T.C.

YEDİTEPE UNIVERSITY

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EVALUATION OF PHENOLIC PROFILE, BOTANICAL ORIGIN, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF TURKISH PROPOLIS

MASTER OF PHYTOTHERAPY THESIS

HACER TUĞBA DEĞİRMENCİOĞLU, Pharm

İSTANBUL- 2018

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APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated 22.06.2018..... and numbered 2018/11-07

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Director of Institute of Health Sciences

DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

19.06.2018

HACER TUĞBA DEĞİRMENCİOĞLU

DEDICATION

The study behind this thesis is dedicated to my husband with love…

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

Akt/GSK-3ß:	Glycogen Sytnhesize Kinase
$AlCl_3$:	Aluminium Chloride
AP-1:	Activated Protein-1
BCRP:	Breast Cancer Resistance Protein
B-type:	Blue Type
BV2:	Microglia Cells
CAPE:	Caffeic acid Phenethyl Ester
CH_2Cl_2:	Dichloromethane
COX:	Cyclooxygenase
ddY mice:	Mouse with Superior Reproductive Performance
H_2O:	Distilled water
DMSO:	Dimethylsulfoxide
DPPH•:	2,2-diphenyl-1-picrylhydrazyl
EtOH:	Ethanol
Fe-NTA:	Ferric Nitrilotriacetate
GAE:	Gallic Acid
GC-MS:	Gas Chromatography-Mass spectrometry
GSH:	Glutathione
H_2O_2:	Hydrogen Peroxide
HPLC:	High Performance Liquid Chromatography
HPTLC:	High Performance Thin Layer Chromatography

IL-2:	Interleukin
IR:	Infrared
LC:	Liquid Chromatography
LDL:	Low-Density Lipoprotein Cholesterol
LTN:	Leukotriene
LPS:	Lipopolysaccharide
MeOH:	Methanol
MIC:	Minimum Inhibitory Concentration
MMP:	Matrix Metalloproteinases
MPLC:	Medium Pressure Liquid Chromatography
MS:	Mass Spectrometry
$NaCO_3$:	Sodium Carbonate
$NaNO_2$:	Sodium Nitrite
NaOH:	Sodium Hydroxide
NF-κB:	Nuclear Factor Kappa B
NMR:	Nuclear Magnetic Resonance
NP:	Natural Product
O_2:	Oxygen
OH:	Hydroxyl
OPLS:	Orthogonal Partial Least Squares
O-type:	Orange type
PCR:	Real Time-Polymerase Chain Reaction
PEG 400:	Polyethyleneglycol 400

PHP:	Patient Hygiene Performance
QE:	Quercetin
3MQ:	3-*O*-methylquercetin
RNS:	Reactive Nitrogen Species
ROS:	Reactive Oxygen Species
RPHPTLC:	Reversed Phase High Performance Thin Layer Chromatograpy
TFC:	Total Flavonoid Content
TLC:	Thin Layer Chromatography
TNF-α:	Tumour Necrosis Factor Alpha
TNF/ NF-κB:	Tumour Necrosis Alpha/Nuclear factor kappa B
TPC:	Total Phenolic Content
UHPTL:	Ultra High Performance Thin Layer
UPLC-MS:	Ultra Performance Liquid Chromatography-Mass spectrometry
UV:	Ultraviolet

ABSTRACT

Değirmencioğlu, HT. (2018). Evaluation of Phenolic Profile, Botanical Origin, Antioxidant and Antimicrobial Activities of Turkish Propolis. Yeditepe University, Institute of Health Science, Department of Pharmacognosy, MSc thesis, Istanbul.

Propolis is a bee product having complex chemical composition and wide spectrum of biological activities. This study evaluated the phenolic profile of Turkish propolis by using an high performance thin-layer chromatographic (HPTLC) method. Also, the botanical origins of propolis samples were determined by comparison of HPTLC fingerprints of propolis samples and plant bud extracts. As a result, Turkish propolis could be categorized into 3 main types: orange (O) type, blue (B) type and 3-*O*-methylquercetin (3MQ) type. O and B-types originated from *Populus nigra* L., *P. tremula* L., respectively. In addition, HPTLC combined with 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) test was used to evaluate antioxidant activity of separated compounds on the HPTLC plate. The results of HPTLC-$DPPH^{\cdot}$ showed that separated compounds in O-type of propolis had a higher radical scavenging effect than the other types when compared the zone areas which had an antioxidant capacity. CAPE (Caffeic acid phenethyl ester), caffeic acid, galangin, kaempferol and quercetin were contributed propolis antioxidant activity. Moreover, comparative antimicrobial activity against strains of *Streptococcus aureus*, *Pseudomonas aureginosa*, *Escherichia coli* and *Candida albicans* was determined by disc diffusion test and minimum inhibitory concentration (MIC) assay on O-, B- and 3MQ-types. Consequently, P17 (B-type) and P19 (O-type) showed the highest antimicrobial activity against *E. coli* and *C. albicans*. Lastly, total phenol content (TPC) and total flavonoid content (TFC) of propolis samples were evaluated by spectrophotometry. TPC and TFC values were found to be highest in O-type.

Key Words: Turkish propolis, High performance thin-layer chromatography **(**HPTLC), Antioxidant activity, Antimicrobial activity, Total phenolic content, Total flavonoid content

ÖZET

Değirmencioğlu, HT. (2018). Türk Propolisinin Fenolik Profili, Botanik Kökeni, Antioksidan ve Antimikrobiyal Aktivitelerinin Değerlendirilmesi. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Farmakognozi ABD, Master Tezi, İstanbul.

Propolis karmaşık kimyasal bileşime ve geniş kapsamlı biyolojik aktiviteye sahip bir arı ürünüdür. Bu çalışmada, Türk propolisinin fenolik profili yüksek performanslı ince tabaka kromatografisi (HPTLC) yöntemi kullanılarak değerlendirildi. Ayrıca propolis örneklerinin ve bitki tomurcuğu ekstrelerinin HPTLC parmak izlerinin karşılaştırılması ile propolislerin botanik kökeni belirlenmiştir. Sonuç olarak, Türk propolisinin (O), (B) and (3MQ) tipi olmak üzere 3 ana tipe ayrılabileceğini göstermiştir. O ve B tiplerinin botanik orijinleri sırasıyla *Populus nigra* L., *Populus tremula* L. bitkileri olarak bulunmuştur. Ayrıca, plak üzerinde ayrılmış her bir bileşiğin antioksidan aktivitesinin değerlendirilmesi için HPTLC, 2,2-difenil-1-pikrilhidrazil ($DPPH^{\bullet}$) ile kombine edilerek tanımlama yöntemi kullanılmıştır. HPTLC-$DPPH^{\bullet}$ sonuçları, O tipi propolisin diğer türlerin antioksidan alanlarının karşılaştırılmasına göre daha yüksek bir radikal süpürücü etkiye sahip olduğunu göstermiştir. CAPE, kafeik asit, galangin, kaempferol ve kersetin bileşikleri propolisin antioksidan aktivitesine önemli ölçüde katkıda bulunmuştur. Ayrıca (O), (B) and (3MQ) tipi propolislerin *Streptococcus aureus*, *Pseudomonas aureginosa*, *Escherichia coli* ve *Candida albicans* suşlarına karşı karşılaştırmalı olarak antimikrobiyal aktivitesi disk difüzyon ve minimum inhibitör konsantrasyonunun (MIC) ölçülmesiyle saptanmıştır. P17 (B-tipi) ve P19 (O-tipi) *E. coli* ve *C. albicans'a* karşı en yüksek antimikrobiyal aktivite göstermiştir. Son olarak, propolis örneklerinin total fenol miktarı (TPC) ve total flavonoid miktarı (TFC) spektrofotometri ile değerlendirilmiştir. TPC ve TFC değerleri O-tipinde en yüksek bulunmuştur.

Anahtar Kelimeler: Türk propolisi, Yüksek performanslı ince tabaka kromatografisi (HPTLC), Antioksidan aktivite, Antimikrobiyal aktivite, Total fenol miktarı, Total Flavonoid miktarı

1. INTRODUCTION and AIM

There is a system of social caste in each beehive in the bee's world. This life style of bees has attracted people's attention and aroused throughout history. In the archeological records of 5000 years ago and in the wall portraits, the queen bee, who ruled the hive, was represented as the 'mother goddess'. Besides, thousands of honey bees work for protection and provide continuity of the hive. They mainly produce honey and also royal jelly, pollen and propolis (1).

Propolis (bee glue) is a sticky, dark colored product collected by bees from living parts of plants such as buds, leaves, flowers and pollen grains. These materials are formed into complex with mandibular secretion of bees (2, 3). Propolis name came from two ancient Greek wordings; pro- (in front of, at the entrance to) and –polis means (city, community) (4). Propolis is used to prevent intrusion of hives such as insects, snakes and lizards, or to protect from bad weather conditions (wind and rain), and to cover interior walls to prevent the formation of fungus and bacteria (5).

Up to know, more than 300 compounds were detected in propolis and content of propolis has a complex mixture. Contrary to the products obtained from medicinal plants, the propolis composition is extraordinary; different botanical and geographical origins may have completely different chemical compositions. In addition, the chemical content of propolis depends on saliva secretion regions and enzymes secreted by the bees (6, 7). This diversity is a major problem for the medical use, quality control and standardization of propolis. Hence, it is very important to know the origin of propolis (4). There is no single plant exudate in the propolis composition. The sources of propolis are *Populus* spp. (poplar), *Salix alba* (willow), *Betula pubescens*, *B. pendula* and *B. verrucosa* (birch), *Ulmus glabra* (elm), *Alnus glutinosa* (alder), *Fagus sylvatica* (beech), *Abies* and *Pinus* spp. (conifer), and *Aesculus hippocastanum* (horse-chestnut) trees (8, 9, 10). However, it is named according to the major plant, which is high in the composition of the propolis type (11)

Propolis has been proven to exert various biological effects such as antibacterial, antifungal, antiviral, antiinflammatory, antiulcer, antioxidant, antitumor, hepatoprotective, immunostimulant (8, 12, 13).

Recent studies have identified the benefits of antioxidants for health because of their effects in disease prevention, such as cancer, cardiovascular diseases and aging. Phenolic compounds found in propolis such as Caffeic acid phenethyl ester (CAPE), caffeic acid and galangin mostly contribute its antioxidant activity (14).

The chemical content of propolis is very important both for public health and evaluation of biological activity study results. Up to now, the chemical composition of propolis has been evaluted by using gas chromatography coupled with mass spectrometry (GC-MS), high performance liquid chromatography (HPLC), ultra high performance liquid chromatography coupled with mass spectrometry (MS) (UPLC-MS) and high performance thin layer chromatography (HPTLC) (10, 15, 16).

The aims of this study were assessed as follows; 1) investigation of HPTLC phenolic profile of 24 Turkish propolis samples collected from different locations, 2) determination of botanical origin of Turkish propolis by simultaneous profiling of different bud extracts as potential botanical sources, 3) evaluation of each antioxidant compounds in Turkish propolis samples directly on the chromatogram using HPTLC-$DPPH^{\cdot}$ assay, 4) identification of a possible marker compound found in new (N) type of propolis according to the HPTLC result by Nuclear Magnetic Resonance (NMR) 5) comparative antimicrobial activity determination on different propolis types 6) investigation of total phenolic (TPC) and total flavonoid contents (TFC) in propolis samples.

2. GENERAL DESCRIPTION

2.1. Theoretical Chapter

2.1.1. Literature review on propolis

2.1.1.1. Chemical Compounds

Although the content of propolis varies depending on geographical location and botanical origin, it is generally composed of resins (%50), wax and fatty acids (%30), essential oils (%10), polyphenols and flavonoids (%10), pollen (%5), vitamins and minerals (%5) approximately (17, 18). The compound groups in propolis are flavonoid aglycones, phenolic acids and its esters, phenolic aldehydes, alcohols, ketones, sesquiterpenes, coumarins, steroids, amino acids and inorganic compounds (6, 19). The most well-known pharmacologically effective chemical compounds in propolis are the flavonoids, isoflavonoids, phenolic acid, terpenes, xanthones which have antimicrobial, anti-inflammatory, antioxidant, antiviral, antifungal and anticancer effects (20).

Table 1. Analytical studies on propolis

Propolis Types	**Extracts**	**Method**	**Major Compounds**	**Plant Source**	**Geographical Origin**	**Ref.**
Brazilian Red Propolis	Ethanolic Extract	HPLC LC-Orbitrap-FTMS	Flavons, Isoflavons, Chalcons and Aurons, Triterpenoids, Catechins	*Dalbergia ecastophyllum*	Brazil	20
Yellow Propolis	Ethanolic Extract	GC-MS NMR	Triterpenoids (oleonane, lupane, ursane and lanostane)		Brazil	21
Red Propolis	Hydroalcoholic Extract (%80)	GC-MS RP-HPLC	Isoflavonoids, Medicarpin, 3-hydroxy-8,9-dimethoxypterocarpan	*D. ecastophyllum*	Brazil	22, 23
Green Propolis	Hydroalcoholic Extract (%95)	GC-MS	Flavonoids (rhamnocitrin, acasethin) Terpenoids (lupeol, ß amyrin)	*Baccharis dracunculifolia*	Brazil	24
Brown Propolis	Hydroalcoholic Extract (%95)	GC-MS	Fatty acids (oleate, palmitate) It doesn't contain flavonoids.	*Hyptis divaricate*	Brazil	24
Turkish Propolis	Hydroalcoholic Extract (%70-80)	GC-MS HPTLC	Pinocembrin, pinobanksin, pinobanksin-3-*O*-acetate, chrysin, galangin, ferulic acid, phenethyl caffeate, cinnamyl cinnamate	*P. nigra* *P. euphratica* *P.tremula*	Turkey	10, 25
Poplar Type	Ethanolic Extract	GC-MS HPLC	Flavons, flavonone, cinnamic acid and esters (CAPE)	*P. nigra* , Some Coniferae types Populus types of Aigeiros section	North America, New Zealand and Europe	26, 27, 28

Table 1. Continued

Propolis Types	**Extracts**	**Method**	**Major Compounds**	**Plant Source**	**Geographical Origin**	**Ref.**
Polish Propolis	Hydro alcoholic Extract(%70)	GC-MS TLC	Polyphenols (Flavonoid aglycons, phenolic acids and esters)	*P. nigra*, *B. pendula*, *A. glutinosa*, *Salix* sp., *Pinus sylvestris*	Poland	29
Portuguese propolis	Hydroalcoholic Extract (%80)	HPLC LC -MS	Caffeic acid, ferulic acid, quercetin, pinocembrin, chrysin, CAPE, galangin, salicylic acid, apigenin, kaempherol-3-*O*-glucoside, kaempherol-3-*O*-rutinoside, acacepin	*Populus x Canadensis*	Portugal	30
German Propolis	Ethanolic Extract	TLC TLC-MS	Apigenin, quercetin, kaempherol, chrysin, caffeic acid, naringenin, CAPE, galangin, pinocembrin	*Populus xcanadensis P.tremula*	Germany	31
Mediterranean		GC-MS NMR	Diterpens (labdane type acids)	Cupressaceae family	Sicilia, Greek,Creta,Maltha	32, 33
Clusia	Hydroalcoholic Extract (%70)	HPLC GC-MS NMR	Polyisoprenylated benzophenones	*Clusia* species	Cuba, Venezuella	34, 35
Pasific	Ethanol Extract	HPLC	C-prenyl flavanones, furo-furan lignans	*Macaranga tanarius*	Taiwan,Okinawa,Indonesia, Pasific Ocean	35, 36, 37

Table 1. Continued

Propolis Types	**Extracts**	**Method**	**Major Compounds**	**Plant Source**	**Geographical Origin**	**Ref.**
Italıan Propolis	Hydroalcoholic Extract (%85)	UHPLC	Chrysin, galangin, pinocembrin and cafeic acid phenethyl ester		Italy	38
Birch			Flavons, flavonols (Different from poplar type)	*B. verrucosa*	Russia	39
Kashmir Himalaya Propolis	Ethanolic Extract	UHPLC	Hydroxy cinnamic acid derivatives, flavonoids(flavonol, flavonone and its derivatives)		India	40
India Propolis	Ethanolic Extract	HPLC	Quercetin, caffeic acid, CAPE, apigenin	*Brassica campestris*, *Eucalyptus* sp., *Cocos nucifera*, *Punica grantanum*	India	41
Greek Propolis	Hydroalcoholic Extract	HPLC-PDA-ESI/MS GC-MS	Pinocembrin, chrysin		Greece	42
Spanish Propolis	Ethanolic Extract	LC-PDA-MS	Pinobanskin 3-acetate, pinocembrin, chrysin, galangin and pinobanskin		Spain	43

2.1.1.2. Botanical Origin of Propolis Type

Information about botanical origin has been provided by comparing the chemical components contained in propolis and the components contained in the plant (4). Botanical origin of Turkish propolis samples were detected by HPTLC fingerprints of propolis samples collected from different localities in Turkey with that of plant bud extracts for comparison together. As a result of the study, Turkish propolis could be categorized under three major types; i.e. Blue (B) (originated from *P. tremula*), orange (O) (originated from *P. nigra*) and nonphenolic types (10). Existence of dominant orange colored bands together with few number of light blue and pale green bands demonstrated O-type propolis, while dominant and light blue bands and green and pale orange bands indicated B-type. Dominant orange bands are kind for flavonoids as quercetin, blue bands for CAPE, caffeic acid, galangin and green bands characterize to apigenin, naringenin (31, 44, 45). Botanical origin of O-types of propolis samples were *P. nigra* buds extract. Therefore, B-type was discovered to be connected prevalently to *P. tremula*. Moreover, in both types have been found *A. hippocastanum* components (46).

