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M.Sc. THESIS

EFFECTS OF VARIOUS EXTRACTION AND FERMENTATION PROCESSES ON THE ANTIOXIDANT CAPACITY OF HONEY, PROPOLIS AND POLLEN SAMPLES

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FOREWORD

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbol	Explanation
$O_2^{\cdot -}$	: Superoxide anion
$OH^{\cdot}$	: Hydroxyl radical
$AlCl_3$	: Aluminum chloride
Ca_2CO_3	: Calcium carbonate
H_2O_2	: Hydrogen peroxide
HOCl	: Hypochlorous acid

Abbreviation	Explanation
A. cerana	: *Apis cerana*
A. mellifera	: *Apis mellifera*
AOX	: Antioxidant(s)
ATP	: Adenosine triphosphate
CUPRAC	: Cupric reducing antioxidant capacity
DNA	: Deoxyribonucleic acid
DPPH	: 1,1-Diphenyl-2-picrylhydrazyl
DW	: Dry weight
DWE	: Dry weight of extract
EtOH	: Ethanol
FRAP	: Ferric reducing antioxidant power
GAE	: Gallic acid equivalent
GPX	: Glutathione peroxidase
MDA	: Malondialdehyde
MeOH	: Methanol
MPO	: Myeloperoxidase
NADPH	: Nicotinamide adenine dinucleotide phosphate
ORAC	: Oxygen radical absorbance capacity
QUE	: Quercetin equivalent
ROS	: Reactive oxygen species
SOD	: Super oxide dismutase

TFC : Total flavonoid content

TPC : Total phenolic content

ÖZET

ÇEŞİTLİ EKSTRAKSİYON VE FERMENTASYON İŞLEMLERİNİN BAL, PROPOLİS VE POLEN ÖRNEKLERİNİN ANTİOKSİDAN KAPASİTESİ ÜZERİNE ETKİLERİ

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Çeşitli balarısı ürünleri besleyici özellikleri ve antioksidan, antimikrobiyal, antikanser vb. aktiviteleri gibi sağlığa yararlı etkileri nedeniyle gıda, farmasötik ve kozmetik endüstrisinin büyük ilgisini çekmektedir.

Bu çalışmanın amacı, farklı çözücüler ve ortam sıcaklıklarında uygulanmış farklı ekstraksiyon süreçlerinin bal, propolis ve arı poleni örneklerinin antioksidan kapasitesi üzerine etkilerini belirlemek ve bazı gıda ürünlerinin aktif bileşenlerinde kalite ve miktar bakımından artışa ve/veya dönüşüme yol açarak yararlı etkilerini artırdığı bilinen fermentasyon işleminin, en aktif örneğin antioksidan kapasitesini değiştirip değiştirmediğini değerlendirmektir.

Meşe balı örneği, iki (sonbahar ve ilkbahar) propolis örneği ve polen örneği, Kırklareli ilindeki (Türkiye) yöresel bir arıcıdan temin edildi. Örnekler tek tek farklı çözücülerle [%100 (v/v) metanol, %70 (v/v) etanol ve distile su] ve üç farklı sıcaklık koşulunda (24 saat oda sıcaklığında, 24 saat 45^0C'da ve 24 saat 45^0C'da + 2 saat kaynatmayla) ekstre edildi. Örneklerin radikal süpürme aktivitesini, total fenolik içeriğini (TPC) ve total flavonoid içeriğini (TFC) belirlemek için, sırasıyla DPPH, Folin-Ciocalteu ve alüminyum klorür yöntemleri kullanıldı. En yüksek radikal süpürme aktivitesi, çözücü olarak etanolün ve sıcaklık koşulu olarak 24 saat 45^0C +2 saat kaynatmanın kullanıldığı ekstraksiyon yöntemiyle elde edilen sonbahar propolis ekstresinde tespit edildiğinden, bu örnek *Lactobacillus brevis*

ile fermente edildi ve fermente propolisin antioksidan kapasitesi ölçüldü. Hem aynı tip ürün için hem de farklı tip ürünler için alınan sonuçlar karşılaştırıldı ve tartışıldı.

Bal örnekleri arasında en yüksek TPC, kaynatarak hazırlanan su ekstresinde, 0.90± 0.09 mg gallik asit eşdeğeri (GAE)/g kuru ekstre ağırlığı (DWE) olarak bulunurken, en yüksek TFC oda sıcaklığında hazırlanan metanol ekstresinde, 0.055 ± 0.002 mg kersetin eşdeğeri (QUE)/g DWE olarak belirlendi. Bu ekstre aynı zamanda en düşük IC_{50} değeriyle (157.0 ± 9.8 mg/mL) en yüksek radikal süpürme aktivitesini gösterdi.

Propolis örnekleri içinde en iyi TPC, TFC ve IC_{50} değerleri sonbaharda toplanan propolisin etanolde 24 saat 45^0C'da + 2 saat kaynatılmasıyla elde edilen ekstrede, sırasıyla 340.4 ± 34.5 mg GAE/g DWE, 103.5 ± 5.2 mg QUE/g DWE ve 0.011 ± 0.001 mg/mL olarak belirlendi. Aslında bu ekstre, üç arı ürünün bütün örnekleri içinde de en aktif olanıydı, dolayısıyla bu çalışmada fermente edilendi.

Polen örnekleri arasında en iyi TPC, TFC ve IC_{50} değerleri, oda sıcaklığında hazırlanmış etanol ekstresinde, sırasıyla 22.8 ± 1.2 mg GAE/g DWE, 4.68 ± 0.30 mg QUE/g DWE ve 5.8 ± 0.8 mg/mL olarak belirlendi.

Propolisin fermentasyonu 6 günde gerçekleştirildi ve fermentasyonun ikinci, dördüncü ve altıncı günlerine ait çeşitli örneklerde santrifüjlemeden önce ve sonra TPC, TFC ve antioksidan kapasite testleri gerçekleştirildi. En iyi TPC, TFC ve IC_{50} değerleri fermente edilmemiş (deney koşullarında bakteri eklenmemiş) pastörize sonbahar propolis örneğinde, üçüncü ve altıncı günlerde birbirine oldukça yakın, sırasıyla ortalama 268.15 mg GAE/g DWE, 23.9 mg QUE/g DWE ve 0.047 mg/mL olarak saptandı. Santrifüjlemeden sonra fermente propolis için en yüksek TPC, TFC ve en düşük IC_{50} değerleri fermentasyonun ikinci gününde, sırasıyla 46.7 ± 1.1 mg GAE/g DWE, 9.65 ± 1.5 mg QUE/g DWE and 0.220 ± 0.005 mg/mL olarak bulundu. Fermentasyona ilişkin veriler propolisin antioksidan aktivitesinin ve başlıca sorumlu fenolik maddelerinin fermentasyon sırasında günden güne belirgin şekilde azaldığını gösterdi.

Tüm bulgular, ekstraksiyon sırasında kullanılan çözücü tipinin ve sıcaklığın olduğu kadar, fermentasyonun da balarısı ürünlerinin antioksidan kapasitesini ve kimyasal bileşimini etkileyebileceğini ortaya koymaktadır. Ayrıca, etanoldeki dekoksiyonu yüksek fenolik ve flavonoid içeriği nedeniyle etkin bir radikal süpürme aktivitesi gösterdiğinden, özellikle sonbaharda toplanmış propolis, doğal bir antioksidan veya yeni antioksidanların kaynağı olarak en iyi arı ürünlerinden biri gibi durmaktadır.

Bu çalışmanın insan sağlığı ve refahı için faydalı yeni balarısı ürünlerini veya ilgili maddeleri saptamak veya üretmek hedefiyle gelecekte yürütülecek çalışmalara yol göstermesi ve bu ürünlerin ekonomik değerini artırmaya yönelik girişimlere katkıda bulunması beklenmektedir.

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Anahtar kelimeler: bal, propolis, polen, antioksidan aktivite, fermentasyon

SUMMARY

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M.Sc. THESIS

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Various honeybee products attract great attention of food, pharmaceutical and cosmetics industries due to their nutritive properties and healthful effects such as antioxidant, antimicrobial, anticancer etc. activities.

The aim of current study is to determine the effects of different extraction processes utilizing different solvents and ambient temperatures onto the antioxidant capacity of honey, propolis and bee pollen samples, and to evaluate whether fermentation process, which is known to enhance beneficial effects of some food products by increasing and/or transforming the active constituents both in quality and quantity, changes the antioxidant capacity of the most active sample.

Oak honey sample, two (autumn and spring) propolis samples and pollen sample were obtained from a local beekeeper in Kırklareli province (Turkey). The samples were extracted individually with different solvents [100% (v/v) methanol, 70% (v/v) ethanol and distilled water] at three different temperature conditions (room temperature for 24 hour, 45ºC for 24 hour and 45ºC for 24 hour + boiling for 2 hour). DPPH, Folin-Ciocalteu and aluminum chloride methods were performed to assess the radical scavenging activity, total phenolic

content (TPC) and total flavonoid content (TFC) of the samples, respectively. Since the highest radical scavenging activity was detected in the extract of autumn propolis obtained by the extraction method utilizing ethanol as a solvent and 45ºC for 24 hour + boiling for 2 hour as temperature condition, this sample was fermented using *Lactobacillus brevis*, and antioxidant capacity of fermented propolis was measured. The results obtained for the same type of product as well as for different groups of products were compared and discussed.

Among honey samples, the highest TPC was found in the aqueous extract prepared by boiling, as 0.90 ± 0.09 mg gallic acid equivalent (GAE)/g dry weight of extract (DWE) while the highest TFC was detected in methanol extract prepared in room temperature as 0.055 ± 0.002 mg quercetin equivalent (QUE)/g of DWE. This extract also showed the highest radical scavenging activity with the lowest IC_{50} (157.0 ± 9.8 mg/mL).

Among propolis samples, the best TPC, TFC and IC_{50} values were found in the extract obtained by extraction of autumn propolis with ethanol at 45 ºC for 24 hour + boiling for 2 hour, as 340.4 ± 34.5 mg GAE/g DWE, 103.5 ± 5.2 mg QUE/g DWE and 0.011 ± 0.001 mg/mL, respectively. In fact, this extract was also the most active one among all samples of three honeybee products, and hence the fermented one in this study.

Among pollen samples, the best TPC, TFC and IC_{50} values were found in ethanol extract prepared at room temperature, as 22.8 ± 1.2 mg GAE/g DWE, 4.68 ± 0.30 mg QUE/g DWE and 5.8 ± 0.8 mg/mL, respectively.

Fermentation of propolis was acheived in 6 days, and TPC, TFC and antioxidant capacity tests were carried out in various samples of second, fourth and sixth days of fermentation, before and after centrifugation. The best values for TPC, TFC and IC_{50} were determined in unfermented (no bacteria were added in assay conditions) pasteurized propolis sample, quite close each other at the second and sixth days, as a mean 268.15 mg GAE/g DWE, 23.9 mg QUE/g DWE, 0.047 mg/mL, respectively. The highest TPC, TFC and the lowest IC_{50} values in fermented propolis after centrifugation were found for the second day of fermentation as 46.7 ± 1.1 mg GAE/g DWE, 9.65 ± 1.5 mg QUE/g DWE and 0.220 ± 0.005 mg/mL, respectively. The data related to fermentation indicated that antioxidant capacity and principal active phenolic substances of propolis significantly decreased during the fermentation process day by day.

Overall findings reveal that solvent type and temperature in extraction as well as fermentation may affect the antoxidant capacity and the chemical composition of honeybee products. Besides, particularly autumn propolis seems to be one of the the best bee products as a natural antioxidant, or as a source of new antioxidants, since its decoction in ethanol exhibits an effective radical scavenging activity because of its high phenolic and flavonoid content.

This study is expected to be a guide for further studies targeting to exhibit or produce new honeybee products and related substances beneficial for human health and welfare, and to contribute to the attempts for increasing the industrial value of these products.

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Keywords: honey, propolis, pollen, antioxidant activity, fermentation.

1. INTRODUCTION

Since ancient times, honeybee products, such as honey, pollen, propolis, royal jelly, bee wax and bee venom had been used in folk medicine due to their beneficial effects on human health (Nakajima et al., 2009; Baso et al., 2016), such as anti-inflammatory, antifungal, antiviral, immune boosting and antioxidant activities. They have also inhibitory effect on the growth of cancer cells (Sforcin et al., 2017).

Owing to their high nutritional values and healing properties, they are being used extensively in food products and beverages (Toreti et al., 2013). Their usage also has attracted attention in various industrial fields such as pharmaceutics and cosmetics (Cornara et al., 2017).

Therapeutic properties of honeybee products have been attributed particularly to their content of phenolic acids and flavonoids (Sun et al., 2015). Several studies have shown a positive relationship between polyphenolic content of bee products and their antioxidant capacity. Therefore, it is one of the priorities to study the antioxidant effects of polyphenols found in honeybee products (Boulanouar et al., 2017).

Apart from product type, origin and collecting time, extraction method and solvent used for extraction during *in vitro* studies are known to have quantitative and qualitative influence on the phenolic content and antioxidant capacity. For example, one study on propolis indicated that the water/ethanol solvent is superior to the aqueous solvent in the extraction of polyphenols (Sun et al., 2015). Another study compared the effectiveness of various solvents in the extraction of phenolic compounds of algerian propolis, revealed that Ethyl acetate and n-butanol are the best solvents for extraction (Narimane et al., 2017).

On the other hand, fermentation process had been used since ancient times to preserve several foods, and to get high quality products, especially in flavour. In addition, this process has gained more attention since its ability to change the bioactive compounds and biological properties of food products. For example, fermentation is considered as a good process to increase the antioxidant activity of plant-based foods (Hur et al., 2014).

This study aimed to describe an efficient method for extracting antioxidant compounds from honeybee products, namely honey, propolis and pollen, and to investigate whether fermentation process changes the antioxidant capacity, with the purpose of producing more useful honeybee products for human health. As the radical scavenging activity, which is one of the features that reflects the antioxidant capacity was measured in the extracts prepared with different methods from honey, propolis and pollen samples. Detailed information and literature survey on each product and the main producer as well as theoretical approaches related to hypothesis have been given in the following sections

1.1. HONEYBEES

Honeybees are classified as social insects belonging to Hymenoptera, genus *Apis* (Cornara et al., 2017) as illustrated in Table 1 (Yilmaz et al., 2017). Currently, there are two species of honeybees; the first originates from Europe, Asia and Africa, and is called the Western bee (*Apis mellifera*), the other race is the eastern bee (*Apis cerana*), which spreads in the South and Southeast of Asia (Cornara et al., 2017). Turkey had a limited history in beekeeping but after the Second World War, beekeeping has increased significantly (Sirali, 2002).

Table 1.1: Scientific classification of honeybee (Yilmaz et al., 2017).

Phylum	Artropoda
Class	Insecta/Hexapoda
Ordo	Hymenoptera
Subordo	Apocrita
Superfamily	Apoidea
Family	Apidae
Genus	*Apis*
Species	*Apis mellifera*

Turkey occupies the third place of bee colonies (Aksoy et al., 2017), and it has about 4.3 millions of them (Sirali, 2002). Besides, it takes the second place for honey output over the world (Aksoy et al., 2017). Turkey has a wide range of honeybee subspecies, and that is due

to its floral and climate diversity (Sirali, 2002). For instance, *Apis mellifera Anatoliaca* is one of the most important subspecies in Turkey, it spreads in the center of Anatolia and it is predominant in Marmara region, while *Apis mellifera Caucasica* spreads in the Northeast region of Anatolia (Sirali, 2002).

Furthermore, *Apis mellifera Meda* and *Apis mellifera Syriaca* are the local honeybees of the Southeastern of Anatolia (Sirali, 2002). Honeybee species producing the honey, propolis and pollen samples used in this study is *A. mellifera Anatoliaca*, since it is common for Northwestern of Turkey (Marmara region) (Sirali, 2002).

1.2. HONEYBEE PRODUCTS

There are several types of natural products such as honey, propolis, pollen, royal jelly, bee wax and bee venom that have been produced by honeybees (Sforcin et al., 2017).

European Comission has declared that honeybee colonies ensuring plant reproduction by pollination are not only fundamental for farming and rural development but also environment. As the world's second most important honey producer after China, the European Union (EU) confers various apiculture products not only honey, but also pollen, propolis, royal jelly and beeswax (https://ec.europa.eu/agriculture/honey_en). Turkey has appeared in the third place in honey production by 2012 (Figure 1.1), although it is still out of exporter countries.

In particular, honey, propolis and pollen have various activities including antimicrobial, antioxidant, anticancer, antiaging, immune modulatory and anti-inflammatory activity (Pasupuleti et al., 2017). Honeybee products are rich in unique compounds including polyphenols, which are responsible for their different therapeutic activities. The wide variety of these compounds makes the products not applicable in clinical settings. However, many compounds have been isolated and studied pharmacologically (Pasupuleti et al., 2017; Cornara et al., 2017). Prominent mutual activities of honey and propolis are probably due to flavonoids, such as apigenin, acacetin, quercetin, galangin, pinocembrin, chrysin, fisetin and caffeic acid phenyl ester that have been identified in both honey and propolis. Thus, it is not a surprise these products possess similar activities. However, each product also contains special constituents that give rise to different activities.

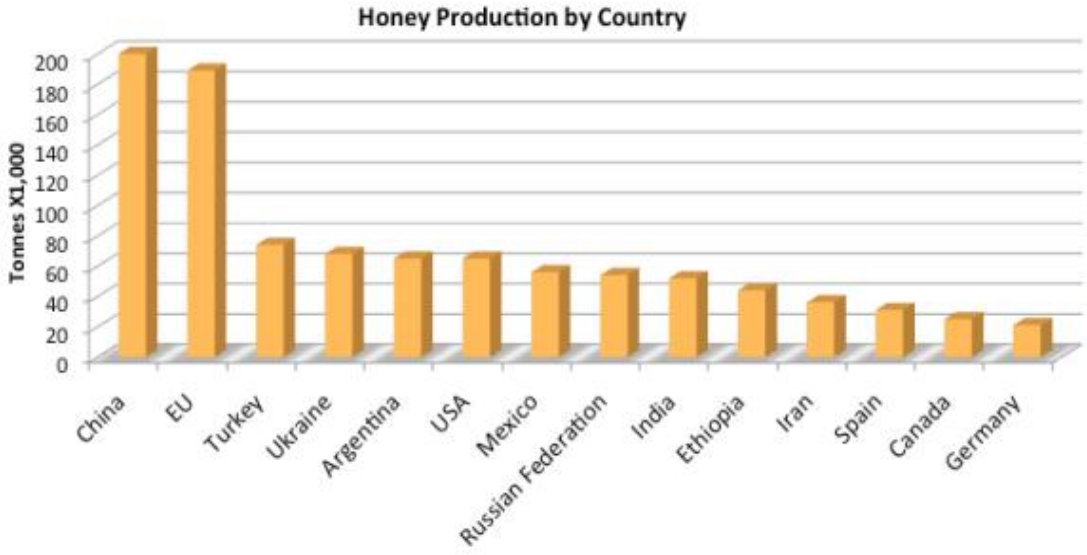

Figure 1.1: World's top honey producers by country and amount in 2012 (Thompson, 2012).

