INVESTIGATION OF BIOACTIVITY AND CHEMICAL CONTENT OF TURKISH PROPOLIS FROM ANKARA PROVINCE

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ABSTRACT

INVESTIGATIN OF BIOACTIVITY AND CHEMICAL CONTENT OF TURKISH PROPOLIS FROM ANKARA PROVINCE

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Propolis is one of the important bee product which is a sticky resin collected by bees. Honey bees (*Apis mellifera*) collect and metabolize the propolis, then line their hives with it to protect the hive. It is so effective that the interior of bee hive has been found to be safer than most operating rooms. The name propolis derived from the Greek meaning (before the city) signifying that propolis defended the entire community from external threat. Propolis has a 5000- years of history. The Asyrians and Greeks used propolis to help maintain good health and the Egyptians used in mummification.

In this study it was aimed to characterize propolis produced by bees dwelling in Ankara (Mamak-Kıbrıs village), focusing on the determination of antioxidant potential, antimicrobial activity, cytotoxic activity and its chemical content. The propolis was obtained from a local beekeeper from Ankara province of Turkey and extracted with ethyl alcohol. The yield of extraction was found as 20.30 ± 0.01 % (w / w). The phenolic composition of ethanolic extract of propolis and

possible effects of those constituents in antimicrobial activity and cancer were investigated. The propolis extract showed high Radical Scavenging Activity against DPPH and ABTS radicals. Total flavonoid content was found as 0.742 ± 0.014 mg QE / mg extract and total phenolic content was 10.712 ± 0.007 mg GAE / mg extract. Detected phenolics were including p-coumeric acid, caffeic acid and vanillic acid also quantitated and validated with LC / MS.

Antimicrobial potential of propolis screened on bacterial and fungal strains, and promising activity is detected on fungal a strain. Cytotoxic activity of propolis checked by MTT assayfor dose and time-dependancy.

Keywords: Propolis, Phenolic, flavonoids, antioxidant.

ÖZ

ANKARA İLİNDEN ELDE EDİLEN TÜRK PROPOLİSİNİN KİMYASAL İÇERİĞİ VE BİYOAKTİVİTE İNCELEMESİ

Yurteri, Ümmügülsüm
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Önemli bir arı ürünü olan propolis, arılar tarafından bitkilerin farklı kısımlarından toplanarak elde edilir. Bal arıları (*Apis mellifera*) propolisi toplayıp metabolize ettikten sonra kovanlarını dış etkenlerden korumak için propolisle kaplarlar. Bu çok etkili madde sayesinde kovanın içi tamamen sterildir. Propolis adını antik Yunandan alır ve „şehir önü „anlamındadır. Propolisin 5000 yıllık tarihi olduğu düşünülmektedir. Asurlar ve Yunanlılar propolisi sağlıklarını korumak için Mısırlılar ise mumya yapımında kullanmışlardır.

Bu çalışmada amaç olarak, Ankara propolisinin biyoaktivitesi, yüksek ısının propolis üzerine etkisi, sitotoksik aktivitesi ve kimyasal içeriğinin belirlenmesine odaklanılmıştır. Bu amaçla, kullanılacak olan propolis Ankara"dan (Kıbrıs Köyü) yerel bir arıcıdan temin edilmiş ve etil alkol ile ekstraksiyonu yapılmıştır. Ekstraksiyon verimi 20.30 ± 0.001 % (w / w) olarak hesaplanmıştır. Propolisin etil alkol ekstresinin fenolik içeriğin ve bu içeriğin olası antimikrobiyal aktivite ve kanser üzerine etkileri araştırılmıştır. Propolis ekstresi DPPH ve ABTS

radikallerine karşı yüksek sönümleme aktivitesi göstermiştir. Toplam fenolik içerik 10.7 ± 0.007 mg GAE / mg ekstre ve toplam flavonoid içerik 0.742 ± 0.014 mg QE / mg ekstre olarak hesaplanmıştır. Önemli fenolik asitler ve bu asitlerin miktarı , p-kümerik asit, kafeik asit ve vanilik asit, LC /MS / MS kullanılarak tayin edilmiştir.

Ankara propolisinin antimikrobiyal potansiyeli bakteri ve mantar (fungus) türleri üzerinde test edilmiş ve en yüksek aktivite *C. albicans* türü üzerinde belirlenmiştir. Propolisin sitotoksik aktivitesinin tespiti için MTT yöntemi zamana ve uygulanan doza bağlı olarak ölçülmüştür.

Anahtar kelimeler: propolis, antioksidanlar, fenolik maddeler, flavonoid maddeler, sitotoksik aktivite.