Isidorov et al. (47) reported that ether extracts of propolis from 11 countries of Europe and Asia along with extracts of the buds of their major plant precursors were prepared and searched by GC-MS. Chemical compositions of the exudates of *P. tremula, B. pubesces and B. pendula* buds were defined. In addition, exudates of 2 black poplar buds and *P. szechuanica* (Sichuan) poplar buds were searched by GC-MS. The examination of plant precursors of propolis was developed with regard to the data on the content of components individually and their groups. Chemical compounds of *B. pendula, B. pubescens, P. tremula* buds exudates were determined. Plant precursors of propolis have been examined. When the contents of propolis samples were examined, the amount of *P. nigra* exudates was reduced from located in the south towards to located in the north. The bees are selective attitude against the plant exudate. Two species of *Betula* sp. on the Eurasian continent were found major. The obtained data showed that only one exudate from the *B. pendula* could be found in the composition of northern propolis. None of the dammaradien-3-one, dipterocarpol, triterpenoids characteristic of the *B. pendula* tree of the propolis samples taken from different parts of Russia have been detected, showing selective attitude of these bees (47).

Chemical contents of propolis samples from various *Populus* species were investigated by spectroscopic methods. One of the main reasons for the variety in *Populus* type propolis was found to be the altitude of collection sites. NMR, Ultraviolet (UV), Infrared (IR) spectroscopy, Orthogonal partial least squares (OPLS) and two-way OPLS were carried out. The main compound found in the samples of propolis collected at altitudes over 500 m in temperate climates were phenolic glycerides from *P. tremula*. Flavonoids were primary compounds in the propolis samples collected under 400 m altitude and originated from *P. nigra* and *P. xuramericana* buds (48).

Propolis samples taken from different Portuguese regions and some plant sources were compared with the phenolic content of *Populus* x *Canadensis* Moench buds and *Cistus ladanifer* to establish geographical and botanical origins. The Portuguese propolis has produced a phenolic profile with marked differences in concentrations, the dominant in respect to flavonoids being widespread in all regions. The *Populus* sp. propolis compounds, which were common in temperate regions, was similar to the north, central coast, Azores propolis samples observed while central interior and southern specimens of propolis seen rich in kaempferol derivatives similar to *Cistus ladanifer* exudates. The kaempferol-3,7-dimethyl-ether compound, which was not found in *Populus* sp. propolis, has been evaluated as important in distinguishing these two types of propolis. As a result, the Portuguese propolis samples exhibited a similar phenolic composition with important differences in their concentrations. *Populus* x *Canadensis* bud exudates with the same chemical content profile, high phenolic substance and flavonoid content were observed with samples from North, central coast, Azore. Fewer phenolic and flavonoid contents were found in the samples taken from Madeira. In some samples taken from the central Interior and the south, the amount of kaempferol-3,7-di-methyl-ether, which is rich in kaempferol derivatives, was found to be dominant and similar to the *C. ladanifer* exudates. Unlike the others in the from central interior one propolis sample, quercetin-tetramethyl-ether, luteolin and chrysoeriol-methylether, these compounds originate from *Origanum* sp (30).

Studies have been carried out on buds, such as *Populus* spp. (*P. alba*, *P. tremula* and *P. nigra*), as primary sources of propolis in the continents of Asia, Europe, Australia and North America. Flavonoids and phenolic acids are abundant in propolis (49). Secondary important sources of poplar species propolis include *B. pendula*, *Acacia* sp., *A. hippocastanum*, *A. glutinosa*, *Pinus* sp. and *S. alba* (31). *Prunus* spp. (*P.*

cerasifera Ehrh., *P. armeniaca* L., *P. avium* L. and *P. cerasus* L.) were also considered to be botanical propolis resources with respect to Crane (50). It has been accepted the existence of two major subtypes of European propolis, O and B-types. (44, 51). Former studies related between Serbian propolis and the European poplar type propolis have shown that Serbian propolis were characterized by a nearly same pattern with European type, while profiles of blue subspecies samples were almost completely different from one another (45, 52). Moreover, 50 different Serbian propolis samples from 14 different plant sources were analyzed for phenolic compounds and antibacterial activities, providing a theoretical basis for investigating the chemical composition and activity of Serbian propolis. They included resins from trees from the Salicaceae family (*S. alba* and *Populus* sp.), fruit trees from Rosaceae family (*Prunus* sp.) and less other type. Extensive plant resins phenolic profile was conducted using ultra-high-performance liquid chromatography (UHPLC) and HPTLC interconnect with hybrid mass spectrometry (MS) (53).

2.1.1.3. Biological Activity

2.1.1.3.1. Antioxidant Activity

Free radicals are atoms or molecules that have more unpaired electrons in one atom in atomic or molecular orbitals. These unshared electron (s) give great reactivity to the free radical. Free radicals are small molecules, have low activation energy and short-lived. Smaller sizes allow easy passage through cell membranes (54). Oxidative stress is the imbalance between reactive oxygen species or other free radicals and the antioxidant system, and this imbalance can cause irreversible damage to the cell. The negative effects of oxidative stress on human health have been an important research topic. The imbalance between reactive oxygen species such as superoxide anion ($O_2^{\cdot}$), hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2) and enzymatic or non-enzymatic antioxidant compounds formed by metabolic pathways or by the influence of exogenous factors is caused by oxidative stress. Antioxidants are molecules that generally have phenolic functions in their structure, preventing the formation of free radicals or damaging the cell by sweeping the formed radicals (55). Antioxidants are produced by the body's cells or they can also be taken by food. Vitamins (vitamins E, C and A), flavonoids, carotenoids and polyphenols are the main natural antioxidants found in foods that protect human body from harmful free radicals (56). The antioxidant capacity of propolis may be related to some biological effects, including chemical precautions. Flavonoids of propolis are potent antioxidants which scavenging free radicals and thus protect the cell against extreme lipid peroxidation (57). Furthermore, reactive oxygene species (ROS) and reactive nitrogen species (RNS), along with other factors, are associated with cellular aging and death in conditions such as cardiovascular disease, arthritis, cancer, diabetes, Parkinson's disease and Alzheimer's disease (58, 59). Propolis H_2O_2 and NO may decrease cellular levels that play a role in antiinflammatory effects (60). The different compounds present in the propolis compound have been identified as potent inhibitors of oxidative stress. It is well known that the propolis composition is variable, but one of the major components, CAPE, inhibits ROS production in a variety of systems (61). CAPE has also been identified as one of the largest cancer chemopreventive and antiinflammatory compounds in propolis. It has been shown that propolis *in-vitro* inhibits the peroxidation of low-density lipoprotein cholesterol (LDL) and the nitration of proteins. *In-vivo*, propolis may increase antioxidant capacity in

animals and in humans reduce lipid peroxidation strongly associated with the risk of cardiovascular disease (62, 63, 64, 65). Turkish propolis inhibited H_2O_2 induced damage to DNA in cultured fibroblasts (66). Antioxidant activity of phenolic compounds in Turkish propolis may reduce H_2O_2-induced DNA damage, which may be associated with chemically inhibitory activity. Red propolis from Cuba is thought to have protective effects in alcohol-induced liver damage models due to antioxidant properties (67). It has been shown that inhibition of macrophage apoptosis is mediated through effects on propolis, glutathione (GSH) and nuclear factor kappa B (TNF / NF-κB) pathway (68, 69). Propolis is rich in flavonoids and phenolics have strong antioxidant properties (70, 71). One of the most commonly used methods for measuring antioxidant activity is the free radical scavenging effect is measurement of $DPPH^{\cdot}$ radical consumption depends on the ability of a substance or complex mixture to transfer hydrogen atoms or electrons to this reactive species in a homogeneous system.

TPC and TFC values were found to be high in propolis water (H_2O) extract collected from 3 different regions of Egypt. The total amount of polyphenol and flavonoid in 100 grams of freeze dry extract was 5.7- 8.79 g and 3.05-4.85 g, respectively. Depending on the amount of total polyphenol and total flavonoids, all propolis showed high antioxidant effect compared to antioxidant methods with beta carotene bleaching and $DPPH^{\cdot}$ radical scavenging effect. Freeze-dried propolis extract can be used as a natural antioxidant compared to butylated hydroxytoluene (72). Oxidative stress adversely affects liver function. In an *in-vitro* study, propolis extracts have been shown to protect liver function against oxidative damage (73). CAPE is an important xanthine oxidase inhibitor, superoxide radical scavenger effect and lipid peroxidation inhibitory effect, compared with galangin, the effect of CAPE was found to be higher (74).

2.1.1.3.2. Anti-inflammatory Activity

Brätter et al. (75) demonstrated efficacy on propolis immune system and inflammation. Arachidonic acid is the main pathway in the formation of inflammation. Propolis inhibited the synthesis of leukotriene (LTN) and prostaglandin by suppressing the lipoxygenase enzyme and the cyclooxygenase (COX) enzyme. Propolis suppresses the expression of transcription factors of nuclear factor (NF-kB) , which plays an important role in inflammation and activator protein-1 (AP-1) so that reduces the levels of inflammatory cytokines and interleukins (75). Fan et al. (76) reported the effects of ethanolic extract of propolis on chronic inflammation were evaluated using rat adjuvant arthritis. In the chronic inflammatory animal model, arthritis index was prevented by ethanolic extract treatments (50 mg-100mg /kg / day). Moreover, the physical weakness caused by chronic disease states was developed as predominantly depend on ethanolic extract of propolis-treated groups. Some studies have concluded that propolis ethanolic extract has significant anti-inflammatory effects in both chronic and acute inflammation (76).

CAPE has a significant anti-inflammatory effect. The immunosuppressive effect of CAPE in human T cells has been examined. CAPE is an inhibitory effect in T cell activation. This phenolic compound specifically inhibited İnterleukine (IL-2) gene transcription and IL-2 synthesis in stimulated T cells. In addition describe the inhibitory mechanisms of CAPE at the transcriptional level, they investigated the DNA binding and transcriptional activities of NF-kB, nuclear factor of stimulated cells and stimulator protein-1 transcription factors in Jurkat cells. CAPE inhibited NF-κB-dependent transcriptional activity without affecting the degradation of the cytoplasmic NF-κB inhibitory protein (77). These results will provide new information on the molecular mechanisms of immunomodulatory and anti-inflammatory activities of the natural compound (77, 78).

Although technology is more developed in vaccine production, the effect of vaccine is related to the adjuvant substance. Propolis is considered as an immunological adjuvant in vaccine production. *In-vivo* experiment, CAPE has been shown to induce antibody formation in mice. Like these experiments, some types of propolis have been shown to induce antibody formation (71, 76, 79, 80, 81).

There are several studies on the anti-allergic activity of propolis. Shinmei et al. (82) and Shinmei et al. (83) reported propolis significantly inhibited pruritus by reducing histamine release and inhibiting vascular permeability. In another *in-vivo* experiment, propolis was administered to rats with nasal itching and sneezing, and histamine release was reported to be beneficial for prolonged use (82, 83). Flavonoids such as chrysin, galangin, kaempferol, 3-*O*-methyl-kaempferol have been reported to have the strongest inhibition of antigen-induced mast cell degranulation and the lowest deleterious effects in RBL-2H3 mast cell lines. The antiallergic and antiinflammatory effects of propolis have been shown to be caused by synergistic effects of polyphenols and different phenolic compounds. The major antiallergic compounds of the propolis ethanol (EtOH) extract are chrysin and kaempferol (84, 85).

Pinocembrin was investigated for ovalbumin-induced allergic airway inflammation in mice. In conclusion, it was found that allergic asthma findings such as increased pulmonary eosinophil infiltration, mucus secretion and airway sensitivity were inhibited (86). Pinocembrin inhibited the expression of matrix metalloproteinases (MMP)-1, MMP-3 and MMP-13 in both mRNA and protein levels in human chondrocytes. Nuclear factor kappa-light chain enhancer stimulation of tumor necrosis factor-alpha (TNF-α) -activated B cells (NF-kB) has been shown to be inhibited by pinocembrin administration. Also, pinocembrin block TNF-α induced p65 nuclear translocation. Studies have shown that pinocembrin is a protective effect against osteoarthritis (87). Propolis supports cartilage and chondrocyte repair properly (88, 89).

2.1.1.3.3. Antimicrobial Activity

2.1.1.3.3.1. Antibacterial Activity

Most studies have shown that propolis extracts has a broad spectrum of activity against gram positive (+) bacterial strains, while the effect on gram negative (-) bacteria is weaker (90, 91, 92). Oliveira et al. (93) conducted in Portugal, the effects of different types of propolis against gram (+) and gram (-) bacteria were examined by agar diffusion. As a result, the propolis extracts showed susceptibility to dose dependence (93). A comparative study of chlorhexidine with a mouthwash containing propolis was performed. The dental plaque was analyzed with the patient hygiene performance (PHP) index. People had a similar PHP index with those who used propolis mouth wash for 14

consecutive days and those who used chlorhexidine alone. The propolis product showed antibacterial activity *in-vitro* and *in-vivo* in inhibiting dental plaque formation (93). Uğur et al. (95) showed that antimicrobial properties of 45 different Turkish propolis samples (Muğla, Turkey) were reported to be increased antimicrobial properties in a dose dependent. Propolis was the most sensitive microorganism, *Shigella sonnei* in Gram (-) and *Streptococcus mutans* Gram (+). Antibiotics were applied and the results showed that these propolis samples had more or approximately same inhibitory effect on *Streptococcus mutans*, *Salmonella typhi*, *Pseudomonas aeruginosa* and *Shigella sonnei* (95). Propolis has been reported to exhibit antibacterial effects by protein synthesis, cell division, bacterial growth inhibition, and cytoplasmic membrane, making the cell wall more dispersed (96). However, it was thought that the activity of antimicrobial effect on *Candida albicans* and *Streptococcus* can be achieved with the participation of flavonoids (pinocembrin, galangin), phenolic acids (CAPE, cinnamic acid) and glycosyl transferase enzyme (97).