1.2.1. Honey

Honeybees produce a natural sweet liquid by mixing floral nectar with their saliva. One kg of honey is produced by approximately 120 000 bees, and a single bee is able to excrete not more then a tablespoon of honey during its whole life (Thomas and Schumann, 1992). Honey is well known as a rich source of energy and nutrients; it has been used for a long time in traditional medicine for its valuable ingredients (Khalil et al., 2010). The color of honey varies depending on its plant origin as well as its chemical content (Khalil et al., 2010), the darker the honey is, the higher the total phenolic content (Figure 1.2) (M.Alvarez-Suarez et al., 2014).

Figure 1.2: Different colors of honey.

Honey can be categorized into four classes according to its origin: blossom honey, monofloral honey, honeydew honey and multifloral honey. Blossom honey is formed mainly from flower nectar. Monofloral honey is formed from one type of plant. Honeydew honey is formed from honeydew which is secreted by insects. Multifloral honey is formed from several types of plants (M.Alvarez-Suarez et al., 2014). Honey consists mainly of carbohydrates (glucose and fructose) and other compounds including vitamins, phenolic acids, minerals, enzymes, proteins, and flavonoids (Khalil et al., 2010).

The antioxidant capacity and the quality of honey vary according to its botanical origin, geographical area, insect's species, harvesting process and storage conditions (Babarinde et al., 2011; Beng-Kek et al., 2018). Several studies have revealed the importance of physiochemical properties of honey in disease prevention (Khan et al., 2014). For example, its acidic pH, high osmolarity and presence of hydrogen peroxide make the honey capable of killing bacteria (Khan et al., 2014).

Moreover, its osmosis property helps in wound healing by pulling water out of the wound, thus preventing the bacterial growth. In addition, honey can also be used in the treatment of ulcer, diarrhea (Khan et al., 2014), acne, and serves as a great antioxidant (Isla et al., 2013).

Turkey is well known for its floral diversity, which in turn leads to produce many types of honey (Yilmaz et al., 2017). Recently, there has been a noticeable increase in honey production in Turkey, up to 17 times (Yilmaz et al., 2017). However, there are limited number of studies on Turkish honeys, although most of them are known to help good daily life and health (Table 1.2).

Table 1.2: Some unifloral Turkish honeys and their beneficial impacts on human health (Yilmaz et al., 2017).

Type of honey (Turkish in brackets)	**Important properties**
Acacia (Akasya)	Protects from cancer and stress, useful for metabolism and diabetes, prevents osteoporosis and deficiency of zinc, selenium and iron.
Alfalfa (Yonca)	Improves the visual and skin system, improves the children growth, removes rheumatism, and treats vascular congestion.
Anzer (Anzer)	Alternative therapy for gastrointestinal disorders (gastritis, peptic ulcer, constipation), hypertension and coronary heart disease.

Table 1.2: Some unifloral Turkish honeys and their beneficial impacts on human health (Yilmaz et al., 2017).

Astragalus (Geven)	Improves nervous system, sedative, blood maker, beneficial for renal diseases protects bladder and prostate from inflammation.
Black cumin (Çörekotu)	Improves activity of nervous and brain system and strengthens the kidney and urine system, good for diabetic patients, lightens the skin and removes blood poisoning.
Chestnut (Kestane)	Relieves from cold and flu, relieves stomach and liver fatigue, and boosts the immune system.
Coriander (Kişniş)	Sedative and sleep adjustment, eliminates muscle spasms, strengthens the digestive system and shortness of breath.
Crazy (Deli)	Alternative therapy in gastritis, gastric ulcer, constipation, hypertension, coronary heart disease and is believed to increase sexual power is used in impotence
Eucalyptus (Okaliptus)	Treatment of lung disorders, asthma, cold
Linden (Ihlamur)	Protects and strengthens the nervous system, calms the body.
Mint (Nane)	Prevents intestinal gas, cures colic, increases pancreatic secretion, and helps digestion.
Orange (Portakal)	Calms the nerves.
Sunflower (Ayçiçeği)	Makes young skin cells, relieves cold, and useful for hoarseness.
Thyme (Kekik)	Serves as antibacterial, anti-cough and anti-cold, useful for diabetic patients, improves the respiratory and digestive systems.

A research study on 60 samples of Turkish honeys reported the existence of 32 phenolic compounds with antioxidant activity including, quercetin, syringic, vanillic, p-coumaric, caffeic and ferulic acids, and it has been found that Carob honey collected from Muğla, Datça, Marmaris had 935.03 mg/kg total phenolic compounds and possessed the highest DPPH scavenging activity (IC_{50} = 54.33 ± 1.69 µg/mL) (Kivrak and Kivrak, 2016).

1.2.2. Propolis

Propolis is a Greek word, which means the material responsible for protection and construction of the hive. Propolis is an apiculture product produced by honeybees as a mixture of plant, buds resins and wax. It is used to seal holes and prevent intruders from entering the hive (Toreti et al., 2013) (Figure 1.3).

In addition to its antiseptic and anti-inflammatory properties, propolis plays a main protective role in the cell (Betances-Salcedo et al., 2017). Propolis contains high phenolic compounds making it one of the most effective bee products (Pratami et al., 2018).

Figure 1.3: Propolis.

Propolis consists mainly of resins, balsams, wax, aromatic oils and pollen as well as vitamins, minerals, sugars, steroids, enzymes and alcohol, and its composition varies according to specific times of the year and phytogeographical diversity of the area (Farooqui and Farooqui, 2012).

Furthermore, solvents and extraction process can affect the chemical composition and the antioxidant capacity of the product (Sun et al., 2015). Due to its varied composition and diverse plant origin, propolis possesses a wide spectrum of biological activities as shown in Table 1.3 (Toreti et al., 2013).

Table 1.3: Various active compounds found in propolis (Toreti et al., 2013).

Chemical compounds	**Activities**
Acacetin	Anti-inflammatory
Apigenin	Anti-inflammatory
Artepillinc	Antimicrobial, antioxidant, antitumor
Caffeic acid phenylester	Antitumor, anti-inflammatory
Chrysin	Anti-inflammatory
Caffeic acid	Antibacterial, antifungal, antiviral
Cinnamic acid	Anti-inflammatory
Dicaffeoylquinic acid derivatives	Hepato protective

Table 1.3: Various active compounds found in propolis (Toreti et al., 2013).

Ferulic acid	Anti-inflammatory
Galangin	Anti-inflammatory
Gallic acid	Anti-inflammatory
Moronic acid	Anti-HIV
Isoferulic acid	Anti-inflammatory
Pinostrobin	Local anesthesia
Protocatechuic acid	Anti-inflammatory, antifungal, antibacterial
Pinocembrin	Antimold, local anesthesia
Propofol	Antioxidant
p-Coumaric acid	Antibacterial
m-Coumaric acid	Anti-inflammatory
o-Coumaric acid	Anti-inflammatory, antiviral
Quercetin	Antihistamine, ulcer healing, capillary strengthening
Volatile constituents (phenols, esters, terpenoid, etc.)	Antibacterial

Propolis can fight against cancer; it can prevent the growth of pancreatic cancer cells and inhibit tumor-induced angiogenesis. Propolis plays an important role in preventing inflammation and treating wounds by enhancing collagen (Krol et al., 2013).

Due to its high phenolic compounds, propolis protects the skin from external factors such as, UV radiation. Hence, it protects skin from aging (Krol et al., 2013).

Moreover, propolis contributes to prevent many diseases including liver and heart diseases (Krol et al., 2013). Although studies on Turkish propolis are limited, some researches reflect its ability to fight against bacteria and fungi, and to inhibit the growth of specific cancer types. The abundance of propolis phenolic content makes it an excellent raw material in pharmaceutical and food industry (Ristivojevic et al., 2018; Barlak et al., 2011; Katircioglu and Mercan, 2006).

1.2.3. Bee Pollen

Bee pollen is an apiculture product characterized by different colors and shapes as shown in Figure 1.4. Pollen is reproductive microspores with diameter ranges from 2.5 to 250 µm.

Pollen grain is surrounded by two cellular layers, the outer layer provides the protection from the physicochemical factors (Komosinska-Vassev et al., 2015).

Honeybees collect pollen from the male flower seeds as a main source of protein and use it for the development of the brood (Ismail et al., 2013) by mixing it with nectar and enzymes from their salivary gland, and roll it into small balls; thereafter, it is placed in honeycombs and covered with a thin surface layer of honey for subsequent use in the production of beebread and royal jelly (Komosinska-Vassev et al., 2015). Beekeepers collect about 50-250 g of pollen daily and up to 7 kg yearly from each bee colony (Komosinska-Vassev et al., 2015).

Figure 1.4: Bee pollen.

Pollen consists of 250 components including essential amino acids, proteins, sugars, lipids, vitamins (C, B1, B2, B6, folic acid, biotin), β-carotene, phenolic compounds and bio elements (Komosinska-Vassev et al., 2015).

The chemical composition of pollen depends significantly on the plant origin, geographical area (Komosinska-Vassev et al., 2015), storage and some environmental factors exposured (Nogueira et al., 2012).As summarized in Figure 1.5, the valuable chemical composition of pollen gives many therapeutic properties, such as, anticancer, antioxidant, antibacterial, antifungal and anti-inflammatory activities (Denisow and Denisow Pietrzyk, 2016).

Some studies exhibit the ability of pollen to increase the lipid metabolism and decrease the triacylglycerol and total lipids of the blood serum. Furthermore, administration of bee pollen can improve blood flow and prevent the atherosclerotic changes of the blood vessels in human (Komosinska-Vassev et al., 2015).

In addition, the high content of flavonoids and phenolic acids play a significant role in detoxifying process by lowering the amount of toxic substances and protecting the liver tissue from damage (Komosinska-Vassev et al., 2015).

The high nutritional value of Turkish pollen was proved by a study conducted on 20 samples of pollen collected from various regions in Turkey. The study showed that the samples contain a high amount of minerals and heavy metals (Altunatmaz et al., 2017). Another study conducted on Turkish chestnut pollen showed that it contains polyphenols including, gallic acid, syringic acid, pinocembrin, kaempherol and chrysin, which are believed to contribute in the prevention of DNA oxidation by 11% (Karkar et al., 2018).

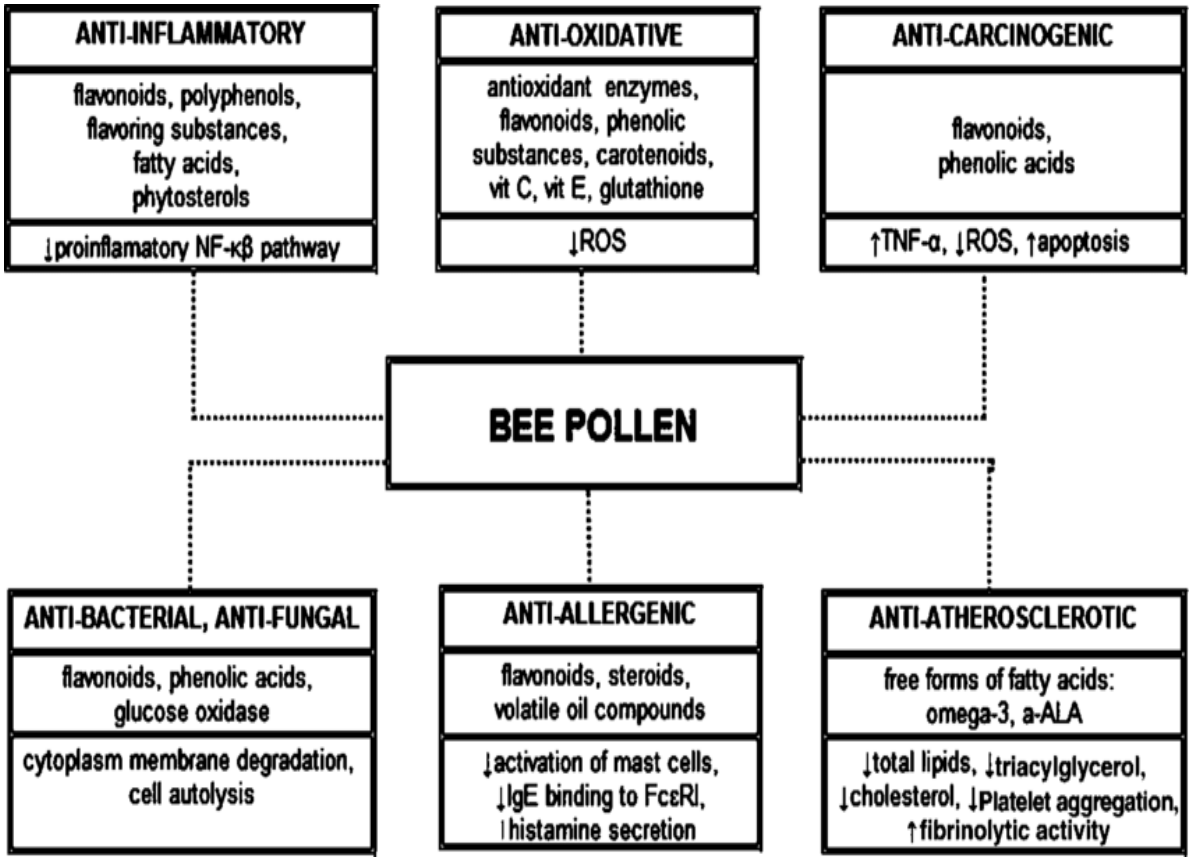

Figure 1.5: Mechanisms of therapeutic properties of pollen compounds (Denisow and Denisow-Pietrzyk, 2016).

1.3. REACTIVE OXYGEN SPECIES

Reactive oxygen species (ROS) are highly reactive molecules formed by cellular metabolism and different external sources (Birben et al., 2012).

ROS level is normally in balance with antioxidans (AOX) in the cell. However, some factors lead to excessive production of ROS resulting in oxidative stress and serious alterations of biochemical molecules (Figure 1.6). The increased level of ROS contributes to severe multifactorial diseases including DNA degradation, neurological disorders, diabetes, hypertension, skin aging, cancers and asthma (Birben et al., 2012).

For this reason, new prospect is to consume natural products as daily supplements to increase the antioxidant level in the human body (Khalil et al., 2010).

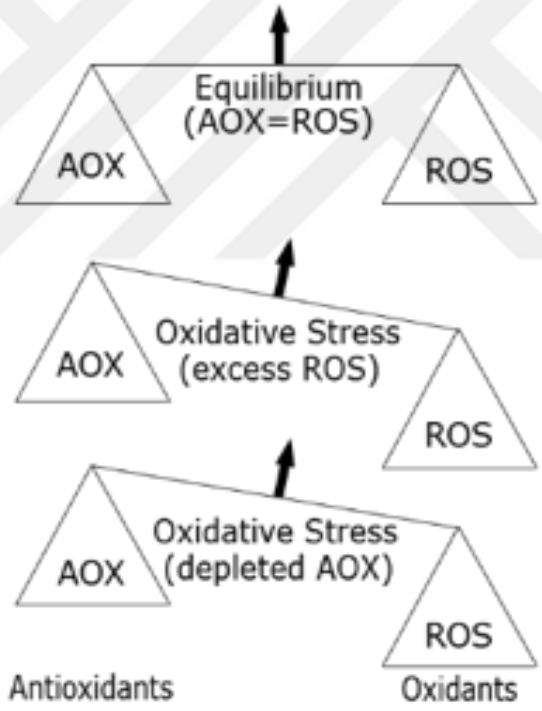

Figure 1.6: The correlation between oxidants and antioxidants (Scandalios, 2005).

Reactive oxygen species are divided into two classes: free radicals and non-radical ROS. Free radicals include superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$) and other substances having unpaired valence electrons whereas non-radicals formed by two free radicals sharing

their electrons comprise molecules that produce free radicals, such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) as shown in Table 1.4 (Birben et al., 2012).

Unpaired electrons increase the molecules' reactivity and lead to many harmful reactions. However, the existence of integrated antioxidant systems in aerobic organisms is effective in inhibiting the harmful effects of the excessive production of ROS within the cells (Birben et al., 2012).

1.3.1. Sources of Reactive Oxygen Species

Mitochondria is believed to be one of the main sources of ROS. During the ATP synthesis through electron chain transport system, 1 up to 3% of electrons leak from the system and form superoxide anion. Furthermore, NADPH oxidase in neutrophils and xanthine oxidase can contribute in $O_2^{\cdot -}$ generation (Birben et al., 2012; Bencheikh., 2012).

Superoxide dismutase SOD enzyme convert $O_2^{\cdot -}$ to H_2O_2, which by itself is not able to react with cellular components. However, the interaction between H_2O_2 and transition metal ions (Fenton reaction and Haber-Weiss reaction) creates hydroxyl radicals, which are the most reactive radicals of ROS and can interact with many cellular components such as lipids, DNA and proteins (Birben et al., 2012; Bencheikh., 2012).

$Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} +$

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + OH^{\cdot}$ Fenton reaction

---

$O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + OH + OH^{\cdot}$ Haber-Weiss reaction

Glutathione peroxidase, catalase enzymes and myeloperoxidase (MPO) in neutrophils can detoxify H_2O_2 by producing HOCl. In addition to endogenous factors, ROS can be generated under a variety of stress conditions including cigarettes smoking, pollution, pesticides and ionizing radiation (Birben et al., 2012; Bencheikh, 2012).

1.3.2. Oxidative Effects on Cellular Components

1.3.2.1. Oxidative Effects on Lipids

During lipid peroxidation, free radicals (especially $OH^{\cdot}$) attack the unsaturated lipids and steal a hydrogen atom from a methylene group ($-CH_2-$). Free radicals attack mainly phospholipids in two positions, the double bond of two carbon atoms and the ester bond. Under the attack of free radicals to unsaturated phospholipids, cell membrane will be degraded by free radicals, which in turn disrupt the membrane function and increase its permeability (Sharma et al., 2012). There are three stages of lipid peroxidation process: initiation, propagation and termination. Abstraction of hydrogen atoms from lipids leads to formation of highly reactive fatty acid radicals, such as malondialdehyde (MDA) and aldehydes, which interact with cellular proteins and alter their function. The level of lipid peroxidation is considered as an important marker to assess the cellular damage by ROS (Birben et al., 2012; Sharma et al., 2012).

Table 1.4: Major endogenous oxidants (Birben et al., 2012).

Oxidant	Formula	Reactive Equation
Superoxide anion	$O_2^{\cdot -}$	$NADPH+2O_2 \leftrightarrow NADP^+ + 2O_2^{\cdot -} + H^+$ $O_2^{\cdot -} + H^+ \rightarrow O_2 + H_2O_2$
Hydrogen peroxide	H_2O_2	Hypoxanthine + H_2O + O_2 ↔ Xanthine + H_2O_2 Xanthine + H_2O + O_2 ↔ Uric acid + H_2O_2
Hydroxyl radical	$OH^{\cdot}$	$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^-$
Hypochlorous acid	HOCl	$H_2O_2 + Cl^- \rightarrow HOCl + H_2O$
Peroxyl radicals	$ROO^{\cdot}$	$R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$
Hydroperoxyl radical	$HOO^{\cdot}$	$O_2^{\cdot -} + H_2O \leftrightarrow HOO^{\cdot} + OH^-$

1.3.2.2. Oxidative Effects on Proteins

Free radicals attack proteins and disrupt their function in several ways, such as alteration of protein electro-charge, oxidation of amino acid specific residues, and fragmentation of protein chains. Thereby, susceptibility of proteins to proteolysis increases (Birben et al., 2012).

