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Phenolic Profiles, Tyrosinase Inhibitory, and Antioxidant Effects of Green Coffee, and Turkish Traditional Coffee

Yeşil Kahve ve Geleneksel Türk Kahvesinin Fenolik Profili, Tirozinaz Enzim İnhibisyonu ve Antioksidan Etkileri

Seyda KANBOLAT[1], Merve BADEM[1], Sıla Ozlem SENER[2], Rezzan ALIYAZICIOGLU[1*]

[1]Karadeniz Technical University, Faculty of Pharmacy, Department of Biochemistry, Trabzon, Turkey.

seydaakkaya@ktu.edu.tr, ORCID: 0000-0001-7261-7067

ecz.mervebadem@gmail.com, ORCID: 0000-0002-1265-5616

*rezzan@ktu.edu.tr, ORCID: 0000-0003-0143-8795

[2]University of Health Sciences, Gulhane Faculty of Pharmacy, Department of Pharmacognosy, Ankara, Turkey.

silashener@hotmail.com.tr, ORCID: 0000-0001-7679-7165

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***Corresponding author** /Yazışılan yazar

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Abstract

Coffee has been drunk for millennia due to its taste and health benefits. High levels of polyphenols, and especially flavonoids and phenolic acids, are found in coffee and contribute significantly to its flavor and health-giving properties. In this study the total phenolic contents, antioxidant, and tyrosinase inhibition of green coffee, and Turkish traditional coffee extracts were evaluated. Antioxidant activities of the coffees were examined by two different methods, radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ferric reducing antioxidant power (FRAP). Total phenolic contents were estimated by using Folin-Ciocalteu reagent as the gallic acid equivalent. The phenolic profiles were investigated by means of reverse phase-high performance liquid chromatography (RP-HPLC). At the same time, tyrosinase enzyme inhibition of extracts has also been worked. The extracts exhibited high levels of antioxidant activities associated with significant antioxidant compound contents. It was determined that the samples contain chlorogenic acid and benzoic acid in the RP-HPLC analysis. It was determined that green coffee extract exhibited tyrosinase enzyme inhibition as effective as kojic acid. The results show that green coffee especially from coffees can be regarded as a potential source of antioxidant compounds and tyrosinase inhibitors of significance in both the pharmaceutical and food industries.

Keywords: Green coffee, Turkish traditional coffee, Antioxidant, Phenolic compounds, Tyrosinase inhibition

Özet

Kahve, tatları ve sağlık yararları nedeniyle binlerce yıldır içilmektedir. Kahvede yüksek düzeyde polifenoller ve özellikle flavonoidler ve fenolik asitler bulunur ve bu bileşenler kahvenin lezzetine ve sağlık verici özelliklerine önemli ölçüde katkıda bulunur. Bu çalışmada, geleneksel Türk kahvesi ve yeşil kahve ekstrelerinin toplam fenolik içerikleri, antioksidan ve tirozinaz inhibitör aktiviteleri değerlendirilmiştir. Kahvelerin antioksidan aktiviteleri, 2,2-difenil-1-pikrilhidrazil (DPPH) ve demir indirgeyici antioksidan gücü (FRAP) olmak üzere iki farklı yöntemle incelenmiştir. Toplam fenolik içerikler, Folin-Ciocalteu reaktifi kullanılarak gallik asit eşdeğeri olarak verilmiştir. Fenolik profiller, ters faz yüksek performanslı sıvı kromatografisi (RP-HPLC) ile çalışılmıştır. Aynı zamanda ekstrelerin tirozinaz enzim inhibisiyonu da çalışılmıştır. Ekstreler, yüksek seviyelerde antioksidan aktiviteler sergilemesi önemli antioksidan bileşenleri ile ilişkilidir. RP-HPLC ile yapılan analizde, örneklerin klorojenik asit ve benzoik asit içerdiği saptanmıştır. Yeşil kahve ekstresinin kojik asit kadar etkili tirozinaz enzim inhibisyonu sergilediği belirlenmiştir. Sonuçlar, özellikle kahvelerden elde edilen yeşil kahvenin, hem ilaç hem de gıda endüstrilerinde potansiyel antioksidan bileşikler ve önemli tirozinaz inhibitör kaynağı olarak kabul edilebileceğini göstermektedir.

Anahtar Kelimeler: Yeşil kahve, Türk geleneksel kahve, Antioksidan, Fenolik bileşikler, Tirozinaz inhibisyonu

1. INTRODUCTION

The coffee plant was first grown in the Kaffa region of Ethiopia, from where it spread to Yemen, Arabia, and Egypt and gradually became part of daily life. After water and tea, coffee is the third most popular drink worldwide (Villanueva et al., 2006). Once coffee berries have ripened, they are dried, roasted at a range of different temperatures until the desired flavor is achieved, and finally ground and brewed. The two most popular coffee berries are harvested from plant species of *Coffea robusta* L. Linden and *Coffea arabica* L.

Various studies have demonstrated an association between tea and coffee consumption and their ability to prevent disease, which has been attributed to their polyphenol contents (Klatsky et al., 2006; Nichenametla et al., 2006). Polyphenols are secondary metabolites that act as a component of the defense system against pernicious environmental factors such as ultraviolet radiation and pathogens.

Flavonoids, particularly flavanols (catechins) and phenolic acids, constitute the major polyphenols identified in coffee. The most plentiful polyphenols identified in coffee are caffeic acid and its derivative chlorogenic acid (a caffeic acid ester of quinic acid). One cup of coffee may contain 70-350 mg of chlorogenic acid (Clifford, 1999). The antioxidant activity exhibited

by coffee is associated with its chlorogenic, ferulic, caffeic, and *n*-coumaric acid contents (Nicoli et al., 1997).

Oxidative stress resulting from disequilibrium between the production and neutralization of pro-oxidants gives rise to numerous human diseases. Oxidative stress is triggered by free radicals including superoxide anions, hydrogen peroxide, nitric oxide and peroxynitrite implicated in injury to various cellular macromolecules (Oyedemi et al., 2010). Copper-containing tyrosinase is responsible for catalyzing melanin synthesis in melanocytes (Vaibhav & Lakshman, 2012). Various tyrosinase inhibitors have been discovered and described so far (Kim & Uyama, 2005; Parvez et al., 2007). Researchers are currently investigating new and potent tyrosinase inhibitors for use in foodstuffs against discoloration and as skin whitening agents.

As part of our research into medicinal plants for new enzyme inhibitors with potential capacity for use as skin whitening agents, we investigated the tyrosinase inhibition potential, phenolic composition, and antioxidant activities of green and Turkish traditional coffees from Turkey.

2. MATERIALS and METHODS

2.1. Chemicals and Instrumentation

DPPH (2,2-Diphenyl-1-picrylhydrazil) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Ethanol, methanol, acetonitrile and acetic acid were purchased from Merck (Darmstadt, Germany). TPTZ (2,4,6-tripyridyl-s-triazine), Trolox (6-hydroxy–2,5,7,8-tetramethylchroman–2-carboxylic acid) and Folin-Ciocalteu were purchased from Fluka Chemie GmbH (Buchs, Switzerland). All absorbance measurements performed in the experiments were made with the A Spectro UV-Vis Double PC-8 automated cell spectrophotometer (Labomed Inc.).

2.2. Determination of Antioxidant Capacity

Samples of green coffee, and Turkish coffee were purchased from herb markets in Trabzon, Turkey, in September 2015. The samples (1 g) were mixed with 10 mL methanol. Each mixture was macerated at room temperature. The suspension was filtrated and concentrated at 40 °C in a rotary evaporator. The samples were dissolved with methanol at a concentration of 10 mg/mL to determine the antioxidant capacity.

The total amount of phenolic substances in the extracts was determined according to the Folin-Ciocalteu method (Singleton & Rossi, 1965). The Folin-Ciocalteu reagent was used

because it is sensitive to reducing compounds, including polyphenols, and gives a blue color after the reaction. This blue color can then be measured spectrophotometrically (Kolayli et al., 2012).

The ferric reducing antioxidant potency (FRAP) test, which is very preferred, was determined according to Benzie and Strain (1996). Results are given as µM Trolox equivalent of g sample.

DPPH radical scavenging activity was performed according to Molyneux (2004). The basis of this method is based on the DPPH cation radical scavenging capacity of the antioxidant. The results were expressed as SC50 (mL per mg sample), which is the concentration of the samples that caused 50% scavenging of the DPPH radical.

2.3. Determination of Phenolic Profiles by RP-HPLC

The extracts were redissolved in HPLC grade methanol and filtered through 0.45-µm membranes. p-hydroxy benzoic acid, vanillic acid, syringaldehyde, p-coumaric acid, sinapic acid, benzoic acid and quercetin as standards were used in RP-HPLC analysis. The phenolic profiles of samples were determinated by validated and modified HPLC method (Korkmaz et al., 2019).

2.4. Tyrosinase Inhibitory Activity

Tyrosinase inhibitory activity (EC 1.14.1.8.1, 30 U, fungal tyrosinase, Sigma) was measured according to the method of Masuda et al. (2005). It uses different concentrations of kojic acid solutions used as standard in this method.

3. RESULTS and DISCUSSION

3.1. Antioxidant Capacity of Coffees

Polyphenols are substances commonly found in plants. Coffee is one of the main sources of polyphenols consumed daily in Turkey. The properties of coffee that make it easier to consume a lot can be attributed to its high amount of antioxidants. Humans can ingest chlorogenic acids from coffee and these are then metabolized by the intestinal flora (Manach et al., 2004; Olthof et al., 2003). High coffee consumption and its bioavailability may play a role in reducing the risk of various diseases.

In this study, total phenolic content (TPC) was determined in comparison with standard gallic acid, and the results were expressed as milligrams of gallic acid equivalents

(GAE) per gram (mg GAE/g) of extract. Measurements showed that the methanolic extract of Turkish traditional coffee had the highest total phenolic content (Table 1). TPC value increases in the order: Turkish traditional coffee > green coffee. Total phenolic contents in methanolic extract of Turkish traditional coffee, and green coffee were 13.9 ± 0.001, and 6.6 ± 0.001 mg of GAE/g, respectively. Fukushima et al. (2009) reported that the concentration of total polyphenols in coffee, was 200 mg/100 mL (Fukushima et al., 2009). Factors such as differences in methodologies used in studies and seasonal variability may cause differences in analytical values (Hertog et al., 1992).

Table 1. The antioxidant activities of methanolic extracts of coffees

Test Compounds	TPC[1]	FRAP[2]	DPPH[3]
Green coffee	6.6 ± 0.001	181 ± 1.140	0.236 ± 0.009
Turkish coffee	13.9 ± 0.001	369 ± 1.000	0.190 ± 0.004
BHT			0.009 ± 0.001

[1] Total phenolic content expressed in mg of gallic acid equivalent (GAE) per gram of dry plant weight.

[2] Expressed as μM trolox equivalents (TE) per gram of dry plant weight.

[3] Concentration of the test sample (mg/mL) required to produce 50 % inhibition of the DPPH radical.

Coffee constitutes a valuable dietary source of antioxidants. Our study reports new data elicited by comparing the in vitro antioxidant/reducing capacities of various types of coffee. FRAP values increase in the order: Turkish traditional coffee > green coffee (Table 1). This study has shown that Turkish traditional coffee has the highest antioxidant power and green coffee has the lowest values. Total antioxidant activity as the FRAP value of Turkish traditional coffee was found 369 ± 1.000 μmol Trolox per gram of sample in methanolic extract. Natella et al. (2002) reported that FRAP values were 96.4 mol Fe^{2+}/L for coffee extract (Natella et al., 2002). Differences in results may vary with geographic regional differences in which coffee is grown, the time of year the leaves are harvested, and differences in subsequent storage conditions (Lin et al., 1996).

When antioxidants interact with DPPH, they neutralize their free radical character by donating an electron or a hydrogen atom to DPPH. The radical scavenging activity of DPPH is expressed as SC_{50}. A lower SC_{50} value indicates higher antioxidant activity. The order of radical scavenging activity of DPPH resulted as follows: Turkish traditional coffee > green coffee. The DPPH scavenging activities of the methanolic extract of Turkish traditional coffee, expressed in terms of SC_{50}, were 0.190 ± 0.004 mg /mL (Table 1). The radical scavenging capacities of the extracts were lower than BHT (0.009 ± 0.001 mg/mL), which is used as a synthetic antioxidant in the food industry.

3.2. Phenolic Profiles by RP-HPLC

RP-HPLC of the methanolic extract was evaluated by comparison with phenolic acid standards (Figure 1).

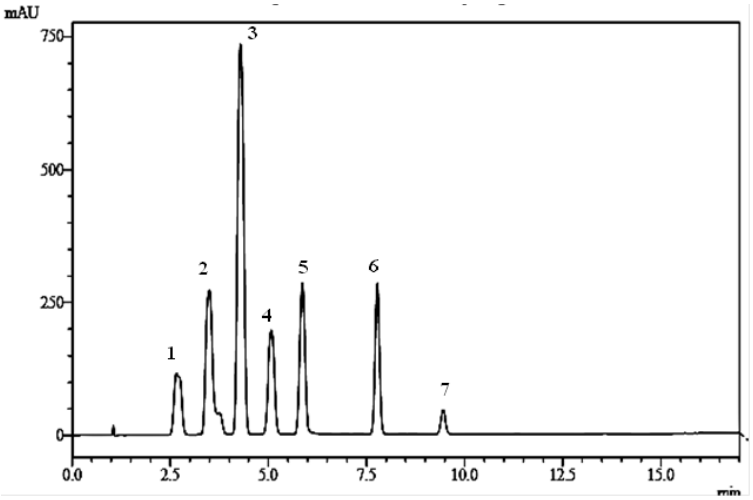

Figure 1. RP-HPLC chromatogram of phenolic standards (25 µM) searched in samples detected at 270 nm by DAD. Waters spherisorp ODS2 -C18 column (4.6 × 250 mm, 5 µm), gradient eluent acetic acid/acetonitrile/water, flow rate 1.2 mL/min. Peak identification: (1) protocatechuic acid, (2) *p*-hydroxy benzoic acid, (3) chlorogenic acid, (4) caffeic acid, (5) vanillin, (6) ferulic acid, (7) benzoic acid.

The concentration of chlorogenic acid is 9.903 mg/g and 9.87 mg/g for Turkish traditional coffee and green coffee, respectively. The concentration of benzoic acid is 7.5 mg/g and 36.007 mg/g for Turkish traditional coffee and green coffee, respectively (Table 2, Figure 2, Figure 3).