2.1.1.3.3.2. Antiviral Activity

According to Kujumgiev et al. (11) and Serkedjiva et al. (98), propolis has different effects on virus types. Much less effect was observed in adenovirus and vesicular stomatitis virus when DNA/RNA viruses showed high activity in virus types such as influenza and herpes polio. They act by inhibiting the enzyme that allows the multiplication of the DNA virus and blocking the entry of the virus into the cell. Chrysin and galangin are the flavonoids found in the propolis and are mainresponsible for the antiviral effect. However, each of the other components is effective on different viruses. 3-methyl 2-enyl caffeate from poplar buds is effective against herpes simplex virus (11, 98)

The effect of 13 ethanolic propolis extracts activity against *in-vitro* and *in-vivo* influenza was investigated. Four different extracts were successful in the first *in-vitro* plaque inhibition. Afterwards, the different ethanolic propolis extracts were given to mice infected with influenza viruses at a dose of 10 mg /kg 3 times a day for 1 week. As a result, one of the ethanolic extract had antiviral effect as strong as oseltamivir (99).

2.1.1.3.3.3. Antifungal Activity

Flavonoids (pinocembrin, sacuretin, pinobanksin), pterostilbenes and caffeic acids are mainly responsible for antifungal effect (97). The effect of propolis on twelve patients with chronic sinusitis induced by *Candida* was investigated. Fungus was found to have poor sensitivity to propolis in eight cases and poor resistance in the other two cases. Patients were treated with propolis in alcohol-oil emulsion. Emulsion was applied to the sinuses. Clinical improvement in nine patients and improvement in the other three patients were observed after 5-8 treatments (100). Antifungal activity on 40 *Candida* spp. and *Trichosporon* spp. strains was investigated in bee products (honey, propolis, etc.) and propolis was shown to be the most effective in *ex-vivo* experiment in blood, sputum, urine, nail and mucus specimens collected from infected patients. This effect is thought to be useful in the treatment of fluconazole-resistant fungal infections (101). Propolis types produced by *Apis mellifera* bees from two different regions of Brazil EtOH extracts and subextracts showed strong anti-Candida effect. It may also be used as a complementary therapy in oral and systemic candidiasis treatment (102).

2.1.1.3.4. Antiprotozoal Activity

Dantas et al. (103) and Salomao et al. (104) showed that strong activity against protozoa *in vitro* studies in most propolis types, especially *Populus* type and Brazil type propolis. Effects of Brazilian propolis on *Leishmania amazonensis*. The *in-vitro* effects of ethanolic extract of propolis sample taken from Adana on *Leishmania tropica* parasite were investigated. It was administered at concentrations of ethanolic propolis (25, 50, 100, 500 and 750 μg/mL) and antileismanial effect was observed at concentrations above 100 μg/mL. *L. tropica* parasites significantly reduced (105).

Duran et al. (106) observed significant *in vitro* antileishmanial effect of propolis samples from Hatay and Bursa against *Leishmania* parasites (*Leishmania infantum* and *Leismania tropica*) species. In addition, the phenolic-rich bolivian propolis sample was considered to have the best antibacterial and antileismanial effects (107).

2.1.1.3.5. Antitumor Activity

The mechanism of anticancer activity in CAPE has been investigated previously and has been shown to activate DNA damage signaling in cancer cells. CAPE has been shown to arrest the development of cells caused by activation of the p53 tumor suppressor protein and down regulation of mortalin. Ishida et al. (108) reported that CAPE-ɣ cyclodextrin complex, which shows great cytotoxicity against a wide variety of cancer cells. The ethanolic extract of Chinese propolis prevent breast cancer proliferation. CAPE is responsible for this impact (109).

Artepillin C was applied to human and mouse malignant tumor cells, artepillin C had been shown to stop tumor cell growth and to have a cytotoxic effect. In addition to suppression of tumor growth, an increase in the ratio of CD4/CD8 T cells and an increase in total helper T cells was observed. Such findings suggested that Artepillin C activates the immune system and exhibited has antitumor activity (110). Kimoto T et al. (111) investigated that using renal carcinogenic ferric nitrilotriacetate (Fe-NTA) in male ddY (mice with superior reproductive performance) mice was induced primary lung cancer in bronchioles and alveolar tissues. 4-Hydroxy-2-nonenal and 8-hydroxy-2'-deoxyguanosine, which are products of oxidative processes in bronchiolar and alveolar cells, have been increased after Fe-NTA application. After oral administration of artepillin C or propolis, these substances decreased in related to the anticancer prophylactic affect of propolis and artepillin C. After oral administration of propolis or artepillin C, adenomas and carcinoma was not developed. Rather than converting to Fe-NTA-induced large-cell cancers in control mice, adenomas have shown that macrophages and local antioxidant activity are increased at a considerable level after treatment with propolis or Artepillin C. By this way, propolis and Artepillin C prevented lipid peroxidation and suppress the development of pulmonary cancers (111).

Cinnamic acid derivatives (such as, Baccharin and drupanin) were examined *in-vivo* antitumor activity by affecting Sarcoma S-180 cells in the mouse. These agents had been shown to kill tumor cells by causing less genotoxicity than anticancer drugs (112).

Chrysin inhibited dose dependently COX-2 protein and mRNA expression induced by lipopolysaccharide (LPS) in a significant dose (113).

3. MATERIALS and METHODS

3.1. Materials

3.1.1. Propolis Materials

Twenty-four different raw propolis samples were collected by beekeepers from different locations (Turkey) in August-November 2017. Each propolis sample was encoded as 'P' from 1 to 24 (Table 2).

Table 2. Collection locations of propolis samples

Sample Code	Location	Propolis Sample
P1	İstanbul (Beykoz-Anadolu Feneri)	
P2	İstanbul (Beykoz-Çatalca)	
P3	Muğla (Marmaris-Turunç)	
P4	Tekirdağ (Süleymanpaşa- Yağcı)	
P5	Kütahya (Aslanapa-Mustafalar)	
P6	Kütahya (Kumarı)	
P7	Denizli (Bekilli- Eldelek)	

Table 2. Continued

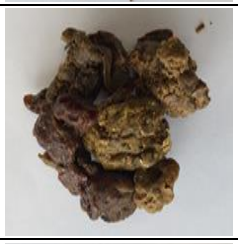
Sample Code	Location	Propolis Sample
P8	Denizli (Hisar)	
P9	Denizli (Bekilli)	
P10	Uşak (Elmadağ)	
P11	Nevşehir (Avanos)	
P12	Isparta (Şarkikaraağaç)	
P13	Ankara (Çamlıdere-Tatlak)	
P14	Isparta (Pirimehmet)	

Table 2. Continued

Sample Code	Location	Propolis Sample
P15	Burdur	
P16	Burdur (Karaçal)	
P17	Kastamonu (İnebolu-Belören)	
P18	Kastamonu (İnebolu-Yenimahalle)	
P19	Yozgat	
P20	Yozgat (Yerköy)	
P21	Manisa (Akhisar-Sırtköy)	

Table 2. Continued

Sample Code	Location	Propolis Sample
P22	Osmaniye (Toprakkale)	
P23	Osmaniye	
P24	Osmaniye (Kadirli)	

3.1.2. Chemicals and Solvents

Table 3. Chemicals, solvents, distributors and lot numbers

Chemicals and Solvents	**Distributors and Lot numbers**
Aluminiumchloride	Merck, UN1726
2- aminoethyl diphenylborinate	Fluka, 10113408
Apigenin	Sigma, WE445301/1
Caffeic Acid	Sigma, 086K1885
Caffeic acid phenethyl ester	BCBC0489V
Chrysin	Sigma, STBD9050V
Copper(II)Sulphate pentahydrate	Merck, UN3077
Dichloromethane	Carlo Erba, N75092
2,2-Diphenyl-1-picrylhydrazyl	Sigma Aldrich, STBH0044
Ethanol	Sigma Aldrich, SZBD3221V
Ethyl Asetat	Merck KGaA, K46450423509
Ferulic Acid	USP, FOJ193
Folin-Ciocalteu Reagent	Sigma Aldrich, BCBV3191
Galangin	Sigma, MKBR5602V
Gallic Acid	Sigma Aldrich, SLBQ0358V
Glacial Asetic Acid	Sigma Aldrich, UN2789
Hydrochloric Acid	Sigma Aldrich, SZBF1530V
Kaempferol	Sigma Aldrich, SLZB3264V
Methanol	Sigma Aldrich, STBG3274V
Naringenin	Sigma Aldrich, STZB1650V
n-Butanol	Fluka,52150
n- Hexane	Sigma Aldrich,STBF2917V
Pinocembrin	Fluka, MKBR9029V
Polyethylene Glycol 400	Merck, S6041785019
Potassium sodium tartrate	Carlo Erba, 6L020156M
Quercetin HPLC >%95	Sigma Aldrich, SLBM7336V
Quercetin Dihydrate	Sigma Aldrich,SZBM6534V
Sephadex LH-20	Sigma Aldrich, LH20100
Sodium Carbonate anhydrous	Riedel-de Haen, 50590
Sodium hydroxide	Sigma Aldrich, STBG9015
Sodium nitrite	Fluka Chemika, 71760
Sulphuric acid 98%	Sigma Aldrich, 30743
Ultrapure Water	Merck Millipore, Simplicity UV
Vanillin	Fluka, 1435805

3.1.3. Chromatographic Plates

Table 4. Plates, manufacturer and lot numbers

Plates	**Manufacturer**	**Lot numbers**
HPTLC glass Silica Gel 60 F_{254} 20 cm x 10 cm	Merck	HX377581
TLC aluminium Silica Gel 60 F_{254} 20 cm x 10 cm	Merck	HX72379454

3.1.4. MPLC Column

Table 5. Column, manufacturer and lot number

Columns	**Manufacturer**	**Lot numbers**
C18	RediSep Rf High Performance Gold Patent Pending 30 g	231518117W

3.1.5. Equipments

Table 6. Equipment and manufacturers

Equipment	**Manufacturer**
Automatic Developing Chamber 2	Camag, Switzerland
Balance	Ohaus Explorer, USA
Centrifuge Labofuge	Thermoscientific ,USA
Centrifuge Tubes (10 mL)	Isolab, Turkey
Chromatogram Immersion Device III	Camag, Switzerland
Control Unit C-620 for MPLC	Buchi, Switzerland
Filter Paper	Munktell, Sweden
Flask (100,250,500 mL)	Isolab, Turkey
Fraction Collector C-660 for MPLC	Buchi, Switzerland
Glass Basic Laboratory Eqiupments	Isolab, Turkey
Hair Dryer	Profilo, Turkey
Lyophilizer	Christ Alpha 2-4LD, Germany
Micropipette (100-1000µL)	Rainin, USA
Micropipette Tips (1000µL)	Rainin, USA
NMR	JEOL Eclipse-Virginia Tech
Oven	Binder, Germany
Pump Module C-605 for MPLC	Buchi, Switzerland
Refrigerator and Deep-freeze	Arçelik, Turkey
Refrigerated Circulator Cool Tech 320	Thermoscientific,USA
Rotary Evaporator	Heidolph,Germany
Sample Syringe for HPTLC (100µL)	Hamilton, Switzerland
Spectrophotometer Multiscan Ascent	Thermo Lab Systems, USA
Spectrophotometer	Spekol 1300
Syringe for single use	Steril Hayat, Turkey
TLC Scanner 3	Camag, Switzerland
TLC Plate Heater	Camag, Switzerland
TLC Visualizer	Camag, Switzerland
Twin Trough Chamber	Camag, Switzerland
Ultrasonic Bath	Sonorex RK156BH, Germany
UV Photometer C-640 for MPLC	Buchi,Switzerland
Vacuum Pump CVC 2000	Vacuubrand, Turkey
Vial (2mL)	Agilent, USA
Vortex	Yellowline TTS 2, USA
WinCATS and Videoscan TLC Evaluation Software	Camag, Switzerland

3.2. Methods

3.2.1. Preparation of Standart Solutions for HPTLC and HPTLC-DPPH˙ analyses

Standart solutions of naringenin, pinocembrin and ferulic acid were prepared as 0.4 mg/mL concentration in methanol (MeOH) whereas, chrysin, CAPE, quercetin dihydrate, kaempferol, apigenin, galangin were prepared as 0.2 mg/mL. In addition, caffeic acid was prepared as 0.05 mg/mL in MeOH. Then, the standart mixture of naringenin, pinocembrin, galangin, ferulic acid, caffeic acid, chrysin, CAPE, quercetin dihydrate, kaempferol and apigenin was prepared in proportion (mL) of 2: 2: 2: 2: 1: 1: 1: 1: 1: 1 and used during the analysis.

3.2.2. Preparation of Sample Test Solutions

3.2.2.1. Preparation of Sample Test Solutions for HPTLC and HPTLC-DPPH˙ Analyses

One gram of each crude propolis samples was accurately weighted and extracted with 10 mL EtOH-H_2O (8:2, v/v) by using sonicator (Sonorex süper RK 156 BH) for 45 minutes. Afterwards, the extract was centrifuged at 5300 rpm for 30 minutes and the supernatant was concentrated in rotary evaporator (Heidolph laborota 4001- efficient). Next, 5 mL of EtOH was added to dissolve residue. The EtOH extract was filtered through the 0.45 μm membrane filter (Syringe Filters Chromafil RC 45/25). Each filtered sample was stored at -20 °C as a stock solution. For analysis, 1/10 and 1/50 diluted solutions were prepared and used during the experiments.

3.2.2.2. Preparation of Sample Test Solutions for Antimicrobial Analysis

Ten grams of each P5, P12, P14, P17, P19 and P22 propolis samples were accurately weighted and extracted by 100 mL of EtOH-H_2O (8:2, v/v) in the ultrasonic bath for 45 minutes. After, the extracts were filtered through a filter paper. The liquid parts of samples were evaporated by rotary evaporator. Then the rest was lyophilized. 0.01024 grams of each lyophilized propolis samples was accurately weighed and dissolved in 10 mL dimethylsulfoxide (DMSO)-H_2O (1:9, v/v).

3.2.3. Extraction of *Populus* species

Each bud samples taken from *P. nigra*, *P. tremula* and *P. alba* were divided into small pieces. It was then treated with 20 mL of EtOH with a heating stirrer at 70 °C. After filtration, rotary evaporator was used to remove the EtOH. The residue was dissolved exactly in 5 mL of EtOH (10).

3.2.4. HPTLC Method

Each propolis sample test solution (2 µL) and standard mixture solution (30 µL) were applied on HPTLC glass plates (20 x 10 cm) covered with silica gel 60 F_{254} using a Linomat V automatic sample spotter fitted with 100 mL Hamilton syringe. For the separation of phenolic and flavonoid compounds, 10 mL of the mobile phase contains n-hexane, ethyl acetate, glacial acetic acid (5:3:1, v/v/v) applied one side of twin trough chamber (20 x 10) and 10 mL of %37 hydrochloric acid applied in the other trough. Before the plate was developed up to a migration distance of 70 mm, the chamber was saturated for 20 minutes. After being developed, the plate was dried by a stream of cold air for 5 minutes. Then, the plates were documented by the TLC visualizer at 254 nm and 366 nm. After, the plates were heated in a TLC heater at 100 ˚C for 3 minutes before being immersed in natural product (NP) and polyethyleneglycol 400 (PEG 400) solutions which were prepared according to Reich & Schibli, 2007. Lastly, the plates were captured at 366 nm after being immersed in NP and PEG solutions, respectively. All documents were run with winCATS program (10).

