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Determination of The Effect of Green Extraction Solvents on The Phenolic Acids and Flavonoids of Propolis

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Abstract

Propolis is an important bee product with many biological activities due to its containing phenolic compounds. The phenolic compounds of propolis vary depending on the plant source, season, altitude, extraction method and solvent. The present study investigated the extraction of phenolic compounds from propolis according to solvent factors. The propolis samples were extracted in four different solvents, which were water, ethanol-water (70%), dimethyl sulfoxide (DMSO), and L-lysine (8%), and were analyzed 36 phenolic compounds by HPLC-DAD. Statistically significant differences in solubility of the phenolic compound at various levels were detected among the solvents ($P < 0.05$). Only water and ethanol-water (70%) more successful than the other solvents were determined in the extraction of phenolic components of propolis. Phenolic acids generally dissolved higher in water, while flavonoids dissolved higher in ethanol were determined. Certain phenolic compounds were detected only in some of the propolis extract: syringic acid and daidzein in water, vitexin, rutin, and epigallocatechin in ethanol, and emodin in DMSO. Consequently, the chemical content is affected significantly depending on the extraction solvent of propolis. Therefore, it is essential to determine the extraction solvent and analyses of propolis before application for therapeutic purposes.

Keywords: Propolis extraction, Water, Ethanol, DMSO, L-lysine, Phenolic compounds.

Introduction

Propolis is a resinous hive product collected by honey bees from tree buds and mixed with secreted beeswax. It is naturally collected by honey bees to seal holes in their honeycombs, smooth out the internal walls and protect the entrance against intruders.[1] Recently, propolis has gained tremendous popularity as a natural health product extensively used in the medical field to improve health and prevent cancer, inflammation, diabetes, and heart diseases.[2]
The active components of propolis play an essential role in biological and physiological behaviour.[3] Propolis contains hundreds of compounds which are phenolic acids, flavonoids, aromatic acids and esters, aldehydes, ketones, terpenoids, phenylpropanoids, amino acids, and vitamins.[4] The flavonoid components of propolis and phenolic acids are predominantly responsible for biological activity.[5] However, it is important to state the chemical compounds concerning flora, geographical origin, honeybee subspecies, collection season, altitudes, and propolis extraction method.[1, 6-10] Since the usage of propolis in its raw form is problematic, and it needs to be purified by extraction using various solvents. Therefore, the solvents used in propolis extraction are also an essential factor in determining the amount and diversity of its active chemical compounds.[11, 12] The active components of propolis exhibit differences depending on the solvent type.[9] Many compounds in propolis are in lipophilic form. It is easy to extract the lipophilic

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form by using alcohol.[9] Ethanol and methanol were better releasing agents for propolis among these solvents.[13, 14] However, for various medical conditions like diabetes and social reasons, including religious beliefs, some people prefer to use water and other green chemical soluble propolis extracts instead of alcohol extraction[15].

In recent years, researchers have started to investigate some extraction solvents that not only succeed in the extraction of propolis as much as ethanol but are also less toxic to the organism, such as DMSO, glycerol, propylene glycol, vinegar, olive oil, and coconut oil.[13, 16] DMSO is one of the most used solvents in the extraction of propolis due to low toxic effects in the biological activity studies that are known as Deep Eutectic Solvents.[17-19] Besides, Deep Eutectic Solvents is defined as a non-toxic green chemical to the organism. The Deep Eutectic Solvents have been widely used to extract phenolic compounds.[19, 20] L-lysine is also an essential Deep Eutectic Solvent that extraction of phenolic compounds.[21, 22]

The current study aimed to investigate the advantages and disadvantages of propolis extraction with water, ethanol (70%), L-lysine (8%), and DMSO for phenolic acids and flavonoids with HPLC DAD.

Materials and Methods

Propolis Collection and Extraction Techniques

The propolis samples were collected with plastic propolis traps placed in the tops of the hives from apiaries of the Düzce University Beekeeping Research, Development and Application Center (DAGEM) in the summer (Figure 1). The samples were kept in a deep freezer (-20 °C), then were ground with a blender for homogenization and protected from light until analysis.