Free radicals can inactivate enzymes, especially those having metal cofactors close to their active sites.

Through divalent cation binding site, metal ion can bind with the protein, this binding lead to interaction between the Fe cofactor and hydrogen peroxide in Fenton reaction producing hydroxyl radicals, which in turn cause protein degradation (Sharma et al., 2012).

1.3.2.3. Oxidative Effects on DNA

Free radicals at high concentration modify DNA strands, leading to deoxyribose oxidation, alteration in purine, pyrimidine and bases. They also cause mutations and strand breakage. Many environmental factors and metals contribute to DNA oxidation and result in carcinogenesis, aging and cardiovascular diseases (Birben et al., 2012).

1.4. ANTIOXIDANT DEFENSE SYSTEMS

The human body possesses antioxidant defense systems, which consist of enzymatic and non-enzymatic antioxidants.

Enzymatic antioxidants can detoxify highly potent free radicals to H_2O_2 and then to water and it includes superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase, and thioredoxine enzymes. Non-enzymatic antioxidants consist of natural antioxidants (vitamins, bioflavonoids, carotenoids, phenolic acids, physiological antioxidants and others) and synthetic antioxidants.

Natural antioxidants have a wide range of biological activities as summarized in Table 1.5 (Nimse and Pal, 2015).

All components of antioxidant defense systems work together to counterbalance the effect of oxidants and contribute in ROS detoxification (Birben et al., 2012).

Table 1.5: Natural antioxidants and some of their properties (Nimse and Pal, 2015).

Natural antioxidants	Some of properties and mechanism of action
Vitamins (C, E, A)	Vitamin C can scavenge free radicals and decrease cellular damage by forming ascorbic radical. Vitamin E (α-tocopherol) breaks the chain of lipid peroxidation and controls some important cellular function such as apoptosis and necrosis. Vitamin A is a lipid-soluble scavenger and it contributes protecting human LDL against copper-stimulated oxidation.
Bioflavonoids (quercetin, isoflavone, myricetin etc...)	Flavonoids can scavenge free radicals and chelate metal ions, thus protect DNA from oxidative degradation. They also prevent lipid oxidation.
Carotenoids	Carotenoids can scavenge peroxyl radicals and prevent the cellular membrane damage.
Phenolic acids	Reduce heart diseases, chelate metal ions and scavenge radicals.
Physiological antioxidants (uric acids, GSH, etc…)	Uric acid scavenges some of free radicals in plasma, such as peroxyl radical. Reduced glutathione (GSH) and related enzymes work together to defense against ROS.

1.5. ASSESSEMENT OF ANTIOXIDANT ACTIVITY

Currently, there is no sufficient method used to evaluate the antioxidant capacity. Thus, it is important to perform several methods to obtain an accurate result (Bertoncelj et al., 2007).

Generally, each assay has its own principle. According to these principles, methods are divided into radical scavenging based assays, redox-potential based methods, metal chelation based methods and assessment of total phenolic content (Zhong and Shahidi, 2015).

1.5.1. Radical Scavenging Based Methods

These methods measure the potential of antioxidants to donate hydrogen atom or electron to the free radicals. Some of these methods are, ORAC (oxygen radical absorbance capacity) and DPPH (2, 2-diphenyl-1-picrylhydrazyl radical scavenging) (Zhong and Shahidi, 2015).

1.5.2. Redox Potential Based Methods

These methods measure the ability of antioxidants to reduce the high valence elements into low valence state. It contains FRAP (ferric reducing antioxidant power) and CUPRAC (cupric reducing antioxidant capacity). FRAP is used to measure the reduction of ferric ion (Fe^{3+}) into ferrous ion (Fe^{2+}) by antioxidants. While CUPRAC assay measure the potential of antioxidants to reduce Cu^{2+} to Cu^{+} (Zhong and Shahidi, 2015).

1.5.3. Metal Chelation Based Methods

These methods measure the ability of antioxidants to chelate the metal ions by forming complexes between metals and antioxidants. Thus, the transition metal ions can no longer stimulate lipid peroxidation process (Zhong and Shahidi, 2015).

Besides, assessment of principle constituents of an active sample can be detected by total phenolic content assay, it is a colorimetric assay based on the reduction of Folin-Ciocalteu reagent by antioxidants under alkaline conditions. (Zhong and Shahidi, 2015). Each of these assays has its own mechanism thus it is necessary to select a proper assays to obtain a reliable results.

1.6. POLYPHENOLIC COMPOUNDS

Polyphenols are secondary plant metabolites, generally consisting of aromatic rings carrying hydroxyl moieties. Due to their redox properties, poly phenols act as free radical scavenger and metal chelator. Specific factors such as, plant genes and environmental agents can affect the amount of phenols in plants (Ozcan et al., 2014).

Polyphenols are found abundantly in fruits, vegetables, chocolate etc... Several studies have proved their ability to lower the risk of diseases. Hence, improving the quality of life (Scalbert et al., 2005). The antioxidant properties of a polyphenol arises from possessing a large number of hydroxyl groups in its structure, and its ability to donate electrons and

modify the outputs of oxidative stress (Nimse and Pal, 2015). Based on the chemical structure, polyphenols are classified into flavonoids, phenolic acids, tannins and stilbenes as shown in Figure1.7 (Ozcan et al., 2014).

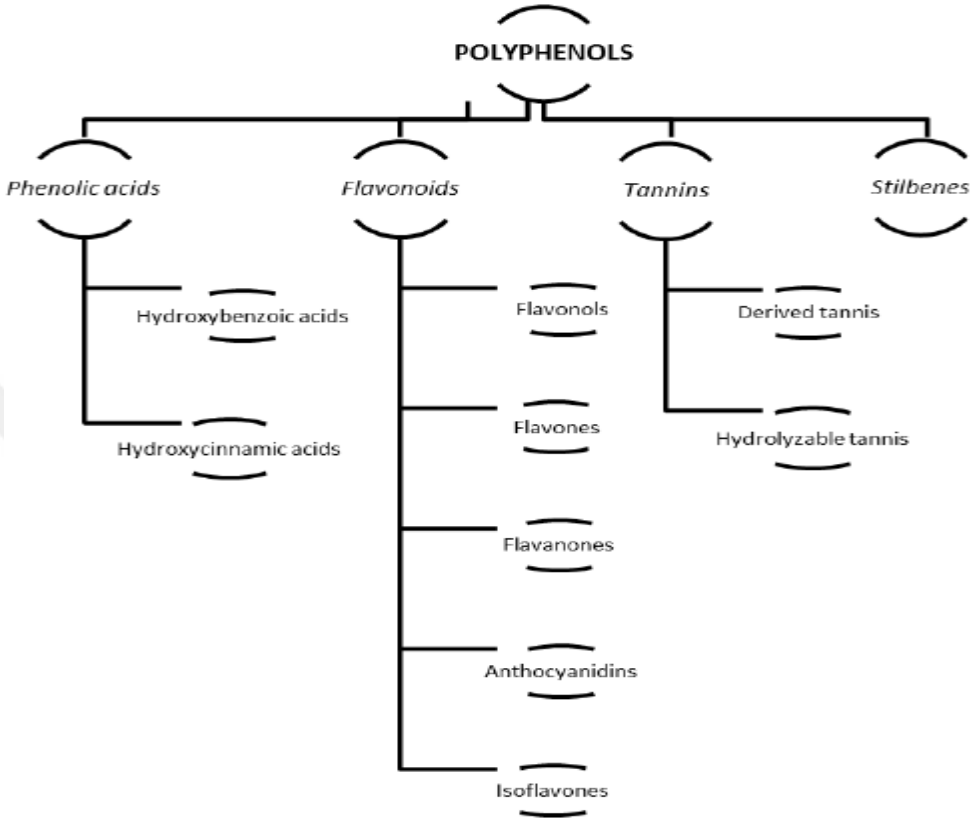

Figure 1.7: Classification of polyphenols (Ozcan et al., 2014).

1.6.1. Phenolic Acids

Phenolic acids have a carboxyl group attached to benzene ring. Based on their structure, phenolic acids are subclassified into two groups, hydroxybenzoic acids and hydroxycinnamic acids (Figure 1.8), and their derivatives (Ozcan et al., 2014), as shown in Table 1.6 (Bencheikh, 2012). Many fruits, such as, blueberry, cranberry, lemon, grapefruit etc. are rich in phenolic acids (Ozcan et al., 2014).

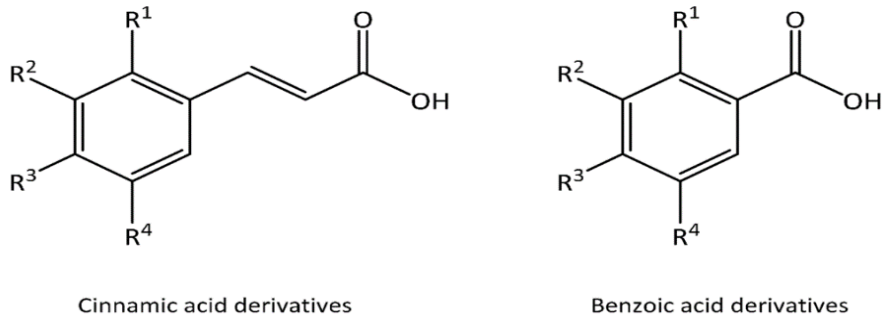

Figure 1.8: Structures of major cinnamic and benzoic acids derivatives (Kaushik et al., 2015).

Table 1.6: Structures of phenolic acids (Bencheikh, 2012).

Name	R^1	R^2	R^3	R^4
Benzoic acid	H	H	H	H
Caffeic acid	H	OH	OH	H
Cinnamic Acid	H	H	H	H
Ferulic acid	H	OCH3	OH	H
Gallic acid	H	OH	OH	OH
Gentisic acid	OH	H	H	OH
m-Coumaric acid	H	OH	H	H
o-Coumaric acid	OH	H	H	H
p-Coumaric acid	H	H	OH	H
p-Hydroxybenzoic acid	H	H	OH	H
Protocatechuic acid	H	OH	OH	H
Salicylic acid	OH	H	H	H
Sinapic acid	H	OCH_3	OH	OCH_3
Syringic acid	H	OCH_3	OH	OCH_3
Vanillic acid	H	OCH_3	OH	H
Veratric acid	H	OCH_3	OCH_3	H

Latest studies on bee derivatives exhibited a strong correlation between the various activities of bee products and their phenolic acids. For instance, a study on Brazilian green propolis showed that cinnamic acids contributes in the antimicrobial activity of the green propolis.

Besides, propolis riches in stilbenes showed a strong antioxidant activity (Huang et al., 2014).

Another study on pollen detected powerful antioxidant effects due to its high phenolic acids (Stojko et al., 2015).

Table 1.7: The main phenolic acids of bee products (Stojko et al., 2015; Huang et al., 2014; Pyrzynska et al., 2009).

Honey	Propolis	Pollen
Gallic acid, vanillic acid, benzoic acid, syringic acid, p-coumaric acid, ferulic acid.	Cinnamic acid, p-coumaric acid, caffeic acid, ferulic acid, lignans, hydroxybenzoic acid, hydroxycinnamic acid.	Gallic acid, protocatechuic acid, caffeic acid, p-cumaric acid, ortho-coumaric acid.

1.6.2. Flavonoids

Flavonoids are characterized by their low molecular weight. The term flavonoid refers to plant pigments. Flavonoids occupy a large part of phenols in plants; they consist of three aromatic rings A, B and C. C ring carries oxygen moiety as shown in Figure 1.9 (Khadem et al., 2010).

Based on the structure of C ring, flavonoids are subclassified into flavonols, flavanols, flavones, flavanones and anthocyanidins as exhibited in Figure 1.10 (Boumerfeg, 2010).

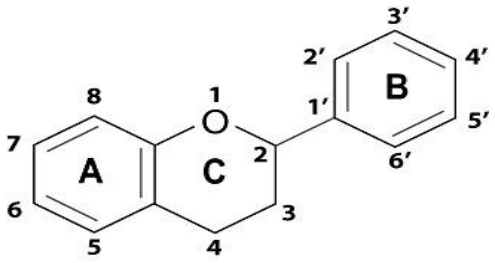

Figure 1.9: Structure of flavonoids (Khadem and Marles, 2010).

Recently, flavonoids have gained more attention due to their significant activities Table 1.8 (Kumars and Pandey, 2013).

Scientists believed that the significant medical value of bee products is related to their high content of flavonoids (Stojko et al., 2015).

Analysis of pollen samples revealed that flavonoids are forming the highest amount of pollen compounds and these in turn gives the pollen its significant activities. However, the abundant presence of flavonol glycosides in bee pollen can reduce its antioxidant activity (Stojko et al., 2015).

Besides, the botanical origin and geographical area affect the content of flavonoids in honey, propolis and pollen.

Propolis and honey have a wide range of flavonoid compounds, which have strong contribution to their medical value. Compared to pollen, propolis has a lesser amount of flavonoids glycosides (Huang et al., 2014; Pyrzynska et al., 2009), which makes it the most active among all bee products (Pratami et al., 2018).

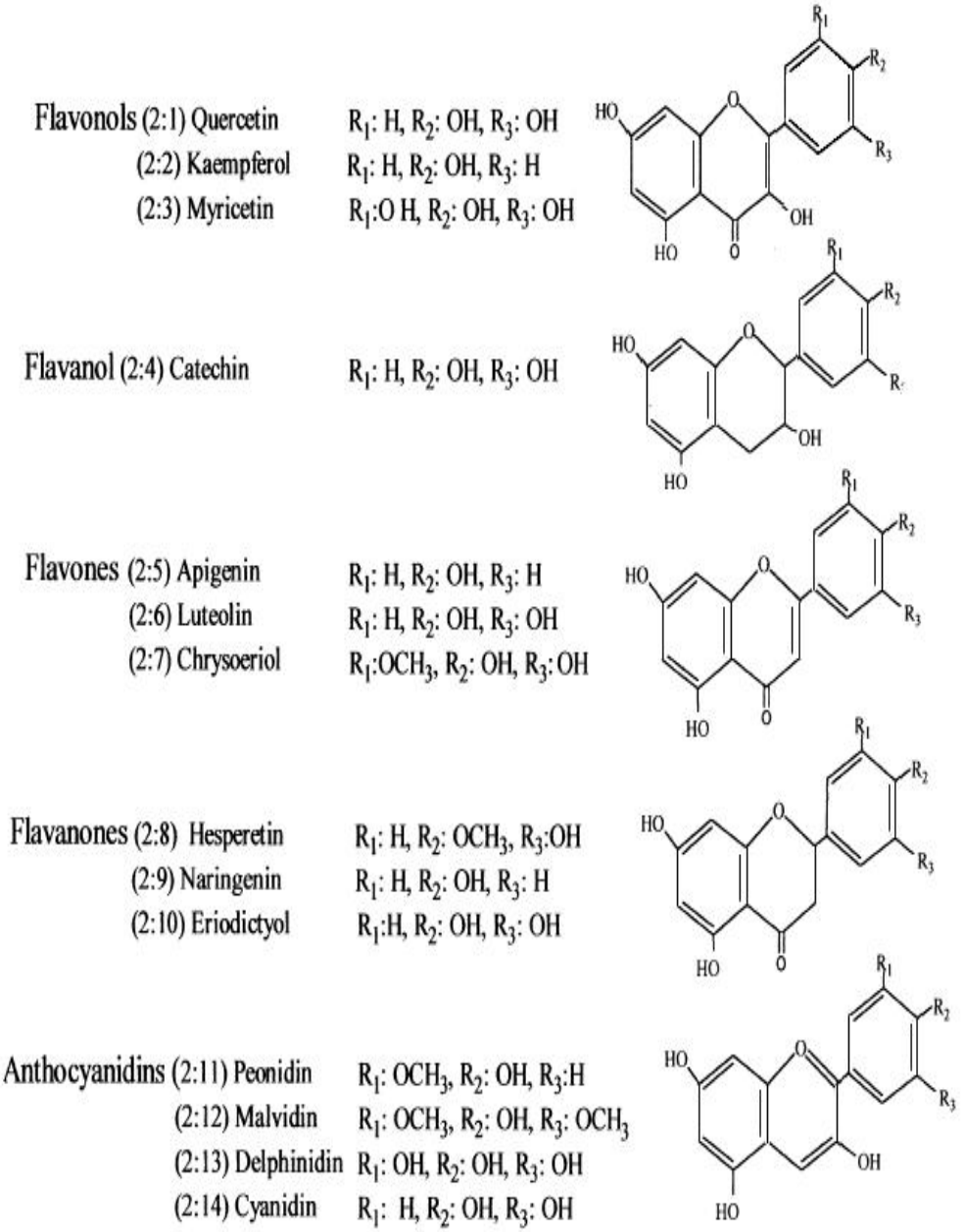

Figure 1.10: Subclassification of flavonoids (Boumerfeg, 2010).

Table 1.8: Flavonoids and their significant activities (Kumars and Pandey, 2013).

Activity	Detected flavonoids
Antioxidant activity	Quercetin, rutin, epicatchin
Hepatoprotective activity	Catechin, apigenin, naringenin, rutin, quercetin, silymarin
Antibacterial activity	Apigenin, galangin, flavone, flavonol glycosides, flavanones, chalcones, catechin
Anti-inflammatory	Hesperidin, apigenin, luteolin, quercetin
Anticancer	Quercetin, flavone -8- acetic acid, robinetin, myricetin, ellagic acid
Antiviral	Catechin, synergism, kaempferol, luteolin, naringin

Table 1.9: The main flavonoids in bee products (Stojko et al., 2015; Huang et al., 2014; Pyrzynska et al., 2009).

Flavonoids	Bee products		
	Honey	Propolis	Pollen
Flavones	Apigenin, luteolin, chrysin	Luteolin, 6-cinnamylchrysin, hexamethoxy flavone, chrysin	Luteolin, apigenin, chrysin
Flavonols	Galangin, kaempherol, quercetin, rutin	Macarangin, quercetin, galangin	Quercetin, rutin, kaempherol, galangin, myricetin
Flavanones	Hesperetin, pinobankins, pinocembrin	Propolin (A,B,E), solophenol A, naringenin, bonannione A, alnustinol, garbanzol, pinobanksin, hesperitin	Naringenin, pinocembrin
Isoflavones	Medicarpin	Odoratin, calycosin, homopterocarpin, medicarpin	Genistein

1.7. FERMENTED FOODS AND PROBIOTICS

Since ancient times, fermentation has been used in the production of food, but there was not sufficient acknowledgment about the whole process. However, in 1850 and after the flourishing of microbiology science, fermentation was well-understood (Blandino et al., 2002). Fermentation was used by ancestors to preserve food and extend its shelf life, and it occured in anaerobic conditions and results in conversion of organic compounds into organic acids or alcohol (Swain et al., 2014). In general, fermentation process includes four different types: alcoholic fermentation, lactic acid fermentation, acetic acid fermentation and alkali fermentation (Blandino et al., 2002).