3.2.5. Sephadex Column Chromatography

According to the HPTLC results, the major compound ($R_F \approx 0.15$) found in P5 and P14 was isolated and identified. 25 grams of P14 crude propolis sample was accuretely weight and extracted by 200 mL of EtOH-H_2O (8:2, v/v) in the ultrasonic bath for 45 minutes 2 times. Then, extracts were combined and evaporated in a rotary evaporator. The residue was weight as 11 grams. 75 grams of Sephadex LH 20 was used for isolation process. 1 gram of P14 extract was weighted and dissolved in 4 mL dichloromethane (CH_2Cl_2)-MeOH (v/v, 1:1). Then, it was applied to the sephadex column. A total of 500 mL of solvent (CH_2Cl_2-MeOH, (1:1 v/v)) was used during the analysis. This procedure was repated in 5 times. Each fractions were analyzed by using TLC. Silica gel plate was used as a stationary phase and the mixture of CH_2Cl_2-MeOH-

H_2O (90:10:1 v/v/v) was used as a mobile phase. The plate was derivatized with vanillin/sulphuric acid.

The obtained fractions related with major compound was combined and then evaporated. The yield was calculated as 105 mg. Further, medium pressure liquid chromatography (MPLC) was performed to obtain the major substance.

3.2.6. Medium Pressure Liquid Chromatography (MPLC) Method

One hundred and five mg extract containing the unknown major component was applied to a C18 column (C18 MPLC, 30 g HP) and gradually increased solvent mixture (MeOH-H_2O) was used to get the major compound (Table 7). According to the TLC results, it was observed that the major compounds were detected in the fractions between 45-48 and 64-66. Fractions from 45 to 48 were named as Pr1 and fractions from 64-66 were named as Pr2 (Figure 5). These fractions were seperately lyophilized for NMR spectroscopy.

Table 7. Solvent composition for MPLC analysis

Start % B (MeOH)	**End %B (MeOH)**	**Minutes**
30	30	10
30	50	30
50	50	15
50	60	20
60	60	10
60	70	10
70	70	20
70	100	20
100	100	15

3.2.7. NMR

NMR was used to elucidate structures of the compounds. NMR (1H, 13C) spectra were operate in CD_3OD and also recorded on JEOL Eclipse 500 MHz NMR (Virginia Tech).

3.2.8. Antioxidant Activity

3.2.8.1 HPTLC-DPPH$^{\cdot}$ Method

HPTLC-DPPH$^{\cdot}$ assay was used to screen propolis components for the presence of the active antioxidative constituents. After HPTLC method was applied in Section 3.2.4, the HPTLC plates were immersed in the % 0.1 DPPH$^{\cdot}$ solution.

3.2.9. Antimicrobial Activity Method

3.2.9.1. Bacterial Culture

To investigate the antibacterial activity both the gram (+) (*Staphyloccoccus aureus* (ATCC 6538)) and gram (-) (*Pseudomonas aeruginosa* (ATCC 15442) and *Escherichia coli* (ATCC 11229)) species were selected. For the antifungal activity *Candida albicans* (ATCC 10231) was used.

3.2.9.2. Disc Diffusion Method

Disc diffusion method was used to examine the antibacterial activity of the lyophilized propolis extracts. Samples were prepared 1024 μg/mL concentration against 4 microorganisms. Ofloxacin 5 μg (anti-bacterial agent) and Nystatin 100 units (anti-fungal agent) as standard discs were used as positive controls, respectively. Bacterial and fungal suspensions which provided the 0.5 McFarland standard were inoculated to Mueller Hinton Agar (bacteria) or Sebouraud 2% Dextrose Agar (fungal) with sterile ecuvion sticks. Blank discs (6 mm in diameter) were impregnated with 20 μL of the extracts and subextracts and located on the inoculated plates. The antimicrobial activity of the extracts were determined by measuring the diameter of zone of inhibition in millimeter after 18-24 h incubation (114).

3.2.9.3. Minimum Inhibitory Concentration (MIC) Assay

Serial tube dilution technique was used to determine MIC of antimicrobial agents. Briefly, ten screw cap test tubes were taken and marked 1, 2, 3, 4, 5, 6 and 7 for extracts and the others were labeled as TM for medium, TMI was labeled for medium and inoculum and TMS for medium and DMSO, respectively. 1 mL of nutrient broth medium were taken in all test tubes and the lyophilized propolis extract (1024 µg/mL) was only added to the tube labelled as number 1 and the tube was shaken for convenient mixing of the content. 1 mL of the content from the 1st tube was added to the tube marked as number 2, that action was operated up to the tubes marked as number 7. After convenient mixing 1 mL content from the 7 marked tube was discarded. 10 µL of the the bacterial and fungal suspensions which provided the 0.5 McFarland standards was added to no 1, 2, 3, 4, 5, 6, 7 and TMI labelled tubes. Only 1 mL of DMSO was added to TMS labelled tube, after shaking 1 mL of the mixture was discarded from the tube. TM labelled tube only contained only 1 mL medium. This process was repeated for all tested substances and microorganisms. All test tubes were subjected to incubation at 37 °C for 18-24h (114).

3.2.10. Total Phenol Content using Folin Ciocalteu Method

TPC of propolis samples were detected by the Folin-Ciocalteu colorimetric method described by Velioglu et al. (115) with slight modifications. Briefly, (%10 w/v) sodium carbonate ($NaCO_3$) and diluted Folin-Ciocalteu reagent (reagent: H_2O, 1:2, v/v) were prepared. The gallic acid standard solutions were prepared in concentration between 31.25-1000 µg/mL. Then, either 30 µL or 250 µL 1 were taken from previously 1/50 diluted propolis samples and each volumes was completed to 2.5 mL with H_2O. Immediately after addition of 0.5 mL Folin-Ciocalteu reagent to each of the 2.5 mL solutions, 1.5 mL of %10 w/v $NaCO_3$ was added. After vortexing, prepared solutions were waited at room temperature in dark for 1 hour. The absorbance was measured at 725 nm by using UV Spectrophotometer. TPC were expressed as mg of gallic acid equivalents (GAE) per g of propolis samples (mg GAE/g propolis) (116).

3.2.11. Total Flavonoid Content

The TFC was detected by using aluminium chloride ($AlCl_3$) colorimetric method in propolis samples. The method described by Zhishen et al. (117) was slightly modified as follows: The quercetin standard solutions were prepared at concentrations between 31.25-2000 µg/mL. 1/50 diluted propolis samples were used during the analysis. From each sample test solutions either 250 µL or 750 µL was taken, then the each volume was completed to 1000 µL with H_2O. After 5 min. of adding 75 µL of sodium nitrite ($NaNO_2$) to each tubes, 150 µL of 10% $AlCl_3$ and then, 500 µL sodium hydroxide (NaOH) were added. Before incubation of all standard and sample test solutions for 15 min., each tube was completed up to 2500 µL with H_2O and vortexed homogenously. The absorbance was measured at 510 nm by spectrophotometer (118).

3.2.12. Preparation of Detection Reagent

Polyethyleneglycol 400 (PEG 400) was prepared in concentration of 5% (w/v) in CH_2Cl_2. NP detection reagent was prepared in concentration of 0.005% (w/v) in ethylacetate (119). In addition, % 0.1 (w/v) dipping solution of $DPPH^{\cdot}$ was prepared in MeOH. Vanillin-sulphuric acid reagent was prepared by dissolving respectively 40 mg vanillin in 10 mL EtOH and 200 µL concentrated sulphuric acid (120).

4. RESULTS

4.1. HPTLC Analysis

The phenolic profiles of 24 propolis samples and their botanical origins were evaluated by using HPTLC fingerprinting. Phenolic profiles of propolis samples were compared using apigenin (green band color, $R_F \approx 0.05$), quercetin (orange band color, $R_F \approx 0.2$), chrysin (green band color, $R_F \approx 0.25$), kaempferol (green band color, $R_F \approx 0.3$), caffeic acid (blue band color, $R_F \approx 0.5$), naringenin (green band color, $R_F \approx 0.55$), CAPE (blue band color, $R_F \approx 0.65$), ferulic acid (blue band color, $R_F \approx 0.67$), galangin (blue band color, $R_F \approx 0.7$) and pinocembrin (blue band color, $R_F \approx 0.77$) standards.

Phenolic compounds such as pinocembrin, CAPE, caffeic acid were present in the intense zones of P1, P2, P4, P6, P7, P8, P9, P10, P11, P12, P16, P18, P19 and P21 propolis samples. In addition, in these propolis samples predominant yellow-orange zones, few number of light blue and faded green zones were found and grouped as O-type (Figures 1 and 2). Galangin, chrysin and apigenin were found in all of the O-types of propolis samples except P21 and P6. Among the O-type of propolis samples, all standards (except naringenin) investigated in this study were detected in P11 and P12 (Table 9).

Due to predominant blue zones, P3, P13, P15, P17, P20, P22, P23 and P24 were grouped as B-type (Figure 1 and Figure 2). Caffeic acid was commonly found in all of the B-type of propolis samples. In contrast, naringenin, kaempferol and quercetin were not found in any B-type of propolis samples (Table 9).

P5 and P14 propolis samples were determined to have characteristic main orange bands. However, these samples could not be grouped under O-type, that is because lacking of some markers belong to O-type such as CAPE, galangin and caffeic acid (observed as fade zone). Therefore, the existance of this type unlike the other Turkish propolis types define in this study indicated a new type propolis which was rich with 3-*O*-methylquercetin (3MQ). So, these propolis samples were grouped under 3MQ-type. A major yellowish-orange band ($R_F \approx 0.15$) belong to these samples was determined. Therefore, further isolation studies to evaluate this compound were planned according to the HPTLC analysis result.

To determine botanical origins of propolis samples, HPTLC fingerprints of plant bud extracts belong to *P. nigra*, *P. tremula* and *P. alba* were compared with HPTLC fingerprints of propolis (Figure 4). Extract of *P. nigra* showed dominant orange, light green and blue bands whereas *P.tremula* bud extract showed dominant light and dark blue bands (Figure 4). As a result of comparison between HPTLC chromatograms of plant bud extracts with propolis, it was found *P. nigra* was the main plant source of P1, P2, P4, P6, P7, P8, P9, P10, P11, P12, P16, P18, P19 and P21. Besides, *P. tremula* was the main source of P3, P13, P15, P17, P20, P22, P23 and P24. In contrast, HPTLC fingerprints of P5 and P14 were not match with these plant sources.

Table 8. Standard compounds with molecular formula, R_F values and band colors

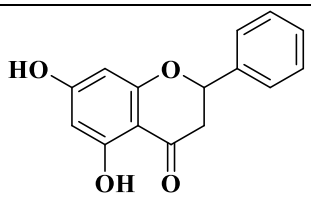
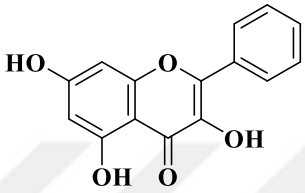
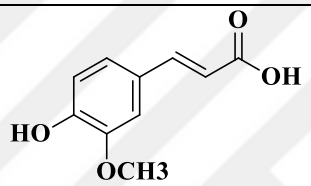
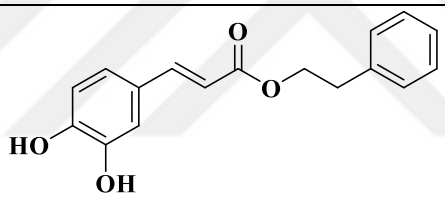
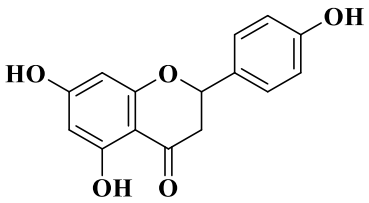
No	Standards	Molecular Formula	R_F (≈) values	Band Color
1	Pinocembrin		0,77	Blue
2	Galangin		0,7	Blue
3	Ferulic Acid		0,67	Blue
4	CAPE		0,65	Blue
5	Naringenin		0,55	Green

Table 8. Continued

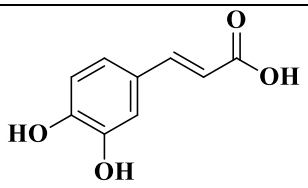
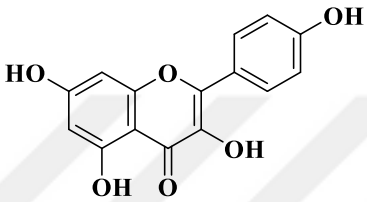
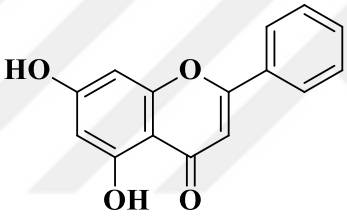
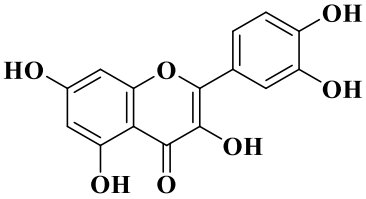
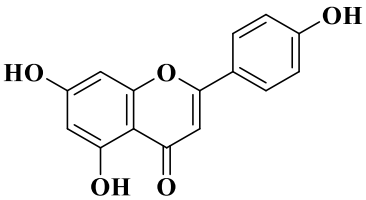
No	Standards	Molecular Formula	R_F (≈) values	Band Color
6	Caffeic acid		0,5	Blue
7	Kaempferol		0,3	Green
8	Chrysin		0,25	Green
9	Quercetin		0,2	Orange
10	Apigenin		0,05	Green

Table 9. Phenolic profile and propolis types

Sample	Type	Pinocembrin $R_F \approx 0.77$	Galangin $R_F \approx 0.7$	Ferulic Acid $R_F \approx 0.67$	CAPE $R_F \approx 0.65$	Naringenin $R_F \approx 0.55$	Caffeic Acid $R_F \approx 0.5$	Kaempferol $R_F \approx 0.3$	Chrysin $R_F \approx 0.25$	Quercetin $R_F \approx 0.2$	Apigenin $R_F \approx 0.05$
P1	O	+	+	-	+	-	+	-	+	-	+
P2	O	+	+	-	+	-	+	+	+	-	+
P3	B	-	-	-	-	-	+	-	-	-	-
P4	O	+	+	-	+	-	+	-	+	-	+
P5	3MQ	-	-	-	-	-	-	-	-	+	-
P6	O	-	+	-	+	-	+	-	-	-	-
P7	O	+	+	-	+	-	+	-	+	+	+
P8	O	+	+	-	+	-	+	-	+	+	+
P9	O	+	+	-	+	-	+	-	+	+	+
P10	O	+	+	-	+	-	+	-	+	+	+
P11	O	+	+	+	+	-	+	+	+	+	+
P12	O	+	+	+	+	-	+	+	+	+	+
P13	B	+	-	+	+	-	+	-	+	-	+
P14	3MQ	-	-	-	-	-	-	-	-	+	-
P15	B	+	-	+	+	-	+	-	+	-	+
P16	O	+	-	-	+	-	+	-	+	-	+
P17	B	-	-	+	-	-	+	-	-	-	-
P18	O	+	-	-	+	-	+	-	+	-	+
P19	O	+	-	+	+	-	+	-	+	-	+
P20	B	+	+	-	+	-	+	-	+	-	+
P21	O	+	-	-	+	-	+	-	+	-	+
P22	B	-	-	+	-	-	+	-	-	-	-
P23	B	-	+	-	-	-	+	-	+	-	+
P24	B	-	+	-	-	-	+	-	+	-	+