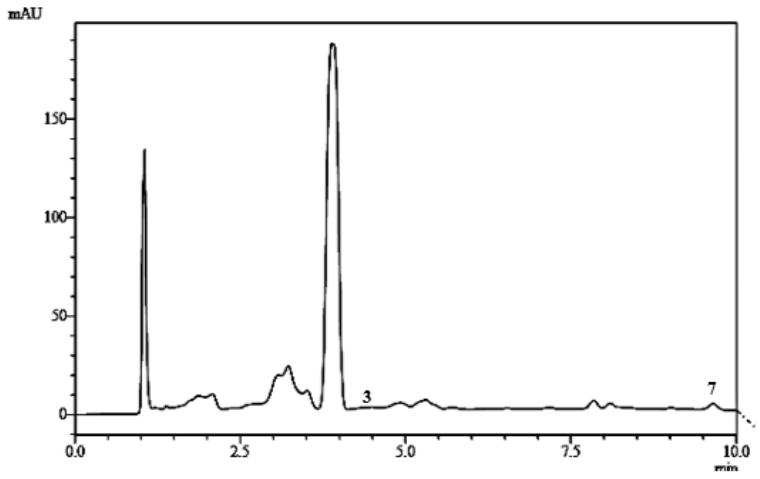

Figure 2. RP-HPLC chromatogram of phenolic standards (50 mg/mL) searched in methanolic extract of Turkish coffee detected at 270 nm by DAD. Waters spherisorp ODS2 -C18 column (4.6×250 mm, 5 µm), gradient eluent acetic acid/acetonitrile/water, flow rate 1.2 mL/min. Peak identification: (3) chlorogenic acid, (7) benzoic acid.

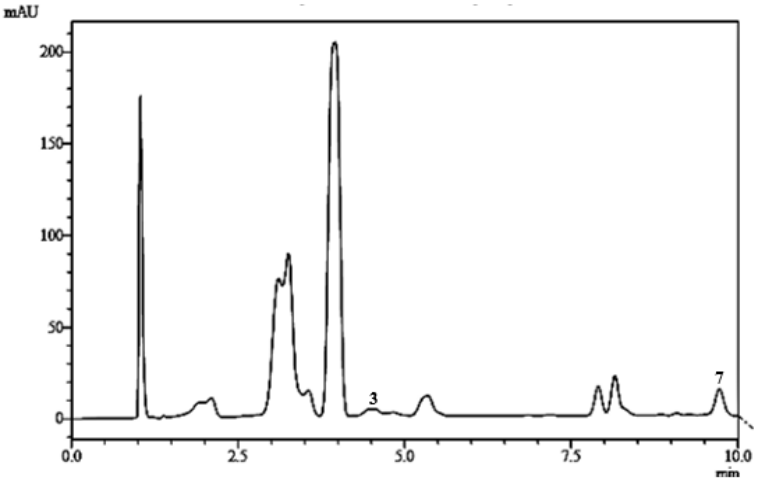

Figure 3. RP-HPLC chromatogram of phenolic standards (50 mg/mL) searched in methanolic extract of green coffee detected at 270 nm by DAD. Waters spherisorp ODS2 -C18 column (4.6×250 mm, 5 µm), gradient eluent acetic acid/acetonitrile/water, flow rate 1.2 mL/min. Peak identification: (3) chlorogenic acid, (7) benzoic acid.

Table 2. Phenolic composition of the methanolic extract of Green Coffee and Turkish coffee

	Amount (mg/ g)	
Compounds	**Green coffee**	**Turkish coffee**
Proto-catechuic acid	-	-
p-hydroxy benzoic acid	-	-
Chlorogenic Acid	9.870	9.903
Caffeic acid	-	-
Vanillin	-	-
Ferulic acid	-	-
Benzoic acid	36.007	7.500

3.3. Tyrosinase Inhibitory Activity of Coffee

According to our literature survey, there are a limited number of studies on Turkish traditional coffee, green cofffee (Erdem et al., 2016; Iwai et al., 2004). Iwai et al. (2004) reported that the dicaffeoylquinic acid isolated from green coffee beans also exhibited more potent (2.0-2.2-fold) tyrosinase inhibitory activities compared to caffeoylquinic acid, arbutin, and ascorbic acid (Iwai et al., 2004). Methanolic extract of green coffee was studied for enzyme inhibitory activity against tyrosinase at 25, 50, 100, and 500 µg/mL concentrations (Table 3, Figure 4). Methanolic extract of green coffee showed a high degree of inhibition against tyrosinase similar to positive control, kojic acid (Table 3).

Table 3. Tyrosinase inhibition % of the methanol extract of the green coffee and the reference (kojic acid) at 25, 50, 100, and 500 µg/mL concentrations.

Sample	Green coffee	Turkish coffee	Kojic acid[a]
I (25 µg/mL)	24.07 ± 2.95	2.56 ± 0.15	23.29 ± 0.34
II (50 µg/mL)	37.04 ± 2.83	10.25 ± 0.29	43.37 ± 0.66
III (100 µg/mL)	64.81 ± 1.85	11.20 ± 0.49	71.48 ± 0.65
IV (500 µg/mL)	92.59 ± 3.26	13.33 ± 0.33	92.77 ± 0.52

Standard error mean (S.E.M.)

[a]Positive control for inhibitory activity against tyrosinase

The IC_{50} values were determined as 63.53 µg/mL for kojic acid, 72.94 µg/mL for green coffee, and over 1000 µg/mL for Turkish coffee according to an equation of graphics (Figure 4).

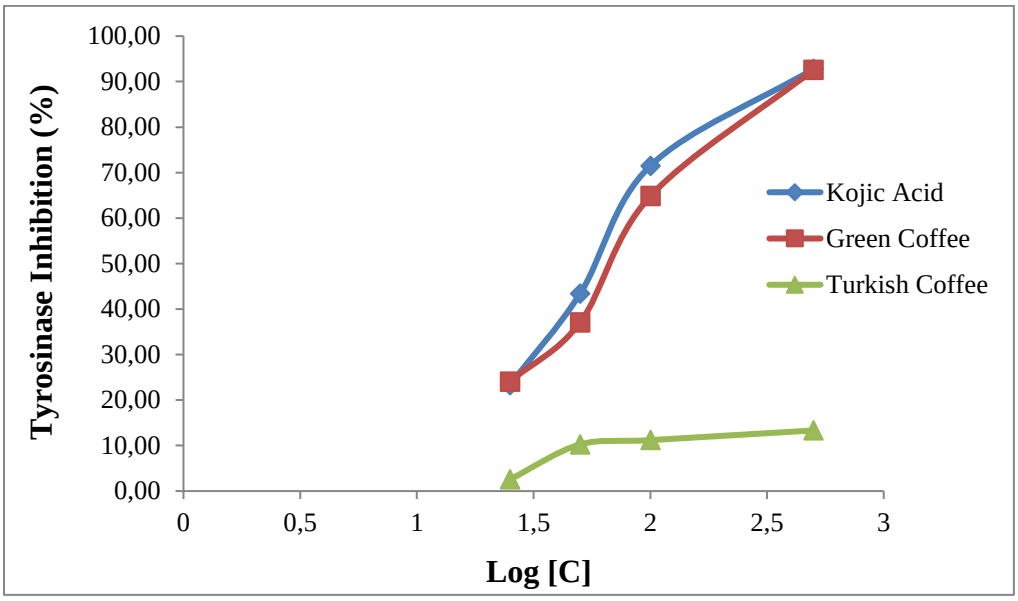

Figure 4. Tyrosinase inhibitions of the methanolic extract

The methanolic extract of green coffee extract possessed a remarkable inhibition against this enzyme (72.94 µg/mL) and was shown to contain chlorogenic acid and benzoic acid, its tyrosinase inhibitory potency might be suggested to be related to the polyphenols (Figure 3). Chlorogenic acid (an ester of caffeic acid and quinic acid) is the most abundant phenolic acid contained in coffee and has been described as a marker or characteristic compound. This has been confirmed by numerous studies investigating chlorogenic acid contents in coffee (Oliveira-Neto et al., 2004). Hence, it can be speculated that chlorogenic acid

found in Turkish traditional coffee, and green coffee may contribute to the skin-whitening effect in cosmetic through its strong antioxidant potential and moderate tyrosinase inhibitory action.

4. CONCLUSION

Our findings revealed that; extracts prepared from green coffee growing in Turkey, appear to have significant tyrosinase inhibitory and antioxidant properties, which might be possibly associated with the rich total phenol content of the green coffee. Thus, green coffee might be used as raw material by pharmaceutical industries for the preparation of natural drugs, in addition to the use in food industries.

DECLARATIONS

All authors declare that they have no conflicts of interest.

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Evaluation of Antileishmanial Activities of a Peganum harmala and Achillea millefolium Essential Oils and Their Combinations Against Leishmania infantum promastigotes

Peganum harmala ve Achillea millefolium Uçucu Yağlarının ve Kombinasyonlarının Leishmania infantum promastigotes'e Karşı Antileishmanial Aktivitelerinin Değerlendirilmesi

Fatemeh AYROM[1], Elsevar ASADOV[2*], Anita DADASHKHANI[1], Shafiqa SULEYMANOVA[2]

[1]Islamic Azad University, Faculty of Veterinary Medicine, Department of Pathobiology, Tehran, Iran.

ayrom1356@gmail.com, ORCID: 0000-0002-4021-7816

anitadadashkhane@gmail.com, ORCID: 0000-0002-8564-1303

[2]Nakhchivan State University, Faculty of Medical, Department of Basic Medical, Nakhchivan, Azerbaijan.

*asadoves@mail.ru, ORCID: 0000-0003-2892-2974

suleymanovasefiqe77@gmail.com, ORCID: 0000-0003-2986-3996

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***Corresponding author**

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Abstract

Medicinal plants and their derivations are used as safe agents for the treatment of parasitic diseases. This preliminary study investigates antileishmanial activities of *Peganum harmala* essential oil (PHEO), *Achillea millefolium* essential oil (AMEO) and their combinations against *Leishmania infantum* (*L. infantum*) promastigotes. A standard strain of *L. infantum* promastigote was cultured in a 96-well Novy-MacNeal-Nicolle (NNN) media culture and antileishmanial activities of glucantime, PHEO, AMEO, an equal ratio of both and 80% PHEO+20%AMEO were investigated in concentrations of 10, 100, 500 and 1000 mg/mL and interval times of 24h, 48h and 72h. The results showed that greatest inhibition was observed in 50% PHEO + AMEO and lowest inhibition was seen in control group. The increased time and increased concentration significantly increased their efficiencies. The analyses showed a significant interaction between time and agents [F (10, 360)=7.84, P=0.000]. The agents showed better effects with increased time. In sum, an equal combination of PHEO and AMEO showed its potential as an antileishmanial safe structure and must be considered for future studies.

Keywords: *Achillea millefolium*, Antileishmanial activity, *Peganum harmala*, Promastigote

Özet

Tıbbi bitkiler ve türevleri paraziter hastalıkların tedavisinde güvenli ajanlar olarak kullanılmaktadır. Bu ön çalışma, *Peganum harmala* uçucu yağı (PHEO), *Achillea millefolium* uçucu yağı (AMEO) ve bunların kombinasyonlarının *Leishmania infantum* (*L. infantum*) promastigotlarına karşı antileishmanial aktivitelerini araştırmaktadır. Standart bir *L. infantum* promastigot suşu, 96 kuyulu bir Novy-MacNeal-Nicolle (NNN) kültür ortamında yetiştirildi ve 10, 100, 500 ve 1000 mg/mL konsantrasyonlarda ve 24 saat, 48 saat ve 72 saat aralık sürelerinde glucantime, PHEO, AMEO, ikisinin eşit oranında ve %80 PHEO+%20 AMEO'nun antileishmanial aktiviteleri araştırıldı. Sonuçlar, en yüksek inhibisyonun %50 PHEO + AMEO'da gözlemlendiğini ve en düşük inhibisyonun kontrol grubunda görüldüğünü gösterdi. Artan zaman ve artan konsantrasyon, verimliliklerini önemli ölçüde artırdı. Analizler, zaman ve ajanlar arasında önemli bir etkileşim olduğunu gösterdi [F (10, 360)=7.84, P=0.000]. Ajanlar, artan süre ile daha iyi etkiler gösterdi. Özetle, PHEO ve AMEO'nun eşit kombinasyonu, antileishmanial güvenli bir yapı olarak potansiyelini göstermiştir ve gelecekteki çalışmalar için dikkate alınmalıdır.

Anahtar Kelimeler: *Achillea millefolium*, Antileishmanial aktivite, *Peganum harmala*, Promastigot

Abbreviations: AMEO, *Achillea millefolium* essential oil; MTT, Methyl thiazole tetrazolium; PHEO, *Peganum harmala* essential oil; NNN, Novy-MacNeal-Nicolle

1. INTRODUCTION

Leishmania infantum is one of the Leishmania species that causes visceral leishmaniasisis (Zheng et al., 2020). The disease is caused by parasitic protozoan and transmitted by the bites of infected female phlebotomine sandflies (Cabral et al., 2020). The protozoa cause serious challenges in all over the world and more than one billion people are at risk for the disease (mondiale de la Santé & Organization, 2021). It causes clinical signs from cutaneous form to the visceral form, fever, splenomegaly (enlargement of the spleen, manifested in the great majority of patients), hepatomegaly (enlargement of liver), pallor (caused by severe anemia), leucopenia (low white blood cell count), and weight loss (Gervazoni et al., 2020). Leishmania has two stages in its life cycle, including promastigote, and amastigote (Tavakoli et al., 2020). Promastigote is developed in sand fly body while amastigote is formed in macrophage (De Queiroz et al., 2014). Various agents are utilized to treat leishmaniasis. Glucantime has traditionally been used for the treatment of leishmaniasis (Lima et al., 2010). The current antileishmanial agents have limitations such as side effects, prolonged treatment period, high costs and induction of parasitic resistance (Herrera et al., 2020). Since antileishmanial drugs have limitations, researchers have sought novel drugs. Herbal medicine and their derivations

such as herbal extracts and essential oils have used as antileishmanial agents (Ayrom et al., 2021; de Paula et al., 2019; Delgado-Altamirano et al., 2017).

Esfand (*Peganum harmala* L.,) belongs to the family Zygophyllaceae and is found in Mediterranean regions such as Iran and Turkey (Asadzadeh et al., 2021). It contains a huge amount of seed (Asgarpanah & Ramezanloo, 2012) β-carboline alkaloids, quinazoline alkaloids, steroids, anthraquinones, flavonoids, and amino acids (Shao et al., 2013). Several studies have reported pharmaceutical properties of *P. harmala* essential oil such antimicrobial activities (Apostolico et al., 2016; Hajji et al., 2020; Khadhr et al., 2017). Studies have reported antileishmanial activity of *P. harmala* against *L. major* (Rahimi-Moghaddam et al., 2011).

Yarrow plant (*Achillea millefolium*) belongs the Asteraceae family and it is found in Asia, European and America (Acar et al., 2020). It is mainly contained amazulene, α-pinene, β-pinene, casticin, 1,8-cineole,cosmosiin and luteolin (Ali et al., 2017). It is known to have some properties such as anti-inflammatory, antipyretic, anthelmintic, antibacterial, antifungal, antitumor, antioxidant and anti-oedematous (Daniel et al., 2020). Studies have reported antileishmanial activity of *A. millefolium* essential oil (Santos et al., 2010).