First, alcoholic fermentation is carried out by yeast and uses in the production of beers and wine. Second, lactic acid type is done by lactic acid bacteria and uses in the production of milk and cereal based fermented products (Blandino et al., 2002). Third, acetic acid process is carried out by acetic acid bacteria and resulted in the conversion of alcohol to acetic acid. Last, alkali fermentation, it is used to ferment fish and seeds (Blandino et al., 2002).

Probiotics are microorganisms (bacteria, yeast) that can promote human health. They can enhance the growth of intestinal flora and improve the body defense system (Swain et al., 2014). Lactic acid bacteria is considered one of the most important probiotics due to their benefits on health and the ease of their employment in food products (Swain et al., 2014).

Recently, fermentation based foods have gained an increased attention because of their positive impact on human body by inhibiting pathogens and increasing the safety of food (Swain et al., 2014) and improving the nutritional value of some products, for instance, fermented cereal has revealed an enhancement of amino acid and vitamins levelsas well as improvement of the texture, flavor, taste, shelf life and aroma of the product (Blandino et al., 2002).

Based on previous studies, polyphenols are considered main constituents responsible for antioxidant activity. In addition, many studies reported that fermentation could enhance the release of flavonoids from some plant products, which in turn, increase the antioxidant activity (Hur et al., 2014). Moreover, microbial enzymes could be synthesized such as cellulase, amylase, esterase and lipase, which allow digesting of starch and breaking down of the plant cell wall. Consequently, improve the extraction of polyphenols (Hur et al.,

2014). Furthermore, the use of lactic acid bacteria in controlled conditions can improve antioxidant activity by converting complex phenolic compounds into simple compounds (Hur et al., 2014).

One study on soybeans showed that conversion of glycosylated isoflavones into aglycones during fermentation increases the antioxidant properties while fermentation negatively affects green tea and reduces its properties by converting catechines to theaflavins and thearabigins (Hur et al., 2014). Consequently, fermentation and some secondary factors including pH, water content, microorganism species, solvents, temperature and fermentation time can affect the antioxidant capacity (Hur et al., 2014).

Fermentation based foods contribute in human health, they can protect from cancer, hypertension, cholesterol, altered vascular reactivity and diabetes as summarized in Figure 1.11 (Farhad et al., 2016). For example, fermented milk can protect from hypertension, also, some fermented foods rich in potassium can affect the blood pressure. Furthermore, probiotics improve the immune system and inhibit the development of colon cancer.

Due to the importance of fermentation in human nutrition, many civilizations used meat fermentation as a safe way to conserve meat and consume it for longer. In addition, the Southern countries of Asia have fermented fish, which are the most important source of food in those areas, in order to increase its shelf life and decrease its cooking time (Rosma et al., 2015).

Turkey takes a place in traditional fermentation of foods, especially, fermentation that carried out by lactic acid bacteria. The most common fermented foods in Turkey are cereal based fermented foods (tarhana, boza), fruits and vegetables based fermented foods, and milk based fermented products (ayran, yoghurt) (Kabak and Dobson, 2011).

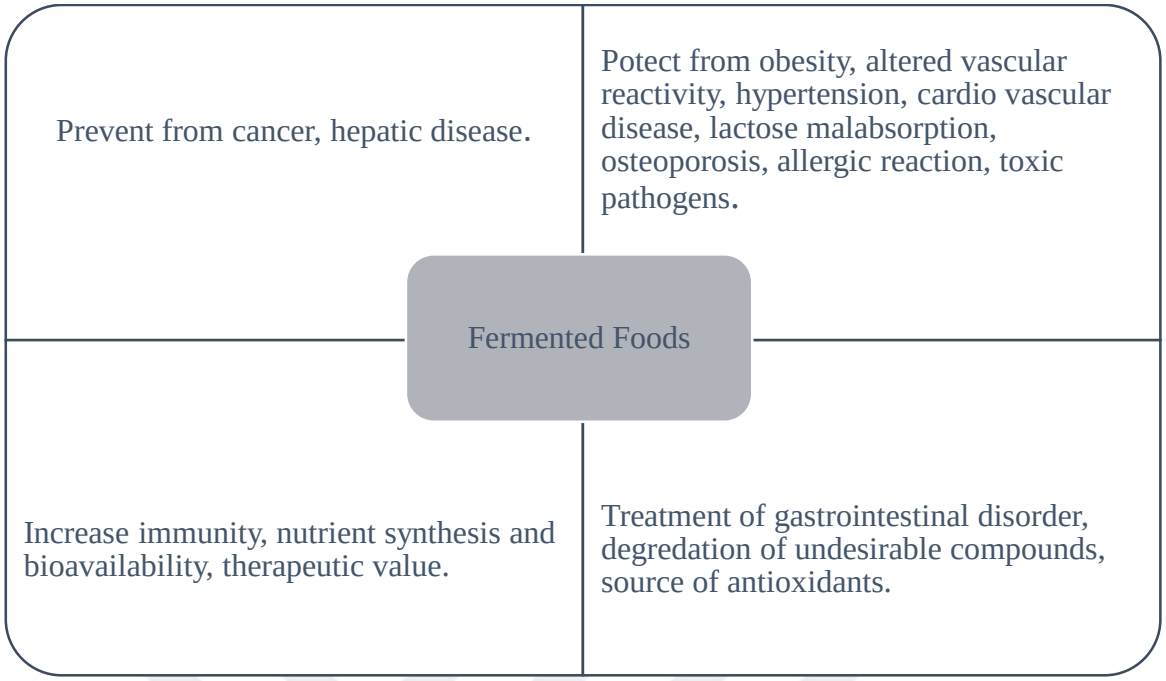

Figure 1.11: The medical benefits of fermented foods (Farhad et al., 2010).

2. MATERIALS AND METHODS

2.1. CHEMICALS AND REAGENTS

Ethanol (EtOH), methanol (MeOH), 1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid (GA), quercetin (QU), aluminum chloride ($AlCl_3$), potassium acetate (CH_3COOK), sodium carbonate (Na_2CO_3), Folin-Ciocalteu reagent were analytical grade, and obtained from Sigma and Aldrich, St. Louis, USA. Type 1 quality pure water (distilled in Millipore Direct-5 UV Remote Water Purification System) was used for the preparation of aqueous solutions.

2.2. HONEYBEE PRODUCTS

Oak honey and spring forest pollen samples from Apis mellifera were provided from a local beekeeper in Kurudere village, Kırklareli Province, Turkey between the years of 2016-2017.

Autumn- and spring-collected propolis samples were purchased in autumn 2016 and spring 2017 from a local beekeeper Igneada Demirkoy village, Kırklareli Province, Turkey.

Propolis and pollen samples were powdered using a grinder. The powder was then sieved to remove large fragments. All samples were covered and stored at -20 ^{o}C for further studies.

2.3. EXTRACTIONS

Honey, propolis and pollen samples were extracted individually with three different solvents [either in 100% (v/v) methanol (MeOH), 70% (v/v) ethanol (EtOH) or pure water] at 3 different temperatures [either at room temperature (RT) for 24 h, at 45 ^{o}C for 24 h, or at 45 ^{o}C for 24 h + boiling for 2 h] as shown in Figure 2.1, 2.2 and 2.3.

30 g of honey (because honey needs more grams to give the required efficacy), 3 g of propolis and 3 g of pollen samples (autumn or spring) were dissolved in 30 mL of each solvent [100% (v/v) MeOH, 70% (v/v) EtOH, pure water] then, each mixture was subjected to three different temperatures. After that, all samples were centrifuged at 5000 rpm for 5 min and filtrated twice with Whatman papers. Then, EtOH and MeOH extracts were evaporated until dryness in a rotary evaporator at 40 ^{o}C whereas aqueous extracts were lyophilized. Each dried extract, was then dissolved in its extraction solvent for the next use.

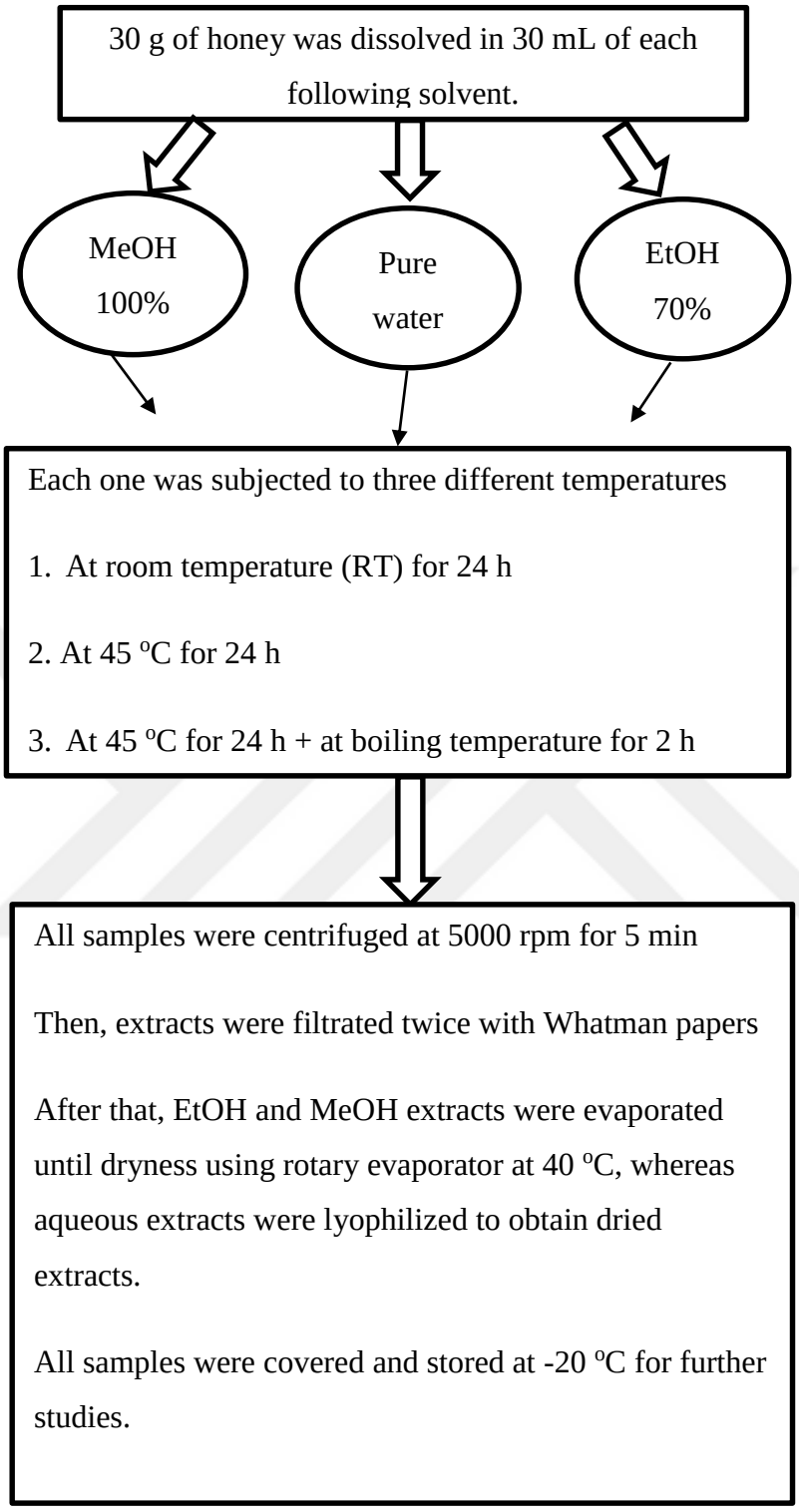

Figure 2.1: Honey extraction processes.

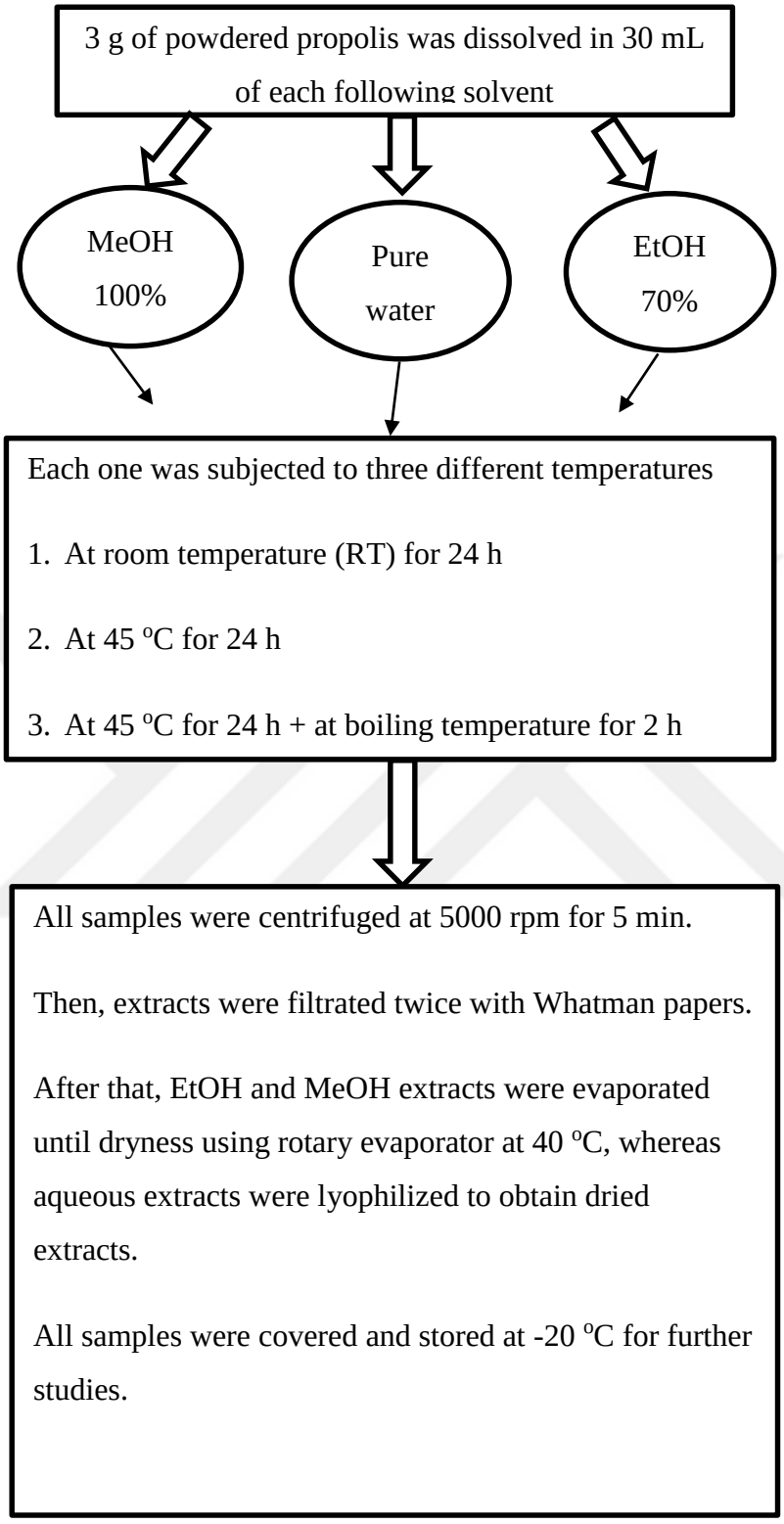

Figure 2.2: Propolis extraction processes.

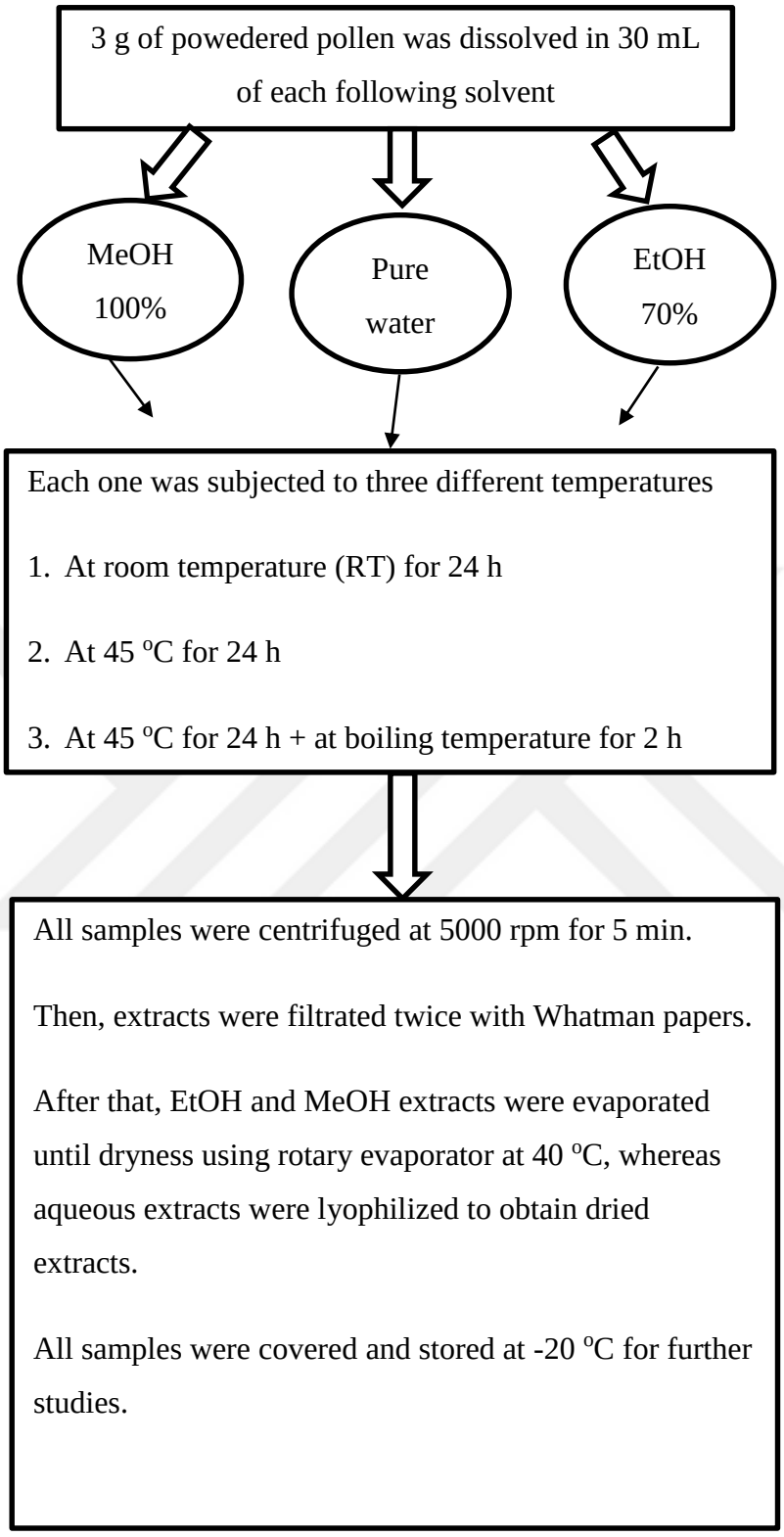

Figure 2.3: Pollen extraction processes.