Extractions

The ethanol extract propolis (EEP), water extract propolis (WEP), dimethyl sulfoxide extract propolis (DMSOEP), L-lysine extract propolis (LLEP) were prepared according to Sorucu and Oruç 2019 with some modifications.1 Propolis extractions were carried out using ethanol/water (70/30), ultra-pure water, DMSO, and L-lysine (8%). The homogenized propolis samples were weighed 10 g per solvent. The propolis was mixed with 100 ml of extractions solvents and shook for four h at 300 rpm with a shaker (Wise Shake, Korea). The extracts were filtered with Whatman No-1 paper to remove wax and bee parts. After each extract was dried in a tube via SpeedVac Vacuum Concentrator (Thermo Fisher Scientific), the dry resins were kept in the refrigerator until HPLC analyses.

Chemical and Solvents

In this study, the HPLC-grade methanol and ethanol were purchased from Merck (Darmstadt, Germany). DMSO and L-lysine were purchased from Sigma-Aldrich (Germany). The compounds were used as standards in HPLC-DAD analysis which galangin (GL), rutin trihydrate (RT), kaempferol (KF), quercetin hydrate (KRC), quercitrin (KCT), p-coumaric acid (p-Q), trans-chalcone (KL), caffeic acid phenethyl ester (CAPE), trans-ferulic acid (FR), trans-cinnamic acid (SA), luteolin (LT), pinocembrin (PN), caffeic acid (KA) and gallic acid (GA) were purchased from Sigma-Aldrich), m-coumaric acid (m-Q) was purchased from Fluka, protocatechuic acid (PCA), trans-isoferulic acid (IFR), daidzein (DZ), rosmarinic acid RA, syringic acid (SYA), (±)-catechin (KT), (±)naringenin (NR), 3-4 dimetoxycinnamic acid (DMCA), apigenin (AP), benzoic acid (BA), ellagic acid dehydrate (EA), emodin (EM), pinobanksin (PNB), vitexin (VT), (±)epicatechin (EKT), (±)epigallocatechin (EGK), isorhamnetin (ISR), chrysin (CR), methyl syringate (MYS), naringin (NG) and myricetin (MR) were purchased from Santa-Cruz biotechnology. Ultrapure water was obtained from ELGA® LabWater, Purelab Flex.

Analysis of Propolis Extracts by HPLC-DAD

The HPLC-DAD analysis method applied a modified version of Yang et al. 2013.23 The analysis was performed using the HPLC-DAD (Shimadzu Kyoto, Japan) system with a pump (LC-20AD), auto-sampler (SIL 20 AC), detector (SPD-M20A). The separation was carried out using Intersil ODS (5 µm 4.6 ×150 mm) column with mobile phase A (deionized water, 0.1% formic acid) and mobile phase B (acetonitrile). The UV wavelength was set at 270 nm, with an injection volume of 5 µl. The gradient elution of mobile phases flowing ramp is presented in Table 1. The phenolic compounds chromatogram was presented in Figure. 2. All extract propolis chromatograms were given in Figure 3.

HPLC method validation: The phenolic compounds' calibration curves showed good linearity (R2 > 0.948). The limit of detection (LOD) and limit of quantitation (LOQ) of the method were realized by MFC serial dilution and by using the equations 3 S/N (signal to noise ratio) and 10 S/N, respectively. The limit of detection (LOD) and limit of quantification (LOQ) ranged from 0.1 to 2.4 µg/ml and 0.4 to 9.1 µg/ml, respectively. Mix phenolic compounds (MFC) were analyzed five different days five times for the accuracy and repeatability of the method. The coefficient of variation (relative standard deviation:

RSD) in result defined less than 4.6 % and in retention time RSD less than 3.1 %. The propolis samples spiked three different levels with MFC (20, 10, 5 µg/g). Recovery of the phenolic compounds was found between 82 and 112%, which differences between spike and blank propolis samples by analyzing.

Table 1. The Mobile phases gradient elution following ramp.

