Chemical Classification of Propolis Samples Collected from Different Regions of Turkey in Geographical Region Base

Türkiye'nin Farklı Bölgelerinden Toplanan Propolis Örneklerinin Coğrafik Bölge Bazında Kimyasal Olarak Sınıflandırılması

Research Article

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ABSTRACT

This study aims to determine chemical compounds of the propolis samples gathered from different regions of Turkey in geographical region-base. Gas Chromatography-Mass Spectrometry (GC-MS) analysis, was performed on 21 propolis samples acquired from different geographical regions of Turkey. Samples were collected from five different geographical regions including Black Sea, Central Anatolia, East Anatolia, Marmarean and Mediterranean Regions (Three different phytogeographic regions: European-Sibaerian, Irano-Turanian and Meditteranean phytogeographic regions). So, by this study the chemical compositions of different propolis samples gathered from three distinct phytogeographical regions will be compared. Accordingly, "1-Nonadecene", "pinocembrin", "phenylethyl alcohol", "2-propen-1-one,1-(2,6-dihydroxy-4-methoxy phenyl)-3-phenyl" and particularly " 2-methoxy-4-vinyl phenol", "ethyl oleate" were determined as mostly found compounds in collected propolis samples. According to the GC-MS analysis results, five geographical regions are distinguished from each other according to chemical compound groups. "Benzoic acid "and "17-Pentatriacontene" were found as possible markers of Black Sea Region, "chrysin" for Marmarean region, "2-buten-1-ol,2-methyl", "Z-12-pentacosane", "1-Hexacosane" for Central Anatolia, "Nonadecane", "Octadecane", and "2-Nonadecanone" for East Anatolia Regions. Besides these for the chemical compositions of Mediterranean Region samples, no clear distinction could be found.

Key Words
Propolis, GC-MS, phytogeographic region, chemical compound, marker compound

ÖZET

Bu çalışma, Türkiye'nin farklı bölgelerinden toplanan çeşitli propolis örneklerinin kimyasal içeriklerini coğrafik bölge bazında belirlemeyi amaçlamıştır. Türkiye'nin farklı coğrafik bölgelerinden toplanan 21 propolis örneği üzerinden Gaz Kromatografisi-Kütle Spektrometresi (GC-MS) analizi gerçekleştirilmiştir. Örnekler Karadeniz, İç Anadolu, Doğu Anadolu, Marmara ve Akdeniz Bölgelerini içeren beş farklı coğrafik bölgeden (üç farklı fitocoğrafik bölge; Avrupa-Sibirya, İran-Turan ve Akdeniz fitocoğrafik bölgeleri) toplanmıştır. Böylelikle bu çalışmayla, üç farklı fitocoğrafik bölgeden toplanan propolis örneklerinin kimyasal içerikleri kıyasalanmış olacaktır. Sonuçlara gore, "1-Nonadesin", "pinosembrin", "feniletil alkol", "2-propen-1-on,1-(2,6-dihidroksi-4-metoksi fenil)-3-fenil" ve özellikle " 2-metoksi-4-vinil fenol", "etil oleat" toplanan propolis örneklerinde en sık rastlanılan bileşikler olarak saptanmıştır. GC-MS analiz sonuçlarına göre beş coğrafik bölge kimyasal bileşik grupları bakımından birbirinden ayrılmıştır. "Benzoik asit" and "17-Pentatriakonten" Karadeniz bölgesi için, "krisin" Marmara bölgesi için, "2-büten-1-ol,2-metil", "Z-12-pentakosan", "1-Hekzakosan" İç Anadolu Bölgesi için, " Nonadekan", "Oktadekan", ve "2-Nonadekanon" Doğu Anadolu Bölgesi için olası belirleyici bileşikler olarak bulunmuştur. Bunların yanında Akdeniz Bölgesi örneklerinde kimyasal içerik bakımından net bir ayrım bulunamamıştır.

Anahtar Kelimeler
propolis, GC-MS, fitocoğrafik bölge, kimyasal bileşik, belirleyici bileşik

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Figure 1. The collected areas of propolis samples are shown with black circles.

INTRODUCTION

Propolis (bee glue) is a sticky dark-coloured material that honeybees collect from living plants, mix with wax and use in the construction and adaptation of their nests. Its resistance against micro-organisms is an essential characteristic of propolis and it has been used by human beings since ancient times for its pharmaceutical properties [1]

Chemical composition of propolis depends upon the phytogeographic characteristics of the site of collection. Although it is of plant origin, propolis is a bee product. Bees collect it from different source plants in different ecosystems by choosing appropriate representatives of the local flora. Different plant choices of bees as a source of propolis in different habitats complicate propolis standardization [2].