2.4. FERMENTATION PROCESS

The most active sample of honeybee products was chosen to carry out the fermentation. Autumn propolis sample extracted with 70% (v/v) ethanol at 45 ^{o}C for 24 h + boiling temperature for 2 h was fermented as follows, using a probiotic bacteria, *Lactobacillus brevis* (heterofermentative Gram-positive bacteria) obtained as a gift from Prof. Dr. Gürhan Çiftçioğlu, Istanbul University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology.

Eight grams of propolis extract was mixed with water, completing to 80 mL (the ratio was 0.1 g/mL) followed by pasteurization at 70 ^{o}C for 15 minutes.

Later, fermentation tube was prepared by adding of 10^{6}- 10^{8} power *Lactobacillus brevis* to a 10 mL of pasteurized propolis/water mixture. Two control groups were prepared to check the effects of bacterial growth:

Control 1: 10^{6}- 10^{8} power bacterial suspension in 10 mL water (no propolis extract was added)

Control 2: 10 mL of pasteurized propolis extract in water (no bacteria were added)

All tubes were kept from 1 to 6 days in an incubator at 35 ^{o}C, 100 rpm. The tubes including fermented samples and Control 1 were taken from incubator on the 2nd, 4th and 6th days of fermentation while the one including Control 2 on the 3rd and 6th days. All samples were kept at -20 ^{o}C until further studies. Samples were collected directly (before centrifugation) and after centrifugation (supernatant) in lyophilized form.

All samples were dissolved in distilled water instead of EtOH to avoid insolubility of propolis extract, and tested for their AOA, TPC and TFC. Mixtures obtained in every step of sampling designated as follows were also checked with biochemical tests:

2fP : Fermented sample before centrifugation at the 2nd day

2fPC : Fermented sample after centrifugation at the 2nd day

2B : Bacterial suspension (Control 1) before centrifugation at the 2nd day

2BC : Bacterial suspension (Control 1) after centrifugation at the 2nd day

4fP, 4fPC, 4B, 4BC and 6fP, 6fPC, 6B, 6BC abbreviations refer the samples of the 4th and 6th days, similarly to those of the 2nd day.

Control 2 (10 mL of pasteurized propolis extract in water, no bacteria were added) was only studied at the 3rd and 6th days of fermentation and and designated as follows:

3P : Propolis extract (Control 2) before centrifugation at the 3rd day

3PC : Propolis extract (Control 2) after centrifugation at the 3rd day

6P : Propolis extract (Control 2) before centrifugation at the 6th day

6PC : Propolis extract (Control 2) after centrifugation at the 6th day

2.5. MEASUREMENT OF ANTIOXIDANT CAPACITY AND PHENOLIC COMPOUNDS

2.5.1. DPPH Free Radical Scavenging Assay

DPPH is an assay used to measure the ability of antioxidants to donate electron or more to the free radicals. The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity of the samples was measured according to the method described by Hamad et al. (2010). 40 µL of each test material was added to 160 µL of 0.2 mM DPPH in MeOH. The mixtures were left in the dark for 10 minutes and the absorbance was monitored at 520 nm against the blank. Following equation was used to caluculate radical scavenging activity.

Scavenged DPPH (%) = $[1 - (A_{sample} - A_{sample\ blank})/ A_{control}] \times 100$

where $A_{control}$ was the absorbance of 160 µL DPPH mixed with 40 µL of solvent (MeOH, EtOH or water)], A_{sample} was the absorbance of 160 µL DPPH mixed with 40 µL of extract and $A_{sample\ blank}$ was the absorbance of 160 µL of MeOH mixed with 40 µL of extract.

The half maximal inhibitory concentration (IC_{50}) defined as the concentration of the sample that gives 50% of DPPH inhibition was calculated for each sample. Linear regression of the concentration of the sample was created to calculate IC_{50} value. The serial concentrations of

extracts lie between 500-31.23 mg/mL, 0.25-0.0078 mg/mL and 50-3.125 mg/mL, for honey, propolis and pollen, respectively. IC_{50} values were expressed as mg/mL.

2.5.2. Determination of Total phenolic content (TPC)

Total phenolic content (TPC) in the samples was measured as described by Vuong and Hirun (2013) with a minor modification. The principle of this method is based on the transfer of electrons from antioxidants (phenols) to form a blue complex. This transition is achieved in alkaline pH (Ainsworth and Gillespie, 2007).

A 20 µL of each sample extract in a concentration of 1 mg/mL was mixed with 100 µL of 10% (w/v) Folin-Ciocalteu reagent, 80 µL of 7.5% (w/v) Ca_2CO_3, and shaked for two min followed by incubated in the dark at room temperature for one hour. The absorbance was measured at 760 nm against a methanol blank. The amount of total phenolic content in the extracts was detected from a standard curve of gallic acid (250-3.9 µg/mL) (Figure 2.4).

The results were expressed as milligram of gallic acid equivalents per gram of dry weight of extract (mg GAE/g DWE). All measurements were performed in triplicate.

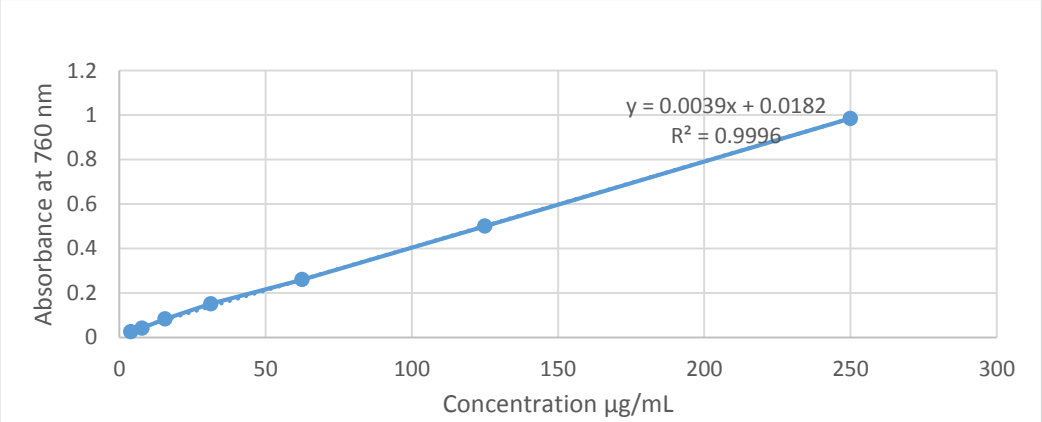

Figure 2.4: Standard curve of gallic acid for the assessment of TPC in the samples.

2.5.3. Determentaion of Total Flavonoid Content (TFC)

The total flavonoid content (TFC) was assessed using aluminum chloride assay as described by Chang et al. (2002), with a slight modification. This method is a colorimetric method based on the ability of $AlCl_3$ to form stable complexes with the hydroxyl groups of

flavonoids (Bag et al.,2015). Briefly, 100 µL of standard or sample was mixed with 300 µL of 95% (v/v) ethanol, 20 µL of 10% (w/v) $AlCl_3$, 20 µL of 1 M potassium acetate and 560 µL of distilled water. Following the incubation for 30 min in room temperature, the absorbance of the mixture was monitored at 415 nm against 80% (v/v) ethanol blank. The amount of $AlCl_3$ was substituted by the same amount of distilled water in blanks. Quercetin standard was prepared to make the calibration curve. One milligram of quercetin was dissolved in 80% (v/v) ethanol and diluted to 100, 50, 25, 12.5, 6.25 µg/mL to prepare standard curve (Figure 2.5).

The results were expressed as milligram of quercetin equivalents per gram dry weight of extract (mg QUE/g DWE). All measurements were performed in triplicate.

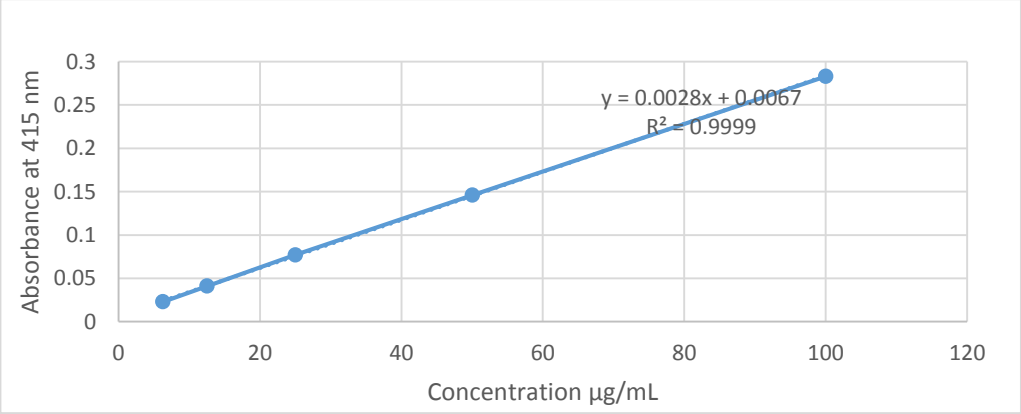

Figure 2.5: Standard curve of quercetin for the assessment of TFC in the samples.

2.6. STATISTICAL ANALYSES

All tests for the detection of TPC, TFC and antioxidant (DPPH scavenging) activity of the extracts were performed in triplicate, and the results were expressed as mean ± SD using GraphPad Software (verison Prism 7.0). The significance of differences in each extract depending on temperature or solvent was determined by a one-way ANOVA with Tukey's post-test. P value of < 0.05 was taken as the criteria of statistically significance.

3. RESULTS

3.1. THE YIELDS OF EXTRACTS

Various bee products were extracted using various solvents differ in their polarities [100% (v/v) MeOH, 70% (v/v) EtOH, pure water] at different temperatures (24 h/RT, 24 h/45 ^{o}C and 24 h/45 C° + 2 h boiled). The yields of extracts in relation to the total weight (3 g of propolis, 3 g of pollen, and 30 g of honey) were presented in Table 3.1. Ethanol (70%) seemed to be the best extraction solvent for propolis and pollen, especially under boiling condition for propolis and RT for pollen, although water is better for honey.

Table 3.1: The yields of extracts from bee products as % of dry weight.

	Extraction time/ temperature	Propolis (A*)	Propolis (S**)	Pollen	Honey
MeOH	24 h/RT	37.6	26.6	32	48.6
	24 h/45 ^{o}C	38.9	27	30	48.5
	24 h/45 ^{o}C + 2 h boiled	39	27	22.3	49
70% EtOH	24 h/RT	59	48.3	**35**	48.4
	24 h/45 ^{o}C	60	49	34	45
	24 h/45 ^{o}C+2 h boiled	**61**	**50**	30	49.6
Water	24 h/RT	0.66	0.5	23	62
	24 h/45 ^{o}C	1.9	1.3	21.6	64.1
	24 h/45 ^{o}C + 2 h boiled	4.5	3.3	20	**65.3**

A*: Autumn

S**: Spring

3.2. TOTAL PHENOLIC CONTENT (TPC)

Total phenolic content of honey, pollen and propolis extracts was assessed using Folin reagent. This method was performed to detect the amount of phenols in different extraction conditions as shown in Table 3.2. Besides, it was performed to detect the amount of phenolic compounds of the samples collected during the fermentation of propolis, and the results were exhibited in Table 3.3.

Autumn propolis extracted with 70% EtOH at 45 ^{o}C for 24 h + boiled for 2 h showed the highest TPC among all tested samples. In fact, propolis extracts were significantly rich in TPC than other bee products for all extraction processes applied. Surprisingly, TPCs of each honey extract was very low, independent of extraction method.

The highest TPC value for each bee product was written as bold, in order to underline the best extraction process for phenolics.

Table 3.2: Total phenolic content (TPC) of pollen, honey and propolis extracts.

Extracts	Extraction time/temperature	TPC (mg GAE/g DWE)			
		Pollen	Honey	Propolis (A*)	Propolis (S**)
MeOH	24 h/RT	20.2 ± 1.2	0.82 ± 0.02	240.0 ± 17.7	219.0 ± 14.5
	24 h/45 ^{o}C	16.9 ± 1.7	0.81 ± 0.01	261.0 ± 13.3	230.0 ± 43.1
	24 h/45 ^{o}C + 2 h/boiled	17.0 ± 2.4	0.88 ± 0.03	280.0 ± 16.7	220.0 ± 29.1
70% EtOH	24 h/RT	**22.8 ± 1.2**	0.77 ± 0.02	314.5 ± 33.4	274.0 ± 31.2
	24 h/45 ^{o}C	17.3 ± 1.3	0.76 ± 0.04	328.3 ± 27.7	284.0 ± 22.2
	24 h/45 ^{o}C + 2 h/boiled	16.1 ± 2.0	0.81 ± 0.01	**340.4 ± 34.5**	**302.0 ± 25.7**
Water	24 h/RT	13.2 ± 1.8	0.77 ± 0.07	249.6 ± 10.0	233.0 ± 34.3
	24 h/45 ^{o}C	11.2 ± 0.8	0.83 ± 0.01	277.6 ± 56.8	258.0 ± 11.7
	24 h/45 ^{o}C+ 2h/boiled	11.5 ± 0.7	**0.90 ± 0.09**	297.0 ± 25.3	290.0 ± 14.7

*A: Autumn
**S: Spring
Each value represents the mean ±SD (n=3).

TPCs of different samples collected during the fermentation of propolis were presented in Table 3.3. Initial numbers represented the days when the samples were taken during the fermentation process in this table. Fermented sample before centrifugation, fermented sample after centrifugation, bacterial suspension (Control 1, no propolis were added) before centrifugation and bacterial suspension after centrifugation were designated with fP, fPC, B and BC symbols followed by the number indicating the day, respectively. Control 2 (10 mL of pasteurized propolis extract in water, no bacteria were added) before centrifugation and Control 2 after centrifugation were given as P and PC symbols followed by the number indicating the day, respectively.

Table 3.3: Total phenolic content (TPC) of fermented propolis samples.

	TPC (mg of GAE /g DWE) of samples written on darker rows			
2nd Day	2fP	2fPC	2B	2BC
	37.3 ± 0.5	**46.7 ± 1.1**	n.d.	n.d
4th Day	4fP	4fPC	4B	4BC
	30.6 ± 1.5	38.3 ± 1.5	n.d.	n.d.
6th Day	6fP	6fPC	6B	6BC
	36.0 ± 1.9	26.3 ± 1.5	n.d.	n.d.
3rd Day	3P	3PC	6P	6PC
	275.1 ± 13.6	275.2 ± 17.4	269.3 ± 9.0	253 ± 8.1

Each value represents the mean ±SD (n=3).
n.d.: Not detected

As seen in Table 3.3, TPC decreased during the fermentation as the highest TPC value was detected in 2fPC (46.7 ± 1.1 mg GA /g DW of fermented propolis) whereas the lowest one in 6fPC (26.3 ± 1.5 mg GA/g DW of fermented propolis). Measurement of TPC after centrifugation seemed to be more accurate, since insoluble materials before centrifugation might interfere the measurement, and solubilization was rather problematic in some samples. All samples of Control 1 (bacterial suspension) had no phenolic compounds, as expected. Control 2 samples (10 mL of pasteurized propolis extract in water without bacterial growth, for 3rd and 6th days) were detected to contain similar amount (more than 250 mg GAE/g DWE) of phenolic compunds, as in the regular propolis samples given in

Table 2.2. Thus, significant decrease in TPC after the fermentation process was occurred, as TPCs of Control 2 samples (3P, 3PC, 6P and 6PC) were much higher than fermented samples. For example, the decrease was approximately 6 fold in 2fPC with respect to 3PC.

3.3. TOTAL FLAVONOID CONTENT (TFC)

Flavonoid content of bee products and fermented propolis was assessed with $AlCl_3$ method using quercetin as a standard, and the results were presented in Table 3.4 and 3.5, respectively. The highest TFC value for each bee product was written as bold, in order to underline the best extraction process for flavonoids.

Table 3.4: Total flavonoid content (TFC) of pollen, honey and propolis extracts.

Extracts	Extraction time/temperature	TFC (mg of QUE/g of DWE)			
		Pollen	Honey	Propolis (A*)	Propolis (S**)
MeOH	24 h/RT	3.72 ± 0.06	**0.055 ± 0.002**	72.0 ± 12.2	67.0 ± 28
	24 h/45 ºC	2.90 ± 0.30	0.052 ± 0.002	72.8 ± 17.0	69.0 ± 9.7
	24 h/45 ºC + 2 h/boiled	3.30 ± 0.80	0.053 ± 0.003	70.7 ± 4.8	59.0 ± 11.1
70% EtOH	24 h/RT	**4.68 ± 0.30**	0.037 ± 0.002	82.2 ± 3.2	75.9 ± 4.4
	24 h/45 ºC	3.90 ± 0.20	0.035 ±0.001	97.8 ± 7.9	86.8 ± 1.8
	24 h/45 ºC + 2 h/boiled	4.40 ± 0.40	0.037 ±0.001	**103.5 ± 5.2**	**90.4 ± 4.6**
Water	24 h/RT	1.54 ± 0.30	0.039 ± 0.006	14.0 ± 2.1	8.4 ± 1.4
	24 h/45 ºC	0.65 ± 0.20	0.039 ± 0.009	19.5 ± 0.5	16.7 ± 0.3
	24 h/45 ºC + 2h/boiled	0.11 ± 0.04	0.037 ± 0.002	21.0 ± 1.1	24.7 ± 2.6

A*: Autumn
S**: Spring
Each value represents the mean ±SD (n=3).

As seen in Table 3.4, 70% (v/v) EtOH was the best solvent to extract TFC in propolis and pollen. Autumn propolis extracted with EtOH at 45 ºC for 24 h+ boiled for 2 h had the highest TFC (103.5 ± 5.2 mg of QUE/g of DWE) among all samples. Ethanol extract of pollen at RT had the highest amount of TFC (4.68 ± 0.30 mg of QUE/g of DWE) among the pollen extracts. Methanol extract of honey prepared at RT had the highest TFC (0.055 ±

0.002 mg of QUE/g of DWE) among all honey extracts. However, honey samples were very poor in TFC in comparison to other products.

In general, it seemed that 70% EtOH was the most effective solvent and high temperatures facilitated the extraction of TFC, for at least bee products tested here.

TFC values of the samples collected during the fermentation process were presented in Table 3.5. Initial numbers represented the days when the samples were taken during the fermentation process in this table. Fermented sample before centrifugation, fermented sample after centrifugation, bacterial suspension (Control 1, no propolis were added) before centrifugation and bacterial suspension after centrifugation at indicated days are designated with fP, fPC, B and BC symbols, respectively. Control 2 (10 mL of pasteurized propolis extract in water, no bacteria were added) before centrifugation and Control 2 after centrifugation were given as P and PC followed by the number indicating the day, respectively.