Methyl thiazole tetrazolium (MTT) colorimetric methodies used cytotoxicity analyzes to human or animal cells (Ayrom et al., 2021).

A combination of both plants can have better antileishmanial activity in against *Leishmania infantum* promastigote. This preliminary study investigates antileishmanial activities of *P. harmala* essential oil (PHEO), *A. millefolium* essential oil (AMEO) and their combinations against *L. infantum* promastigotes.

2. MATERIALS AND METHODS

2.1. The Preparation of Essential Oils

The aerial parts of the *A. millefolium* and *P. harmala* seeds were prepared from a local market in the West-Azerbaijan province of Iran and identified by an expert botanist in Biology Department in Islamic Azad University, Urmia Branch. A. millefolium was prepared as reported by previous studies (Daniel et al., 2020). Briefly, aerial parts were dried, ground, and extracted by hydro distillation in Clevenger apparatus. *P. harmala* essential oil was prepared by hydro distillation in Clevenger apparatus as reported by previous studies (Yang et al., 2020). The prepared essential oils were dried over sodium sulfate anhydrous and kept at 0ºC after filtration. The aerial parts yielded for *A. millefolium* and *P. harmala* oils were 1.10% and 1.32% dry weight of the plant material, respectively.

2.2. Cultivation of *L. infantum* promastigote

A standard strain of *L. infantum* (MCAN/IR/96/LON49)) promastigote was provided from Urmia University of Medical Science and cultured in a 96-well Novy-Mac Neal-Nicolle (NNN) medium containing antibiotics as reported by previous studies (Ayrom et al., 2021).

2.3. MTT Test

The tests were conducted based on previous studies (Ayrom et al., 2021). Summary, promastigotes were cultured and incubated. MTT material was added it, incubated, removed and loaded with 100 µL DMSO (Toray Fine Chemicals Co., Ltd.). Densities were investigated by ELISA reader (Stat fax 2100, USA) at the wavelength of 570 nm. The most appropriate concentration of promastigote was 106 parasites/ml. Following dilution of promastigotes with liquid media of 1640 RPMI (Thermo Fisher Scientific Company), they were transferred into plates containing media culture and investigated in smear form. Various concentrations (10, 100, 500 and 1000 mg/mL) of PHEO, AMEO, Glucantime, 80%PHEO+20% AMEO (80PHEO+AMEO) and 50%PHEO+50% AMEO (50PHEO+ AMEO) were tested in time intervals of 24, 48 and 72 hours. We also considered wells lack of essential oil and Glucantime as control. Five replications were considered for each treatment in specific time points.

2.4. Data Analysis

The data were analyzed for normality by Kolmogorov-Smirnov test and the data were normal. The data were analyzed in a factorial arrangement with six agents (control, Glucantime, PHEO, AMEO, 80PHEO+AMEO, and 50PHEO+ AMEO), four concentrations (10, 100, 500 and 1000 mg/mL) and three interval times (24, 48 and 72 h). Main effects and interactions were investigated by SPSS software (version of 24). A $p<0.05$ was considered as significant.

3. RESULTS and DISCUSSION

Figure 1 shows the effects of commercial agent of glucantime and essential oils on inhibition percentage. The results showed that greatest inhibition was observed in 50PHEO+AMEO and lowest inhibition was seen in control group. Glucantime and 80PHEO+AMEO showed greatest antileishmanial activity after 50PHEO+AMEO and did not show significant differences (P=0.721). PHEO had better antileishmanial activity compared to AMEO.

An equal ratio of both essential oils had the best activity while 80% PHEO and 20% AMEO had lower effects. Glucantime showed lower activity compared to an equal ratio of both essential oils. The results for antileishmanial activities of AMEO and PHEO are similar to

results reported by previous studies (Rahimi-Moghaddam et al., 2011; Santos et al., 2010). Pharmacological activity of AMEO is attributed to its active compounds including sesquiterpene lactones, azuleneand flavonoids (Benedek et al., 2006). The inhibitory effects of PHEO could be attributed to its compounds such as β-carbolines and quinazoline derivatives (Mirzaie et al., 2007). β-carboline derivatives have antiparasitic activities. It was reported that other compounds of PHEO such as harmaline have in vivo antileishmanial activity (Evans & Croft, 1987). Other studies have reported antileishmanial activity of β-carboline alkaloids such asharmine and harmane (Di Giorgio et al., 2004). It was reported that plant active compound such as harmine and harmaline prevent mono-amino oxidase type A enzyme and cause psychological disorders such as hallucination (McKenna et al., 1984). An equal combination of PHEO and AMEO had better effects compared to single form and 80% combination that might be attributed to synergistic effects of PHEO and AMEO. A combination of PHEO and AMEO had equal and better effects with commercial agent of Glucantime that are parallel with results reported by previous studies (Ayrom et al., 2021).

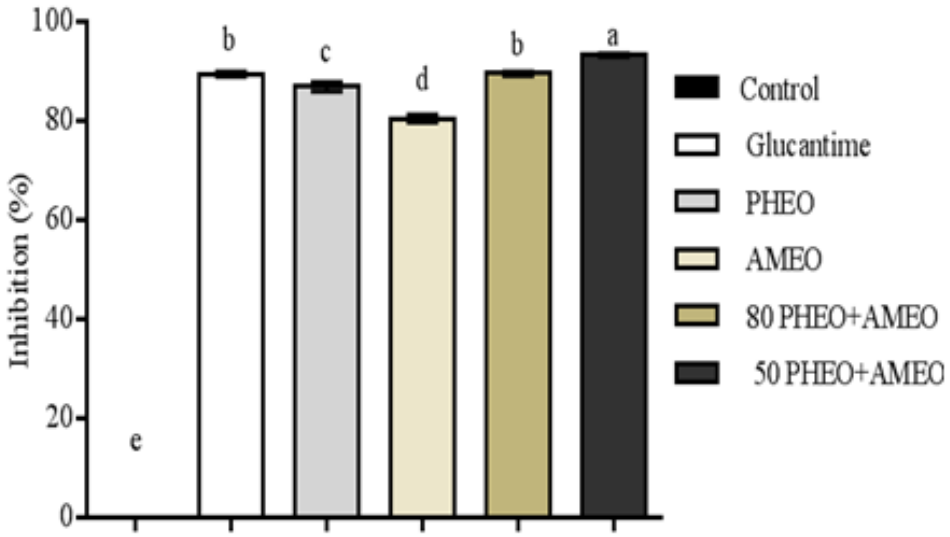

Figure 1. Inhibitory effect of the agents against leishmanial promastigotes. Different letters (a-e) on figures show significant differences between groups.

The results for the effects of different concentrations are shown in Figure 2. The results showed that the lowest antileishmanial activity was observed in concentration of 10 mg/mL and greatest inhibitory effects were seen in concentration of 1000 mg/mL ($P<0.05$). Significant differences were not seen between concentrations of 100 and 500 mg/mL ($P=0.061$). It means that increased concentration increases inhibitory effects. Similar to our findings, previous studies have reported that increased concentration raises antileishmanial activity of essential oils (Ayrom et al., 2021). As mentioned, essential oils show their activities via active

compounds. Having more active compounds causes that essential oils efficiently show their effects. Higher concentrations provide more synergism interaction effects for influencing on Leishmania.

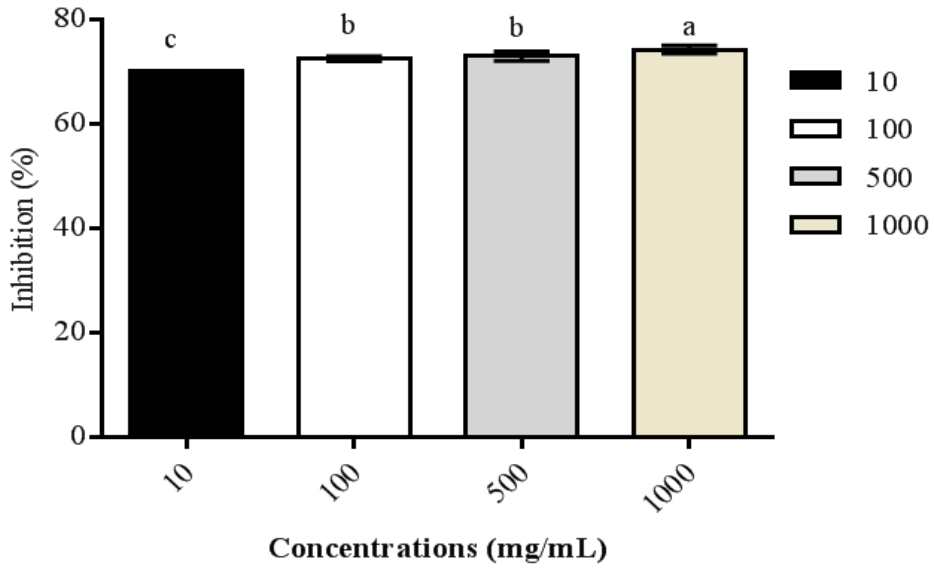

Figure 2. Inhibitory effects of different concentrations against leishmanial promastigotes.

Different letters (a-c) on figures show significant differences between groups.

Figure 3 shows inhibitory effects of treatments in different times against *L. infantum* promastigotes. The results showed that increased time raises inhibitory effects against *L. infantum* promastigotes. The lowest inhibitory effects were seen in time of 24 h while the greatest effects were observed in time of 72 h ($P<0.05$).

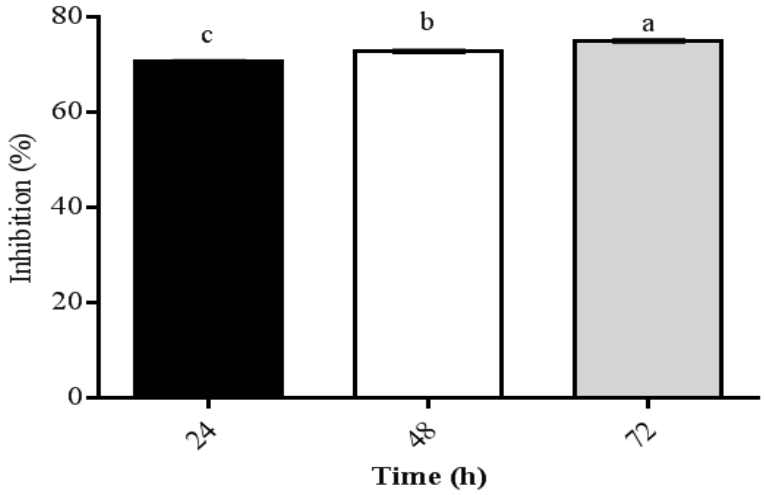

Figure 3. Inhibitory effects of treatments in different times against leishmanial promastigotes.

Different letters (a-c) on figures show significant differences between groups.

The results for the effects of time on inhibition of *L. infantum* are in agreement with previous studies (Ayrom et al., 2021). Seemingly, agents need more time for affecting on parasites and increased time improves its efficiency.

The results for interactions did not show significant differences for interaction between agents and concentration [F (15, 360)=0.836, *P*=0.637], for interaction between time and concentration [F (6, 360)=0.266, *P*=0.952], and also for interaction between agent, time and concentration [F (30, 360)=0.211, *P*=1.00]. The analyses showed a significant interaction between time and agents [F (10, 360) =7.84, *P*=0.000]. The agents showed better effects with increased time (Figure 4).

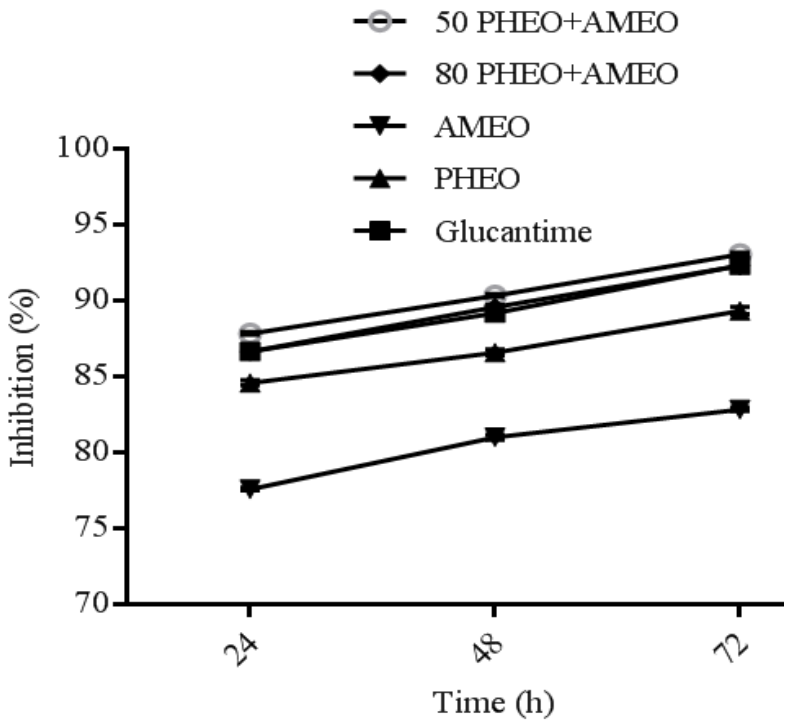

Figure 4. Interaction between treatments in different times of 24, 48 and 72 h.

4. CONCLUSION

In conclusion, a combination of AMEO and PHEO in an equal ratio had the best antileishmanial activity against *L. infantum* promastigotes. An equal ratio of both AMEO and PHEO could compete with synthetic agent of Glucantime and is a safe structure for the treatment of leishmaniasis.

DECLARATIONS

All authors declare that they have no conflicts of interest.

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Rheumatoid Arthritis – Is There a Role for Apitherapy? Analysis of Books Written by Apitherapists Shows that Most Recommendations are Not Evidence-Based

Romatoid Artrit – Apiterapinin Rolü Var mı? Apiterapistler Tarafından Yazılmış Kitapların Analizi, Önerilerin Çoğunun Kanıta Dayalı Olmadığını Gösteriyor

Karsten MÜNSTEDT

Ortenau Clinic Offenburg-Kehl, Offenburg, Baden-Württemberg, Germany.

karsten.muenstedt@web.de, ORCID: 0000-0003-4273-5964

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***Corresponding author** /Yazışılan yazar

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Abstract

Apitherapy is a therapeutic approach based on the use of beehive products. It is frequently suggested for the treatment of rheumatoid arthritis. This study was to assess the recommendations of apitherapists regarding the treatment of rheumatoid arthritis. 129 books written by apitherapists were reviewed regarding their recommendations for rheumatoid arthritis. These recommendations were compared to the findings of preclinical and clinical studies on the subject. Sixty-eight (52.7%) of the books mention the topic of rheumatoid arthritis and there were 44 different recommendations. They include all bee products (honey, pollen, propolis, etc.) but mainly bee venom (15 times) and royal jelly (5 times). Bee venom acupuncture (apipuncture) is mentioned only once. Compared to analyses from the scientific literature, only bee venom but mainly apipuncture are supported. This analysis shows that the majority of apitherapeutic books do not provide adequate information. However, some reports supported the scientific evidence that bee venom and apipuncture could be an interesting means of treatment of rheumatoid arthritis but more and higher quality clinical investigations are necessary.