Time	Mobile phase	Concentration %
0.01	Start	
0.02	B.Conc	3
3.00	B.Conc	10
30.00	B.Conc	13
60.00	B.Conc	16
70.00	B.Conc	17
80.00	B.Conc	18
120.00	B.Conc	30
135.00	B.Conc	35
170.00	B.Conc	40
172.00	B.Conc	45
182.00	B.Conc	60
202.00	B.Conc	90
203.00	B.Conc	90
205.00	B.Conc	30
206.00	B.Conc	3
207.00	Stop	

Figure 1. Propolis was collected by plastic trap

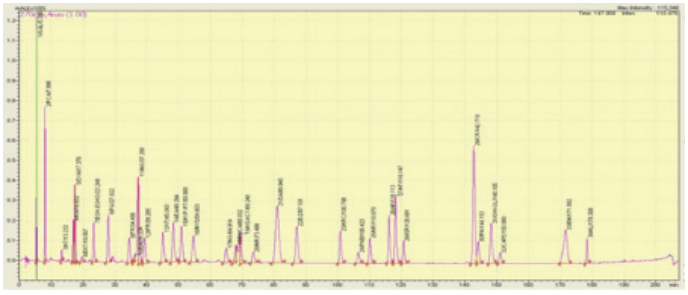

Figure 2. The chromatogram of the mixed phenolic compounds.
The retention time of phenolic compounds; Gallic acid: 5.338, Protocatechuic acid: 7.996, Catechin: 13.232, Caffeic acid: 16.832, Syringic acid: 17.376, Epicatechin: 20.196, Epigallocatechin: 23.248, p-Coumaric acid: 27.822, trans-Ferulic acid: 34.456, Benzoic acid: 36.224, m-Coumaric acid: 37.28, trans- Isoferulic acid: 39.265, Vitexin: 45.043, Ellagic acid: 48.294, Rutin: 50.9, Naringin: 64.914, Quercitrin: 69.248, Methyl syringate: 54.603, DMCA: 68.032, Myricetin: 73.486, Rosmarinic acid: 78.344, trans-Cinnamic acid: 80.945, Daidzein: 87.101, Quercetin: 100.798, Luteolin: 102.368, Pinobanksin: 106.423(±)-, Naringenin: 110.07, Apigenin: 116.113, Kaempferol: 118.147, Isorhamnetin:120.681, Chrysin: 142.719, Pinocembrin: 144.153, Galangin: 148.185, CAPE: 150.99, Emodin: 171.582, trans-Chalcone178.308

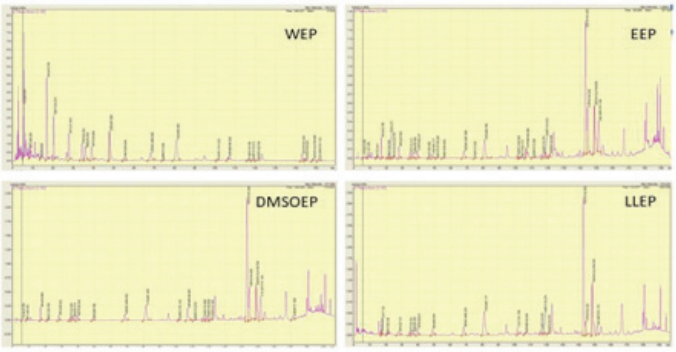

Figure 3. The chromatograms of the propolis extracts.
WEP: Water extract propolis, EEP: Ethanol extract propolis, DMSOEP: Dimethyl sulfoxide extract propolis (DMSOEP), LLEP: L-lysine extract propolis.

Statistical Analysis: The statistical analysis was performed using SPSS 15.0 software for Windows (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± SE. One-way ANOVA and the Duncan test were used for differences regarding the solvent, with $P < 0.05$ considered statistically significant.