Table 3.5: Total flavonoid content (TFC) of fermented propolis samples.

	TFC (mg of QUE /g DWE) of samples written on darker rows			
2nd Day	2fP	2fPC	2B	2BC
	3.7 ± 0.7	**9.7 ± 1.5**	n.d.	n.d
4th Day	4fP	4fPC	4B	4BC
	3.4 ± 0.8	7.6 ± 0.6	n.d.	n.d.
6th Day	6fP	6fPC	6B	6BC
	2.0 ± 0.6	1.2 ± 0.3	n.d.	n.d.
3rd Day	3P	3P C	6P	6PC
	24.3 ± 3.1	23.7 ± 2.6	24.2 ± 3.0	23.7 ± 2.5

Each value represents the mean ±SD (n=3).
n.d.: Not detected

The highest TFC was found as 9.7 ± 1.5 mg QUE/g of DWE for 2fPC whereas the lowest one was detected as 1.2 ± 0.3 mg QUE/g of DWE in 6fPC among the fermented samples. As seen in Table 3.5, TFC content decreased during the fermentation as the highest TFC value was detected in 2fPC whereas the lowest one in 6fPC. Measurement of TFC after centrifugation seemed to be more accurate, since insoluble materials before centrifugation

might interfere the measurement, and solubilization was rather problematic in some samples.

All samples of Control 1 (bacterial suspension) had no flavonoids, as expected. Interestingly, Control 2 samples (10 mL of pasteurized propolis extract in water without bacterial growth, for 3rd and 6th days) contained similar amount of flavonoids in comparison to the regular propolis samples extracted with water, not EtOH at 45 ^{o}C for 24 h+boiled for 2 h, as given in Table 3.4. Significant decrease was also occurred in TFC after the fermentation process, as TFC of Control 2 samples (3P, 3PC, 6P and 6PC) was much higher than fermented samples. For example, the decrease was approximately 2.5 fold in 2fPC with respect to 3PC.

3.4. FREE RADICALS SCAVENGING ACTIVITY

DPPH assay measures the ability of antioxidant to scavenge DPPH radical. This reaction is based on the hydrogen donating by antioxidants. The lower of IC_{50} value is the higher of scavenging activity.

The results obtained for bee products and fermented propolis were given in Table 3.6 and Table 3.7, respectively.

The antioxidant profiles of bee products showed a dose-dependent activity in scavenging of DPPH radical. Honey sample extracted with MeOH at RT (IC_{50} = 157.0 ± 9.8 mg/mL) was the sample exhibited the highest antioxidant activity among honey samples.

The autumn propolis sample extracted with 70% EtOH at 45 ^{o}C for 24 h+boiled for 2 h had the highest antioxidant activity (IC_{50}= 0.011 ± 0.001 mg/mL) among all samples. Thus, propolis seemed to be the most active bee product as an antioxidant. Ethanol extract of pollen at RT possessed the highest antioxidant activity (IC_{50} = 5.8 ± 0.8 mg/mL) among pollen samples.

Table 3.6: DPPH scavenging activity (IC_{50} value) of pollen, honey and propolis extracts.

Extracts	Temperatures	IC_{50} (mg/mL)			
		Pollen	Honey	Propolis (A*)	Propolis (S**)
MeOH	24 h/RT	7.4 ± 0.5	**157.0 ± 9.8**	0.028 ± 0.002	0.033 ± 0.003
	24 h/45 ºC	14.5 ± 0.7	180.0 ± 14.5	0.025 ± 0.001	0.031 ± 0.001
	24 h/45 ºC + 2 h/boiled	15.7 ± 1.1	170.0 ± 10.0	0.024 ± 0.001	0.039 ± 0.008
70% EtOH	24 h/RT	**5.8 ± 0.8**	185.0 ± 5.0	0.016 ± 0.002	0.027 ± 0.006
	24 h/45 ºC	13.5 ± 0.5	178.6 ± 6.5	0.013 ± 0.002	0.025 ± 0.005
	24 h/45 ºC + 2 h/boiled	15.1 ± 1	170.6 ± 3.5	**0.011 ± 0.001**	**0.023 ± 0.001**
Water	24 h/RT	21.7 ± 1.1	183.0 ± 10.4	0.048 ± 0.002	0.066 ± 0.004
	24 h/45 ºC	24.2 ± 0.6	175.3 ± 6.5	0.037 ± 0.020	0.039 ± 0.005
	24 h/45 ºC + 2 h/boiled	25.3 ± 0.3	160.3 ± 5.5	0.029 ± 0.001	0.034 ± 0.009

A*: Autumn
S**: Spring
Each value represents the mean ±SD (n=3).

Table 3.7: DPPH scavenging activity (IC_{50} value) of fermented propolis samples.

	IC_{50} (mg/mL)			
2nd Day	2fP	2fPC	2B	2BC
	0.25 ± 0.001	0.220 ± 0.005	n.d.	n.d
4th Day	4fP	4fPC	4B	4BC
	0.38 ± 0.001	0.37 ± 0.007	n.d.	n.d.
6th Day	6fP	6fPC	6B	6BC
	0.47 ± 0.003	0.513 ± 0.001	n.d.	n.d.
3rd Day	3P	3PC	6P	6PC
	0.045 ± 0.005	0.047 ± 0.006	0.048 ± 0.004	0.048 ± 0.004

Each value represents the mean ±SD (n=3).
n.d.: Not detected

Table 3.7 illustrated that the antioxidant activity of propolis decreased during the fermentation process since the lowest activity was detected in 6fPC representing fermented

sample after centrifugation at the 6th day (IC_{50} = 0.513 ± 0.001 mg/mL). The highest radical scavenging ability was detected in 2fPC. All samples of Control 1 (bacterial suspension) had no antioxidant activity, as expected. The highest scavenging ability was observed in 3P sample representing Control 2 (pasteurized propolis extract in water, no bacteria were added) (IC_{50} = 0.045 ± 0.005 mg/mL) at the third day of fermentation. In fact, radical scavenging activities of all Control 2 samples were similar (mean 0.047 mg/mL), and higher than that of fermented propolis. These results reflected that propolis extract lost its antioxidant activity during fermentation process.

3.5. STATISTICAL EVALUATIONS

There was a strong negative correlation (R within the range of -0.9 to - 0.5) between TPC as well as TFC and IC_{50} values for most of the samples, such as 70% EtOH extracts of propolis, fermented propolis and pollen, indicating that higher TPC and TFC resulted with lower IC_{50} or higher antioxidant capacity. However no correlation or moderate correlation (R= -0.4) was found between these parameters in some samples, such as MeOH extract of honey between TPC and IC_{50} and between TFC and IC_{50}, respectively. Surprisingly, there was a strong positive correlation between TFC and IC_{50} in aqueous and ethanolic extracts of honey, probably due to antioxidant activity depended on other compounds rather than flavonoids.

The effect of temperatures and solvent types on TPC, TFC and scavenging activity of bee products were determined, and the results were evaluated with following statistical data produced for each bee product.

3.5.1. Significant Differences in Autumn Propolis Extracts

There was no significant effect of temperature on TPC values of water and 70% EtOH extracts, but the TPC in MeOH extract slightly increased by boiling compared to RT (Figure 3.1A) ($P<0.05$). Solvent type affected TPC values, with EtOH preference especially to MeOH at RT and boiling conditions (Figure 3.1B) ($P<0.05$).

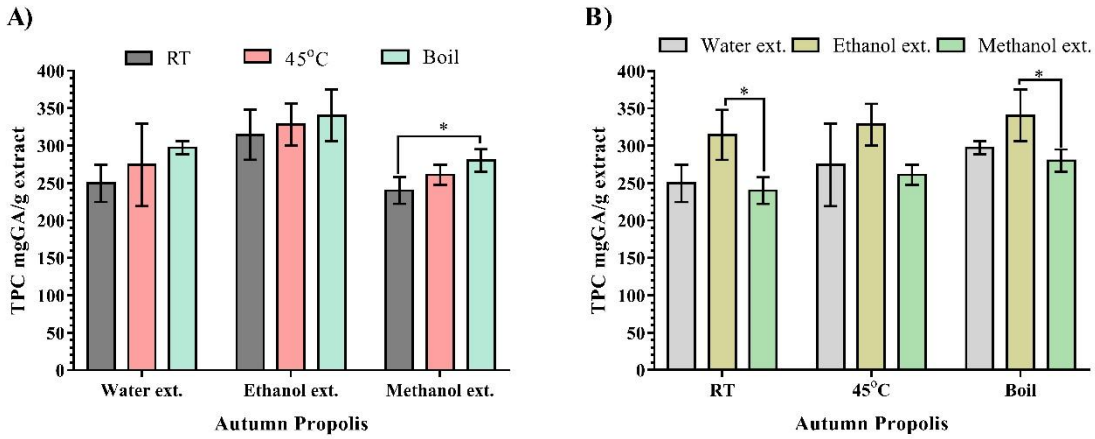

Figure 3.1: The significant differences in TPC values of autumn propolis extracts, A) dependence to extraction temperature, B) dependence to extraction solvent.

TFC in water and 70% EtOH extracts was slightly high when these solvents were heated, but there was no significant effect of temperature on TFC of MeOH extracts (Fig 3.2A) ($P>0.05$). However, there were significant differences between the efficacies of the solvents used in extraction on TFC at different temperatures ($P<0.01$, $P< 0.001$) in favour of alcohols, especially 70% EtOH (Fig 3.2B).

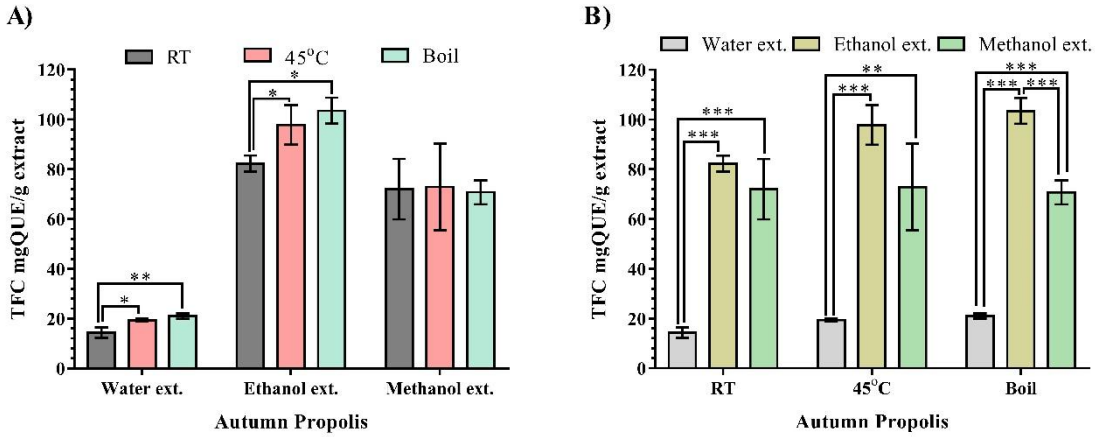

Figure 3.2: The significant differences in TFC values of autumn propolis extracts, A) dependence to extraction temperature, B) dependence to extraction solvent.

Raising the temperature increased the antioxidant capacity significantly when water was used as extraction solvent ($P<0.01$, $P<0.001$). There was no effect of high temperatures on

the antioxidant activity of MeOH extract (P>0.05), while difference between RT and boiling for 70% EtOH extract was also significant (P<0.05), in favour of boiled one (Figure 3.3A). Antioxidant capacity prominently depended on extraction solvent at each ambient temperature (P<0.01, P< 0.001) (Figure 3.3B). There was only a slight difference between the antioxidant capacity of boiled water and boiled MeOH extracts. The highest activity was detected in boiled EtOH.

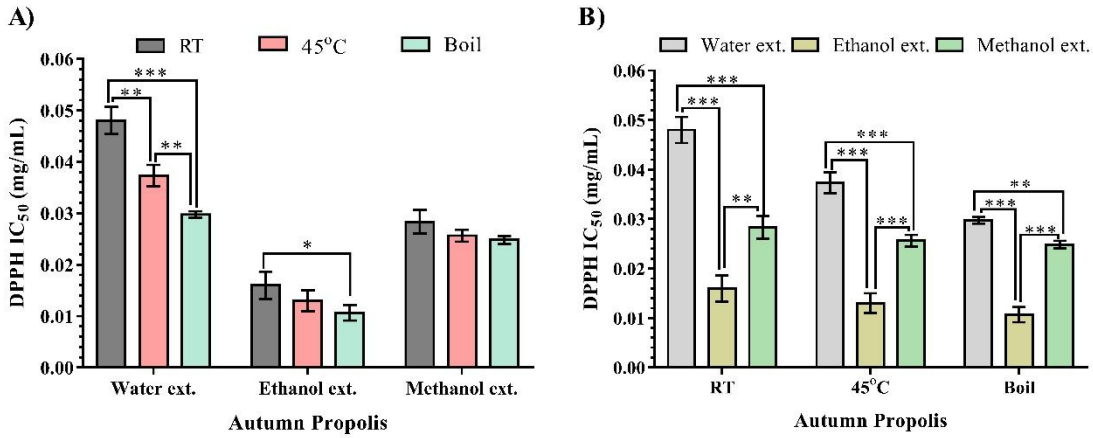

Figure 3.3: The significant differences in antioxidant activities of autumn propolis extracts, A) dependence to extraction temperature, B) dependence to extraction solvent.

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3.5.2. Significant Differences in Spring Propolis Extracts

There was a significant difference between RT and boiling condition in the extraction of TPC when only water was used for the extraction (Figure 3.4A) (P<0.01). Raising the extraction temperature also enhanced the effectiveness of water for extracting phenolics, since TPC increased as higher temperature was used. Moreover, the efficacy of boiled water became closer to that of ethanol. However, ambient temperatures did not affect the TPC when 70% EtOH or MeOH was used as solvent. MeOH seemed to be the less efficient solvent for extracting the phenolics as it produced lower TPC than aqueous EtOH and water in all conditions (Figure 3.4B). Thus 70% EtOH and water seemed to be convenient solvents for the extraction of phenolics since those extracts exhibited more TPC in all conditions than MeOH.

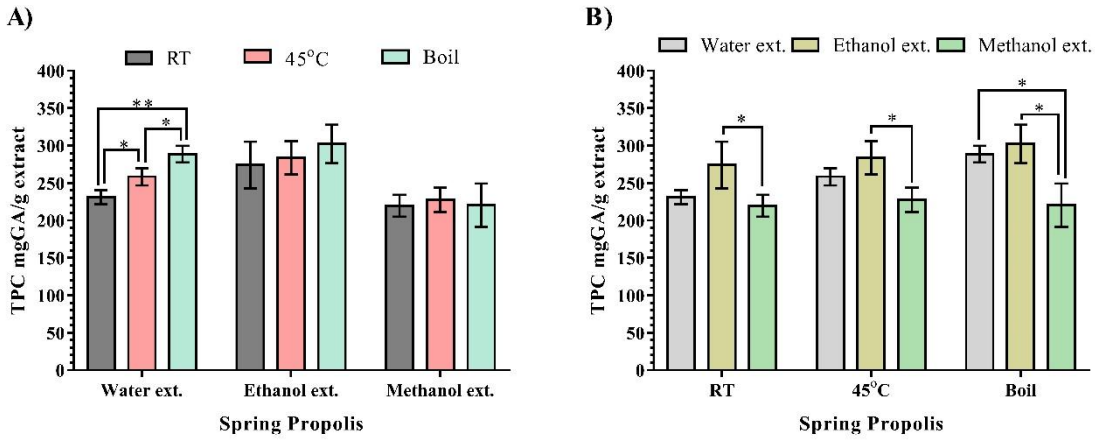

Figure 3.4: The significant differences in TPC values of spring propolis extracts, A) dependence to extraction temperature, B) dependence to extraction solvent.

Only the TFC in water and 70% EtOH extracts increased by raising the extraction temperature (Figure 3.5A), but not in MeOH extract. However, water seemed to be an inconvenient solvent for total flavonoid extraction, since TFC was distinctly lower in aqueous extract rather than alcoholic extracts in all conditions. (Figure 3.5B). The 70% EtOH was the most efficient solvent in all conditions for flavonoid extraction, and the highest TFC was detected in boiling condition.

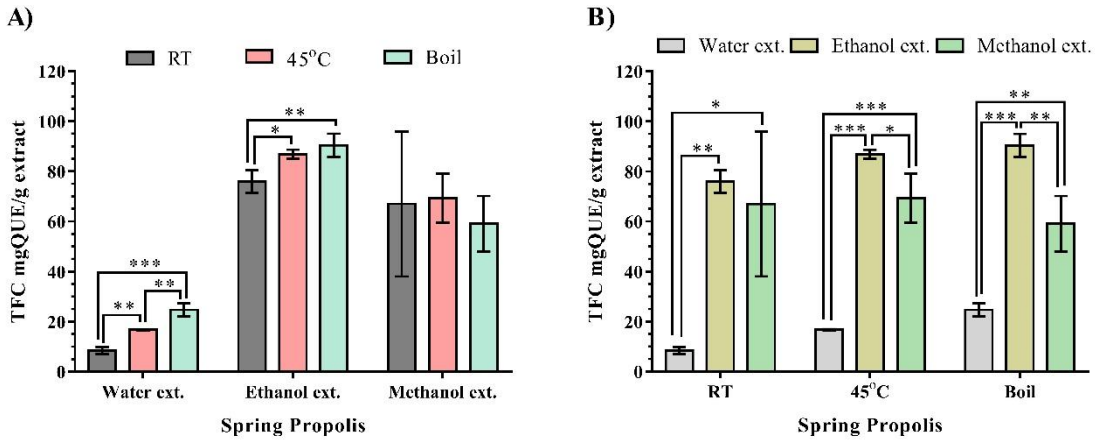

Figure 3.5: The significant differences in TFC values of spring propolis extracts, A) dependence to extraction temperature, B) dependence to extraction solvent.

Heating and/or boiling increased the scavenging activity of the extracts significantly, comparing to RT when water or 70% EtOH was used as solvent ($P<0.001$), while there was no effect of temperature on the antioxidant activity of MeOH extract ($P>0.05$) (Figure 3.6A). However, water was distinctly less efficient than EtOH and MeOH at RT, but than only EtOH at 45 ^{o}C (Figure 3.6B). Although there were no significant differences between the antioxidant activities in all extracts obtained boiled solvents, that of 70% EtOH was prominent.

