AB}	0.290	310.90 ± 56.43 ^B	0.263	0.037
	Summer	647.72 ± 93.69		547.79 ± 119.05		426.4 ± 77.41		0.4
	Autumn	611.60 ± 101.61 ^A		550.01 ± 102.71 ^A		441.15 ± 84.82 ^B		0.03
NR	Spring	1068.56 ± 97.15	0.08	897.12 ± 82.63	0.507	867.39 ^a ± 69.44	0.010	0.254
	Summer	1061.46 ± 103.02		859.98 ± 89.39		1056.95 ^b ± 59.23		0.344
	Autumn	1192.74 ± 110.70 ^A		781.49 ± 118.44 ^B		949.18 ^{ab} ± 79.22 ^{AB}		0.005
PN	Spring	8056.99 ^a ± 771.99	0.055	6506.91 ^a ± 823.26	0.049	6399.22 ± 978.25	0.458	0.281
	Summer	7878.48 ^{ab} ± 1000.35 ^A		4779.62 ^b ± 547.25 ^B		6041.38 ± 686.55 ^{AB}		0.05
	Autumn	7180.38 ^b ± 936.69 ^A		5395.64 ^{ab} ± 916.61 ^B		5862.82 ± 820.04 ^{AB}		0.006
p-COU	Spring	2445.12 ± 327.30 ^A	0.588	2028.93 ± 402.87 ^{AB}	0.224	1100.49 ^a ± 184.55 ^B	0.046	0.041
	Summer	2175.43 ± 302.89		1891.55 ± 370.75		1599.46 ^{ab} ± 193.98		0.662
	Autumn	2385.92 ± 317.85 ^A		1552.43 ± 322.93 ^B		1764.15 ^b ± 271.11 ^B		0.001
RT	Spring	142.00 ± 100.00	0.183	90.10 ± 45.67	0.113	139.06 ± 65.24	0.104	0.907
	Summer	20.80 ± 5.64		27.29 ± 15.73		22.79 ± 11.14		0.885
	Autumn	10.04 ± 5.23		21.48 ± 10.21		22.32 ± 8.17		0.947
t-CA	Spring	2029.08 ^a ± 284.05	0.003	2025.40 ^a ± 479.42	0.022	2155.39 ± 610.22	0.704	0.975
	Summer	1686.070 ^{ab} ± 261.71		1198.56 ^b ± 323.34		2099.53 ± 342.64		0.394
	Autumn	1354.40 ^b ± 251.30		1102.13 ^b ± 354.20		1809.33 ± 361.50		0.206

SE standard error, AP apigenin, CAPE caffeic acid phenethyl ester, FA *trans*-ferulic acid, GA gallic acid, GL galangin, CA caffeic acid, KF kaempferol, QE quercetin, LT luteolin, m-COU *m*-coumaric acid, NR naringenin, PN pinocembrin, p-COU *p*-coumaric acid, RT rutin, t-CA *trans*-cinnamic acid

^{a,b}Different superscripts indicate statistical difference at the same row

^{A,B}Different capital letters indicate statistical difference at the same line

Bold data is statistical differences

(Supplementary Table 3). Although there were differences in the general comparison of seasons and inter-seasonal of the altitudes prevalence, no statistical differences were found general comparison of altitudes and inter-altitudes of the season's prevalence ($P < 0.05$) (Supplementary Table 3). Andelkovic et al. [17]. And Goethevac et al. [21] determined higher of prevalence flavonoids in low altitudes and phenolic glycerides in high altitudes. In the current study, there was no difference about prevalence flavonoid or phenolic acid of the low altitudes.