Keywords: Apitherapy, Bee venom, Honey, Propolis, Rheumatoid arthritis

Özet

Apiterapi, arı kovanı ürünlerinin kullanımına dayalı bir tedavi yaklaşımıdır. Romatoid artrit tedavisi için sıklıkla önerilmektedir. Bu çalışma, apiterapistlerin romatoid artrit tedavisine ilişkin önerilerini değerlendirmek amacıyla yapılmıştır. Apiterapistler tarafından yazılan 129

kitap, romatoid artrit önerileri açısından gözden geçirilmiştir. Bu öneriler, konuyla ilgili klinik öncesi ve klinik çalışmaların bulgularıyla karşılaştırılmıştır. Kitapların 68'i (%52.7) romatoid artrit konusuna değinmektedir ve 44 farklı öneri bulunmaktadır. Bu öneriler tüm arı ürünlerini (bal, polen, propolis vb.) içermekle birlikte başlıca arı zehiri (15 kez) ve arı sütüne (5 kez) değinilmiştir. Arı zehri akupunkturundan (apipunktur) sadece bir kez bahsedilmiştir. Bilimsel literatürdeki analizlerle karşılaştırıldığında, sadece arı zehri ancak ağırlıklı olarak apipunktur desteklenmektedir. Bu analiz, apiterapötik kitapların çoğunun yeterli bilgi sağlamadığını göstermektedir. Bazı araştırmalar arı zehri ve apipunkturun romatoid artrit tedavisine yönelik farklı bir tedavi yaklaşımı olabileceğine yönelik bilimsel kanıtları desteklemekte olup bununla birlikte bu konuda daha fazla ve daha kaliteli klinik araştırmalara ihiyaç vardır.

Anahtar Kelimeler: Apiterapi, Arı zehri, Bal, Propolis, Romatoid artrit

1. INTRODUCTION

Rheumatoid arthritis (RA) is defined as a usually chronic autoimmune disease that is characterized especially by pain, stiffness, inflammation, swelling, and sometimes destruction of joints. The chronic inflammation of the synovial membranes leads to the destruction of cartilage and the adjoining bone, with the destruction of affected joints. Typical symptoms include pain, restriction in mobility, and fatigue, as well as inflammations of tendon sheaths, blood vessels, and internal organs. According to the 2010 American College of Rheumatology/European League Against Rheumatism, the classification criteria for rheumatoid arthritis are based on a score based on four criteria (Aletaha et al., 2010):

- Joint involvement (number of joints involved, especially small joints)
- Serology (positivity of rheumatoid factor (RF) and/or anti-citrullinated protein antibody (ACPA)
- Acute-phase reactants (abnormal C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR)
- Duration of symptoms (< 6 weeks or ≥ 6 weeks)

Rheumatoid arthritis is the most common chronic inflammatory joint disorder in industrial countries. Its prevalence varies between 0.5% to 1.0% in the adult population. Women are more frequently affected than men. A study in the UK found the minimum population prevalence of RA is 1.16% in women and 0.44% in men (Institute for Quality and Efficiency in Health Care, 2015; Littlejohn & Monrad, 2018).

Early diagnosis and initiation of treatment are of great importance. If rheumatoid arthritis is diagnosed and treated within 3 to 6 months after the onset of symptoms, the immunological process can be influenced significantly; otherwise, the course of the disease

may vary greatly (Institute for Quality and Efficiency in Health Care, 2015; Littlejohn & Monrad, 2018).

Because of the importance of the disease, treatment guidelines have been issued by various national societies, e.g. Deutsche Gesellschaft für Rheumatologie (German Rheumatology Association) (Fiehn et al., 2018). The main aspects of these guidelines include that:

- Patients with functional restrictions should receive physiotherapy in order to maintain physical fitness and for the short-term reduction in pain. These patients should receive occupational therapy.
- Methotrexate is considered to be the treatment of the first choice.
- Administration of glucocorticoids is suggested until initial therapy achieves an effect. Afterward, glucocorticoids should be reduced as rapidly as feasible.
- In more complicated cases (intolerance to methotrexate, high disease activity, failure to respond to tocilizumab) patients should receive TNF-α antagonists, tocilizumab, a monoclonal antibody against the interleukin-6 receptor or tofacitinib, a Janus kinase inhibitor or rituximab.
- For the treatment of pain and joint stiffness, the use of non-steroid anti-inflammatory drugs (NSAIDs) or cyclooxygenase (COX)-2 inhibitors are recommended.

Although there are many treatment options in conventional medicine, many patients consider and use methods from so-called integrative, complementary or alternative medicine (CAM). User rates and the dominant CAM methods vary greatly between countries. In India, only 26.2% of patients with rheumatoid arthritis used CAM, mainly Ayurveda (Bhalerao et al., 2013). Of the patients in Turkey, 46.9% used herbs taken orally, nutritional supplements, and mind-body therapies (Tokem et al., 2014), whereas in Korea 82% of the patients had used CAM, mainly traditional Oriental medical treatments, plant- and animal-derived over-the-counter healthcare products, and manual therapies (Lee et al., 2008).

A recent systematic review of randomized controlled trials identified and analyzed 60 good-quality trials using CAM as an intervention for patients with rheumatic diseases. In the main, acupuncture was considered effective in the many trials relating to acupuncture, ayurvedic treatment, homeopathic treatment, electricity, natural products, megavitamin therapies, chiropractic or osteopathic manipulation, and energy healing therapy (Phang et al.

2018). However, different authors consider far more therapies to be potentially beneficial (Fernández-Llanio et al., 2016): fasting followed by a vegetarian diet, Mediterranean diet, fish oil, virgin olive oil, vitamin D, probiotics, herbal medicinal products (*Tripterygium wilfordii*), physical activity, yoga and tai chi, meditation/mindlessness, acupuncture, homeopathy, and balneotherapy/hydrotherapy.

Today's literature largely ignores a very old type of treatment – apitherapy. It is defined as the use of substances produced by honeybees (such as venom, propolis, or honey) to treat various medical conditions. According to other publications, the use of bee venom for rheumatism or arthritis has a long history that can be traced back to ancient Egypt, Greece, and China (Chen et al., 2010). Today, apitherapy is widely promoted by apitherapeutic societies all over the world and beekeepers (https://apitherapy.com/addresses/societies/; accessed April 2nd, 2020). It must be noted that there are two different directions: scientific apitherapy and holistic apitherapy. Holistic apitherapy can be considered as a part of complementary and alternative medicine and is largely promoted in books, apitherapeutic congresses, and beekeeping congresses. However, personal experiences and treatment concepts of holistic apitherapists are mainly published as books being guidelines for other practitioners since there are no uniform concepts for the education of apitherapists.

So far, there have been no detailed reviews on the potential benefits of holistic apitherapy for rheumatoid arthritis. Therefore we

- analyzed the recommendations in various apitherapy books
- analyzed the scientific evidence of bee venom therapy
- tried to answer the question of whether apitherapy could be a reasonable means of treatment of rheumatoid arthritis for patients who wish to use methods from integrative, complementary, or alternative medicine.

2. MATERIALS and METHODS

Using the term "apitherapy" on PubMed and the selection "books and documents" no results were retrieved. Therefore, we had to look for different search strategies.

On bookseller platforms and the JUSTfind system of the Justus-Liebig-University Gießen, Germany, which comprises 337 databases from the EBSCO Discovery Service we identified 131 books on apitherapy. Apart from the search terms "apitherapy", "apitherapie" and "apithérapie" we search for possible book issues by opinion leaders, such as former and

current presidents of the Apimondia Scientific Commission on Apitherapy and presidents of societies for apitherapy. Therefore, we also included one book in Italian because the author was Dr. Mateescu, the current president of Apimondia's commission on apitherapy. In all, 129 books on apitherapy could be obtained and were analyzed regarding the recommendations concerning rheumatoid arthritis. There was no selection of the books except for restrictions to English, French, and German language. An overview of the languages in which the books were published and the countries of origin of the authors are given in Table 1. All books were analyzed in detail for apitherapeutic recommendations regarding rheumatoid arthritis. We are aware of the fact that there are significant contributions published in other languages such as Spanish, Romanian and Russian. However, the main impetus regarding apitherapy and rheumatoid arthritis came from Filip Terč (1844–1917), Charles Mraz (1905–1999), and Bodog F. Beck (1871–1942). Their contributions are included in this work.

Table 1. Authors' nationalities and languages of the various analysed books.

	Authors' nationalities [n; (%)]	Language of the book [n; (%)]
German	52 (40.9)	91 (70.5)
English	8 (6.2)	27 (20.9)
French	14 (10.9)	10 (7.8)
Italian	2 (1.6)	1 (0.8)
USA	23 (17.8)	
Romanian	8 (6.2)	
Austrian	5 (3.9)	
Russian	3 (2.3)	
Bulgarian	3 (2.3)	
Ukrainian	2 (1.6)	
Dutch	1 (0.8)	
Swiss	1 (0.8)	
Lithuanian	1 (0.8)	
Algerian	1 (0.8)	
Yugoslavian	1 (0.8)	
Iraqi	1 (0.8)	
Isrealian	1 (0.8)	

n: Total number

Additionally, we looked at whether there is scientific evidence regarding the use of bee products for rheumatoid arthritis, using PubMed, Scopus, and JUSTfind. We used the

search term 'rheumatoid arthritis' combined with the names of various bee products. All retrieved articles were read in full except for the studies published in languages other than English, German, and French. However, it was not our intention to do a systematic review on the topic. These results were only meant to be compared with the suggestions of the books of holistic apitherapy.

3. RESULTS and DISCUSSION

3.1. Historic Data

In 1888 Filip Terč published a case series of 173 patients who received 39,000 bee stings for rheumatoid arthritis. Later in 1912, his son Rudolf Tertsch summarized the experiences of his father and reported that 82% (544/663) could be cured with the help of bee stings (Terč, 1888; Tertsch, 1912).

3.2. Analysis of Apitherapy Books

Sixty-eight out of 129 books (52.7%) mention the topic of rheumatoid arthritis. From these, there are 44 different recommendations. Among these, the recommendation for bee venom was found 15 times and royal jelly five times. All other recommendations were promoted once or twice (Table 2). Interestingly, in only five books (7.3%) we found detailed recommendations regarding the exact dosage and administration of bee products.

Table 2. Recommendations of apitherapists for the treatment of rheumatoid arthritis.

	Frequency	Percent
Bee venom, bee venom acupuncture	1	1.5
Bee venom	15	22.1
Honey, propolis, bee venom	2	2.9
Propolis, royal jelly, bee venom, bees wax	2	2.9
Honey, propolis, bee venom ointment, bee venom acupuncture, bee venom ultrasound administration	1	1.5
Honey (external use), bee venom, bees wax, oxymel	1	1.5
Honey, bee venom	1	1.5
Honey, honey massage, pollen, perga, royal jelly, bee venom, apilarnil	1	1.5
Honey massage, bee venom	1	1.5
Propolis, royal jelly, bee venom, aromiel	1	1.5

Propolis, propolis ointment, propolis compresses, bee venom, stinging nettle	1	1.5
Propolis, bee venom, aromiel	1	1.5
Royal jelly, bee venom	1	1.5
Bee venom, apipressure with honey, propolis, pollen, perga, royal jelly, bee venom, bees wax and apilarnil	1	1.5
Bee venom, bee venom ointment, bees wax compresses	1	1.5
Bee venom, honey compresses	1	1.5
Bee venom, honey compresses, baths, advice against bee venom ointment	1	1.5
Bee venom, bees wax compresses	1	1.5
Royal jelly	5	7.4
Propolis ointment	2	2.9
Propolis	2	2.9
Pollen	2	2.9
Pollen, tea, baths	2	2.9
Forrest honey	1	1.5
Honey, propolis, propolis ointment and tea	1	1.5
Honey, propolis, pollen, perga, royal jelly, bees wax, apilarnil, chitosan	1	1.5
Honey, propolis, propolis ointment, royal jelly	1	1.5
Honey, diet	1	1.5
Honey, cinnamon	1	1.5
Honey massage	1	1.5
Honey massage, diet	1	1.5
Honey compresses, camphor	1	1.5
Honey bath, honey with plant additives (juniper berries)	1	1.5
Propolis ointment, tree gum	1	1.5
Propolis, pollen, propolis compresses, baths	1	1.5
Propolis, royal jelly	1	1.5
Propolis, bees wax	1	1.5
Propolis compresses	1	1.5

Royal jelly, propolis combined with royal jelly	1	1.5
Royal jelly, diet baths	1	1.5
Royal jelly, bee venom ointment, homoeopathy – bee venom	1	1.5
Royal jelly, bee venom ointment, bee cellular therapy, honey compresses	1	1.5
Bee venom ointment, bee venom embrocation	1	1.5
Bee venom ointment, honey compresses, propolis ointment, bees wax packing	1	1.5
Total	68	100.0

As Figure 1 shows, the early publications of Terč (1888) and Tertsch (1912) should have allowed all authors who cover the topic of rheumatoid arthritis to mention bee venom. It is comprehensible that all books published in the 20th century did not mention apipuncture, the combination of bee stings, and acupuncture.