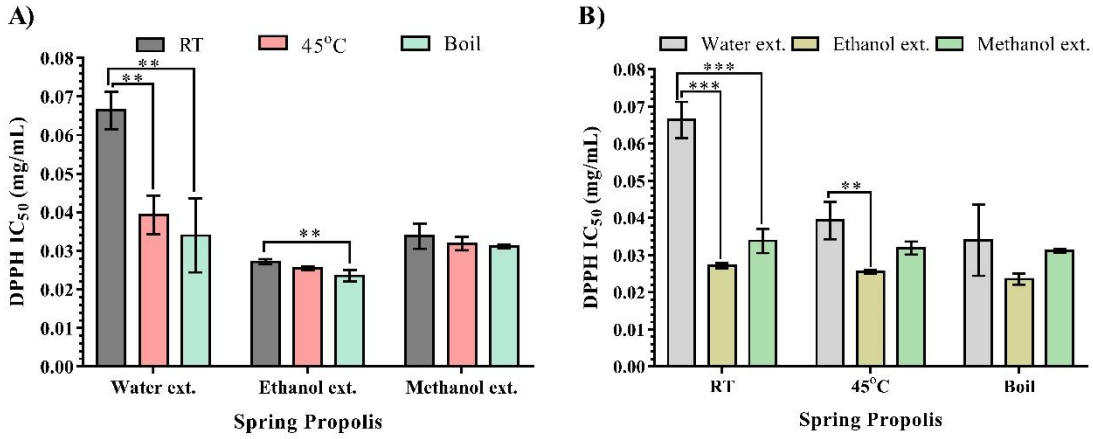

Figure 3.6: The significant differences in antioxidant activity of spring propolis extracts, A) dependence to extraction temperature, B) dependence to extraction solvent.

3.5.3. Significant Differences in Bee Pollen Extracts

Raising the extraction temperature had a negative effect on TPC of pollen extract, if 70% EtOH was used as a solvent. However, no differences were observed for other solvents for all conditions ($P>0.05$), as shown in Figure 3.7A. The results showed that TPC strongly depended on the extraction solvent, and alcohols, primarily 70% EtOH at room temperature was the most appropriate solvent (Figure 3.7B).

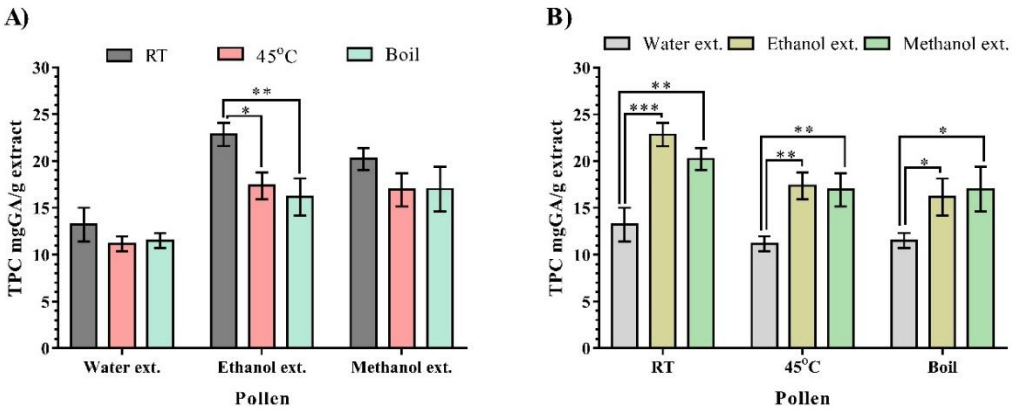

Figure 3.7: The significant differences in TPC values of bee pollen extracts, A) dependence to extraction temperature, B) dependence to extraction solvent.

Extraction temperature had no effect on TFC of alcoholic extracts (P>0.05). However, TFC of water extract was significantly reduced by boiling (P<0.05) (Figure 3.8A). TFC of pollen extracts significantly depended on the extraction solvent. However, water was not a suitable solvent for the extraction of flavonoids from pollen, since TFC of aqueous extracts significantly lower than that of alcoholic extracts for all conditions (P<0.01, P<0.001), as shown in Figure 3.8B. The 70% EtOH seemed to be the best solvent for total flavonoid extraction from pollen for all conditions.

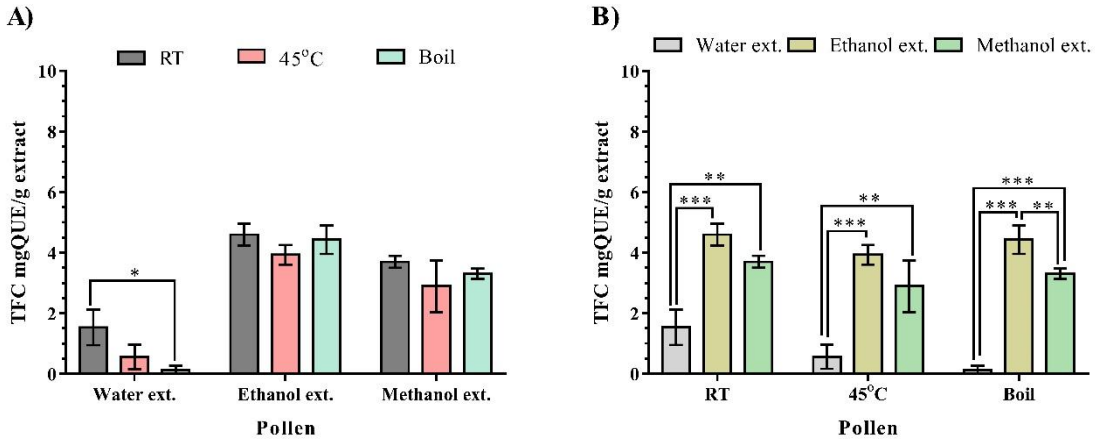

Figure 3.8: The significant differences in TFC values of bee pollen extracts, A) dependence to extraction temperature, B) dependence to extraction solvent.

In accordance the data resulted from TPC in 70% EtOH extracts and TFC in water extracts, high temperatures, especially boiling significantly ($P<0.05$, $P<0.01$) reduced the scavenging activity, since IC_{50} values increased in warmer extracts (Figure 3.9A). Although differences in neither TPC nor TFC of MeOH extracts were statistically meaningful, DPPH scavenging activity surprisingly decreased in warmer conditions. This finding probably arised from experimental variations and/or inconsistency of statistical method used here. Water extracts were distictly possessed lower DPPH scavenging activity than alcoholic extracts in all conditions ($P< 0.001$) (Figure 3.9B), due to their low amount of TPC and TFC. The highest antioxidant activity was absolutely detected in 70% EtOH extract prepared at RT.

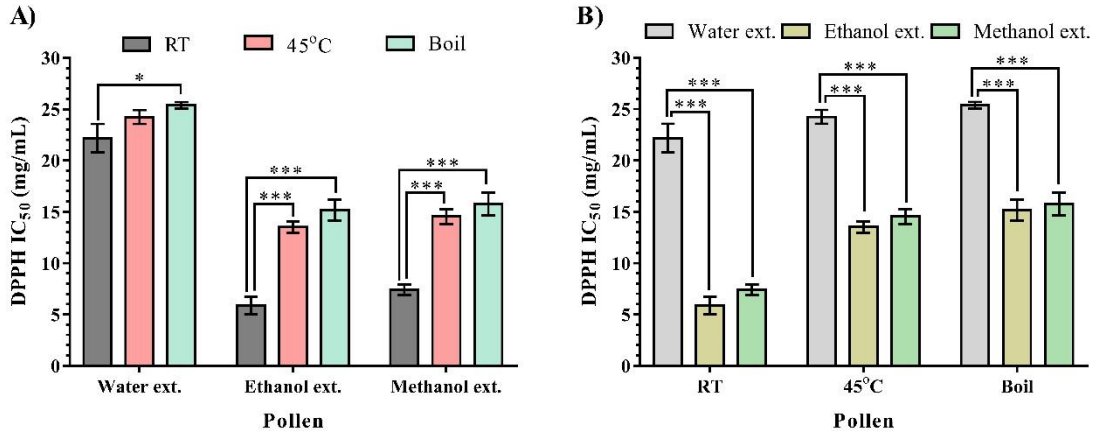

Figure 3.9: The significant differences in antioxidant activity of bee pollen extracts, A) dependence to extraction temperature, B) dependence to extraction solvent.

3.5.4. Significant Differences in Honey Extracts

Heating of extraction solvent to 45°C or more did not affect the TPC of honey extracts (Figure 3.10A). Similarly, there were no significant differences between the TPCs of honey extracts prepared with different solvents in all conditions ($P>0.05$) (Figure 3.10B). TPC of all extracts ranged between 0.76 and 0.90 mg GAE/g DWE, and were distictly lower than those of other two bee products.

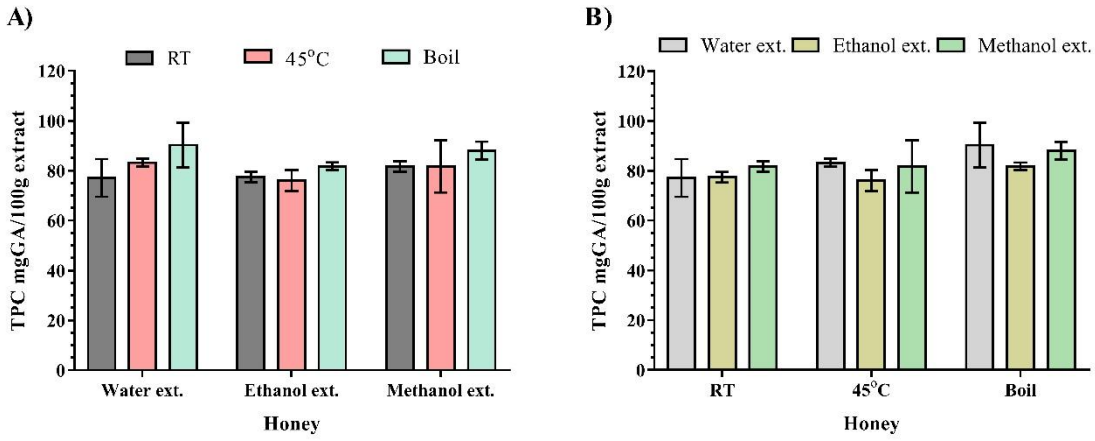

Figure 3.10: The significant differences in TPC values of honey extracts, A) dependence to extraction temperature, B) dependence to extraction solvent.

Extraction temperature did not change the TFC of each honey extract for all solvents used in this study (Figure 3.11A). However, TFCs of honey extracts significantly depended to the extraction solvent ($P<0.05$, $P<0.01$), and the most effective solvent was MeOH (3.11B). MeOH was significantly more efficient than 70% EtOH in all conditions ($P<0.05$, $P<0.01$) and than water at RT ($P<0.05$).

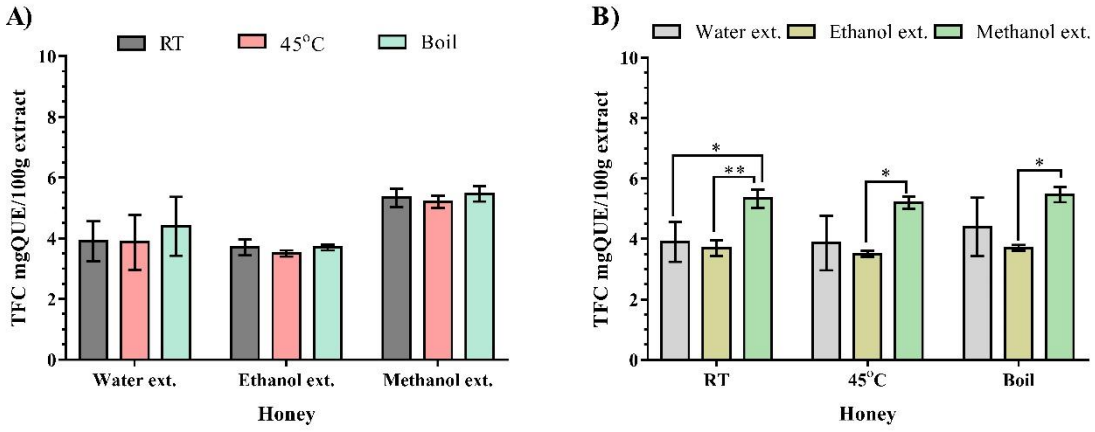

Figure 3.11: The significant differences in TFC values of honey extracts, A) dependence to extraction temperature, B) dependence to extraction solvent.

DPPH radical scavenging activities of honey extracts obtained with MeOH in all conditions were similar (Figure 3.12A). However, there were slight differences, in favor of high

temperature, between RT and boiling temperature for water and 70% EtOH extracts ($P<0.05$). All solvents exerted similar DPPH scavenging activity at high temperatures. However, MeOH significantly produced more active honey extract than water and 70% EtOH extracts prepared at RT ($P<0.05$) (Figure 2.12B).

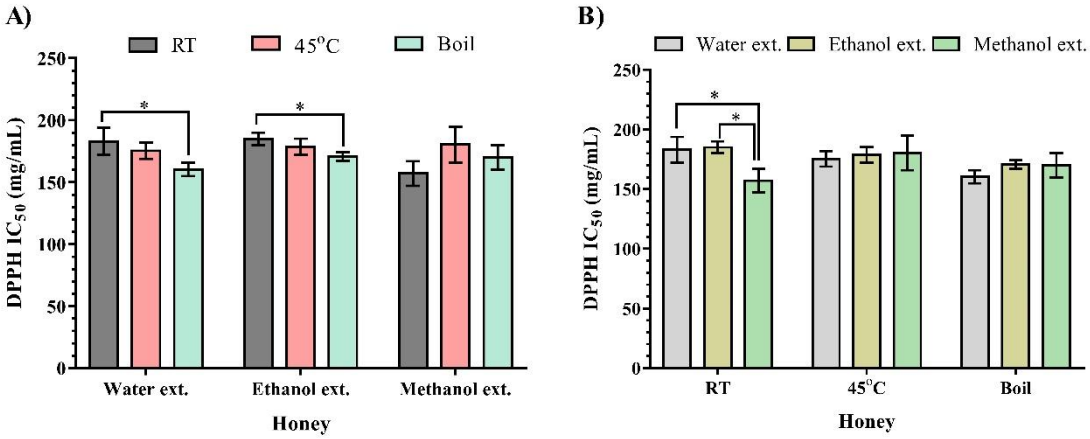

Figure 3.12: The significant differences in DPPH scavenging activity of honey extracts, A) dependence to extraction temperature, B) dependence to extraction solvent.

3.5.5. Significant Differences in Fermented Autumn Propolis Samples

There were significant differences in TPC, TFC and antioxidant activity between the regular propolis and fermented propolis ($P<0.001$), in favor of excess quantities of all in unfermented sample, and between the fermented samples ($P<0.001$), downwardly day by day (Figure 3.13).

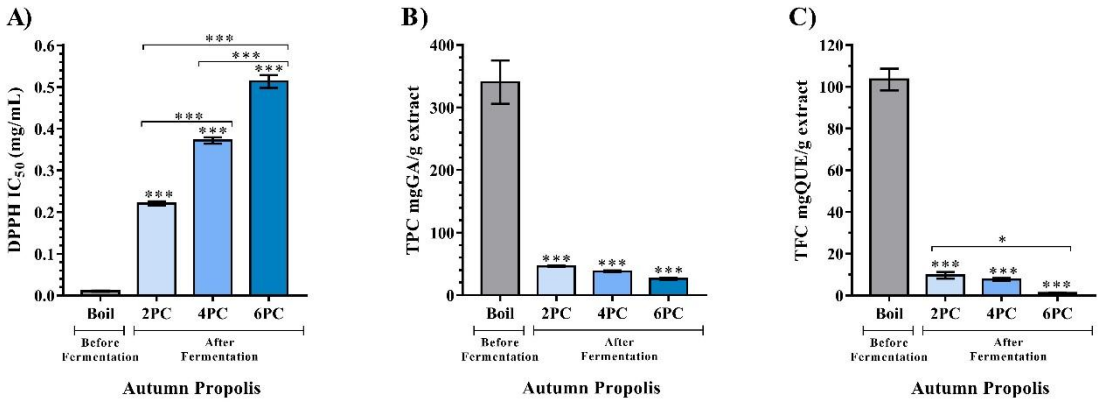

Figure 3.13: Comparison of A) TPC, B) TFC and C) DPPH scavenging activity (IC_{50} values) of regular and fermented propolis samples.

4. DISCUSSION

Honey bee products are well known for their benefits on human health, and these benefits are related to composition of the product. For a better understanding as well as enhancement of the biological activities, molecular basis underlying the benefits and bio-availability of active substances, various extracts of bee products should be studied *in vitro* for both their activities and their chemical constituents.

There are many reports on the antioxidant activity and related chemical composition (mainly phenolics and flavonoids, water-soluble vitamins in some samples) of bee products in the literature (Carpes et al., 2007; Bertoncelj et al., 2007; Gülçin et al., 2010; Kurek-Gorecka et al., 2012; Chua et al., 2013; Sun et al., 2015; Bakchiche et al., 2017; Uçar et al., 2017). Several extraction methods utilizing different solvents at different temperatures, and various antioxidant activity tests, such as oxidation of β-carotene and linoleic acid, ferric thiocyanate method, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) and ferric reducing antioxidant power (FRAP) assays have been used in these studies. In addition, correlation between antioxidant activity and total phenolic content (TPC) or total flavonoid content (TFC) has been investigated.

In general, honey samples have been studied directly (Chua et al., 2013), or after dilution with distilled water (Bertoncelj et al., 2007). However, Bakchiche et al. (2017) have used honey extracts prepared with 50% ethanol (EtOH) at 37 ^{o}C for 96 h. Pollen samples have been extracted with aqueous EtOH (60, 70 and 80%) at 70 ^{o}C for 30 min (Carpes et al., 2007), with water or dimethylsulphoxide (DMSO) at 60 ^{o}C for 24 h (Uçar et al., 2017), and propolis samples with boiling water for 15 min (Gülçin et al., 2010), with 35, 55 and 75% EtOH consecutively or individually (Kurek-Gorecka et al., 2012), with water or EtOH (25, 50, 75, 95 or 100%) (Sun et al., 2015) and with 80% EtOH at 37 ^{o}C for 96 h (Bakchiche et al., 2017). There are also some other extraction methods described for honey bee products in the literature. Hence, there is no a universal solvent recommended for honey bee products to obtain a highly active antioxidant extract with the highest contents of phenolic and flavonoid substances, although EtOH has been considered as the most suitable solvent for this purpose in a herbal material (*Limnophila aromatica*) (Do et al., 2014).

On the other hand, a recent study has proved that increasing the temperature improves the antioxidant activity and TPC by decreasing solvent viscosity, facilitating the movement of molecules and preventing dissolution of phenolic compounds (Hur et al., 2014). However, extremely high temperature may have negative effects on antioxidant activity by evaporating the solvent and oxidizing the phenolic acids (Reblova, 2012).

Here, three different polar solvents [100% (v/v) MeOH, 70% (v/v) EtOH and distilled water] at three different temperatures (room temperature for 24 h, 45ºC for 24 h and 45ºC for 24 h + boiling for 2 h) were used to evaluate the impact of various extraction conditions on the antioxidant activity and related compounds, phenolics and flavonoids of oak honey, two types of propolis (autumn-collected and spring-collected) and pollen samples provided from Kırklareli province-Turkey. Antioxidant activity of the extracts was measured with DPPH assay, which is an accurate, reliable and easy method. Its principle is based on the measurement of the reduction of DPPH radical by antioxidants (Macdonald-Wicks et al., 2006). Since radical scavenging activity of natural products mainly originates from phenolics and flavonoids (Sun et al., 2015), TPC and TFC of the samples were also determined. TPC assay is based on the transfer of electrons from polyphenols forming a blue complex. This transition is achieved in alkaline pH (Ainsworth and Gillespie, 2007). TFC was measured with a colorimetric method based on the ability of $AlCl_3$ to form stable complexes with the hydroxyl groups of flavonoids (Bag et al., 2015).