The common determined plants around the apiaries which are probable of propolis sources were *Salix* spp., *Populus* spp., *Quercus* spp., *Tilia* spp., *Pinus* spp., *Castanea* spp. and *Juglans* spp. as shown in Supplementary Table 1 (respectively). In temperate climate zones which middle of Europe, part of Asia and North America the main sources of propolis are different poplar bud exudate. Moreover, in this zone's predominant phenolics are flavonoid aglycones (quercetin, pinocembrin, naringenin, kaempferol, galangin etc.) and hydroxycinnamic acids and esters (CAPE, ferulic acid, caffeic acid, p-coumaric acid, m-coumaric acid etc.) [2, 32]. Similarly, *Populus* spp. (poplar) were identified as one of the most common propolis sources (available 42 of 58 constant apiaries) in the present study (Supplementary Table 1). This parallel finding is probably related to the location of Turkey as it is between Europe and Asia. In addition, the highest levels of among the phenolic compounds were galangin, caffeic acid and pinocembrin (Table 2). When the results of the present study were compared to the previous studies in Turkey; Aliyazıcıoğlu et al. [33] indicated the presence of caffeic acid (between 1446.8 and 4658.1 $\mu\text{g/g}$), p-coumaric acid (between 381.7 and 4579.8 $\mu\text{g/g}$) and gallic acid (between 8.7 and 70.1 $\mu\text{g/g}$) levels were lower; ferulic acid (between 223.3 and 7126.9 $\mu\text{g/g}$) and rutin (between 32.9 and 550.4 $\mu\text{g/g}$) were similar; and quercetin (between 776.2 and 16,516.8 $\mu\text{g/g}$) levels were higher than the present study's result (Table 2) [33]. In addition, another study reported levels of the following phenolics as: caffeic acid (between 284 and 546 $\mu\text{g/g}$), p-coumaric acid (between 17 and 88 $\mu\text{g/g}$), gallic acid (between 13 and 35 $\mu\text{g/g}$) and luteolin (between 31 and 79 $\mu\text{g/g}$), which were lower than the present study's result (Table 2).

Significant differences were found in the mean levels of pinocembrin, galangin, naringenin, luteolin, quercetin, caffeic acid, p-coumaric acid, trans-cinnamic acid, m-coumaric acid and CAPE in propolis across the seasons and altitudes ($P < 0.05$). However, no differences were found mean levels of rutin, apigenin, kaempferol, ferulic acid and gallic acid as shown in Table 2.

There were 10 significant differences found in same season according to different altitudes which six of them in autumn, two of them in spring and two of them in summer (presented in the inter altitude column of Table 2). There

were 15 significant differences also found in same altitudes according to different seasons which eight of them spring and autumn, five of them between spring and summer, and two of them between summer and autumn (presented in the inter season columns of Table 2). Majority of the significant differences were determined CAPE and PN in the phenolic compounds as shown in Table 2

Evaluation of spring according to altitudes: The amount of m-COU acid and p-COU acid decreased significantly from 0–200 to 500 m and above altitudes ($P = 0.037$ and $P = 0.041$, respectively) as shown in Table 2.

Evaluation of summer according to altitudes: There was a considerable increase in the amount of CAPE from 0–200 to between 200–500 and 500 m and above altitudes ($P = 0.02$); however, PN decreased appreciably from 0–200 m to between 200–500 m altitudes ($P = 0.05$) as shown in Table 2.

Evaluation of autumn according to altitudes: The amount of m-COU decreased considerably from 0 to 200 and between 200–500 and 500 m and above altitudes ($P = 0.03$); NR and PN decreased significantly from 0–200 to 200–500 m altitudes ($P = 0.005$ and $P = 0.006$, respectively), and in addition GL and p-COU decreased considerably from 0–200 to between 200–00 and 500 m and above altitudes ($P = 0.025$ and $P = 0.001$, respectively) as shown in Table 2.

Evaluation of between 0 and 200 m altitudes according to seasons: There was an important increase in the amount of CAPE from summer to autumn ($P = 0.049$); GL and LT from spring to autumn ($P = 0.008$ and $P = 0.011$, respectively), in addition to considerable increase the amount of QE from spring to summer ($P = 0.04$). Moreover, the amount of PN and t-CA decreased significantly from spring to autumn ($P = 0.05$ and $P = 0.003$, respectively) as shown in Table 2.