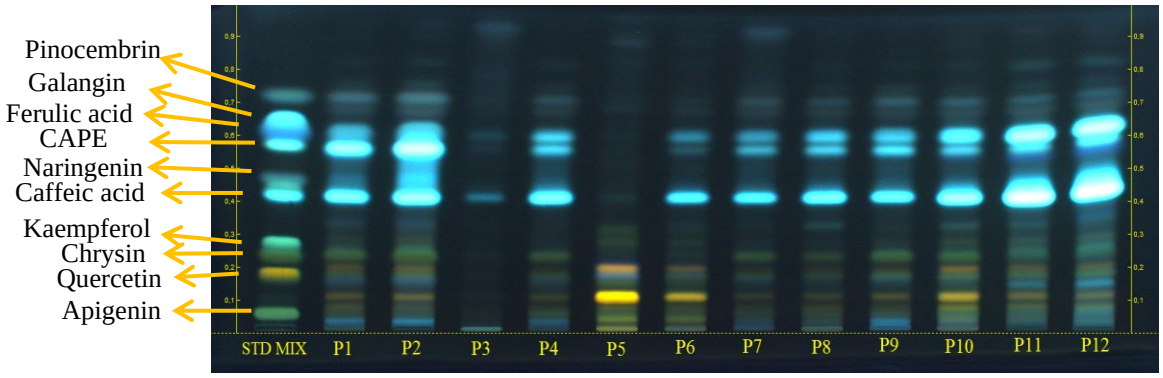


Figure 1. HPTLC chromatogram of P1-P12 propolis extracts at 366 nm, developing solvent system *n*-hexane-ethyl acetate-glacial acetic acid (5:3:1 v/v/v), derivatization NP/PEG 400

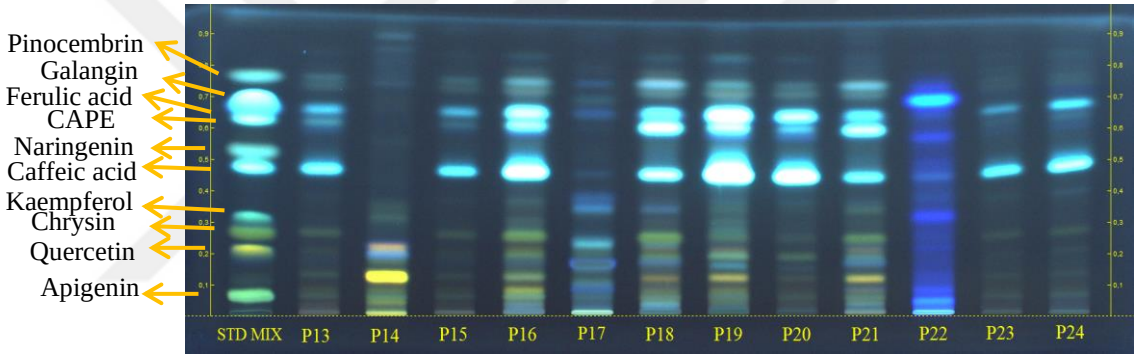


Figure 2. HPTLC chromatogram of P13-P24 propolis extracts at 366 nm developing solvent system *n*-hexane-ethyl acetate-glacial acetic acid (5:3:1 v/v/v), derivatization NP/PEG 400

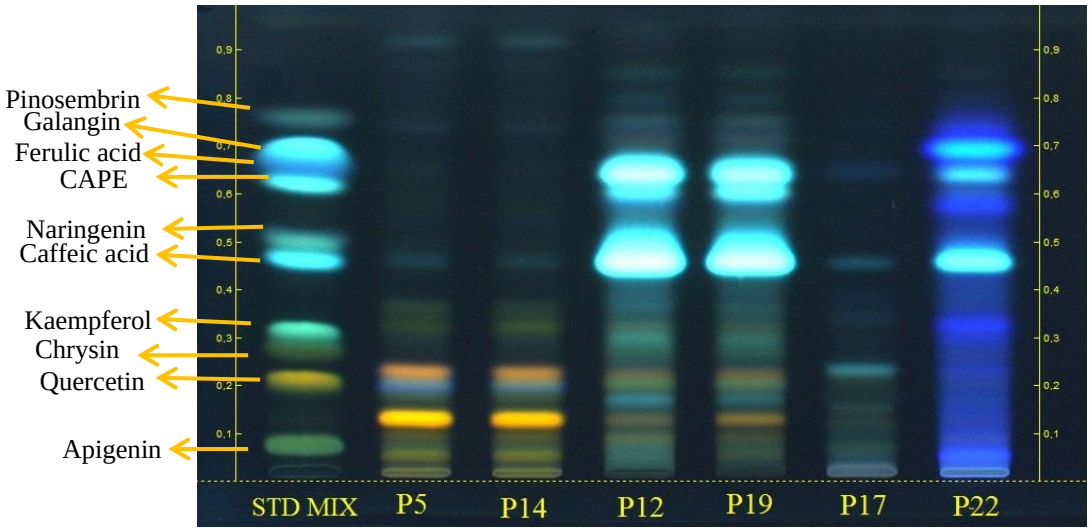


Figure 3. HPTLC chromatogram of different types of propolis samples at 366 nm developing solvent system *n*-hexane- ethyl acetate- glacial acetic acid (5:3:1 v/v/v), derivatization NP/PEG 400

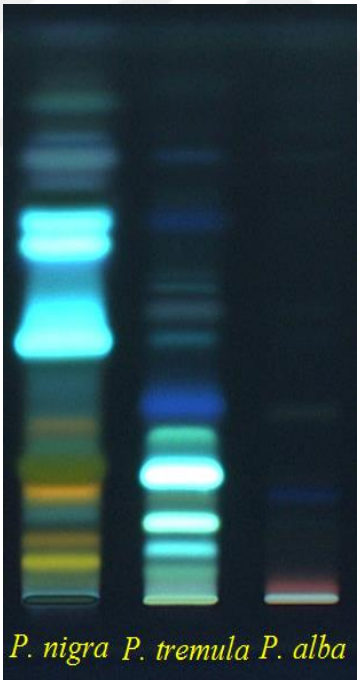


Figure 4. HPTLC chromatogram of comparison plant species at 366 nm developing solvent system *n*-hexane-ethyl acetate-glacial acetic acid (5:3:1 v/v/v), derivatization NP/PEG 400

4.2. Phytochemical Analysis

4.2.1. Phytochemical Results

The structure of the isolated compounds was determined by spectroscopic methods namely; ^{1}H- NMR, ^{13}C-NMR and MS. According to the NMR results, previously named as Pr1 and Pr2 were identified as 3-*O*-methylquercetin (3MQ) and rhamnetin, respectively (Figure 5). The NMR results of the 3MQ and rhamnetin were given in the Tables 9 and 10. In addition, the molecular formula, molecular weight and structure of the 3MQ and rhamnetin were given in Figures 6 and 9, respectively.

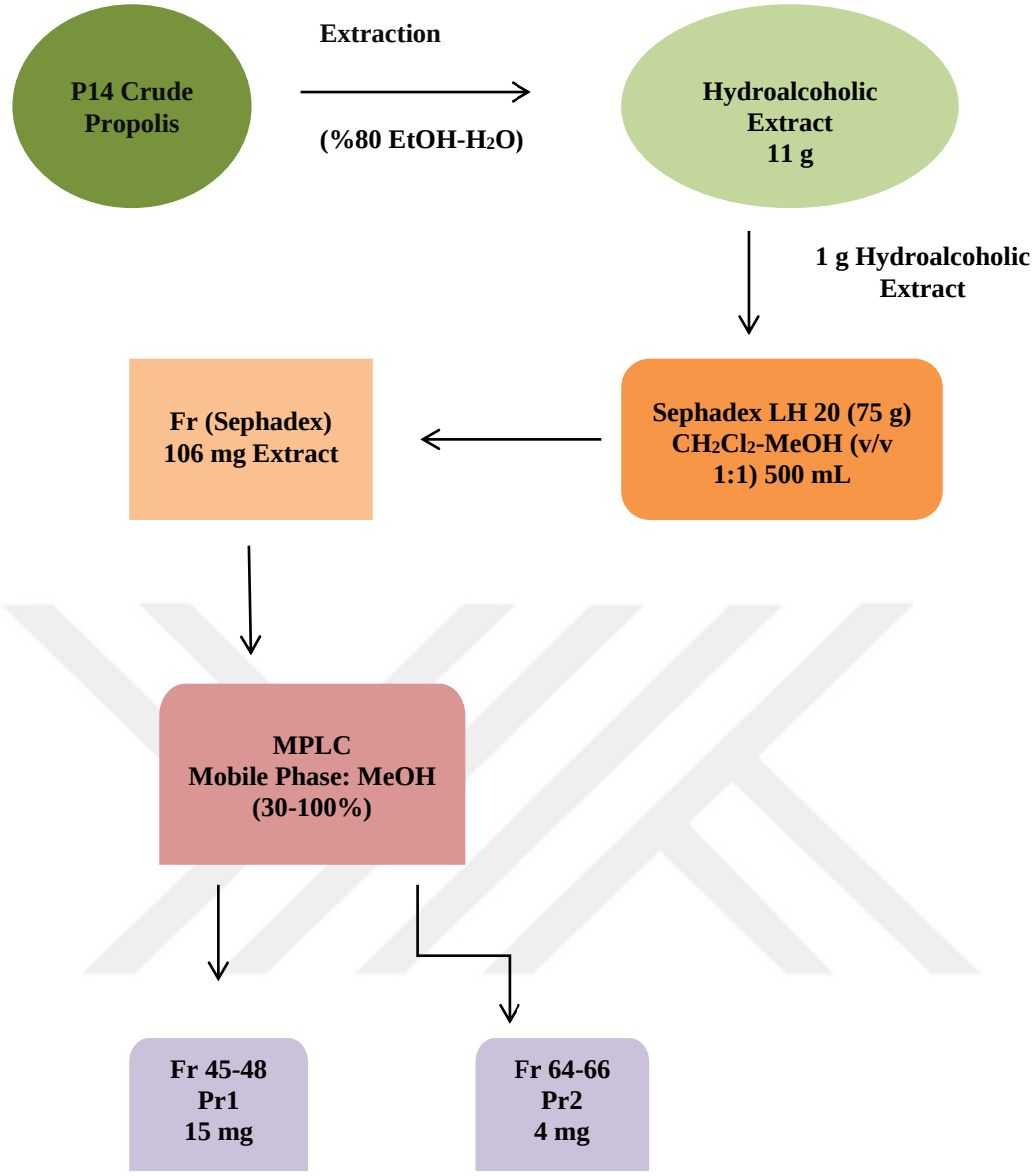


Figure 5. Isolation schema of Pr1 and Pr2

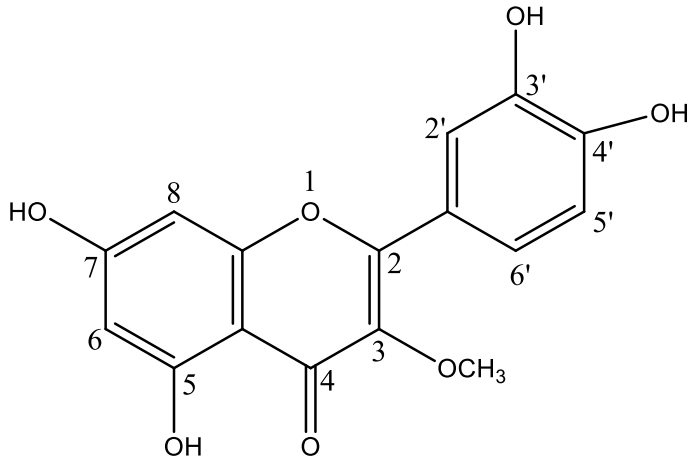


Figure 6. Structure elucidation of 3MQ (Pr1)

Molecular Formula: $C_{16}H_{12}O_7$

Molecular Weight: 317.3

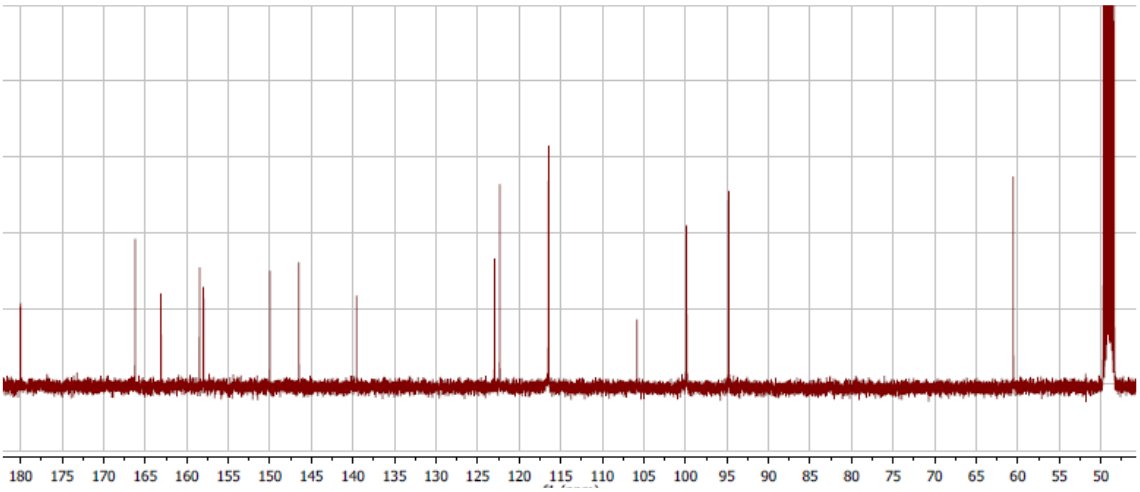


Figure 7. ^{13}C NMR for Pr1

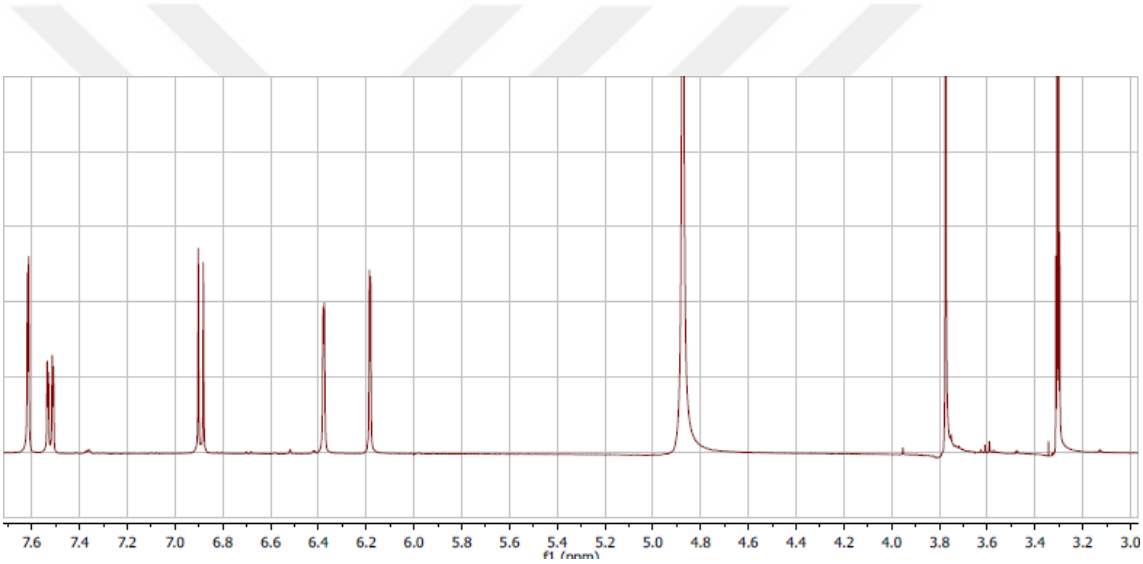


Figure 8. ^{1}H NMR for Pr1

Table 10. ^{1}H (300MHz, Methanol) and ^{13}C-NMR datas of Pr1 (3MQ)