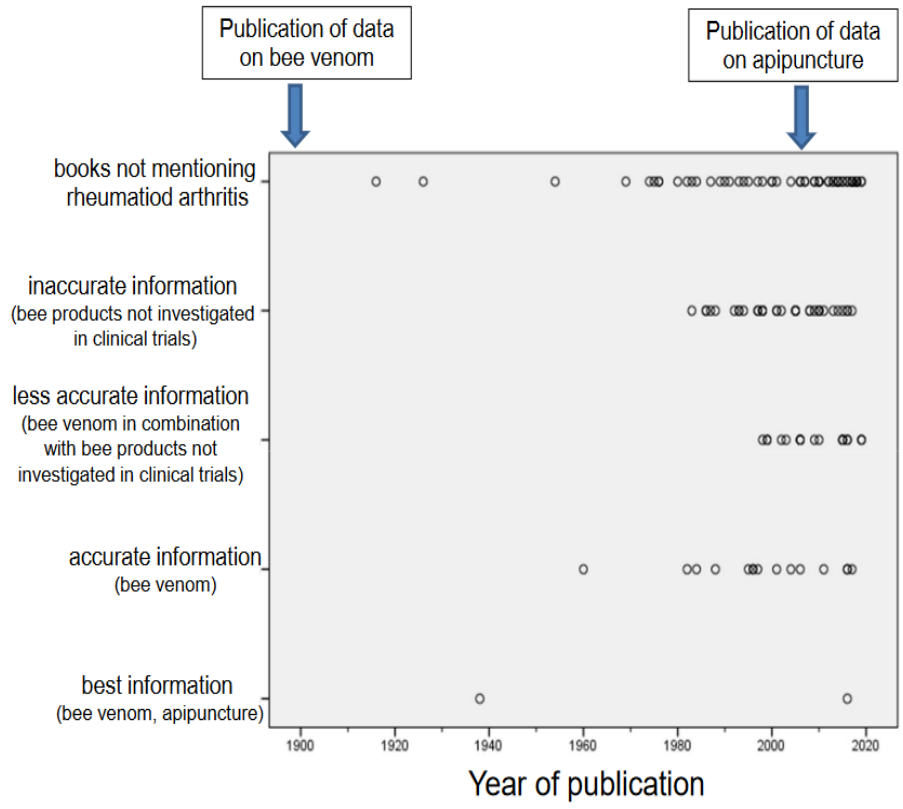

Figure 1. Correctness of apitherapeutic publications depending on the year of publication

3.3. Analysis of the Scientific Literature

The search on PubMed, Scopus, and JUSTfind did not reveal any relevant publications on the topic of rheumatoid arthritis and bee products except for bee venom. Thus, to the best of our knowledge, there are no works, in vitro studies, animal trials, or clinical trials on honey, pollen, propolis, royal jelly, beeswax, apilarnil, and all other bee products. The only study which used honey was an ayurvedic study that used Sidh Makardhwaj, an ayurvedic formulation on the basis of *swarna* (gold), *parada* (mercury), and *gandhaka* (sulphur), which was administered with honey (Kumar et al., 2015). This study was not analyzed in detail because honey was not the main focus of the study. However, recent work suggests that some bee products (honey, propolis) could positively influence the disease (Jagua-Gualdrón et al., 2020). However, this conclusion is only derived from the positive effects of bee products in the treatment of other diseases than RA, and clinical evidence is lacking. Due to the lack of clinical trials on any bee product except bee venom, it is not possible to do a systematic review on apitherapy. The evidence regarding bee venom is described below.

3.4. Evidence Regarding the Use of Bee Venom and Bee Venom Acupuncture (Apipuncture)

The literature search using PubMed, Scopus, and JUSTfind identified basically two fields of interest: bee venom and the combination of bee venom with acupuncture, also called apipuncture, which means that bee stings are applied to the respective acupuncture points.

3.5. Use of Bee Venom

Apart from the reports by Filip Terč and his son, there are few clinical trials on the subject. In 1966 a study of 50 cases was presented, which reported an 84% benefit for bee venom therapy (Steigerwaldt et al., 1966). Apart from this study, there is only in vitro data on the subject. It seems that melittin exerts multiple positive effects on various pathways resulting in a potent anti-arthritis and anti-inflammatory effect by inhibiting sodium nitroprusside, IKK activity, IkB release, the activation of NF-kB, and inflammatory mediators such as iNOS, COX-2, NO, and PEG2, and inactivating the JNK pathway (Zhang et al., 2018).

3.6. Bee Venom Acupuncture

In 2014, Lee and co-workers undertook a systematic review and concluded from one eligible randomized, controlled trial that compared with placebo, bee venom acupuncture may effectively improve joint pain, swollen joint counts, tender joint counts, erythrocyte sedimentation rate, and C-reactive protein, but was not shown to improve morning stiffness

(Lee et al., 2014). Another clinical trial was not included in the systematic review for reasons that we cannot explain (Lee et al., 2014). This study, which assessed five treatment options in 75 patients, concluded that bee venom is an effective treatment for rheumatoid arthritis because it has anti-inflammatory properties with the inhibition of nitric oxide and prostaglandin production (Abdel-Rahman et al., 2013).

Another trial not included in the systematic review is that by Liu et al. (2008) which compared bee venom acupuncture in combination with methotrexate, sulfasalazine, and meloxicam (Western medicines) versus methotrexate, sulfasalazine, and meloxicam only and found that the combined application of bee venom therapy and medication is superior to the simple use of medication. The authors consider that the bee-sting therapy may allow a dose reduction of Western medicines and lower relapse rates (Liu et al., 2008).

Recently another Chinese trial compared apipuncture to standard therapy and found improvements regarding the duration of morning stiffness, arthralgia index, swollen joint count index, joint tenderness index, 15-minute walking time, and various other parameters, with no differences between the groups. The authors found no differences between bee venom acupuncture and standard therapy (Chen et al., 2018). Apart from this clinical study, there have been only animal trials and a case report that provide evidence for the use of bee venom (Tekeoglu et al., 2016; Yamasaki et al., 2015; Zhao et al., 2016).

3.7. Comparison of Apitherapeutic Recommendations and Clinical Evidence

In summary, the current scientific data provide evidence that only bee venom and bee venom acupuncture could be reasonable treatment options for rheumatoid arthritis. As mentioned in the introduction, acupuncture alone could be a reasonable treatment option (Phang et al., 2018).

There is no evidence whatsoever regarding any other bee product. In this respect, only recommendations of bee venom or bee venom acupuncture can be considered reasonable. Therefore, only one book (1.5%) by Hainbuch may be considered to provide the most accurate information mentioning both apipuncture and bee venom, whereas 15 books (22.0%) mention bee venom only Hainbuch, 2016. Recommendations that include bee venom must be considered of little accuracy since the co-administration of bee products may cause ‘drug’ interactions and result in side effects due to the bee products (18 books, 26.5%). Recommendations that do not include bee venom or apipuncture must be considered inaccurate (31 books, 45.6%).

This work shows that the vast majority of recommendations issued in apitherapeutic books must be considered inaccurate or of little accuracy when compared with the scientific

evidence. As shown, there is no scientific background for the use of honey, aromiel, apipressure with honey, propolis (including ointment and compresses), royal jelly, beeswax, oxymel, honey massage, pollen, perga, apilarnil, chitosan, and bee cellular therapy for the treatment of RA. There is the only evidence on bee venom and bee venom acupuncture (apipuncture). Therefore, recommendations of apitherapists must be regarded with great caution. Their practices are not evidence-based and possibly unsafe.

Acknowledging the fact that Terč published his observations in the 19th century and that bee venom acupuncture first became popular at the beginning of the 21st century it is clear that not all books could have provided the optimal information regarding bee venom and apipuncture. However, the question remains why a considerable number of books that mention the topic of rheumatoid arthritis recommend one or more bee products that have never been investigated in this context and often do not mention bee venom and apipuncture despite scientific data. This question cannot be answered by this study. However, the great variety of recommendations shows that there is no uniform treatment concept behind holistic apitherapy. The recommendations appear to be arbitrary and it seems that apitherapists have not compared their recommendations with the results of scientific investigations. This calls for the necessity of education of apitherapists.

However, even for bee venom and bee venom acupuncture, there are several problems that need to be solved before both can be put into clinical practice:

1. Can bee venom be regarded as clinically efficient for the treatment of rheumatoid arthritis?
2. Is bee venom acupuncture superior to bee venom treatment alone?
3. Should bee venom be used in the light of its potential allergic side effects?

3.8. Can Bee Venom Be Regarded As Clinically Efficient For The Treatment of Rheumatoid Arthritis?

Most studies indicate that bee venom and bee venom acupuncture could be interesting treatment options (Lee et al. 2014). Systematic reviews and meta-analyses for other diseases have also confirmed the efficacy of bee venom. These diseases include post-stroke shoulder pain, musculoskeletal pain, and acne vulgaris (Coao et al., 2005; Lee et al., 2008; Lim & Lee, 2015). In particular, the positive effects on post-stroke shoulder pain and musculoskeletal pain allow the conclusion that there are common working mechanisms responsible for positive effects.

3.9. Is Bee Venom Acupuncture Superior to Bee Venom Treatment Alone?

To answer the question, it is important to understand the effects of acupuncture. A meta-analysis on hot flushes in menopausal women with breast cancer concluded that there is insufficient evidence to affirm the effectiveness of traditional Chinese acupuncture compared to sham acupuncture and that there may be slight superiority in the effectiveness of traditional Chinese acupuncture (Lopez-Júnior et al., 2016). Another review confirmed that many clinical trials and experimental studies show that sham acupuncture is as effective as traditional Chinese acupuncture (Zhang et al., 2016). This review concluded that it is important to set standards for acupuncture studies in the future before firm conclusions on the efficacy of acupuncture can be drawn (Zhang et al., 2016). Likewise, it is important that future randomized trials on apipuncture will have an appropriate comparative arm in order to meet current scientific standards.

3.10. Should Bee Venom Be Used in The Light of its Potential Allergic Side Effects?

Treatment with bee venom is associated with the risk of an allergic reaction. According to an analysis by Park et al., bee venom acupuncture shows a 261% increased relative risk for the occurrence of adverse events compared to saline in randomized controlled trials (Park et al., 2015). However, the authors were not sure whether this risk is over-or underestimated because of poor reporting quality in the included studies. As shown, there is a wide range of possible side effects after treatment with bee stings. These include ulnar nerve injury, immune thrombocytopenia, Guillain-Barré syndrome, hepatotoxicity, and many others (Abdulsalam et al., 2016; Alqutub et al., 2011; Lee et al. 2015; Park et al., 2017). To reduce these problems, the use of sweet bee venom, which is comprised of mellitin only, was suggested. However, systemic hypersensitive reactions have already been reported with this concept (Jo & Roh, 2015).

3.11. Limitations

Since we were able to analyze only books in English, French, and German language we may have missed books published in other languages. However, books from famous apitherapists from Russia, Romania, and other countries have been translated into English, French, and German, as indicated in Table 1. Thus, we believe that we did not miss relevant publications. Furthermore, we are aware of the fact that the current practices of apitherapists may differ from what is written in the books. It is also possible that we missed publications on apitherapy and bee products regarding RA which were not listed in the referred search engines.

4. CONCLUSION

According to this analysis, the vast majority of apitherapeutic books do not provide adequate information. Use of the bee products honey, aromiel, apipressure with honey, propolis (including ointment and compresses), royal jelly, beeswax, oxymel, honey massage, pollen, perga, apilarnil, chitosan, and bee cellular therapy cannot be recommended for the treatment of RA. Also, the usefulness of bee products together with other methods and procedures has not yet been shown in clinical studies, as well as an integrative approaches. This negative recommendation is not only because of a lack of scientific evidence but also due the fact that bee products are frequently associated with allergic reactions. Overall, there is evidence that bee venom could be an interesting means of treatment of rheumatoid arthritis. However, especially bee venom is frequently associated with allergic reactions which will limit its use. As shown, bee venom must be considered effective for the treatment of several conditions, e.g. low back pain and post-stroke pain (Lim & Lee, 2016; Shin et al., 2012). However, more and higher quality clinical studies are necessary in order to establish its value for RA either alone or in combination with acupuncture (apipuncture).

DECLARATIONS

The author declares that has no conflicts of interest.

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Chemical and Palynological Properties of Ayder (Camlihemsin/Rize) Honeys

Ayder (Çamlıhemşin/Rize) Ballarının Kimyasal ve Palinolojik Özellikleri

Esra DEMIR KANBUR[1*], Vagif ATAMOV[2]

[1]Recep Tayyip Erdogan University, Faculty of Arts and Sciences, Department of Biology, Rize, Turkey.

*esra.demir@erdogan.edu.tr, ORCID: 0000-0002-0222-1865

[2]Recep Tayyip Erdogan University, Central Research Laboratory Aplication and Research Center, Rize, Turkey.

vagif.atamov@erdogan.edu.tr, ORCID: 0000-0002-6718-7979

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***Corresponding author** /Yazışılan yazar

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Abstract

Total number of pollen, pollen recognition, fructose, glucose, sucrose and moisture were examined in 41 honey samples collected in Ayder Plateau in August 2012. Following the pollen recognition in the samples, 36 taxa were detected. Castanea sativa Miller. pollen was found in all samples in a dominant. The total number of pollen (TPN-10 g) in 10g honey varieted between 3438 and 87056. It has been determined that honey samples were normal and dominant pollen. The moisture content in the samples ranged between 16.4-20.9%. In honey samples, fructose ranges from 14.05 to 52.82%, while glucose ranges from 14.31 to 49.24%.

Keywords: Ayder Plateu, Honey, Pollen analysis, TPN-10g, Sugar analysis

Özet

Ayder Yaylası'nda Ağustos 2012'de toplanan 41 bal örneğinde toplam polen sayısı, polen tanıma, fruktoz, glukoz, sükroz ve nem bakıldı. Örneklerde polen tanılamanın ardından 36 takson tespit edildi. *Castanea sativa* Miller. polen örneklerinin tamamında baskın olarak bulunmuştur. 10 gram baldaki toplam polen sayısı (TPN-10 g) 3438 ile 87056 arasında çeşitlenmiştir. Bal örneklerinin normal ve baskın polen olduğu belirlenmiştir. Numunelerdeki nem içeriği %16.4-20.9 arasında değişmiştir. Bal örneklerinde fruktoz %14.05 ile %52.82 arasında, glukoz ise %14.31 ile %49.24 arasında değişmektedir.

Anahtar Kelimeler: Ayder yaylası, Bal, Polen analizi, TPS-10g, Şeker analizi

Abbreviations: TPN, Total Pollen Number.

1. INTRODUCTION

Pollen and nectar from plants provide the raw materials for the food of honeybees, and the collection and processing of these raw materials are executed by these insects in an exciting manner, exhibiting an intricate pattern of behavior. The plant preferences of the honeybees can be elucidated from pollen contained in the honey and also from pollen loads (denoting perhaps the plants visited for pollen collection alone). Moreover, the bee pollen load analysis indicates the behavioral pattern and sense of selection of plants for food (Sharma & Abrol, 2005).

Ayder Plateau, with natural beauties, authentic architecture of the region and scientifically proven thermal spa, which has been proven to be healing for many diseases, has become the center of attention of local and foreign tourists. Also, its attractiveness increased with the Heliski sport, which was realized for the first time in our country in 2004. The Ayder Plateau is located on the left branch of the Firtina Valley and covers subalpine-alpine bushes and meadows at 1500-2300 m altitude (Figure 1). On the other hand, since the alpine belt consists of rash areas, it is relatively poor in terms of floristry (Demir & Atamov, 2019).