Results

In the present study, the phenolic compounds which have a biological effect on propolis were extracted with water, ethanol, DMSO, and L-lysine. Thirty-six bioactive phenolic compounds in propolis, of which 14 phenolic acids and 22 flavonoids, were analyzed by HPLC-DAD. While the primary phenolic acids of the propolis extracts were KA, BA, DMCA, and CAPE, the primary flavonoids were PNB, CR, PN, GL, and EKT. Water and ethanol were more successful than the other solvents in extracting the phenolic components of propolis (Tables 2 and 3). Phenolic acids dissolved at a higher rate than flavonoids in all solvents (Tables 2 and 3). Phenolic acids generally dissolved higher in water, while flavonoids dissolved higher in ethanol (Tables 2 and 3). While KA, p-Q, FR, BA, IFR, EA, DMCA, SA, PNB, AP, KF, ISR, CR, PN, GL, and EKT were found in all extraction solvents, m-Q, KL, NG, and KCT were not found (Table 2 and 3). There were determined statistically significant differences in solubility of phenolic compounds among the extraction solvents of propolis ($P < 0.05$) (Tables 2 and 3). GA, KA, p-Q, IFA, BA, FA, MYS, MR, KT, DZ, and EKT were significantly higher in the aqueous extract than in the other extracts, while DMCA, CAPE, RA, VT, RT, EGK, KRC, LT, NR, AP, KF, ISR, and PN were significantly higher in ethanol extract (Table 2 and 3). EM and PNB were markedly higher in DMSOEP than the other extracts, whereas EA, SA, PN, and GL were considerably higher in L-lysine ($P < 0.05$, Tables 2 and 3).

Table 2. Phenolic acid constituents of propolis in the extraction solvents determined by HPLC-DAD

	Compounds (µg/ml)	RT	Water	Ethanol	DMSO	L-lysine
1	Gallic acid	5.338	74.7 ± 0.02^{a}	7.13 ± 0.01^{b}	3.05 ± 0.03^{c}	nd
2	Protocatechuic acid	7.996	27.79 ±1.16^{a}	3.04 ± 1.08^{b}	1.35 ± 0.01^{b}	nd
3	Caffeic acid	16.832	616.39 ± 1.36^{a}	204 ± 0.05^{b}	151.70 ± 0.14^{c}	3.29 ± 1.36^{d}
4	Syringic acid	17.376	0.63 ± 0.09	nd	nd	nd
5	*p*-Coumaric acid	27.822	167.47 ± 0.09^{a}	99.28 ± 1.21^{b}	51.02 ± 0.11^{c}	68.11 ± 2.10^{d}
6	*trans*-Ferulic acid	34.456	205.10 ± 0.65^{a}	84.72 ± 0.30^{b}	46.12 ± 0.07^{c}	79.59 ± 3.11^{b}
7	*m*-Coumaric acid	37.280	nd	nd	nd	nd
8	Benzoic acid	36.224	424.83 ± 0.92^{a}	195.05 ± 0.81^{b}	15.97 ± 0.06^{c}	224.64 ± 0.90^{d}
9	*trans*- Isoferulic acid	39.265	146.45 ± 0.38^{a}	103.82 ± 0.47^{b}	85.87 ± 0.01^{c}	121.82 ± 2.40^{bd}
10	Ellagic acid	48.294	19.68 ± 0.25^{a}	74.57 ± 0.14^{b}	27.77 ± 0.03^{c}	93.11 ± 0.01^{d}
11	*DMCA*	68.032	254.10 ± 0.7^{a}	641.18 ± 0.50^{b}	305.80 ± 0.02^{c}	392.81 ± 0.07^{d}
12	Rosmarinic acid	78.344	0.02 ± 0.10^{a}	0.205 ± 0.01^{b}	nd	nd
13	*trans*-Cinnamic acid	80.945	106.98 ± 0.55^{a}	124.71 ± 0.03^{b}	121.35 ± 0.08^{b}	197.22 ± 2.61^{c}
14	CAPE	150.990	52.49 ± 1.18^{a}	1256.99 ± .09^{b}	1207.27 ± .43^{c}	386.90 ± 1.50^{d}

Data are presented as ± SD, nd: none-detected, the abbreviations on the different letters a,b, c, and d mean significantly different (P <0.05), RT: Retention time.