Position		**δ_H,ppm (J, Hz)**	**δ_C, ppm**
2	C		158.0
3	C		139.5
4	C		180.0
5	C		163.1
6	CH	6.18 (d, J=2.1Hz)	99.8
7	C		166.2
8	CH	6.38 (d, J=2.1Hz)	94.7
9	C		158.4
10	C		105.8
1’	C		122.3
2’	CH	7.62 (d, J=2.1Hz)	116.5
3’	C		146.5
4’	C		150.0
5’	CH	6.89 (d, J=8.3)	116.5
6’	CH	7.52 (dd, J= 8.3 and 2.1 Hz)	122.9
3’-OMe		3.77 singlet	60.5

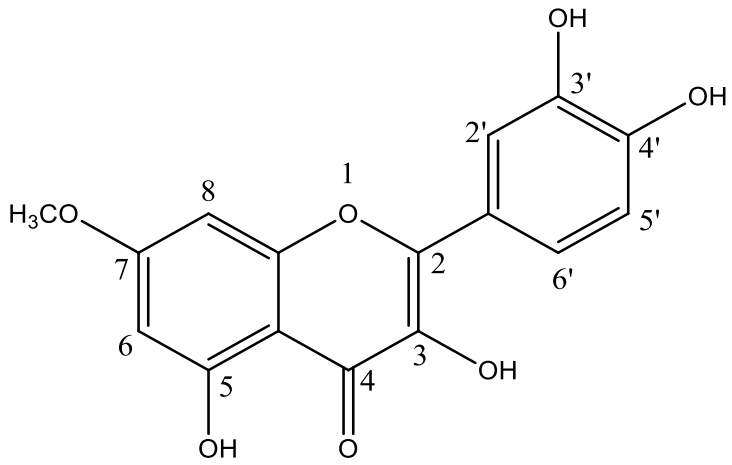


Figure 9. Structure elucidation of Rhamnetin (Pr2)

Chemical Formula: $C_{16}H_{12}O_7$

Molecular Weight: 316.26

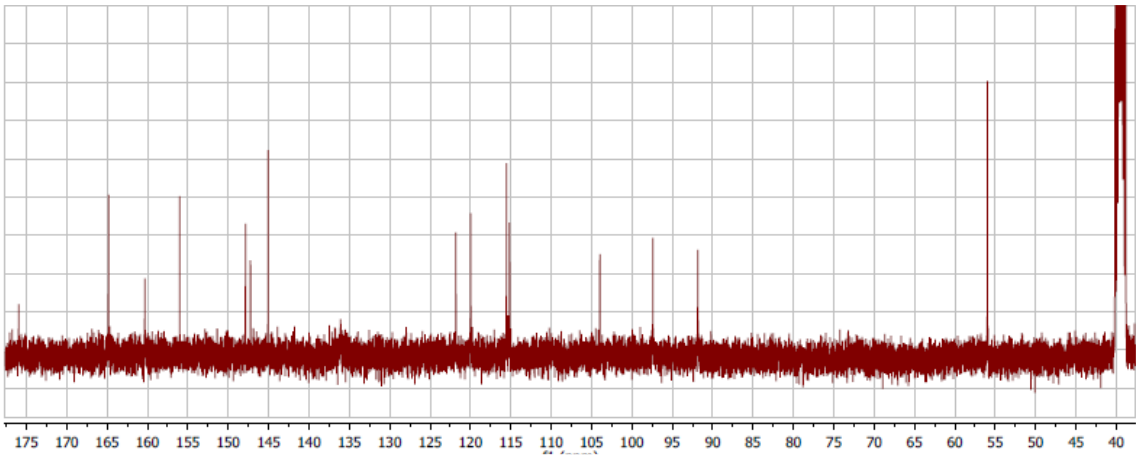


Figure 10. ^{13}C NMR for Pr 2

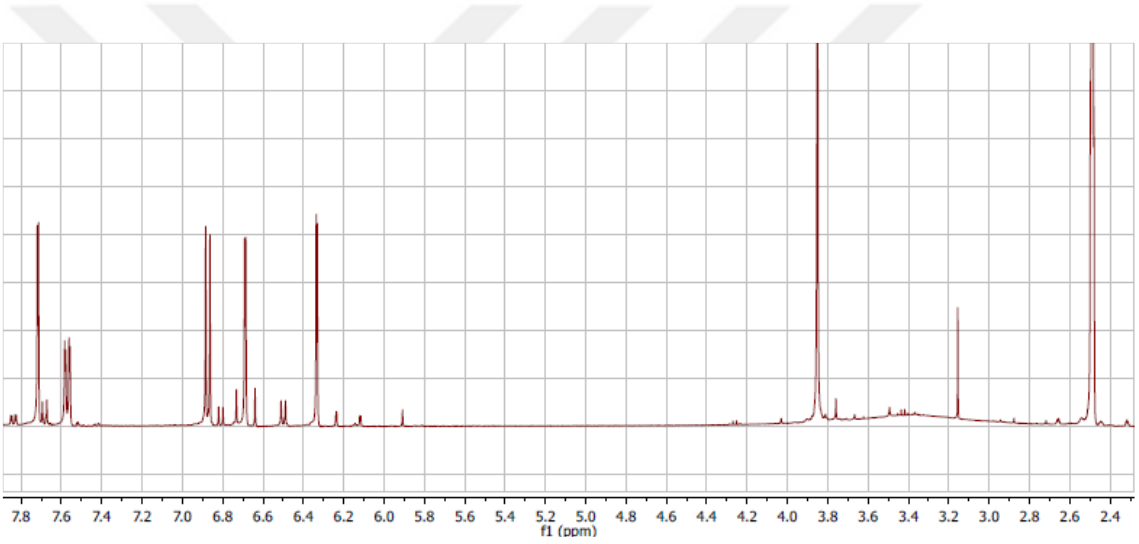


Figure 11. ^{1}H NMR for Pr 2

Table 11. ^{1}H (300MHz, Methanol) and ^{13}C-NMR datas of Pr2 (Rhamnetin)

Position		**δ_H,ppm (J, Hz)**	**δ_C, ppm**
2	C		147.3
3	C		136.1
4	C		175.9
5	C		160.3
6	CH	6.33 (d, J=2.1 Hz)	97.4
7	C		164.8
8	CH	6.69 (d, J=2.1 Hz)	91.8
9	C		156.0
10	C		103.9
1’	C		121.8
2’	CH	7.72 (d, J=2.0 Hz)	115.2
3’	C		145.0
4’	C		147.8
5’	CH	6.87 (d, J=8.4)	115.6
6’	CH	7.57 (dd, J=8.4, 2.0 Hz)	120.0
7-OMe		3.85 singlet	55.9

4.3. HPTLC-DPPH$^{\cdot}$ Analysis

DPPH$^{\cdot}$ assay coupled with HPTLC is a rapid scanning technique which supplies determination of each phenolic compounds with antioxidant activity. Compounds with antioxidant activity distiguishes from compounds without antioxidant activity on the plate. Antioxidant compounds could be determined by detection of light yellow color bands on the purple background.

The color change from purple to yellow was observed on CAPE, caffeic acid, kaempferol, quercetin and galangin standards separated on HPTLC plate, indicating the antioxidant activity of these compounds. However, color change was not observed in some propolis samples which had CAPE, caffeic acid, kaempferol, quercetin and galangin. The reason could be because of the concentration of the compounds separated on the plate. Concentrations of the components in the standard mixture on the plate were determined as pinocembrin (1.71 μL/band), ferulic acid (1.71 μL/band), and naringenin (1.71 μL / band), galangin (0.86 μL/band), chrysin (0.43 μL / band), CAPE (0.43 μL / band), quercetin (0.43 μL/band), kaempferol (0.43 μL /band), apigenin (0.43 μL/band) and caffeic acid (0.11 μL/band).

According to the separated compounds on HPTLC plate, major yellow zones were determined in P1, P2, P10, P11, P12, P16, P18, P19, P20 and also pale yellow zones were determined at the R_F value of 3MQ compound in P5 (3MQ-type) and P14 (3MQ-type). Besides, yellow zones both separated compounds on the HPTLC plate and at application position were determined in P2, P11, P12, P18 and P19. Only, yellow zone at application position was determined in P17. These results indicated that not only phenolic compounds separated on the plate had an antioxidant activity but also the compounds which were not separated on the plate had antioxidant capacity (Figures 12 and 13).

When different types of propolis were compared, yellow zones were mostly detected in O-type (Figure 14). That could be also because of the major contribution of galangin, caffeic acid and CAPE to propolis antioxidant activity.

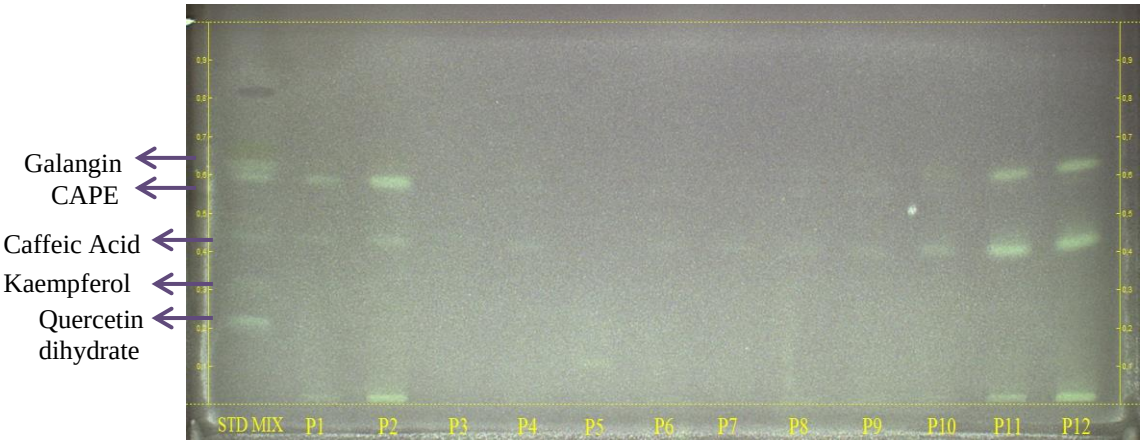


Figure 12. HPTLC- DPPH$^{\cdot}$ of P1-P12 propolis samples captured with white RT, developing solvent system *n*-hexane-ethyl acetate-glacial acetic acid (5:3:1 v/v/v), immersed in 0.1% DPPH$^{\cdot}$ solution

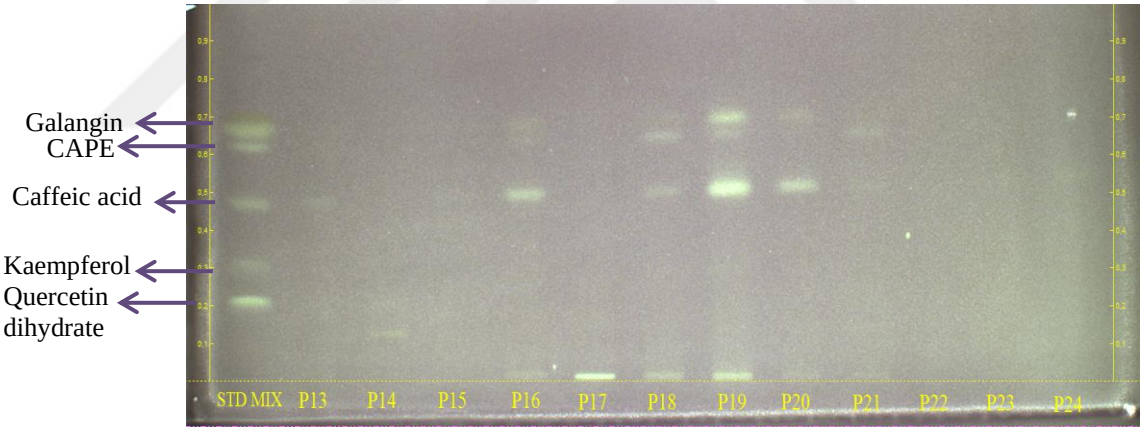


Figure 13. HPTLC- DPPH$^{\cdot}$ hydroalcoholic P13-P24 propolis samples captured with white RT, developing solvent system *n*-hexane-ethyl acetate-glacial acetic acid (5:3:1 v/v/v), immersed to 0.1% DPPH$^{\cdot}$ solution

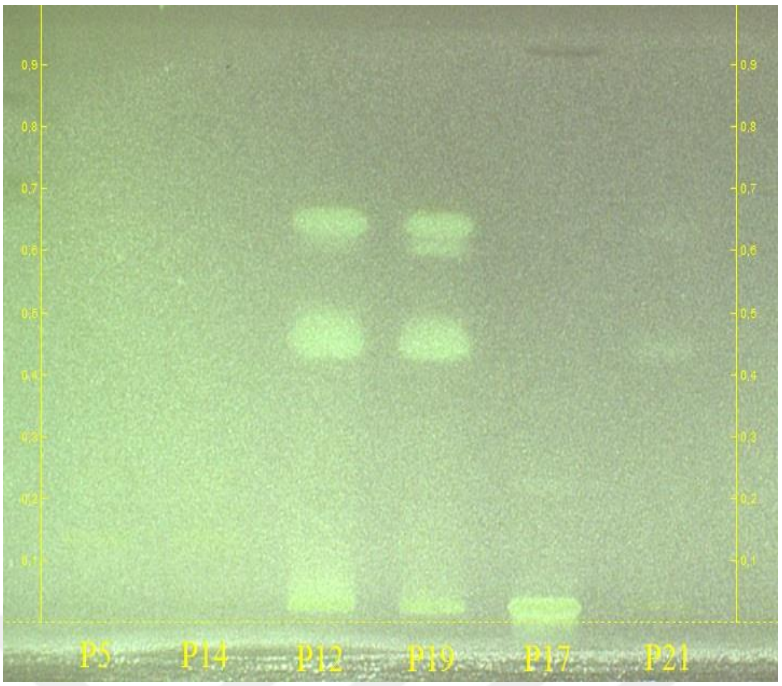


Figure 14. HPTLC- DPPH$^{\cdot}$ chromatogram of comparison of different types of propolis samples captured with white RT, developing solvent system *n*-hexane-ethyl acetate-glacial acetic acid (5:3:1 v/v/v), immersed in 0.1% DPPH$^{\cdot}$ solution

4.4. Antimicrobial Analysis

The antibacterial activity of the different type of propolis samples were evaluated by the disc diffusion method and MIC assays against the gram (+) and the gram (-) bacteria.

Ofloxacin (5 μg) which was used as a standard in disc diffusion method showed inhibition against *S. aureus*, *E. coli* (inhibition zone: 30 mm) and against *P. aureginosa* (inhibition zone: 28 mm). 100 units of nystatin was tested for antifungal activity as standard against *C. albicans* strain (inhibition zone: 32 mm) (Table 12).

P17 (B-type) at a concentration of 1024 μg/mL showed the highest inhibition zone (11 mm) against *S. aureus*. However, P5 (3MQ-type), P12 (O-type) and P22 (B-type) (1024 μg/mL) showed the least inhibition zone (8 mm) against *S. aureus*. While P17 (B-type) propolis showed the highest inhibition (11 mm) against *S. aureus* and *P. aureginosa* strains, the P14 (3MQ-type) and P19 (O-type) were found to have equal inhibitory effect (10 mm). P17 (B-type) (1024 μg/mL) showed the highest inhibition zone (11 mm) against *P. aureginosa* whereas P5 (3MQ-type), P12 (O-type) and P22 (B-type) (1024 μg/mL) showed the least inhibition zone against investigated bacteria. P17 (B-type) and P19 (O-type) (1024 μg/mL) showed the highest inhibition zone (12 mm) against *E. coli*. On the other hand, P5 (3MQ-type), P12 (O-type) and P22 (B-type) (1024 μg/mL) showed the least inhibition zone (9 mm) against *E. coli* (Table 12)

When the antifungal activity of different types of propolis was evaluated, it was found that P17 and P19 (1024 μg/mL) showed the highest inhibition zone (12 mm) against *C. albicans*. While P19 (O-type) and P17 (B-type) propolis showed equal inhibition (12 mm) against *E. coli* and *C. albicans*, P14 (3MQ-type) was found to be less effective (inhibition zone: 11 mm) than P19 (O-type) and P17 (B-type). Contrarily, P5 (3MQ-type), P12 (O-type) and P22 (B-type) (1024 μg/mL) showed the least inhibition zone (9 mm) against this fungus (Table 12).