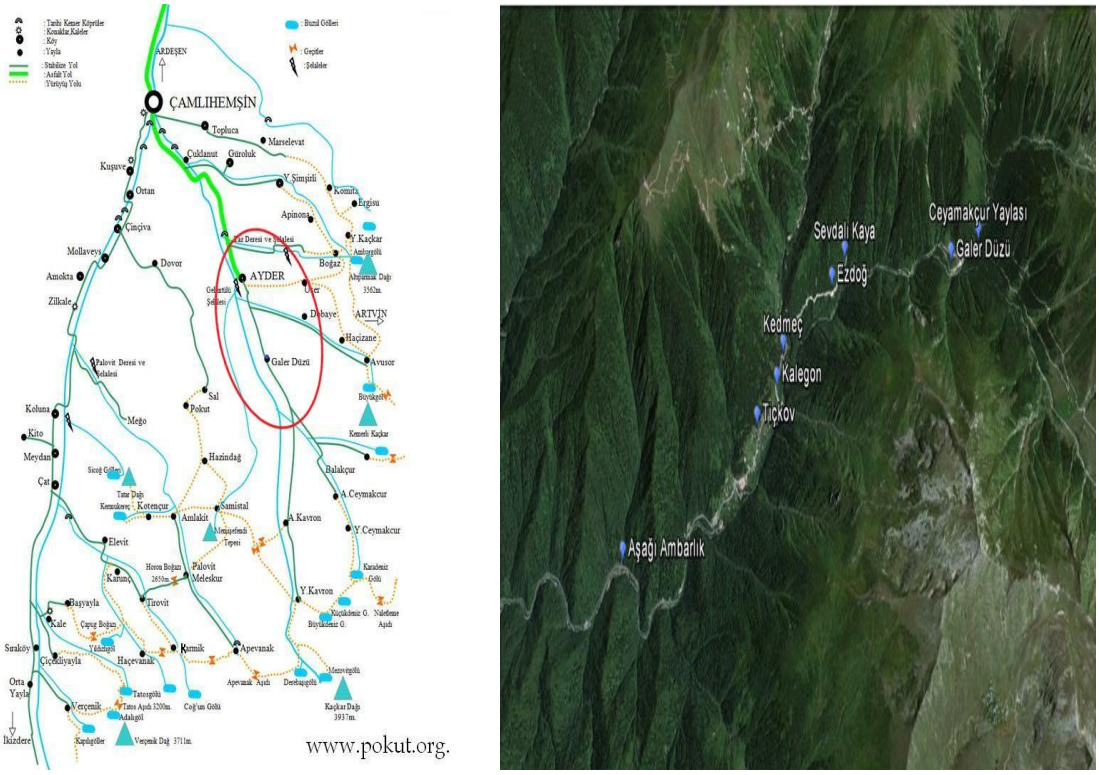

Figure 1. Ayder Plateau location and stations where samples were collected (Anonymous, 2022)

Typically, the verification of the geograpical origin of honey is realized by analysis of the pollen (mellisopalinology) in honey (Çelemli et al., 2021). This method is based on the identification of pollen by microscopic analyses. It requires an expert, it is very time consuming

and dependent on the expert's ability and judgments (Howels, 1969). Other methods that could be more widely used for characterizing honey have been sought for many years (Radovic et al., 2001).

This study was aimed to determine chemical and palynological properties of Ayder Plateau honey samples.

2. MATERIALS and METHODS

In this study, pollen analysis was performed on 41 honey samples collected in 2011 from hives in 8 different regions in Ayder Plateau and Galer Düzü locations. In addition, the pollen spectrum of the samples and the total number of pollen (TNP-10 g) was examined, moisture content and sugar analyses were also performed.

The honey samples were collected from different beehives in the month of July-August in 2011. During the field studies, herbarium materials were collected. Reference pollen slides were made from the herbarium materials.

The preparation of the honey samples was done using the method defined by the International Bee Research Association (Oddo et al., 2004). Preparations were made from each honey sample for identification of pollen. After identification, 200 pollen samples were counted in each preparation. Source books (Aytug et al., 1971; Sorkun, 2008), reference pollen slides were used during the pollen analyses. Nikon Eclipse E100 microscope was used for the analyses. Total pollen number (TPN-10g) analyses was done according to (Sorkun, 2007).

The amount of moisture in honey samples stored at 20 °C was measured with portable refractometer.

3. RESULTS and DISCUSSION

The average amount of TPN-10 is higher in honey samples collected from the Asagı Ambarlik, Tickov and Kalegon localities. The presence of *Castanea sativa* Miller. in these regions caused the total number of pollens to be high (Figure 2).

As a result of determining the amount of TPN-10 in 41 samples collected from different regions in Ayder Plateau, the total number of pollen in 10 g honey was found to be the lowest in the honey sample 34 and the highest in the sample 12 (Table 1). As a result of the determination of the amount of TPN-10 in 41 samples collected from different regions in Ayder Plateau, the total number of pollen in 10 g honey value was determined to be the minimum 3.438 in sample 34 and the maximum value was determined to be 87.056 in sample 12. Bayram

et. al. (2019) determined TPN values of 10 grams of honey between 16.024 and 90.126 in Bayburt Honeys. Ozler (2018) reported that TPN-10 values ranged from 332 to 42.496. The total number of pollen in 10 g honey value was determined to be the minimum 3.438 in sample 34 and the maximum value was determined to be 87.056 in sample 12. Çelemli et al. (2021) reported nearly same results.

Sorkun and Sahin (2000) reported that starch from pollen can be found in honey and this is a natural result. No starch grains were found in any of the 41 samples we collected.

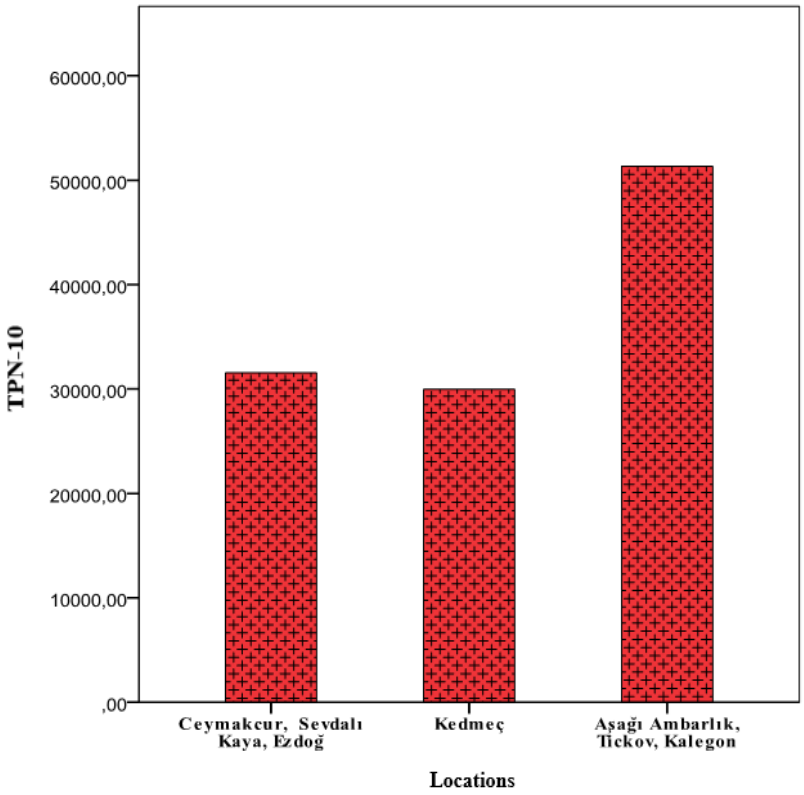

Figure 2. Average amounts of TPN-10 g according to the regions where the honey samples were collected

Table 1. Collected Area, TPN-10 g Values, Pollen Spectrum (*Dominant pollen,**Secondary Pollen,***Minor Pollen,****Rare Pollen)

Sample No	Collected Area	TPN-10 g	Pollen Spectrum *(*Dominant pollen,**Secondary Pollen,***Minor Pollen,****Rare Pollen)*
1	Tickov	27.225	* *Castanea sativa* ** - *** Brassicaceae, Fabaceae, Rosaceae **** Rosaceae, Fabaceae, Boraginaceae, Ericaceae, Caryophyllaceae, Pinaceae, *Onobrychis* sp., *Zea mays* ve *Carex* sp.

2	Tickov	15.050	* *Castanea sativa* ** - *** Brassicaceae, Boraginacea,Fabaceae **** Rosaceae, Ericaceae, Apiaceae, Chenepodiaceae, *Rumex* sp., *Onobrychis* sp., *Carex* sp., *Tilia* sp.
3	Kedmec	25.635	* *Castanea sativa* ** - *** Fabaceae **** Scrophulariaceae, Rosaceae, Brassicaceae, Ericaceae, Asteraceae, *Myosotis* sp.
4	Kedmec	21.135	* *Castanea sativa* ** - *** Brassicaceae, Ericaceae, Fabaceae **** Apiaceae, Rosaceae, Boraginaceae, *Salix* sp., *Rumex* sp., *Carex* sp., Liliaceae, Poaceae, Caryophyllaceae, Unidentified
5	Tickov	26.174	* *Castanea sativa* ** - *** Ericaceae, Rosaceae **** Brassicaceae, Rutaceae, Fabaceae, Caryophyllaceae, Boraginaceae, *Salix* sp., *Gentiana* sp.
6	Tickov	15.926	* *Castanea sativa* ** - *** Rosaceae, Brassicaceae, Ericaceae **** -
7	Kedmec	5.806	* *Castanea sativa* ** - *** Brassicaceae **** Rosaceae, Fabaceae, Ericaceae, Caryophyllaceae, *Salix* sp., *Onobrychis* sp., *Lotus* sp., *Myosotis* sp.
8	Kedmec	27.218	* *Castanea sativa* ** - *** Ericaceae, Rosaceae, Brassicaceae **** Fabaceae, Boraginaceae, Geraniaceae, Pinaceae, *Onobrychis* sp.
9	Kedmec	22.489	* *Castanea sativa* ** - *** Rosaceae, Brassicaceae **** Fabaceae, Ericaceae, Boraginaceae, Cistaceae, *Carex* sp., *Myosotis* sp.
10	Asagi Ambarlik	50.168	* *Castanea sativa* ** - *** - **** Ericaceae, Rosaceae

11	Asagi Ambarlik	54.908	* *Castanea sativa* ** - *** Ericaceae, Rosaceae **** Brassicaceae, Boraginaceae , Apiaceae, *Carex* sp., Unidentified
12	Asagi Ambarlik	87.056	* *Castanea sativa* ** - *** Ericaceae ve Rosaceae **** Brassicaceae, Caryophyllaceae, *Tilia* sp., *Gentiana* sp.
13	Kedmec	33.808	* *Castanea sativa* ** - *** Ericaceae **** Brassicaceae, Rosaceae
14	Sevdali Kaya	15.392	* *Castanea sativa* ** - *** Rosaceae, Brassicaceae, Ericaceae **** Pinaceae, Rhamnaceae, Fabaceae, Poaceae, *Verbascum* sp. , *Rumex* sp., *Geranium* sp.
15	Asagi Ambarlik	69.197	* *Castanea sativa* ** - *** Ericaceae **** Brassicaceae, Rosaceae, Unidentified
16	Tickov	53.303	* *Castanea sativa* ** - *** Rosaceae, Ericaceae ****Boraginaceae, Fabaceae, Apiaceae, Cistaceae, *Anchusa* sp.Brassicaceae, *Campanula* sp.
17	Asagi Ambarlik	14.109	* *Castanea sativa* ** - *** Ericaceae **** Rosaceae, Brassicaceae, Boraginaceae, Fabaceae, *Salix* sp., *Campanula* sp.
18	Kedmec	52.258	* *Castanea sativa* ** - *** Rosaceae **** Fabaceae, Cistaceae, Scrophulariaceae, Ericaceae, Asteraceae, Cyperaceae, Brassicaceae, *Rumex* sp., *Myosotis sp.*, *Onobrychis sp.*,Unidentified
19	Ceymakcur	35.172	**Castanea sativa* ** - *** Rosaceae, Brassicaceae, Ericaceae, Unidentified **** Scrophulariaceae, *Rumex* sp.

20	Kedmec	85.285	*Castanea sativa ** - *** - ****Boraginaceae, Brassicaceae, Fabaceae, Rosaceae, Ericaceae, *Rumex* sp., *Vicia* sp.,
21	Kedmec	26.477	*Castanea sativa ** *** Rosaceae, Brassicaceae, Ericaceae, Unidentified **** Pinacaea, *Salix* sp.
22	Kedmec	25.557	*Castanea sativa ** - *** Rosaceae, Ericaceae **** Cistaceae, Chenepodiaceae, Scrophulariaceae, Amaryllidaceae, Brassicaceae, *Veronica* sp.
23	Kalegon	54.683	*Castanea sativa ** - *** Brassicaceae ****Scrophulariaceae, Asteraceae, Lamiacaea, Fabaceae, Ericaceae, Cistaceae, *Salix* sp., *Primula* sp., *Ranunculus* sp.
24	Kedmec	29.323	*Castanea sativa ** - *** - **** Ericaceae, Poaceae, Brassicaceae, Fabaceae, Caryophyllaceae, Rosaceae, *Rumex* sp., Unidentified
25	Kedmec	40.441	*Castanea sativa ** - *** - **** Rosaceae, Ericaceae, ,Brassicaceae, Fabaceae, Ranunculaceae, *Gentiana* sp., Unidentified
26	Kedmec	21.645	*Castanea sativa ** - *** - ****Rosaceae, Ericaceae, Poaceae, Brassicaceae, Fabaceae, Boraginaceae, Scrophulariaceae *Carex* sp.
27	Kedmec	12.723	*Castanea sativa ** - *** Rosaceae, Ericaceae ****Cistaceae, Brassicaceae, Scrophulariaceae, Asteraceae, Caryophyllaceae
28	Sevdali Kaya	5. 073	* *Castanea sativa* ** - *** Brassicaceae, Ericaceae

			**** Rosaceae, Cucurbitaceae, Cistaceae, Fabaceae, Boraginaceae, *Laurus nobilis*, Unidentified
29	Kedmec	44.265	* *Castanea sativa* ** Brassicaceae *** Rosaceae **** Ericaceae, *Geranium* sp., *Primula* sp.,*Verbascum* sp., *Myosotis* sp.
30	Kedmec	52.631	* *Castanea sativa* ** - *** Rosaceae, Ericaceae **** Brassicaceae, Boraginaceae, *Salix* sp., Unidentified
31	Ezdog	18.982	* *Castanea sativa* ** - *** Brassicaceae **** Rosaceae, Ericaceae, Apiaceae, Cucurbitaceae, Fabaceae, Pinaceae, Caryophyllaceae, Boraginaceae, *Carex* sp., *Tilia* sp., Unidentified
32	Kedmec	34.325	* *Castanea sativa* ** - *** Brassicaceae **** Scrophulariaceae, Rosaceae, Ericaceae
33	Kedmec	7.862	* *Castanea sativa* ** - *** Brassicaceae, Ericaceae **** Rosaceae, Boraginaceae, Lamiaceae, Apiaceae, Fabaceae, Pinaceae, Poaceae, Caryophyllaceae, Asteraceae, *Geranium* sp., *Ranunculus* sp.,
34	Kedmec	3.438	**Castanea sativa* ** - ***Brassicaceae, Scrophulariaceae **** Fabaceae, Lamiaceae, Boraginaceae, Asteraceae, *Onobrychis* sp., *Rumex* sp.
35	Ezdog	34.354	**Castanea sativa* ** - ***Ericaceae, Rosaceae ****Fabaceae, Boraginaceae,Caryophyllaceae, Brassicaceae, Asteraceae, *Carex* sp., *Rumex* sp., *Geranium* sp., Unidentified
36	Ezdog	79.977	**Castanea sativa* ** - *** Ericaceae ve Rosaceae ****Fabaceae, Boraginaceae, Lamiaceae, Brassicaceae, Apiaceae, Chenepodiaceae, *Zea mays*, *Carex* sp.