Table 3. Flavonoid constituents of propolis in different extraction solvents determined by HPLC-DAD

	Compounds (µg/ml))	RT	Water	Ethanol	DMSO	L-lysine
1	(±)-Catechin	13.232	29.22 ± 0.18^{a}	6.38 ± 0.03^{b}	nd	nd
2	Epicatechin	20.196	915.47 ± 0.04^{a}	39.89 ± 1.83^{b}	27.95 ± 0.30^{c}	35.47 ± 038^{d}
3	Epigallocatechin	23.248	nd	4.33 ± 0.39	nd	nd
4	Vitexin	45.043	nd	2.57 ± 2.13	nd	nd
5	Rutin	50.900	nd	3.34 ± 2.01	nd	nd
6	Naringin	64.914	nd	nd	nd	nd
7	Quercitrin	69.248	nd	nd	nd	nd
8	Methyl syringate	54.603	20.55 ± 0.62^{a}	4.56 ± 0.22^{b}	nd	nd
9	Myricetin	73.486	15.24 ± 1.20^{a}	4.58 ± 0.19^{b}	nd	nd
10	Daidzein	87.101	0.0098 ± 0.31	nd	nd	nd
11	Quercetin	100.798	14.09 ± 0.50^{a}	37.18 ± 0.57^{b}	18.60 ± 0.70^{c}	nd
12	Luteolin	102.368	nd	2.65 ± 0.75^{a}	nd	25.29 ± 4.50^{b}
13	Pinobanksin	106.423	92.90 ± 0.40^{a}	342.81 ± 0.26^{b}	606.60 ± 0.13^{c}	84.12 ± 0.04^{d}
14	(±)-Naringenin	110.070	5.62 ± 0.12^{a}	16.01 ± 0.04^{b}	5.42 ± 0.03^{a}	nd
15	Apigenin	116.113	5.72 ± 4.71^{a}	38.79 ± 0.01^{b}	32.97 ± 0.01^{c}	32.73 ± 0.03^{c}
16	Kaempferol	118.147	5.42 ± 6.71^{a}	38.91 ± 0.06^{b}	31.60 ± 0.62^{c}	11.90 ± 0.02^{d}
17	Isorhamnetin	120.681	8.43 ± 0.46^{a}	70.25 ± 0.07^{b}	62.57 ± 0.34^{c}	15.48 ± 4.14^{d}
18	Chrysin	142.719	18.06 ±0.85^{a}	448.84 ± 0.10^{b}	80.73 ± 0.41^{c}	562.47 ± 3.21^{d}
19	Pinocembrin	144.153	27.02 ± 1.16^{a}	890.10 ± 0.20^{b}	735.70 ± 2.88^{c}	308.62 ± 0.19^{d}
20	Galangin	148.185	36.72 ± 1.40^{a}	1056.82± 0.11^{b}	86.20 ± 0.12^{c}	1401.48 ± 0.05^{d}
21	Emodin	171.582	nd	nd	6.58 ± 015	nd
22	*trans*-Chalcone	178.308	nd	nd	nd	nd

Data are presented as ± SD, nd: none-detected, the abbreviations on the different letters a,b, c, and d mean significantly different (P <0.05), RT: Retention time.

According to the analysis, the results of phenolic compounds of solubility in the extraction solvents were determined the significant differences (P <0.05, Tables 2 and 3). The differences were from the most amount to the least; p-Q, FR, BA in the WEP>EEP>LLEP>DMSOEP (Table 2), KA in the WEP>EEP>DMSOEP>LLEP (Table 2), EA in the LLEP>EEP>DMSOEP>WEP (Table 2), DMCA in the EEP>LLEP>DMSOEP>WEP (Table 2), CAPE in the EEP>DMSOEP>LLEP>WEP (Table 2), PNB in the DMSOEP>EEP>WEP>LLEP (Table 3), AP in the EEP>DMSOEP=LLEP>WEP (Table 3), KF, ISR, PN in the EEP>DMSOEP>LLEP>WEP (Table 3), CR in the LLEP>EEP>DMSOEP>WEP (Table 3), GL in the EEP>LLEP>DMSOEP>WEP (Table 3), EKT in the WEP>EEP>LLEP>DMSOEP (Table 3), GA in the WEP> EEP>DMSOEP and was not detected in the LLEP (Table 2) and KRC in the EEP>DMSOEP>WEP and was not detected in the LLEP (P <0.05, Table 3). IFR was significantly higher in the WEP than in EEP and DMSOEP (P <0.05, Table 2). PCA and NR were detected except for LLEP (Tables 2 and 3). PCA was significantly higher in the WEP and NR considerably higher in the EEP (P <0.05, Tables 2 and 3). While RA was considerably higher in the EEP than in WEP, MYS, MR, and CT in the WEP were markedly higher than in EEP, and that phenolic compounds were not detected in the DMSOEP and LLEP (P <0.05, Tables 2 and 3). LT was significantly higher in the LLEP than EEP and not detected in the WEP and DMSOEP, and the (P <0.05, Table 3).