For example, while European propolis contains phenolics: flavonoid agylcones (flavones and flavonones), phenolic acids and their esters, the main compound classes found in Brazilian propolis are prenylated derivatives of p-coumaric acid and acetophenone [3].

Frequently found as major constituents in propolis samples are flavonoids, organic acids, phenols and various kinds of enzymes, vitamins and minerals. These compounds give antibacterial, anti-inflammatory, antioxidant, prooxidant, immuno-enhancement and antitumor qualities to propolis. For instance, flavonoids, as antioxidants, prevent oxidative damage of DNA caused by reactive oxygen species [4].

In Turkey the first research on propolis was carried out by Sorkun and Bozcuk [5]. They investigated the effects of propolis on seed germination. Sorkun et al., also investigated the chemical composition of propolis samples taken from Erzurum, Gümüşhane and Trabzon by another study" [6]. More recently, Kolankaya et al., [7] studied fat variation and liver injury in mice that were fed propolis.

Due to the location of Turkey, different climatic conditions and plant covers can be seen in this country. Turkey has three phyto-geographical and seven geographical regions. There are 9222 naturally grown species in Turkey and 3000 of these are endemic [8]. Because of its rich plant cover and different climatic conditions, the content of Turkish propolis varies from one geographical region to another.

MATERIALS AND METHODS

Propolis samples

Propolis samples were obtained from the beehives that were located at different geographical regions of Turkey (Figure 1).

Extraction and sample preparation

Each frozen propolis sample was ground and dissolved in ethanol (96%) with a ratio of 1:3 (w/v). Then the mixture was kept in a tightly closed bottle in an incubator at 30°C for two weeks. Then the supernatant was filtered twice through Whatman (Whatman, Maidstone, England) No. 4 and No. 1 filter papers. The final filtered and concentrated solution (1:10, w/v) designated "ethanol extract of propolis" (EEP) and analysed by gas chromatography and mass spectrometry (GC-MS) [9].

GC-MS analysis

A GC 6890N instrument from Agilent (Palo Alto, CA, USA) coupled with a mass spectrometer (MS5973; Agilent) was used for the analysis of EEP samples. Experimental conditions of the GC-MS system were as follows: a DB 5MS column (30 m x 0.25 mm, 0.25 µm film thickness) was used and the flow rate of the mobile phase (He) was set at 0.7 mL/min. In the GC part, temperature was kept for 1 min at 50°C and then increased to 150°C at 10°C/min intervals followed by 2 min at 150°C. Finally, the temperature was increased to 280°C at 20°C/min intervals and kept at 280°C for 30 min.

Organic compounds in propolis samples were identified in Wiley's NIST Mass Spectral Library, if the obtained comparison scores were higher than 95%. Otherwise, fragmentation peaks of the compounds were evaluated, and the compounds were identified using the memory background for the identification of the compounds that appeared in GC-MS chromatograms. For the quantification of the compounds in the ethanol extract, no internal and external standard was used. Only percentage reports of the compound in the sample were used. This was the standard way to quantify the many organic compounds in the propolis samples. Contents of individual compounds in the ethanol extract are given in percent of the total compounds in the sample. This was the standard way to quantify most organic compounds in the propolis samples. In this case, the relative error could not be higher than 5% [9].

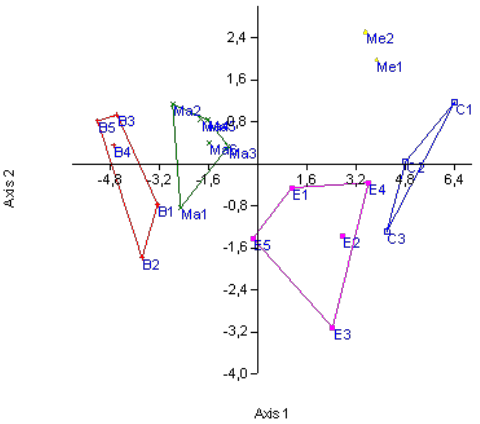

Figure 2. The PCA analysis of 21 propolis samples in geographical region base.

Statistical analysis

PCA (Principal component analysis) technique was applied to chemical data for propolis to evaluate possible similarities and differences among the propolis samples of five different geographical regions. In this context, the cases were the different propolis samples, the variables were the identified volatile compound groups and the input value in the matrix was the compound ratio. The result scores were used for CVA analyse.