37	Ezdog	51.897	**Castanea sativa* ** - *** Ericaceae, Brassicaceae, Rosaceae ****Lamiaceae, Chenepodiaceae, Liliaceae, Pinaceae, Fabaceae, Boraginaceae, *Zea mays*, *Rumex* sp., *Salix* sp.,*Carex* sp.
38	Kedmec	27.444	**Castanea sativa* ** - ***Brassicaceae **** Ericaceae, Chenepodiaceae,Rosaceae, Fabaceae, *Carex* sp.
39	Kedmec	28.725	**Castanea sativa* ** - *** Ericaceae, Rosaceae, Fabaceae ****Malvaceae, Apiaceae, Brassicaceae, Chenepodiaceae, Liliaceae, Cistaceae, *Rumex* sp., *Onobrychis* sp., *Laurus nobilis*, Unidentified
40	Kedmec	30.892	**Castanea sativa* ** - *** Rosaceae and Ericaceae ****Scrophulariaceae, Brassicaceae, Apiaceae, Liliaceae, Chenepodiaceae, Lamiaceae, Boraginaceae, *Onobrychis* sp., *Zea mays*, *Rumex* sp., *Laurus nobilis*, Unidentified
41	Asagi Ambarlik	29.122	**Castanea sativa* ** - *** - ****Brassicaceae, Boraginaceae, Fabaceae, Rosaceae, Cistaceae, Scrophulariaceae, Lamiaceae

Fructose was calculated between 14.05 % and 65.28 % in honey samples. Glucose changed between 14.31-67.47 %. F/G was found between 0.76-1.6. According to the Turkish Food Codex Honey Commission (2020/7), the amount of Fructose + Glucose should be at least 60g per 100g. In our study, Fructose was calculated between 14.05- 68.25% in honey samples. Sajwani et al. (2007) found the amount of fructose of honey samples to be between 19.40-40.75% in their study. The current study glucose changed between 14.31-67.47 %. Ouchmoukh et al. (2007) reported fructose amount 35.99-42.57% and glucose value was found to be between 26.23-34.38%.

While Demir Kanbur et al. (2021) found fructose amount to be an average of 45,38 in their study, the average fructose amount was 29 in our study. Fructose is responsible for many nutritional and physical characteristics of honey. The rate of fructose is determined by the variety of floral sources from which honey samples are originated (Sajwani et.al., 2007).

Glucose, high temperature and storage conditions accelerate crystallization in honey. In honey samples, F/G ratio changed between 0.76 and 1.6 (Figure 3). Moisture value was over the Honey Standards commission (2020/7) in one sample, while 13 samples were calculated below the honey standard (Figure 4).

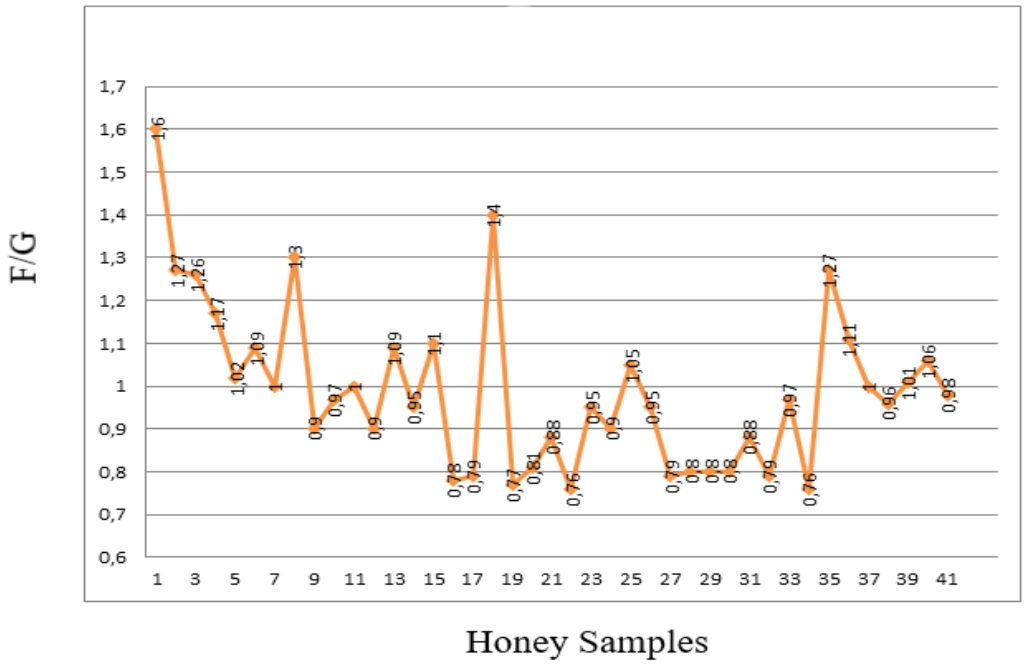

Figure 3. F/G ratios of honey samples

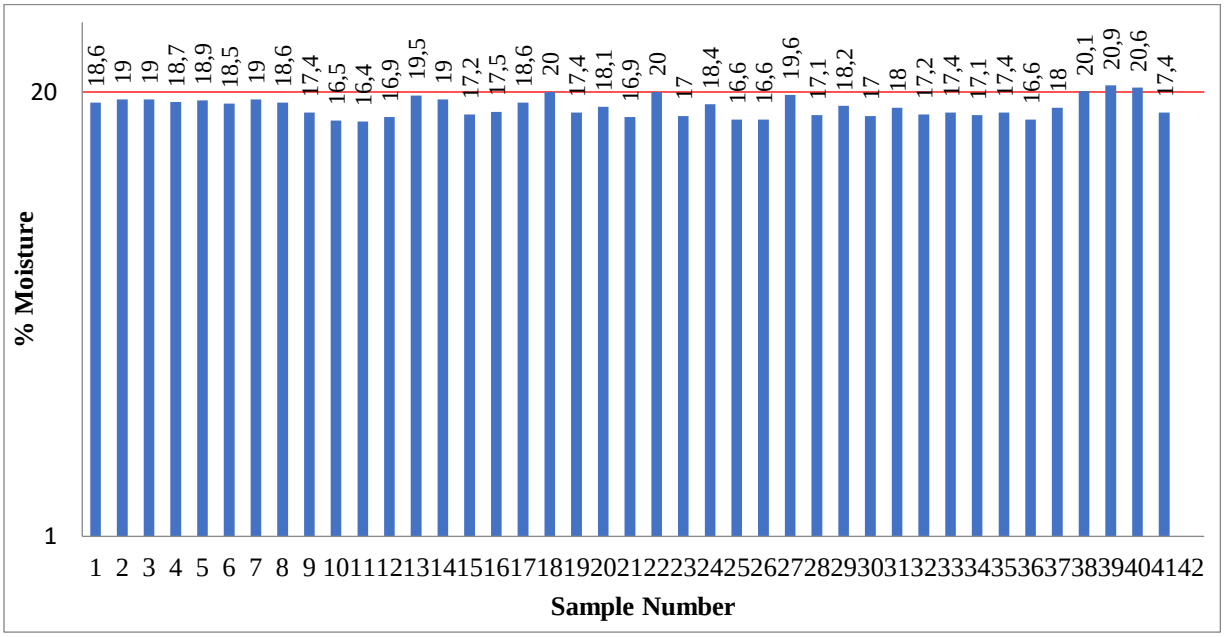

Figure 4. Moisture contents of honey samples

The moisture percentage was high in samples 39 and 40 respectively 20.9 and 20.6. According to the Turkish Food Codex Honey Commission (2020/7), the moisture percentage

was high in samples 39 and 40. There is an increased chance of fermentation in honey with a water ratio higher than 18.5 (Bolukbasi, 2009).

Castanea sativa pollen (Figure 5) was detected dominantly in all honey samples. Pollen belonging to Brassicaceae family was found in a single sample in a secondary amount. While Brassicaceae pollen was not found in sample 10, minor or trace amounts were encountered in all other samples. Secondary and trace amounts of pollen were not found in one sample. Pollen belonging to the Rosaceae family was not found only in the sample 34. While pollen belonging to Ericaceae family was not found in honey samples 34 and 41, it was found in minor or trace amounts in other honey samples. The pollen of Lamiaceae family was found in seven honey samples. During honey pollen analysis, a maximum of 14 taxa were detected, while at least 3 taxa were detected in honey samples. These taxa were *Castanea sativa*, Ericaceae and Rosaceae. Fabaceae pollen was determined in the minor and rare categories. Celemli et al. (2021), reported nine out of twenty samples as chestnut honey in Ayder Plateau. Erdogan (2007) found that *Castanea sativa* pollen is dominant in 25 of 65 samples. Bayram and Demir (2018) reported that C*astanea sativa* pollen is dominant in t Rize and Giresun province. 5 samples were identified as unifloral honey. Bayram (2019) identified *Castanea sativa* and *Astragalus* sp. pollen at dominant rates. Çam (2006) reported that Rosaceae is the most common pollen species in honey samples. In addition, Taşkın (2006) found Rosaceae pollen in 14 of 20 samples in his study. In addition to nectar and pollen yield, bees prefer Rosaceae family more due to its high number of taxa.

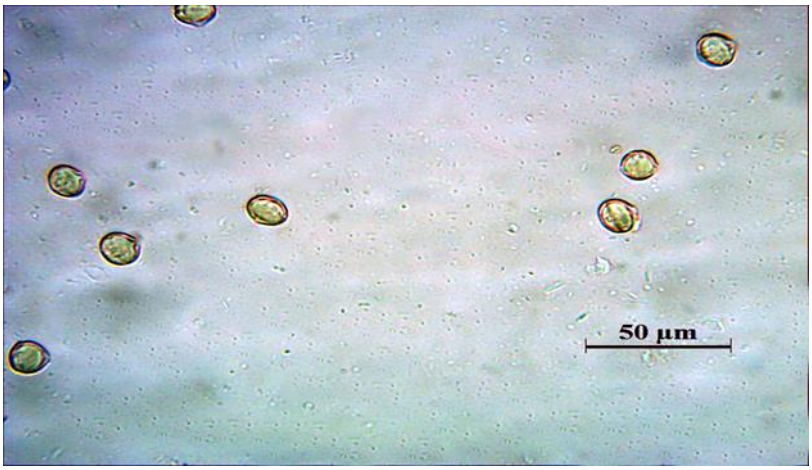

Figure 5. *Castanea sativa* pollen

4. CONCLUSION

In this study, melissopalinological and some chemical analyses of 41 honey samples collected from Ayder Plateau were conducted. Honeybees have preferred to collect *Castanea*

sativa Ericaceae, Chenepodiaceae, Rosaceae, Fabaceae, *Carex* sp. Malvaceae, Apiaceae, Brassicaceae, Chenepodiaceae, Liliaceae, Cistaceae, *Rumex* sp., *Onobrychis* sp., *Laurus nobilis* Scrophulariaceae, Lamiaceae, Boraginaceae, *Onobrychis* sp., *Zea mays*, Asteraceae, *Geranium* sp. pollens. *Castanea sativa* pollen was found to be dominant in all honey samples.

The intensive tourism in Ayder Plateau is negatively affected the floristic structure. Increasing human population and vehicle traffic cause environmental pollution. This affects the bee population and honey yield. For this reason, although there are no broad-leaved woody plants in Ayder Plateau, it has been determined that honeybees fly long distances and reach chestnut flowers. While this is tiring for the bee, the honey yield decreases.

DECLARATIONS

All authors declare that they have no conflicts of interest.

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Klotho Protein and Type 2 Diabetes Mellitus

Klotho Proteini ve Tip 2 Diabetes Mellitus

Eda DOKUMACIOGLU[1*], Hatice ISKENDER[1]

[1]Artvin Coruh University, Faculty of Healthy Sciences, Department of Nutrition and Dietetics, Artvin, Turkey.

*eda_ozcelik@artvin.edu.tr, ORCID: 0000-0002-2223-1331

haticeiskender2011@hotmail.com, ORCID: 0000-0002-8063-4972

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***Corresponding author** /Yazışılan yazar

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Abstract

Diabetes mellitus (DM) is considered an epidemic disease by many countries and shown as one of the leading causes of death in western societies. In the development of the disease, the underlying pathophysiological mechanisms are complex and multifactorial. The frequency of DM increases with age, and the severity of events such as oxidative stress and inflammation increases in patients diagnosed with DM. The Klotho (KL) protein, defined as a new anti-aging protein as a result of the studies on aging mechanisms and it has an important functions on glucose homeostasis and insulin secretion. In this review study, the relationship between KL protein and DM is explained by compiling the information in the articles published in PubMed indexed journals between 2002-2020. In conclusion, a decrease in KL levels plays a role in type 2 DM and the development of nephropathy and vascular diseases caused by type 2 DM.

Keywords: Type 2 Diabetes mellitus, Klotho, Oxidative stress, Inflammation

Özet

Diabetes Mellitus (DM), birçok ülke tarafından epidemik bir hastalık olarak kabul edilmekte ve batı toplumlarında en önde gelen ölüm nedenlerinden biri olarak gösterilmektedir. Hastalığın gelişiminde altta yatan patofizyolojik mekanizmalar kompleks ve multifaktöriyeldir. DM sıklığı yaş ile artmakta ve bununla birlikte oksidatif stres, inflamasyon gibi olayların şiddeti de DM tanılı hastalarda artmaktadır. Yaşlanma mekanizmaları üzerinde yapılan araştırmalar sonucu yeni bir anti-aging protein olarak tanımlanan klotho (KL) proteini glukoz homeostazı ve insulin salgılanmasında önemli fonksiyonlara sahiptir. Bu derleme çalışmasında, 2002-2020 yılları arasında PubMed'de taranan dergilerde yayınlanan makalelerdeki bilgiler derlenerek KL proteini ile DM arasındaki ilişki anlatılmıştır. Sonuç olarak, KL düzeylerindeki azalma tip 2 DM ve buna bağlı olarak gelişen nefropati ve vasküler hastalıklarda önemli rol oynar.