As a result, P17 (B-type) and P19 (O-type) exerted the highest inhibition zone (12 mm) against *E. coli* and *C. albicans* at 1024 μg/mL concentration.

MIC values were determined by serial dilution methods against the same panel of bacteria and fungus. MIC of P14 (3MQ-type), P17 (B-type) and P19 (O-type) extracts against *S. aureus* and *P. aureginosa* were 256 μg/mL. Besides, MIC of P17 (B-type) and P19 (O-type) extracts against *E. coli* and *C. albicans* were 128 μg/mL.

Consequently, P17 (B-type) and P19 (O-type) showed higher antimicrobial activity than the 3MQ-type of propolis.

Table 12. Antibacterial and antifungal activities of propolis samples (inhibition zone).

Sample/Propolis Types	**Conc. of Test Sample**	**Zone of Inhibition (diameter in mm)** ***S. aureus*** **ATCC 6538**	**Zone of Inhibition (diameter in mm)** ***P. aureginosa*** **ATCC 15442**	**Zone of Inhibition (diameter in mm)** ***E. coli*** **ATCC 11229**	**Zone of Inhibition (diameter in mm)** ***C. albicans*** **ATCC 10231**
P5 (3MQ-type)	1024 µg/mL	8	8	9	9
P12 (O-type)	1024 µg/mL	8	8	9	9
P14 (3MQ-type)	1024 µg/mL	10	10	11	11
P17 (B-type)	1024 µg/mL	11	11	12	12
P19 (O-type)	1024 µg/mL	10	10	12	12
P22 (B-type)	1024 µg/mL	8	8	9	9
Ofloxacin	5 µg	30	28	30	-
Nystatin	100 units	-	-	-	32

Table 13. MIC results for P5 (3MQ-type)

Test Tube No	**Sample Solution (µg /mL)**	**Inoculums Added (µL)**	***S. aureus* ATCC 6538**	***P. aureginosa* ATCC 15442**	***E. coli* ATCC 11229**	***C. albicans* ATCC 10231**
1	1024	10	-	-	-	-
2	512	10	-	-	-	-
3	256	10	+	+	+	+
4	128	10	+	+	+	+
5	64	10	+	+	+	+
6	32	10	+	+	+	+
7	16	10	+	+	+	+
TMI*	0	10	+	+	+	+
TMS*	0	10	-	-	-	-
TM*	0	0	-	-	-	-

*TMI: Medium and inoculum; TMS: Medium and solvent; TM: Medium (+): Growth. (-): No Growth.

Table 14. MIC results for P12 (O-type)

Test Tube No	**Sample Solution (µg /mL)**	**Inoculums Added (µL)**	***S. aureus* ATCC 6538**	***P. aureginosa* ATCC 15442**	***E. coli* ATCC 11229**	***C. albicans* ATCC 10231**
1	1024	10	-	-	-	-
2	512	10	-	-	-	-
3	256	10	+	+	+	+
4	128	10	+	+	+	+
5	64	10	+	+	+	+
6	32	10	+	+	+	+
7	16	10	+	+	+	+
TMI*	0	10	+	+	+	+
TMS*	0	10	-	-	-	-
TM*	0	0	-	-	-	-

*TMI: Medium and inoculum; TMS: Medium and solvent; TM: Medium (+): Growth. (-): No Growth.

Table 15. MIC results for P14 (3MQ-type)

Test Tube No	**Sample Solution (µg /mL)**	**Inoculums Added (µL)**	***S. aureus* ATCC 6538**	***P. aureginosa* ATCC 15442**	***E. coli* ATCC 11229**	***C. albicans* ATCC 10231**
1	1024	10	-	-	-	-
2	512	10	-	-	-	-
3	256	10	-	-	-	-
4	128	10	+	+	+	+
5	64	10	+	+	+	+
6	32	10	+	+	+	+
7	16	10	+	+	+	+
TMI*	0	10	+	+	+	+
TMS*	0	10	-	-	-	-
TM*	0	0	-	-	-	-

*TMI: Medium and inoculum; TMS: Medium and solvent; TM: Medium (+): Growth. (-): No Growth.

Table 16. MIC results for P17 (B-type)

Test Tube No	**Sample Solution (µg /mL)**	**Inoculums Added (µL)**	***S. aureus* ATCC 6538**	***P. aureginosa* ATCC 15442**	***E. coli* ATCC 11229**	***C. albicans* ATCC 10231**
1	1024	10	-	-	-	-
2	512	10	-	-	-	-
3	256	10	-	-	-	-
4	128	10	+	+	-	-
5	64	10	+	+	+	+
6	32	10	+	+	+	+
7	16	10	+	+	+	+
TMI*	0	10	+	+	+	+
TMS*	0	10	-	-	-	-
TM*	0	0	-	-	-	-

*TMI: Medium and inoculum; TMS: Medium and solvent; TM: Medium (+): Growth. (-): No Growth.

Table 17. MIC results for P19 (O-type)

Test Tube No	**Sample Solution (µg /mL)**	**Inoculums Added (µL)**	***S. aureus* ATCC 6538**	***P. aureginosa* ATCC 15442**	***E. coli* ATCC 11229**	***C. albicans* ATCC 10231**
1	1024	10	-	-	-	-
2	512	10	-	-	-	-
3	256	10	-	-	-	-
4	128	10	+	+	-	-
5	64	10	+	+	+	+
6	32	10	+	+	+	+
7	16	10	+	+	+	+
TMI*	0	10	+	+	+	+
TMS*	0	10	-	-	-	-
TM*	0	0	-	-	-	-

*TMI: Medium and inoculum; TMS: Medium and solvent; TM: Medium (+): Growth. (-): No Growth.

Table 18. MIC results for P22 (B-type)

Test Tube No	**Sample Solution (µg /mL)**	**Inoculums Added (µL)**	***S. aureus* ATCC 6538**	***P. aureginosa* ATCC 15442**	***E. coli* ATCC 11229**	***C. albicans* ATCC 10231**
1	1024	10	-	-	-	-
2	512	10	-	-	-	-
3	256	10	+	+	+	+
4	128	10	+	+	+	+
5	64	10	+	+	+	+
6	32	10	+	+	+	+
7	16	10	+	+	+	+
TMI*	0	10	+	+	+	+
TMS*	0	10	-	-	-	-
TM*	0	0	-	-	-	-

*TMI: Medium and inoculum; TMS: Medium and solvent; TM: Medium (+): Growth. (-): No Growth.

Table 19. Antibacterial and antifungal activities of propolis samples (MIC)

Sample/Propolis Types	***S. aureus* ATCC 6538 (µg/mL)**	***P. aureginosa* ATCC 15442 (µg/mL)**	***E. coli* ATCC 11229 (µg/mL)**	***C. albicans* ATCC 10231 (µg/mL)**
P5 (3MQ-type)	512	512	512	512
P12 (O-type)	512	512	512	512
P14 (3MQ-type)	256	256	256	256
P17 (B-type)	256	256	128	128
P19 (O-type)	256	256	128	128
P22 (B-type)	512	512	512	512

4.5. Total Phenol Content

Gallic acid was used as a standard in the determination of TPC and a calibration curve was shown in Figure 15.

The TPC was calculated as grams equivalent to gallic acid. As stated in Table 20, TPC values were ordered from the highest to the lowest as follows: P2> P12> P11> P19> P18> P1> P16> P10> P21> P9> P20> P4> P8> P7> P17> P5> P6> P14> P13> P24> P23> P15> P3> P22.

When the TPC was examined, it was seen that the highest value was found as 172.98±8.96 mg/g GAE in P2. On the other hand, the lowest TPC value was determined as 11.24±0.66 mg/g GAE in P22.

Among the different propolis types, the highest TPC value was found as 172.98±8.96 mg/g GAE in P2 (O-type); as 63.99±3.11 mg/g GAE in P20 (B-type); as 40.43±0.92 mg/g GAE in P5 (3MQ-type).

As a result, the highest amount of TPC was found to be in O-type propolis (P2: 172.98±8.96 mg/g GAE) and at least TPC value was found in B-type propolis (P22: 11.24±0.66 mg/g GAE) (Table 20).

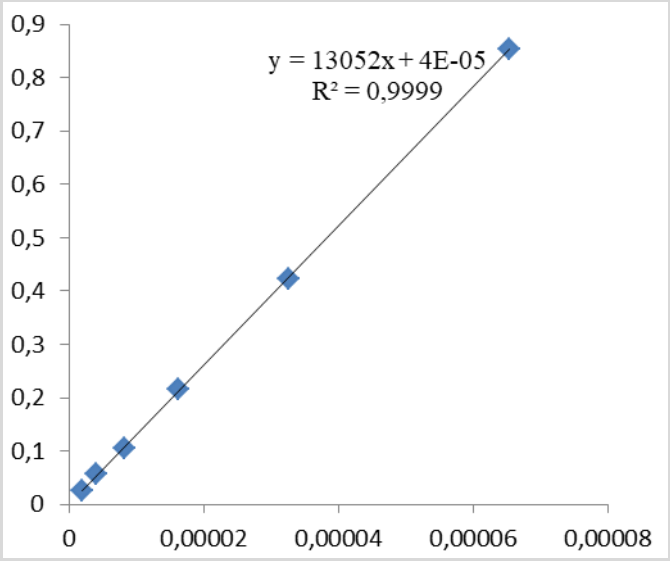


Figure 15. TPC calibration curve (Abs/Concent.)

4.6. Total Flavonoid Content

TFC was detected by $AlCl_3$ procedure and calculated as quercetin equivalent in grams. The calibration curve (Abs/Concent. µg/mL) was shown in Figure 16.

TFC values were listed from the highest to the lowest as follows: P12 > P19 > P2 > P11 > P21 > P6 > P10 > P18 > P20 > P1 > P16 > P8 > P7 > P4 > P9 > P5 > P17 > P24 >P14 > P13 > P22 > P23 > P15 > P3 (Table 20).

When TFC was evaluated, it was observed that the highest value was found as 110.78±11.02 mg/g QE in P12. On the other hand, the lowest TFC value was determined as 8.88±1.35 mg/g QE in P3.

Among the different propolis types, the highest TFC value was found as 110.78±11.02 mg/g QE in P12 (O-type); as 48.27±12.95 mg/g QE in P20 (B-type); as 26.70±2.19 mg/g QE in P5 (3MQ-type).

According to these results, O-type propolis (P12: 110.78±11.02 mg/g QE) had the highest TFC value and B-type propolis (P3: 8.88±1.25 mg/g QE) had the least TFC amount (Table 20).

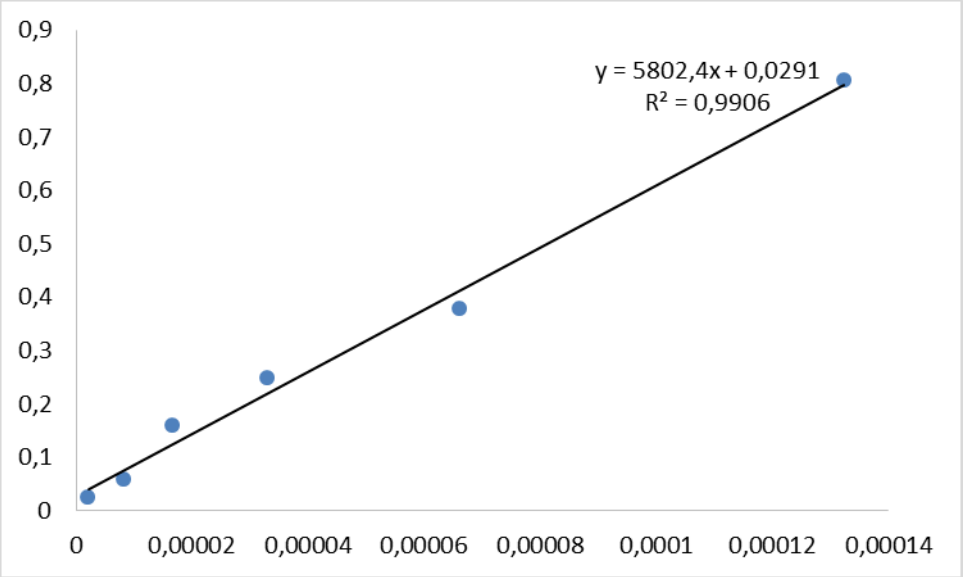


Figure 16. TFC calibration curve (Abs/Concent.)

Table 20. TPC and TFC values in propolis samples

Sample	Propolis Types	Total Phenol (mg/g GAE/Propolis)	Total Flavonoid (mg/g QE/Propolis)
P1	**O**	**98.82± 1.20**	48.05±2.07
P2	**O**	**172.98±8.96**	**90.03±6.17**
P3	B	17.36±0.24	8.88±1.25
P4	O	56.32±2.78	31.56±3.81
P5	3MQ	40.43±0.92	26.70±2.19
P6	O	39.73±0.43	51.87±6.65
P7	O	50.22±3.08	31.82±8.90
P8	O	54.89±0.62	32.47±18.59
P9	O	67.62±1.86	30.47±3.20
P10	O	88.99±2.95	49.49±4.98
P11	**O**	**138.98±9.14**	85.21±10.18
P12	**O**	**169.91±6.81**	**110.78±11.02**
P13	B	26.84±1.50	14.31±2.63
P14	3MQ	36.91±0.65	17.32±1.49
P15	B	19.06±0.80	9.75±0.83
P16	**O**	**95.47±3.76**	46.80±5.24
P17	B	47.15±3.36	22.88±2.56
P18	**O**	**122.01±3.75**	48.88±9.46
P19	**O**	**138.83±6.66**	**94.11±10.62**
P20	B	63.99±3.11	48.27±12.95
P21	O	72.00±1.41	53.57±50.68
P22	B	11.24±0.66	14.15±0.69
P23	B	19.83±0.15	12.24±1.19
P24	B	26.60±0.66	21.69±6.03

5. DISCUSSION

Chemical structure of propolis is variable and complex, while identification of its chemical composition is important for prediction of its biological activity profile. The composition of main plant source determines its major components. Due to synergistic interactions between the propolis constituents, identification of main chemical groups would be a better solution for evaluation of its biological activity instead of quantification of individual propolis components.

HPTLC is a rapid, flexible and cost-efficient technique and enables visual results of many samples on one plate before and after derivatization. In addition, HPTLC combined with bioautography determines biologically active compounds which are separated on the plate.

In this study, the phenolic profiles and botanical origins of Turkish propolis samples via HPTLC fingerprinting were determined comparatively with standard mixture solution and *P. nigra, P. tremula, P. alba* bud extracts, respectively. According to the botanical origin identification result, O- and B-types and one new type were determined. Consequently, structure elucidation studies were conducted to identify major compound belong to new type propolis. Further, HPTLC-DPPH$^{\cdot}$ assay was applied to detect potent antioxidant compounds separated on the plate. The comparative antimicrobial activity against *S. aureus, E. coli, P. aureginosa* and *C. albicans* strains was applied among propolis samples having different botanical origins. Lastly, TPC and TFC values were determined.