All findings related to antioxidant (DPPH scavenging) activity, TPC and TFC of honey, propolis and pollen extracts are discussed in detail for each product, and finally concluded in the following sections.

4.1. THE YIELDS OF EXTRACTS

In the present study, the use of various extraction solvents at different conditions affected the yield of the extract in most cases. Extraction yields exhibited by MeOH, 70% EtOH and water distinctly varied in propolis samples, independently of temperatures. However, MeOH and 70% EtOH produced similar yields for pollen and honey samples. The most convenient extraction solvent seemed to be 70 % EtOH for propolis and pollen, and water for honey in this study. In fact, the highest yield of solubles was detected in water extract of honey

obtained as a result of traditional maceration at 45 ºC for 24 h and boiling for 2 h, among all products for all conditions.

Raising the extraction temperature for all solvents led to slight increase in the yields for propolis and honey samples, but not for pollen sample. Interestingly, very few or no decrease was detected in the yields of pollen extracts for higher temperatures. The 70% EtOH at room temperature yielded the highest percentage (35%) of pollen extract while the boiled water produced the lowest one (20%). The yields were very close (45-49% of dry weight) for alcohols in all conditions for honey samples. Besides, extraction with water at 45ºC for 24 h + boiling for 2 h exhibited the highest yield (65.3%) whereas 70% EtOH at 45 ºC had the lowest yield (45%) for honey. Yields of water extracts from propolis were significantly low in all conditions, indicating the presence of only a few amount of hydrophilic substances. Among propolis samples, generally, the yield of the autumn propolis was superior to that of spring sample. The highest yield was obtained for 70% EtOH extract of autumn propolis prepared at 45ºC for 24 h + boiling for 2 h (61%) while the lowest yield was obtained for the aqueous extract at room temperature (0.66%). Previously, high yield (64%) for 75% EtOH extract of Polish propolis prepared at room temperature has been reported by Kurek-Gorecka et al. (2012), and low yield (1.81) for water extract of Chine propolis obtained under ultrasound (100 W, 40 ºC) by Sun et al. (2015).

Differences in yields of bee products could be due to many reasons including type, origin and composition of bee product, extraction method and extraction conditions such as polarity of the solvent and ambient temperature (Trusheva et al., 2007; Do et al., 2014; Narimane et al., 2017; Pujirahayu et al., 2014).

4.2. TPC, TFC AND ANTIOXIDANT ACTIVITY IN PROPOLIS EXTRACTS

In the current study, autumn propolis extracts exhibited higher TPC levels compared to spring samples. A study reported by Miguel et al. (2014) suggested that significant differences in terms of TPC and TFC levels in two collections of propolis samples (winter and spring) might be due to the collecting time. Our results are in accordence with this finding. According to Bankova et al. (1998), diterpenes appeared in summer reach maximum percentage in autumn. The higher levels of TP and TFC were also observed for

70% EtOH extract of propolis, and this is in line with other studies reported that aqueous EtOH is the best solvent for the extraction of phenolic compounds (Miguel et al., 2014; Do et al., 2014; Narimane et al., 2017).

TPCs of all propolis extracts varied between 219 and 340 mg GAE/g DWE, and they were higher than those of pollen and honey extracts in this study; but the highest one referred to the autumn propolis extracted with 70% EtOH under boiling condition as 340.4 mg GAE/g DWE (or 208.8 mg GAE/g propolis, if the yield of extraction is taken into consideration). This TPC value is superior to those of EtOH extracts of propolis collected from Poland (178 mg GAE/g DWE) (Kurek-Górecka et al., 2012) and South Algeria (23.85 mg GAE/g DWE) (Bakchiche et al., 2017), as well as from China, Beijing (164.2 mg GAE/g propolis) (Sun et al., 2015). In addition, TPC of this extract is distinctly higher than those of 20 Korean and 3 commercial propolis (Australian, Brazilian and Chinese) extracts, varying between 125 and 238.9 mg GAE/g DWE (Wang et al., 2016). TPC in the MeOH extract of Bornes propolis from Portugal (320 mg GAE/g DWE) (Moreira et al., 2008) was very close to TPC detected for EtOH extract of autumn propolis in this study. However, 70% EtOH extract of propolis collected from flooded fields in Fernando Falcao region at Brazil produced much higher TPC (847.5, 30 mg GAE/g DWE) (Batista et al., 2016) than propolis extracts discussed here.

TPC data of this study were also compared with those of other propolis samples collected from different parts of Turkey. The highest TPC detected in propolis during this study was much higher than that of propolis collected in Erzurum by Gülçin et al. (2010) (0.124 mg GAE/ g DWE), probably due to great difference in extraction solvent and condition, since we macerated the sample in 70% EtOH at 45ºC for 24 h, and then at boiling temperature for 2 h, while they prepared the extract with boiled water for 15 min. Ciftci-Yilmaz et al. (2017) also used the maceration method, but employing MeOH at room temperature for 24 h for propolis samples collected in Konya Sakyatan and Kızılören regions, and they found TPCs as 40.83 and 94.54 mg GAE/g DWE, respectively. It seems that propolis samples collected from Igneada Demirkoy village, Kırklareli province for this study are prominent with their higher (minimum two fold) TPCs, since the MeOH extracts of both autumn- and spring-collected propolis samples contain 240 and 219 mg GAE/g DWE, respectively.

TFCs of autumn propolis ranged between 14 and 103.5 mg QUE/g DWE, and were slightly higher than those of spring propolis, which were detected in the range of 8.4-90.4 mg QUE/g DWE (Table 3.4). Even the highest TFC was not more than 24.7 mg QUE/g DWE among all water extracts. Hence, water seemed to be an improper solvent for extracting flavonoids, due to the poor solubility of flavonoids in water, although rising the extraction temperature increased the amount of flavonoids in water extracts. The highest TFC was found in 70% EtOH extract of autumn propolis obtained in boiling condition (45ºC for 24 h + boiling for 2 h), as 103.5 ± 5.2 mg QUE/g DWE) . This value was higher than that of Erzurum-Turkey (8.15 mg QUE/g DWE) (Gülçin et al., 2010), Poland (92 mg QUE/g) (Kurek-Górecka et al., 2012), 14 propolis samples collected from different regions of Korea (36.9-99.8 mg QUE/g) (Choi et al., 2013), 20 propolis samples collected from different regions of Korea (20.8-49.8 mg QUE/g) and reference samples from Australia (38.0 mg QUE/g), Brazilia (3.0 mg QUE/g) and Chine (32.5 mg QUE/g) (Wang et al., 2016).

However, our result was inferior to the results of 4 samples collected from diffrent parts of Korea, reported as 111.4 (Chungbuk-Chungju), 104.4 (Chungbuk-Cheongju), 104.9 (Chungnam-Cheonan) and 108.6 (Gyeongnam-Channgyeong) mg QUE/g DWE by Choi et al. (2013).

In agreement with TPC and TFC, autumn collected propolis extracted with boiled 70% EtOH exhibited the highest radical scavenging activity, since the lowest IC_{50} was detected as 0.011 ± 0.001 mg/mL for this sample. Based on the data obtained here, the antioxidant activity of propolis is directly proportional to TPC and TFC. This finding conforms with those of other studies (Sun et al., 2015; Narimane et al., 2017).

During the extaction with MeOH or EtOH, raising the temperature had no significant influence on TPC of both propolis samples. However, boiling after 45ºC enhanced the TPC in water extract of spring propolis. TFC was only augmented by heating the extraction temperature when 70% EtOH or water was used as solvent in both propolis samples. Appropriately, the high temperature increased the radical scavenging activity of EtOH and water extracts. The efficiency of solvents was found to be decreased in the following order, 70% EtOH> Water at 45 ºC and boil> MeOH>Water at RT.

As a summary, 70% EtOH was the most effective solvent for extracting phenols and flavonoids from propolis, and raising the temperature gradually up to boiling temperature

(e.g. as in this study, 45ºC for 24 h + boiling temperature for 2 h) during the extraction resulted in high DPPH scavenging activity, depending on TPC and enhanced TFC in the extract.

4.3. TPC, TFC AND ANTIOXIDANT ACTIVITY IN POLLEN EXTRACTS

TPCs of all pollen extracts varied between 11.2 and 22.8 mg GAE/g DWE, and they were higher than those of honey extracts, but much lower than those of propolis extracts in this study. Interestingly, the highest TPC was found in 70% EtOH extract obtained at room temperature, as 22.8 ± 1.2 mg GAE/g DWE (or 8 mg GAE/g pollen, when the yield is taken into consideration). According to our finding, TPC was significantly lower than those obtained in MeOH extracts of Sinop pollen-Turkey (64.02-103.8 mg GAE/g DWE) (Avşar et al., 2016), as well as than those obtained in MeOH extracts of Anzer pollen collected in both summer and autumn in Turkey (44.07-124.1 mg GAE/g pollen) (Ulusoy and Kolayli, 2014), of Zonguldak pollen-Turkey (28.87 mg GAE/g pollen) (Yıldız et al., 2013), in DMSO extract of a mix sample containing pollens collected from Balıkesir, Bayburt, Erzurum and Trabzon-Turkey (18.86 mg GAE/g pollen) (Ucar et al., 2017) and in EtOH extract of Southern Brazil pollens (19.28-48.90 mg GAE/g pollen) (Carpes et al., 2009). However, highest TPC value of Kırklareli pollen detected in this study was close or greater than those reported in MeOH extracts of several pollen samples collected in and around Douro International Natural Park-Portugal (12.9-19.8 mg GAE/g extract) (Feás et al., 2012) as well as in EtOH extracts of Alagoas and Parana pollens-Southern Brazil (3.6-10.9 mg GAE/g pollen) (Carpes et al., 2007), and in water extract of a mix sample containing pollens collected from Balıkesir, Bayburt, Erzurum and Trabzon-Turkey (5.29 mg GAE/g pollen) (Ucar et al., 2017).

The greatest value of TFC was observed in 70% EtOH extract of pollen at room temperature, as 4.68 ± 0.3 mg QUE/g DWE (or 1.64 mg QUE/g pollen). Compared with previous reports, this value was lower than those of 70% EtOH extracts of Brazilian samples (2.10-28.33 mg QUE/g pollen) (Carpes et al., 2009), of water extract from above mentioned mix Turkish sample (2.27 mg QUE/g pollen) (Ucar et al., 2017), of DMSO extract of above mentioned mix Turkish sample (5.66 mg QUE/g pollen) (Ucar et al., 2017) and of MeOH extract from Zonguldak pollen (8.07 mg QUE/g pollen) (Yıldız et al., 2013) .

Pollen sample extracted with 70% EtOH at RT had the strongest antioxidant activity (IC_{50} = 5.8 ± 0.8 mg/mL), in accordance with its high TPC and TFC. Studying the correlation between TFC, TPC and the antioxidant activity indicated that the antioxidant activity was proportional to TPC and TFC. This finding is consistent with other study reported by Kim et al. (2015).

The low amount of phenolics and flavonoids in the aqueous solutions probably depended on the poor solubility of polyphenols in water, hence resulted in lower antioxidant activity.

Solvent- and temperature-dependencies of TPC, TFC and DPPH were in agreement with previous reports (Carpes et al., 2007; Kim et al., 2015), which indicated that solvents and temperature greatly affect the level of phenolics and the antioxidant capacity of bee pollen.

On the other hand, high temperature may break down the nutritive components of bee pollen, which explains the sharp decrease in the concentration of polyphenols at 45^{o}C and boiling condition. Hur and coworkers (2014) reported that extremely high temperature had negative effect on antioxidant activity because of evaporation of the solvent and oxidation of the phenolic compounds.

4.4. TPC, TFC AND ANTIOXIDANT ACTIVITY IN HONEY EXTRACTS

Among different solvents and temperatures, water subjected to boiling degree resulted in the highest TPC in honey sample, as 0.90± 0.09 mg GAE/g DWE (corresponded to 58 mg GAE/100 g honey). However, the greatest value of TFC referred to MeOH extract prepared at RT (0.055± 0.002 mg QUE/g DWE or 3.6 mg QUE/100 g honey).

A study from Turkey conducted on MeOH extracted honey samples revealed higher TPC in Chesnut (98.26 mg GAE/100 g honey), Heather (105.46 mg GAE/100 g honey), Oak (120.04 mg GAE/100 g honey) and Pine honey (61.42 mg GAE/100 g honey) (Can et al., 2015) than our sample, while Kırklareli honey studied here contained more phenolics than other samples. Besides, our sample had significantly more phenolics than pine honey extracts (35.36-365.94 mg GAE/kg honey) reported by Özkök et al. (2010). Our sample had also more phenolics than Light and Ambar honeys from Porteguese (226.16 and 406.23 mg GAE/kg honey, respectively) (Ferreira et al., 2009) and a unifloral sample (honey 1-*Z. lotus*)

from Algeria (38 mg GAE/100 g honey) (Bakchiche et al. 2017). However, three monofloral honey (Chestnut, Bayburt and Anzer) harvested from Turkey had greater (at least 3 fold) TPC, ranged from 170 to 430 mg GAE/100 g honey (Kolayli et al., 2008). TPC of multifloral honey harvested from Tenosique Mexico (134.02 mg GAE/100 g honey) (Ruiz-Navajas et al., 2011), 3 types of honey harvested from Malaysia (110.394, 159.743 and 196.500 mg GAE/100 g honey) (Chua et al., 2013), a unifloral sample (honey 2-*P. harmala*) (86 mg GAE/ 100 g honey) (Bakchiche et al. 2017) and Dark honey from Portuguese (727,77 mg GAE/kg honey) (Ferreira et al., 2009) were higher than our TPC value.

On the other hand, the highest TFC detected in MeOH extract in our honey sample at RT (0.055 mg QUE/ g DWE, or corresponded to 3.6 mg QUE/100 g honey) was much higher than most of the samples, except Chesnut honey (8.1 mg QUE/100 g honey) and Heather honey (5.84 mg QUE/100 g honey) reported by Can et al. (2015). In addition, our sample contained more phenolics than several pine honey samples from Turkey, but less than some others, reported as in the range of 4.80-54.78 mg QUE/ kg honey by Özkök et al. (2010). These findings on TPC and TFC of honey gave rise to tought that they depended on the origin of honey samples.

The greatest IC_{50} obtained for MeOH extract of honey at RT in this study was 157 ± 9.8 mg/mL, in correlation with high TFC, since TPCs were similar for all solvents at all conditions. DPPH radical scavenging activity of honey sample in this study was close or not more than other samples those TPCs and TFCs were discussed before, and unfortunately was not an attractive bee product.

4.5. TPC, TFC AND ANTIOXIDANT ACTIVITY IN FERMENTED PROPOLIS EXTRACTS

Fermentation was conducted on 70% EtOH extract of autumn propolis prepared at high temperature (45 ^{o}C for 24 h + boiling temperature for 2 h), the product having the highest antioxidant capacity among tested samples, in order to check the effects of fermentation on the components and antioxidant activity. This extract was fermented by *Lactobacillus brevis*. During six days of fermentation, the antioxidant activity, TPC and TFC of fermented samples were decreased significantly in all fermented samples (see Tables 3.3, 3.5 and 3.7).

Decreasing of TPC and TFC during the fermentation gave rise to thought that most of these compounds disappeared, probably due to decomposition, degradation or oxidation of phenolic acids and flavonoids somehow during the process. Previously, negative effects of fermentation on the antioxidant activity have been reported in a study on fermented dark honey (Dezmirean et al., 2012), which was in agreement with our result. However, a study on cereal showed opposite results, fermentation increased the TPC from 50.7 to 53 mg GA/g DWE, and the antioxidant capacity from 82.5% to 86% for buckwheat (Đordevic et al., 2009). Consequently, the effects of fermentation on bioactivity of natural products may alter according to the material type and its chemical composition, as well as fermentation process related with several parameters such as microorganism, medium, temperature etc. For honeybee products, fermentation seemed to have a negative effect on the antioxidant activity and on the bioactive compositions.

5. CONCLUSION AND RECOMMENDATIONS

Overall results reveal that propolis, especially the one collected in autumn has the highest antioxidant capacity, at least amongst the bee products studied here. In addition, boiled EtOH (70%) seems to be the most appropriate extraction solvent for the isolation of phenolics and flavonoids, and accordingly for the preparation of a crude extract having high ability of radical scavenging from propolis and pollen, especially when it is used in the extraction method described here. However, MeOH or water, preferentially at room temperature should be used for honey instead of 70% EtOH.

In this study, raising the extraction temperature did not change TPC of extracts in most cases. Significant increase in TPC by using warmer extraction solvent was only detected in two extracts (water extract of honey and spring propolis) (see Figures 3.1, 3.4, 3.7 and 3.10). On the contrary, TPC in EtOH extract of pollen decreased by heating, probably due to destruction of phenolic compounds at different temperatures (Hur et al., 2014), probably depending on structure, characteristics and localization of related compounds as well as their interaction with the solvent. Besides, extraction with warmer water and 70% EtOH resulted with more TFC in only propolis samples, not in other samples (see Figures 3.2, 3.5, 3.8 and 3.11). Warmer solvents produced similiar TFCs in honey extracts, while boiled water significantly reduced the TFC of pollen.

In accordance with TPC and TFC values, bee products are listed as autumn propolis>spring propolis>pollen>honey, in descending order of their average radical scavenging capacities. Hence, this study confirms previous studies reported that DPPH scavenging activity of the extracts were highly correlated with the concentration of phenolic substances (Carpes et al., 2007; Choi et al. 2013), and propolis is the best bee product. Superiority of autumn collected propolis indicates that not only collection season but also localization is critical to achieve high-capacity propolis preparates, as in the previous studies reported that antioxidant activity and phenolic compounds may differ accordingly (Pujirahayu et al., 2014; Miguel et al., 2014; Do et al., 2014; Narimane et al. 2017).

According to our findings, fermentation is not an advisable strategy for obtaining more active propolis preparates, since TPC and TFC, hence the antioxidant activity of propolis extract decrease during the fermentation process.

Bee products have significant biological properties, but their activities change significantly according to the extraction of related compounds. Thus it is important to find the proper extraction method to get maximum efficacy. Although diverse extraction and detection methods complicate the comparison of antioxidant capacity of honey bee products, especially those coming from different origins reported in the literature, the data is expected to contribute current knowledge on the antioxidant capacity of bee products, interms of extraction and detection methods.

For future studies, we recommend performing HPLC analyses of the most active extracts to detect the phenolic and flavonoid compounds individually. In addition, studies on the other biological activities, such as antibacterial and cytotoxic activities, bioavailability and standardization could be useful for assessing the honey bee products.

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