Discussion and Conclusion

The concentration of flavonoids and phenolic acids in the propolis depends on some factors, which are the seasons and altitudes of region, origin and plant source, as well as on the extraction method used.[1, 9, 13, 16, 20.] The current study indicated that the type of solvent significantly affects the chemical constituents of the propolis as well as the factors.

Studies that investigate the content of propolis with both extraction methods and some extraction solvents have determined that solvents directly affect the chemical content of propolis, and its biological activity.[7, 9, 13, 14, 20, 22, 24-26] Rocha et al. 2013 examined some phenolic compounds, including KA, p-Q, SA in the EPP, and WEP. SA and p-Q were found to be high in EEP that similar to the present study, but KA was found to be high in WEP.20 Çakıroğlu 2010 determined that propolis dissolves best in DMSO and ethanol when it examines DMSO, ethanol, acetone, glycerol, and water in his master's thesis.[27] On the contrary, the present study indicated that the phenolic compound in the propolis dissolved the most successfully in water and ethanol, apart from EM and PNB, which dissolve better in DMSO.[27] Park and Ikegaki 1998 compared the solubility of some flavonoids in propolis extraction with different combinations of water and ethanol.[7] The study indicated that as the ethanol content increased in solvent content, KRC, KF, ISR, and

PN phenolic compounds measured amount increased.[7] A significant increase in the phenolic compounds also was found in comparison with water to ethanol extract in the present study. When pinocembrin values were examined, a severe increase in ethanol extraction was found very similar in our study (Table 3). Funari et al. 2019 emphasized that L-lysine may be an alternative to water and ethanol extraction of propolis analyzing of artepilin C. However, in the present study, L-Lysine was not successful in extracting propolis as much as water and ethanol in terms of other phenolics.[22] Silva et al. 2012 methanol and ethanol (80%) total phenolic and flavonoid contents were compared, and EEP was high in both.[14] Pujirahayu et al. 2014 also examined the entire flavonoid content of EEP and WEP, and the total flavonoid content of EEP was determined higher than WEP.[13]

Similarly, in the present study, flavonoids were found high in EEP[13, 14] Mello and Hubinger 2012 examined the content of flavonoids and polyphenol in water and ethanol extract propolis at different pH values.[25] EEP was found higher than water in both parameters.[25] Similarly, the present study of results reveals that the flavonoids were high in EEP. Sun et al. 2015 found the most successful results in a 75% ethanol/water combination that analyzed flavonoids and phenolic acid of the propolis extracts with different mixtures of water and ethanol. While flavonoids were similar to our study, phenolic acids were found higher in EEP than in WEP. [28]

When we consider the previous studies, more water and alcohol extracts were emphasized for propolis, but also an alcohol derivative, polyethene glycol, is used. In studies conducted, alcohol solvent propolis extract was evaluated as the highest phenolic compound among all extract types. At the same time, overall propylene glycol could rank second, and water solvent propolis extract was lowest in terms of the phenolic-flavonoid compound.[11, 29-33]
In the study, the solubility of propolis in L-lysine, DMSO, water and ethanol were investigated by HPLC-DAD in terms of quantity both of flavonoids and phenolic acids. The advantages and disadvantages of water, DMSO, and L-lysine, which will be an alternative to ethanol, have been shown up for the phenolic compounds. Although researchers show that the best solvent of propolis is ethanol, this study showed that different solvents dissolve better in terms of some compounds. The present study generally indicated that the phenolic acids of propolis were more soluble in water while flavonoids of propolis were soluble in ethanol. As a result, it is essential to choose the extraction solvent that affects the content by considering the phenolic compounds for the biological activity studies to be carried out.

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