RESULTS

According to the results, compounds belong to alcohols, aldehydes, aliphatic acids and their esters, carboxylic acids and their esters, cinnamic acids and their esters, ethers, flavonoids, hydrocarbons, ketones and terpenes groups were detected in the collected 21 propolis samples.

Table 1. The propolis samples, collected regions, locations and symbols.

	Europaen Siberian Phytogeographical Region		Irano-Turanian Phytogeographical Region		Mediterranean Phytogeographical Region
Compounds	**Black Sea**	**Marmarean**	**Central Anatolia**	**East Anatolia**	**Mediterrranean**
Alcohols					
2-methoxy-4-vinylphenol	5	6	2	3	
Phenylethyl alcohol	4	5	2		
Benzenemethanol	3	4			
2-buten-1-ol,2-methyl			2		
3-buten-1-ol,3-methyl			2	3	
2-Naphthalenemethanol,1,2,3,4,4a,5,6,8a-octahydro-.alpha.,.alpha.,4a,8-tetramethyl-,(2R-(2.alpha.,4a..alpha.,8a.beta.))			2	3	
Aliphatic acids					
Ethyl oleate	3	6	2	5	
Hexadecanoic acid ethyl ester				4	2
Flavonoids					
4H-1-Benzopyran-4-one,5-hydroxy-7-methoxy-2-phenyl	3	4	2		
Chrysin		4			
2-propen-1-one,1-(2,6-dihydroxy-4-methoxyphenyl)3-phenyl	4		3	3	
Pinocembrin		5	3	4	
Hydrocarbons					
17-pentatriacontene	3				
1-Nonadecene	3	5	3		2
Tricosane		4			
Z-14-Nanacosane			3	3	
Z-12-Pentacosane			2		
1-Hexacosane			2		
Nonadecane				3	
Octadecane				3	
2-Nonadecanone				4	
Carboxylic acids					
Benzoic acid	3				

Table 2. The chemical compound groups and ratios (%) determined in propolis samples by GC-MS.

Comp.	B1	B2	B3	B4	B5	C1	C2	C3	E1	E2	E3	E4	E5	Ma1	Ma2	Ma3	Ma4	Ma5	Ma6	Me1	Me2
Alcohols	0.51	2.54	19.21	12.25	34.6	6.56	4.54	4.3	0.67	12.54	5.87	8.31	4.38	29.6			8.39	31.11	13.34		5.45
Aldehydes	0.3	1.12	0.33	0.38	0.74	-	-	-		0.14	0,03	0.45	0.76	0.47	-	0.19	0.14	0.43	-	-	0.14
Aliphatic acids and their esters	0.75	3.31	-	0.2	1.36	7.38	30.73	1.6	22.5	1.27	2.89	22.65	3.29	4.10	4.31	5.15	4.34	4.19	15.99		0.9
Carboxylic acids and their esters	1.51	0.11	8.3	3.19	7.78	0.65	1.28	-		-	0.54	1.49	0.11	2.47	7.97	9.02	7.3	0.34	2.36	1.33	7.96
Cinnamic acids and their esters	-	3.29	6.17	4.73	6.58	-	-	-	-	-	-	-	-	2.41	0.5	0.48	26.75	0.49	-	1.06	3.47
Ethers	-	0.03	-	-	-	0.5	-	-	-	-	-	-	0.13	-	-	-	-	-	-	-	0.46
Flavonoids	3.6	12.74	14.09	2.72		35.77	7.17	52.52	6.3	47.04	30.71	24.18		22.54	8.36		10.81		4.24	-	31.79
Hydrocarbons	6.21	12.11	6.62	5.35	6.14	9.24	4.84	5.92	16.82	6.29	5.54	5.13	7.19	10.42	3.73	6.72	0.5	6.32	15.73		1.39
Ketones	0.06	0.08	-	-	-	1.71	1.17	-	-	0.97	3.54	1.52	1.82	2.78	-	0.3	0.51	0.16	-	-	0.4
Terpenes	-	0.88	-	-	1.55		0.44	-	-	0.09	0.12		0.12	-	-	0.56	-	0.31	-	-	-

As shown in Figure 2, five geographical regions are distinguished from each other according to chemical compound groups. Figure 2 showed the scores scatter plot, representing the 21 propolis samples.

In general, flavonoids were found in relatively higher amounts in propolis samples of almost all the geographical regions. In the case of lower flavonoid contents, it was observed that these samples include higher aliphatic acids as well as hydrocarbons. According to the GC-MS results, flavonoid group compounds were found in Black Sea, Central Anatolia, East Anatolia and Marmara Region samples in high amounts. Contrary to these samples, in Me1 (Mediterrranean 1) sample flavonoids couldn't be observed. While Me1 sample had no flavonoid content, its aliphatic acid and their esters content were considerably high (40.55%). Although there was not any flavonoid content in Me1 sample, a high amount of flavonoid was observed in the other Mediterranean sample (Me2) (Table 2). Even though flavonoids were found in higher amounts in nearly all of the investigated samples, not all of them are marker compounds of these samples.