Anahtar Kelimeler: Tip 2 Diabetes mellitus, Klotho, Oksidatif stres, İnflamasyon

Abbreviations: Diabetes mellitus (DM); Klotho (KL); α-Klotho (KLA); β-Klotho (KLB); γ-Klotho (KLG); Fibroblast growth factors (FGFs); Insulin-like growth factor-1 (IGF-1); Insulin receptor substrate (IRS); Transforming growth factor-β1 (TGF-β1); 1,25-dihydroxyvitamin D_3 (1,25(OH)2VD3); Reactive oxygen species (ROS); Superoxide dismutase (SOD)

1. INTRODUCTION

With the adoption of a sedentary lifestyle and a type of nutrition with a high glycemic index, chronic diseases with high morbidity and mortality rates are rapidly increasing worldwide. Diabetes mellitus (DM) is considered an epidemic disease by many countries and shown as one of the leading causes of death in western societies (Özdemir et al., Whiting et al., 2011). DM is described by hyperglycemia resulting from certain or relative insufficiency of insülin secretion because of pancreatic β-cell dysfunction or reduction of insulin sensitivity. DM is a chronic disease that influences quality of life and lifetime negatively owing to micro and macro complications (Dokumacioglu et al., 2018; Ighodaro, 2018).

Different pathogenic stage are related in the development of DM. These range from autoimmune demolition of the β-cells of the pancreas with result insulin hormone insufficiency to abnormalities that consequent in resistance to insulin action. DM is a group of metabolic disorders of carbohydrate, lipid and protein metabolism characterized by hyperglycemia (ADA, 2012). Hyperglycaemia is associated with both inadequate insulin secretion and insulin resistance. Reduction of insulin hormone secretion and flaws in insulin action constantly coexist in the same patient, and it is often uncertain which abnormality, if either alone, is the basic reason of the high levels of glucose (Kahn, 2003). Due to the increase in mortality and morbidity rates resulting from diabetes and its negative effects on all organ systems, identification of the molecules playing a role in the pathogenesis of the disease and development of effective treatment strategies are of extreme importance. In the development of the disease, the underlying pathophysiological mechanisms are complex and multifactorial. DM and aging are in close interaction with each other. The frequency of DM increases with age, and the severity of events such as oxidative stress and inflammation increases in patients diagnosed with DM (Banday et al., 2020; Domingueti et al., 2016).

The KL protein, defined as a new anti-aging protein as a result of the studies on aging mechanisms, was named after Klotho, who was one of the three goddesses of destiny and spun the thread of life according to Greek mythology (Kuro-o, 2008). It has been reported that in

case of a mutation or dysfunction in the KL protein expressed in various tissues and organs, mice in which early aging symptoms appeared and the expression of the KL protein was inhibited had short lives. Information indicating the relationship between the KL protein, glucose hemostasis, and insulin secretion in the literature shows that these proteins can be effective in the occurrence of insulin resistance and diabetes (Kuro-o, 2008). In this review study, the information in the literature was compiled, and the relationship between the KL protein and diabetes was explained. Additionally, the relationship between KL protein and DM is explained by compiling the information in the articles published in PubMed indexed journals between 2002-2020.

2. KLOTHO PROTEINS AND BIOLOGICAL FUNCTIONS

The KL gene, first discovered in 1997, is located on the q arm of chromosome 13 in the genome. The human KL gene consists of 5 exons and 4 introns and is located in a region of approximately 50 kbp on chromosome 13 (Arking et al., 2002; Matsumura et al., 1998). The KL protein is almost 130 kDa with a putative signal sequence at the N-terminus, a single transmembrane domain near the C-terminus and a short cytoplasmic domain (Razzaque et al., 2012). KL is a single-pass trans-membrane protein secreted in the brain, kidney, eye, testis, ovary, pancreas, pituitary and parathyroid gland tissues (Kuro-o, 2010; Moos et al., 2020). The KL protein family has three members: alpha, beta and gamma. α-Klotho (KLA) was shown to be an antiaging molecule (Zhang et al., 2017). Mice that exhibited KL insufficiency offered a premature aging phenotype, whereas KL over expression spreaded their lifetime by up to 30% and conserved them against many pathological phenotypes, particularly renal disease (Kuro-o, 2018; Zou et al., 2018). β-Klotho (KLB) is mostly secreted in the liver, but it exists in the kidney, pancreas, adipose tissue, and spleen and regulates bile acid production (Ito et al., 2000; Ito et al., 2005). γ-Klotho (KLG) is expressed in the kidney, testis, and skin. In literature indicated that KLG has a major role in prostate tumorigenesis and it may become a new biomarker for diagnosis and/or a therapeutic target in patients with prostate cancer (Onishi et al., 2020). The KL proteins are compulsory components of fibroblast growth factors (FGFs) receptor complexes and these proteins form a unique endocrine system that manages various metabolic processes in mammals (Kuro-o, 2019; Kurosu et al., 2009). FGF23 synthesized in the bone, particularly in osteocytes, is also produced by the heart, lymph nodes, liver, and thymus. Although FGF23, FGF21, and FGF19 belong to the FGF ligand superfamily, they are collectively named endocrine FGFs. The reason why they are named so is that they function as endocrine factors, contrary to other classic FGFs working as paracrine and autocrine factors

(Degirolamo et al., 2016; Yamashita et al., 2000). FGF23 and FGF21 need the Klotho proteins to show their metabolic activity. This enabled the Klotho proteins to be defined as cofactors to show their effects by binding to the receptors of FGF23 and FGF21 (Nishimura et al., 2000; Stubbs et al., 2007).

KL proteins are determined as having the activity of endocrine, autocrine, and paracrine hormone (Olejnik et al., 2018). KL proteins have been found to arrange energy metabolism, utilize anti-oxidative and anti-inflammatory effects and modulate calcium homeostasis by the restriction of insulin/insulin-like growth factor-1 (IGF-1) and transforming growth factor-β1 (TGF-β1) signaling pathways (Haipeng et al., 2017; Yamamoto et al., 2005). KL proteins have also been reported in vascular disease and various studies propose that KL has a cardioprotective effect (Martin-Nunez et al., 2014). Additionally, KL proteins seems to modulate tissue inflammatory responses to damage or interact with inflammatory mediators (Maekawa et al., 2009; Xie et al., 2012). KL is affected by numerous physiological and pathological terms. Decreased KL level is remarkably changed with numerous physiological processes. For example, KL level decreases in the heart sinoatrial node and the liver with aging (Cararo-Lopes et al., 2017; Jia et al., 2019). KLA is a circulating hormone that can lead to phosphaturia on its own, independently of FGF-23, and exhibit renal-cardio efficacy through endothelial protection (Maltese et al., 2012).

2.1. Klotho Protein Role in Type 2 Diabetes

DM is a general health problem worlwide. This disease having harmful influences on all tissues and causing increased mortality and morbidity levels. At the present time, researchers investigate on identifying molecules that play a role in the pathogenesis of DM (Dokumacioglu et al., 2019). KL protein defends cells against expedited aging and destruction during the course of DM. The oxidative stress, inflammation, degradation of phosphate and calcium metabolism and an increase in the ratio of β-cell and adipocyte loss through apoptosis can be consequence in pathologies of DM (Buchanan et al., 2020; Buendía et al., 2016). Oxidative stress develops as a result of the deterioration of the balance between oxidants and antioxidants. Increased oxidative stress in diabetes leads to loss of membrane integrity, structural or functional changes in proteins, and genetic mutations (Rehman & Akash, 2017). Hepatic glucose production also has a major role in maintaining glucose metabolism (Ohnishi et al., 2011). In the literature, it has been reported that KLA and KLB levels are low in patients with type 2 DM. Nie et al. (2017) found significantly lower α-KL levels in diabetics than in healthy controls, and suggested that such a decrease in α-KL levels could be involved in the pathological mechanism

of type 2 diabetes. It has been underlined that this decrease in the levels of KL proteins negatively influences the physiological processes of diabetic nephropathy and coronary artery diseases caused by DM (Nie et al., 2017). In a study conducted on people above the age of 65, an aging-related decrease in serum KL levels was reported. Moreover, it was stated that this decrease in KL levels would increase the rate of encountering new diabetes cases in this age group (Semba et al., 2011).

KLA is induced by 1,25(OH)2VD3 and restricted by FGF-23 thereby creating an endocrine loop, as circulating KL can stimulate FGF-23-FGFR signaling (Silva et al., 2017; Urakawa et al., 2006). In many clinical studies of chronic kidney disease has been defined has a state of FGF-23 resistance owing to endocrine and renal KL insufficiency (Hu et al., 2013; Ribeiro et al., 2020). There are various opinions on the effect of KL protein on diabetes. KL insufficiency induced the apoptosis of insulin-producing β-cells, which were defended against this process after KL overexpression (Kim et al., 2016; Olauson et al., 2014). Also, KL increased the β-cell function and prevented the progress of type 2 DM (Typiak et al., 2021). Dokumacioglu et al. (2019) put forward that the KL protein levels are a promising bio-indicator in various cases of type 2 diabetes pathology.

Oxidative stress could fast-track the incidence of clinical manifestation of DM. Oxidative stress is playing a major role in the etiology and pathophysiology of DM (Harani et al., 2012). This is because prolonged exposure of both human and animal tissues to hyperglycaemia is recognised to result in non-enzymatic glycation of proteins and the end products such as schiff base and culminates in the generation of free radicals (Hojs et al., 2016). Hyperglycaemia is recognised to be accountable for the damage of DNA, lipids and proteins and the degree of damage has been linked to the degree of hyperglycaemic-induced production of ROS and consequently oxidative stress (Butkowski et al., 2017). KL protein enhances counteraction to oxidative stress at the cellular and organismal level in mammals. KL protein mobilizes the FoxO transcription factors that are unfavorable arranged by insulin/IGF-1 signaling, thereby inducing expression of superoxide dismutase (SOD). In this way facilitates rustication of ROS and prevents oxidative stress (Figure 1) (Kazemi et al., 2021; Lim et al., 2019). Therefore, overexpress of KL levels have higher levels of MnSOD and decreased oxidative stress as demonstrated by lower levels of DNA damage. Secretion of KL can decrease the levels of peroxide induced apoptosis, SOD production and mitochondrial DNA fragmentation (Guo et al., 2018; Ma et al., 2021).

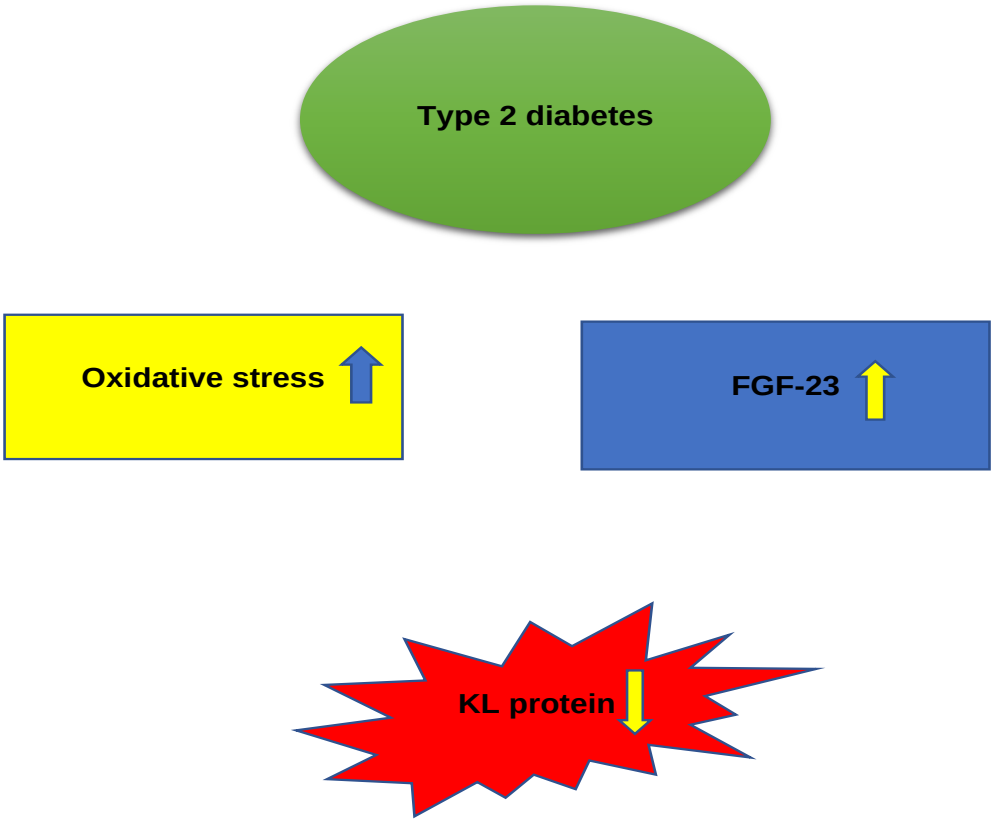

Figure 1. KL/FGF-23/Oxidative stress in Type 2 Diabetes

Inflammation is a significant factor in the progression of DM. Elevated serum levels of various inflammatory cytokines, as well as infiltration of the vascular tissue by immune cells, are features of a low-grade inflammation which can behavior as substrate for the initiation and progression of DM (Oguntibeju, 2019; Tsalamandris et al., 2019). Many researchs on human and animal models ensured further supporting proof for the role of inflammation in the development of DM. Numerous scientific resource reports that activation of pro-inflammatory markers in target cells of insulin action may conduce to obesity, insulin resistance and involved metabolic damages including DM (León-Pedroza et al., 2003; Marques-Vidal et al., 2012).

Inflammatory cytokines can down-regulate KL levels whereas, in the reverse direction, KL is able to modulate inflammation by inhibiting central signaling pathways and the expression of inflammatory involved molecules (Buendía et al., 2015; Maekawa et al., 2009). Some immunotherapeutic treatment protocols for type 1 and type 2 DM, diabetic nephropathy, and other kidney disorders have been suggested. In the literatüre, KL have indicated its possible beneficial as an early biomarker for DM initiation and progression (Hu et al., 2012; Liu et al., 2014). KL proteins may serve as a potential, safe and powerful agent in immunotherapy of DM and its complications. KL protein was newly indicated to function as a hormone that restricts

insulin/insulin-like growth factor-1 (IGF-1) signaling (Rubinek et al., 2016). In the literature, KL knockout mice have declined insulin production with increased insulin sensitivity. Additionally, mice have less energy storage and consumption than controls. KL can restrict IGFRs and insulin receptor substrate (IRS) through indirect pathways (Hasannejad et al., 2019; Utsugi et al., 2000).

3. CONCLUSION

Diabetes mellitus imposes a significant burden on patients due to the loss of working capacity and extremely high treatment costs. It is known that early diagnosis and treatment of type 2 DM are of great significance to delay or prevent the emergence of diabetic complications (Marshall & Flyvbjerg, 2006). It is clear that any new finding that could be an indicator of early diagnosis will be critically important in respect of the diagnosis, follow-up, and treatment of patients. To this end, there is a need for ideal biomarkers for the protection of health, early diagnosis, evaluation of treatment efficacy, and prognosis. The KL proteins are shown as promising markers in many diseases. A decrease in Klotho levels plays a role in type 2 DM and the development of nephropathy and vascular diseases caused by type 2 DM. A decrease in the KL proteins identified at an early stage will contribute to the prevention of diseases caused by type 2 DM and cessation of their progression.

DECLARATIONS

All authors declare that they have no conflicts of interest.

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