Evaluation of between 200 and 500 m altitudes according to seasons: There was a considerable increase in the amount of CAPE from summer to autumn ($P = 0.033$); However, the amount of PN decreased considerably from spring to summer ($P = 0.049$); t-CA from spring to summer and autumn ($P = 0.022$) as shown in Table 2.

Evaluation of 500 m and above altitudes according to seasons: There was a significant increase in the amount of CAPE and from spring to summer and autumn ($P = 0.022$); CA from spring to autumn ($P = 0.004$); NR from spring to summer ($P = 0.01$); and moreover p-COU from spring to autumn ($P = 0.046$) as shown in Table 2.

Generally, evaluation of the altitudes according to seasons that 0–200 m attitudes in significantly results higher than other altitudes (except CAPE). Besides general evaluation of the seasons according to altitudes which 0–200 m altitudes (except PN and t-CA), and 500 m and above altitudes in autumn were significant results higher than other seasons but in 200–500 m altitudes of spring was significantly result higher than other seasons (except CAPE) as shown in Table 2.

There are some reports related to season and altitude (particularly season) and propolis chemical composition and concentrations of phenolic compounds [22–24, 26, 27].

Although these reports have precious scopes and data, they have comparable narrower scope for chemical composition and concentrations of phenolics according to season and altitude (particularly for concentrations of phenolics for altitude). Oruç et al. [14] analyzed two difference altitudes and three seasons of phenolic compounds in 15 samples collected from Bursa in 2012 which 0–600 m and above 600 m, in summer and autumn seasons. Depend on the season; galangin, naringenin, and p-coumaric acid, CAPE phenolic compounds values were found to be higher in the autumn than in the summer. These results differ from the present study's results except for CAPE. Depending on the altitude, galangin, naringenin and p-coumaric acid were determined to be higher than values of above 600 m; these results are generally similar to the present results. Cinnamic acid was found to be higher than the values of above 600 m. In the current study, there was no difference in cinnamic acid values depending on altitude.

Nunes and Guerreiro [23] and Teixeira et al. [26] studies similar to present study result that seasonality influences the chemical composition of propolis. Moreover, Isla et al. [22] stated that the antioxidant activity of propolis depended on the collection month and they detected that propolis had the highest antioxidant capacity in November in Argentina. Nunes et al. [34] also reported that phenolic content of Brazilian red propolis had the highest amounts in February, June, and October, respectively. The study's results concur with the changes of propolis chemical amounts depending on the collection seasons. Besides season, we found out that there were remarkable changes in phenolic compound levels of propolis related to changes in the altitudes. On the other hand, Valencia et al. [27] reported that seasons did not have a significant effect on the major chemical constituents of Sonoran propolis in Mexico, but they reported that seasons influenced anti-proliferative and antioxidant activity. Sforcin et al. [24] also reported that seasons did not affect the antibacterial activity of propolis.

Conclusion

The study indicated that amounts of CAPE, quercetin, luteolin, trans-cinnamic acid, pinocembrin, caffeic acid, naringenin and p-coumaric acid were significantly changed seasonally and m-coumaric acid, p-coumaric acid, CAPE, pinocembrin, galangin, quercetin and naringenin amounts were changed related by altitudes. While the amounts of CAPE, quercetin, pinocembrin, naringenin, and p-coumaric

acid were significantly changed depending both of the altitude and seasons, there was not a significant change in the amounts of apigenin, ferulic acid, gallic acid, kaempferol, and rutin. The effect of seasons or altitudes on phenolic compounds may be related to the synthesis of the phenolic compounds in the vegetation process of the plant. This should be clarified by new future studies. Main phenolic compounds determined were pinocembrin, m-coumaric acid, CAPE, caffeic acid, p-coumaric acid, naringenin, quercetin, ferulic acid, luteolin, and galangin in Marmara region of Turkey. Although certain chemical standardization of propolis could not be possible due to the variants of propolis sources, the study claim that is possible to partially standardize of propolis to detect propolis components and their amounts regarding seasons and altitudes. In addition, if propolis is to be used for medical and research purposes, it should be analyzed with the different analytical method in order to determine its biologically active compounds and their concentrations.

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