HPTLC fingerprinting results showed that propolis samples could be classified under three main groups according to the band colors of the separated compounds on the plates: O-type (dominant yellow-orange zones, few number of light blue zones and faded green zones), B-type (predominant blue zones) and 3MQ-type (major yellowish-orange bands). Propolis samples with standard mixture comparison and structure elucidation studies resulted that CAPE, caffeic acid, pinocembrin, galangin, chrysin and apigenin were mainly found in O-type (P1, P2, P4, P6, P7, P8, P9, P10, P11, P12, P16, P18, P19 and P21), caffeic acid was major compound in B-type (P3, P13, P15, P17, P20, P22, P23 and P24) and 3MQ, quercetin and rhamnetin were determined in 3MQ-type (P5 and P14) (Figures 1 and 2, Table 9).

Visual comparison of the HPTLC chromatograms of Turkish propolis samples together with plant bud extracts have supplied the scanning of different Turkish propolis samples having different geographical origin. Up to now, HPTLC fingerprinting results depending on the band colors of propolis phenolic compounds from Slovenia, Croatia, Serbia, Romania and Germany have been shown the existance of two different types of European propolis which were O-type originated from *P. nigra* (defined with various dominant orange colored bands together with few number of light blue and faded green bands) and B- type originated from *P. tremula* (marked with main blue bands with light orange bands) (31, 44, 45, 51, 121). Dominant orange bands are typical for flavonoids like quercetin, blue bands for caffeic acid, CAPE, galangin, feruloyl and p-coumaroyl derivatives, and green ones match with apigenin, naringenin and chrysin (31, 44, 45). In addition, Ristivojevic et al. (45) determined pinocembrin, galangin, CAPE and chrysin as specific compound for *P. nigra* bud extract. Up to now, only one research was reported conducted the authentication of Turkish propolis samples collected from different localities by using HPTLC fingerprinting. In that study, unlike the other propolis types (O- and B-types), non-phenolic propolis type was discovered (10).

In this study, 24 propolis samples obtained from different localities in Turkey was comparatively evaluated with *P. nigra*, *P. tremula* and *P. alba* plant bud extracts in order to identify their origin plants by using HPTLC fingerprinting. HPTLC fingerprints of plant bud extracts comparison with that of HPTLC fingerprints of propolis samples according to the band colors indicated that P1, P2, P4, P6, P7, P8, P9, P10, P11, P12, P16, P18, P19 and P21 were originated from *P. nigra* (O-type) whereas the botanical source of P3, P13, P15, P17, P20, P22, P23 and P24 were found to be *P. tremula* (B-type). However, P5 and P14 propolis samples were found to be neither O- nor B-types. These samples had main orange bands as O-type. However, the absence of characteristic markers such as CAPE, galangin and caffeic acid distinguished it from O-type. Therefore, the existance of this type unlike the other Turkish propolis types (O-, B- and non-phenolic types) determined by Guzelmeric et al. (10) led to perform further studies. Accordingly, the structure elucidation analyses were shown that main yellowish-orange band with R_F value at 0.15 was found as 3MQ (Figures 1 and 2). This type of propolis could be encoded as 3MQ-type due to having major 3MQ compound without O-type propolis characteristic compounds caffeic acid, CAPE and galangin. 3MQ rich this type was reported for the first time in this study. Quercetin is one of the

main dietary flavonoids possessing to a group of flavonols. It exists mainly as glycosides, however other derivatives of quercetin have been defined as well. Attached substituents alter the biochemical activity and bioavailability of molecules while compared to the aglycone (122). The effects on bioactivity of quercetin derivatives and its impact on human health have been researched up to now (123). Kumar et al. (123) conducted that 3MQ was isolated from the stem bark of *Semecarpus anacardium* and they found that 3MQ protected lung and liver cells from H_2O_2 induced cytotoxicity, ROS formation, membrane and DNA damage. Sadhu et al. (124) reported that the 3MQ compound was a prostaglandin inhibitor and had antioxidant effect. Moreover, Akkol et al. (125) also mentioned 3MQ compound exhibited significant acetylcholinesterase suppression and Bettega et al. (126) reported that 3MQ compound had antiviral and anticancer effects.

As a result of botanical origin determination among the 24 propolis samples investigated in this study, 14 propolis samples were belong to O-type; 8 samples were from B-type and 2 samples were categorized as 3MQ-type. According to these results, O-type was found to be a dominant type among Turkish propolis samples. These findings were also supported by Guzelmeric et al. (10).

Propolis samples of antioxidant activity was possibly consequence of the presence of phenolic components having *O*-dihydroxy phenyl structure that is the main structural requirement for important radical scavenging activity. HPTLC-DPPH$^{\cdot}$ is a rapid, convenient screening technique which supplies determination of separated compound on the plate with potent antioxidant activity. The HPTLC chromatogram of standard mixture after dipping with DPPH$^{\cdot}$ solution indicated that quercetin, 3MQ, kaempferol, caffeic acid, CAPE and galangin had potent antioxidant activity due to color change from purple to yellow (Fig. 13). Accordingly, Guzelmeric et al. (10) also mentioned antioxidant activity of caffeic acid, CAPE, galangin and pinobanksin after HPTLC-DPPH$^{\cdot}$ analysis. Furthermore, Sadhu et al. (124) and Schwingel et al. (127) reported that 3MQ contributed to the antioxidant effect of propolis.

Caffeic acid, kaempferol, galangin, quercetin, cinnamyl caffeate, phenethyl caffeate were mentioned as basic phenolics of propolis with high reducing power (128). Among different types investigated in this study, yellow zones were mostly seen in O-type after immersed in DPPH$^{\cdot}$ solution. Separated compounds on HPTLC plate belong

to P1, P2, P10, P11, P12, P16, P18, P19 and P20 had shown antioxidant activity (Figure 12 and 13). These yellow bands mostly correspond to CAPE and caffeic acid. Although propolis samples of P1, P2, P10, P11, P12, P16, P18, P19 and P20 contained kaempferol, quercetin dihydrate, and galangin, discoloration was not seen on their band zones that could be because of low concentration of these compounds in propolis samples. Accordingly, Guzelmeric et al. (10) reported that O-type Turkish propolis sample which was supplied from Persembe (Ordu) was found to have the highest antioxidant activity among the all tested samples.

Although there are many studies on antimicrobial activity of Turkish propolis, only few studies comparatively evaluated the antimicrobial activity of propolis samples from different botanical origins. In this study, antimicrobial activity of different types of Turkish propolis samples against *S. aureus*, *E. coli*, *P. aureginosa* and *C. albicans* were comparatively evaluated using disc diffusion and MIC assay. P17 at a concentration of 1024 µg/mL showed the highest inhibition zone (11 mm) against *S. aureus* and *P. aeruginosa* (Table 12). In addition, Kartal et al. (129) have been studied on antimicrobial activity of propolis obtained from Ankara and Muğla (Turkey) against *S. aureus*, *P. aeruginosa* and *E. coli.* They found that hydroalcoholic propolis extract (0.1 mg/mL) showed 11 mm inhibition zone against *S. aureus*. However, antimicrobial activity of these propolis extracts against *P. aeruginosa* and *E. coli* were not observed. However, Stepanović et al. (130) found that Serbian propolis high antimicrobial against indicated *S. aureus* (inhibition zone: 13 mm). Besides, Ophori et al. (131) reported that Nigerian propolis (inhibition zone: 24 mm) exerted higher antimicrobial activity against *Streptococcus mutans* at a concentration of 32 µg/mL. P17 (B-type) and P19 (O-type) exerted the highest inhibition zone (12 mm) against *E. coli* and *C. albicans* at 1024 µg/mL concentration.

According to the HPTLC results, ferulic acid and caffeic acid were commonly found in P17 (B-type) and P19 (O-type) samples. Moreover, Borges et al. (132) found that caffeic acid and ferulic acid were existence in nearly all samples with powerfull antimicrobial activity, these compounds are thought to promote antimicrobial efficacy in propolis samples. In addition, Borges et al. (132) and Mirzoeva et al. (133) were reported that ferulic and caffeic acids showed antimicrobial effects on the cell membrane, causing irreversible alterations and damage. Accordingly, it was obvious

that phenolic acids showed higher contribution to the antimicrobial effect of Turkish propolis samples than flavonoids (134).

MIC values were determined by serial dilution methods against the same panel of bacteria and fungus. MIC of P14, P17 and P19 extracts against *S. aureus* and *P. aureginosa* were 256 μg/mL (Tables 15, 16 and 17). Accordingly, Neves et al. (135) found that MIC of Brazilian red propolis extract against *S. aureus* and *P. aeruginosa* were 256 μg/mL. In addition, Georgieva et al. (136) reported that MIC of dichloromethane propolis extract from Pacific against *P. aeruginosa* was 256 μg/mL. However, they found antimicrobial effect against *S. aureus* at 128 μg/mL concentration. Besides, MIC of P17 and P19 extracts against *E. coli* and *C. albicans* were 128 μg/mL (Tables 16 and 17). Furthermore, Sun et al. (137) reported that CAPE contributed antifungal effect significantly. In addition, Georgieva et al. (138) found that MIC of dichloromethane extract of propolis from Pacific against *E. coli* and *C. albicans* were 128 μg/mL. In contrast, Stepanović et al. (130) found that Serbian propolis had no effect against *E. coli* and *P. aureginosa.*

When the TPC and TFC were examined, significant correlations were obtained. The total amount of phenolic compounds was determined from 24 propolis extracts. The TPC was calculated as gram equivalent to gallic acid. As stated in Table 20, among the different types of propolis samples the highest TPC value in O-type was found as 172.98 ± 8.96 mg/g of GAE for P2; the highest TPC value in B-type was found as 63.99±3.11 mg/g of GAE for P20; the highest TPC value was found as 40.43±0.92 mg/g of GAE for P5 in 3MQ-type (Table 20). Among the different types of propolis samples evaluated in this study, it was concluded that the highest TPC amount was found in O-type and the least was found in B-type propolis. Ristivojevic et al. (134) also indicated that O-type of Turkish propolis had higher antioxidant activity than the other types (B-type and nonphenolic type). On the other hand, Moreira et al. (138) Portuguese propolis samples from Bornes region exhibited higher TPC values (329 mg/g of GAE) than propolis samples investigated in this study. In addition, higher TPC values were also found in Chinese samples from Hebei, 302 ± 4.3 mg/g of GAE (139), and Hubei, 299 ± 0.5 mg/g of GAE (128); and Korean propolis from Yeosu, with 212.7 ± 7.4 mg/g of GAE (140) than propolis samples in this study. In addition, TPC values of propolis obtained from Canada and Brazil showed 199.35 mg of GAE/g and 120 ± 3.5 mg/g of GAE (140, 141). Andrade et al. (142) also found that among the red, green and brown

propolis samples prepared hydroalcoholic (%70 EtOH-H2O) extract taken from Brazil (Alagoas and Sergipe), the highest TPC value was seen in red propolis as 91.32 ± 0.49 mg/g of GAE. In contrast, TPC value of Thailand propolis and Canada propolis showed respectively 31.2 ± 0.7 mg/g of GAE and 65.92 mg of GAE/g (128, 141). Moreover, Barra et al. (143) reported TPC values of ethanolic propolis extract taking from Santiago (Buin and Caleu) were 36.4±0.6 and 14.6± 0.4 mg/g of GAE. Miguel et al. (144) also found TPC value of hydroalcoholic propolis extract prepared from Portugal (Algarve) was found to be 6.27 ± 0.19 mg/g of GAE. In addition, Francisco et al. (145) found that TPC value of hydroalcoholic Brazilian propolis extract from Parana was 100.7 ± 6.47 mg GAE/g.

TFC was detected by $AlCl_3$ procedure and calculated as quercetin equivalent in grams. As stated in Table 20, the highest TFC value was found in P12 (110.78±11.02 mg/g QE). Amoung different types of propolis, the highest TFC value was found as 110.78±11.02 mg/g QE in P12 (O-type), 48.27±12.95 mg/g QE in P20 (B-type) and 26.70±2.19 mg/g QE in P5 (3MQ-type) (Table 20). Ristivojević et al. (134) was also concluded that O-type Turkish propolis had higher TFC values than the other types.

TFC value of Turkish propolis was found to be the highest in O-type and the lowest in non-phenolic group by Ristivojević et al. (134), supporting the results found in this study. It has also been reported that the TFC of Turkish propolis in this study was found to be higher when compared with ethanolic extract of Japanese (Okayama) (18.3±1.2 mg/g QE) and Chinese propolis (Yunnan) (8.3±3.7 mg/g QE) (139, 146). In addition, Barra et al. (143) found that TFC values of ethanolic propolis extracts obtained from Chile (Santiago-Buin) and Santiago-Caleu were 14.8±0.4 mg/g QE and 2.1 ±0.2 mg/g QE, respectively. In addition, Andrade et al. (142) found that TFC value of green hydroalcoholic propolis extract showed the highest amount (59.45 ± 0.82 mg/g QE) when compared with red and Brown.

6. CONCLUSION

Turkey has different climates due to its geographical location. It covers intersections of European-Siberian, Iranian-Turan and Mediterrenean regions. As a result of this condition, it is rich in plant diversity. Up to now, almost 10.000 plant species have been found in Turkey.

Propolis is prepared by bees from the secretions collected from plant parts. Eventually, propolis samples collected from different locations are composed of different plant origins, therefore each propolis sample may be composed of different active materials or same active compound at different concentrations. Identification of chemical composition and active compound concentration related with biological activity is very important for the production of healthcare materials and therefore standardization is essential. Since there has not been approved an obligatory standardization method for propolis samples, the propolis products on the market lacks chemical component analysis and standardization. It is essential to use analyzed propolis samples for the expected biological activity.

In this study, phenolic profiles, botanical origins, antioxidant and antimicrobial activities, TPC and TFC values of Turkish propolis from different localities were evaluated. Phenolic profiles of propolis samples with different botanical origins were determined by HPTLC analysis. In addition to the three known (O, B and nonphenolic type) Turkish propolis types, the fourth 3MQ-type of propolis was discovered for the first time in this study. *P. nigra* bud extracts indicated a similar structure substantially to the O-type samples that is most likely to represent the origin of this type. The B-type was found to be associated with a certain extent with the a *P. tremula*. Unlike the other Turkish propolis types, HPTLC analysis showed that 3MQ-type of propolis had extraordinary chemical profile containg the major 3MQ compound. O-type was found to be dominant type among the analyzed propolis samples and showed nearly all phenolic standards in the standard mixture. Considering the separated bands on the HPTLC-DPPH$^{\cdot}$ plate, it was seen that the antioxidant effect of the O-type propolis was the greatest. Most studies have shown that propolis extracts has a broad spectrum of activity against gram positive (+) bacterial strains, while the effect on gram negative (-) bacteria is weaker. However, propolis samples in this study showed broad spectrum of activity against gram (-) strains. The antimicrobial effect against *S. aureus*, *P.*

aureginosa, E. coli and *C. albicans* was determined by disc diffusion and MIC assay. The most active extract against *E. coli* was P17 (B-type) and P19 (O-type) (inhibition zone: 12 mm). The antifungal activity of the extracts were tested against *C. albicans* using nystatin as a standart. P17(B-type) and P19(O-type) exerted the highest antifungal activity agains *C. albicans* (inhibition zone: 12 mm). MIC values were determined by serial dilution methods against the same panel of bacteria and fungus. The MIC of the P17(B-type) and P19(O-type) against *E. coli* and *C. albicans* was 128 μg/mL respectively. Lastly, TPC and TFC values were evaluated and high similarity between the TPC and TFC results was determined. As a result, the highest and the least TPC and TFC values were found in O- and B-type of propolis, respectively.

In conclusion, within the scope of this study it was clearly determined that which type of propolis that is going to be prepared in pharmaceutical formulations would be more effective in terms of human health.

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