When the results were compared according to the geographical region base, it was found that "2-methoxy-4-vinyl phenol" could be considered as a principal compound of the investigated Black Sea Region samples, since it was present in all of the five samples collected from this region. In addition to this, " Phenyethyl alcohol, 2-propen-1-one,1-(2,6-dihydroxy-4-methoxy phenyl)-3-phenyl" were found in four of the five samples

(Table 3). As these compounds were found in the samples belong to the other geographical regions we couldn't consider these compounds as possible marker compounds for the Black Sea Region propolis. and its botanical distinction.

In all the three samples collected from Central Anatolia Region "2-propen-1-one,1-(2,6-dihydroxy-4-methoxyphenyl)-3-phenyl and pinocembrin" flavonoids were observed. Besides these, "1-Nonadecene, Z-14-Nonacosane" hydrocarbons were observed in these three samples, too (Table 3). In the light of these results, it can be said that these two flavonoids and hydrocarbons are principal compounds of the collected Central Anatolian samples."

The identified compounds in more than two of the five East Anatolian samples were reported in Table 3. Of these acknowledged compounds, only ethyl oleate was found in all of the East Anatolian samples. Besides this, "hexadecanoic acid ethyl ester, pinocembrin, 2-Nonadecanone were found nearly in all of the East Anatolian samples (four of five samples).

According to Table 3 "Ethyl Oleate, 2-methoxy-4-vinylphenol" were found in all of the six Marmarean samples. These compounds can be considered as specific to the investigated Marmarean propolis samples. In addition to these compounds "benzenemethanol, phenylethyl alcohol, chrysin, pinocembrin, 4H-1-Benzopyran-4-one,5-hydroxy-7-methoxy-2-phenyl, 1-Nonadecene and tricosane were found in more than three of the six samples.

Only two propolis samples could be investigated from the Mediterranean Region. Even though they were located in the same geographical region, they showed differences from each other in chemical composition base. For example, while the content of aliphatic acids and their esters in Me1 sample is 40.55%, the same content is only 0,9% in Me2 sample. Another variation was observed in flavonoid contents. While Me1 sample had no flavonoid content, Me2 sample had a rather high flavonoid content (31.79%). Although these two samples were situated in the same geographical and phytogeographical region, they showed many differences from each other. Owing to these results, it can be said that except for "Hexadecanoic acid ethyl ester and 1-Nonadecene", there are not many common compounds in the Mediterranean samples (Table 3).

DISCUSSION

There are many propolis types in the world: Pacific (macaranga-derived), Mediterranean (containing mainly diterpenes), South American (Cuban, Brazillian, Mexican) and red propolis (dalbergia-derived) [2]. Similar to these types, by multiplying and improving these kind of researches the

identification of a type for Turkish propolis can be made.

If we look at the results in country-base it can said that firstly, " 2-methoxy-4-vinyl phenol", "ethyl oleate (oleic acid, ethyl ester)" and secondly, "1-Nonadecene", "pinocembrin", "phenylethyl alcohol", "2-propen-1-one,1-(2,6-dihydroxy-4-methoxy phenyl)-3-phenyl" compounds can be considered as possible markers for the collected Turkish propolis samples, since these compounds can be found in more than 10 of 21 investigated samples. From these compounds; Phenylethyl alcohol and 2-methoxy-4-vinyl-phenol are alcohols, ethyl oleate is a kind of aliphatic acid and 1-Nonadecene is a hydrocarbon. Of these marker compounds "pinocembrin and 2-propen-1-one, 1-(2,6-dihydroxy-4-methoxy phenyl)-3-phenyl belong to the flavonoid groups. Previous studies and our researche have showed that Turkish propolis samples are rich for flavonoids [6,9].

As seen in Table 3, some compounds were identified as marker compounds for more than one geographical region. Only a few of these were found as markers in just one geographical region. For example benzoic acid and 17-Pentatriacontene were found as markers of Black Sea Region only. In Marmarean region "chrysin", in Central Anatolia "2-buten-1-ol,2-methyl", "Z-12-pentacosane", "1-Hexacosane", in East Anatolia " Nonadecane", "Octadecane", "2-Nonadecanone" were found as marker compounds that did not overlap with other region markers. In the Mediterranean only "Hexadecanoic acid ethyl ester and 1-Nonadecene were pinpointed as markers but these two compounds were also found as markers in the other regions too. So, no clear distinction could be found for the Mediterranean Region.

Besides detecting principal compounds we noticed that Turkish propolis has a quite high flavonoid content. Similiar to this study Girgin et al., [10] analysed Zonguldak, Artvin and Bursa propolis samples too. They also found flavonoids in different ratios (0.33-19.89%) in these samples just like us.

Pinocembrin; a kind of flavonoid is the most abundant compound in the investigated propolis samples. We found pinocembrin in Çanakkale (Ma6), Tahtaköprü-Bursa (Ma5), Karacabey-Bursa (Ma4), Kırklareli (Ma1), Tekirdağ (Ma3), Kayseri (C2), Ankara (C1), Sivas (C3), Erzincan (E3), Tunceli (E5), Elazığ (E2), Ardahan (E1) samples. Due to the pinocembrin contents of these samples, they can show antimicrobial [11], antioxidant and anti-imflammatory activities [12]. In recent years, a lot of new research were undertaken on this compound, and some findings indicated that pinocembrin had some protective effects on ischemic injury [13]. Also pinocembrin content possibly gives bacteriostatic, anti-mould and anti-mycotic effects in vitro, and anti Candida, local anaesthetic, anti-Helicobacter pylori activities in external use to Turkish propolis. Similiar to our results in the previous studies this compound was found in propolis samples collected from Bursa [14], Erzincan [15], Erzurum [16] and Elazığ [17].

Another flavonoid "chrysin" was identified as a possible marker of Marmarean propolis. It is found in four (Karacabey-Bursa, Tahtaköprü-Bursa, Kırklareli, Tekirdağ) of the six Marmarean samples, and as we had observed, Velikova et al., [14] found chrysin in Bursa propolis in their previous studies. Moreover, tumor cytotoxicity and anti-Helicobacter pylori activity of this compound is also known [18].

From flavonoids "2-Propen-1-one-1-(2,6-dihydroxy-4-methoxyphenyl) 3-phenyl" was observed as a possible principal compound for Black Sea, Central Anatolia and East Anatolia Regions. It was found in 10 of 21 Turkish propolis samples. Also in previous studies carried out on Turkish propolis samples, it is detected in high amounts [19].

Another frequently found compound, after flavonoids, was hydrocarbons. Yet, hydrocarbon content did not give any biological activity to propolis samples [18] contrary to flavonoids.

The other compound observed quite frequently was "ethyl oleate". In this study ethyl oleate was found in 16 of 21 Turkish propolis samples. This number is rather high and remarkable. It was observed in Artvin (B2), Zonguldak (B5), Trabzon (B4), Çanakkale (Ma6), İstanbul (Ma2), Tahtaköprü-

Bursa (Ma5), Karacabey-Bursa (Ma4), Kırklareli (Ma1), Tekirdağ (Ma3), Kayseri (C2), Ankara (C1), Ardahan (E1), Tunceli (E5), Malatya (E4), Erzincan (E3) and Elazığ (E2) samples. Likewise, in the previous studies this compound was observed in samples taken from Ankara, Erzincan [15], Bursa and Trabzon [6].

Another compound frequently found in the investigated propolis samples is hexadecanoic acid ethyl ester. It was found in Ardahan (E1), Tunceli (E5), Erzincan (E3), Elazığ (E2), Hatay (Me2) and Antalya (Me1) samples and also this compound found in Hatay sample previously by Silici [20].

"Benzoic acid " a kind of carboxylic acid, was considered as a basis compound of the Black Sea Region samples only. Similiarly Girgin et al., [10] found this compound in a sample of Black Sea Region too.

A vast number of papers dealing with different aspects of the biological properties of propolis have been published during the last decades. However, a considerable amount of them are of limited usefulness, despite the claims that they have on "strong", "remarkable" or "significant" activity. The major reason is the lack of basis for comparison and scientific evaluation of the results, since they do not refer to the chemical nature of the studied propolis samples [2]. It is deficient for the studies of propolis. So, we aimed firstly to find the chemical composition and the possible marker compounds of Turkish propolis samples. But the samples collected from the different geographical regions are insufficient to standardize the propolis samples for geographical region base.

Although, many studies were done to find the chemical composition of Turkish propolis, there is not any detailed research on the marker compounds of propolis collected from different geographical regions of Turkey. This study will be a step towards the geographical distinction of Turkish propolis. And data will be helpful for further researches to find marker compounds of Turkish propolis in geographical region base.

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