To my parents: Meryem & Hamdi YURTERİ

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LIST OF ABBREVIATIONS

ABTS	2, 2‟-azinobis-(3-ethylbenzothiazoline-6- sulfonic acid)
CRP	C-reactive protein
DMSO	Dimethyl sulfoxide
DPPH	1, 1-diphenyl-2-picrylhydrazyl
EEP	Ethanol Extract of Propolis
EGCG	Epigallocatechin-3-gallate
ER-α	Estrogen receptor alpha
ER-β	Estrogen receptor beta
FBS	Fetal Bovine Serum
GAE	Gallic Acid Equivalent
IC50	Half Maximal Inhibitory Concentration
IL-6	Interleukin 6
LDL	Low- density lipoprotein
MBC	Minimum Bacteriocidal Concentration
MHB	Mueller Hinton Broth
MIC	Minimum Inhibitory Concentration
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NIDDM	Noninsulin-Dependent Diabetes Mellitus

PBS	Phosphate-Buffered Saline
QE	Quercetin Equivalent
RSA	Radical Scavenging Activity
TEAC	Trolox Equivalent Antioxidant Activity
TNF-α	Tumor Necrosis Factor alpha

CHAPTER 1

INTRODUCTION

Bees are insects and they are taxonomically ranked as Hymenoptera and have relation with wasps and ants. (Oldroyd *et al*., 2006). In addition to their significant economical role they have ecological importance in the pollination of plant species. (Pyrzynska and Biesaga, 2009).

Bee classification depends on the morphological differences such as size, color, length of tongue, the shape of wax gland, structure of blood vessels on wing, size of wing, type of hair on bodies. Taxonomic researches show that there are 24 bee species in the world. In Turkey there are 4 different species of bee, they are *Apis mellifera lugistica*, *Apis mellifera carnica*, *Apis mellifera caucasica*, *Apis mellifera anatoliaca* (Kumova *et al*., 2012).

Apis mellifera anatoliaca spp. (**Figure 1.1**) is a typical Anatolian bee and has subspecies. It is dark colored, small body sized bee which is resistant to harsh winter conditions and it has high yield in both honey and youngsters.

Figure.1.1 *Apis mellifera anatoliaca spp***.**

Honey bees harvest resins with antimicrobial properties from various plant species and bring them back to the colony where they are then mixed with varying amounts of wax and utilized as propolis.

The term „Propolis" comes from Greek pro for "in front of" „at the entrance" and polis for „community or city" and means a defence for hive (Basim *et al*., 2006).

The honeybees collect this resinous product from buds and leaves of trees and other plants, mixing with polen and resin as well as enzyme β-galactosidase that is secreted from salivary glands of bees, partially digested and wax added to form the final product (Umthong *et al*., 2011).

The content of wax in propolis changes apparently linked to genetic factors (Custodio *et al*., 2003).

It has been thought that propolis have an impact in the social immunity of honeybees, decresing possibility of infection and parasite transmission to the hive. It could be said that propolis is „self-medicator" of bee colonies (Simone-Findstrom *et al*., 2014). Propolis extracts have activity against honeybee parasites, such as Paenibacillus larvae, the harmful mite *Varroa destructor* and the wax moth *Galleria mellonella* (Garadew *et al*., 2002, Antunez *et al*., 2008; Garadew *et al*., 2008).

Although inner conditions of hive, temperature (34 °C) and the humudity (40- 65 %) constitute a suitable environment for microorganisms to grow up, pathogenic incidents could not evolve because of propolis (Wilson *et al*., 2015).

In doing so, they give the whole colony a form of "social immunity", which lessens the need for each individual bee to have a strong immune system (**Figure 1.2**).

Figure1.2 Honeybees store propolis inside their hives.

In the production of propolis, honeybees may cut fragments from vegetative tissues of plants (Salatino *et al*., 2005). The term „propolis" does not have specific chemical connotation unlike the scientific name of a plant species. Propolis consists of secondary plant metabolites, but they are produced by using different plant species and are not the same through out the world (Bankova *et al*., 2014).

1.1. History of Propolis

Propolis uses has a long history by humans for example Egyptians used it for making embalment because it was acting as plastic material that protected the mummy from viruses,bacteria and fungi. Propolis first discovered by Greeks and used as a natural antibiotic. In ancient times Europians, North Africans and Romans used for both prevent and cure diseases (Castolda *et al*., 2002).

Modern science concentrate on propolis after 1960's. Over last 50 years, several studies have done to understand biological activities of propolis: antioxidant, antimicrobial, cytotoxic antifungal, antiviral, immune modulatory, anti-inflammatory effects (Burdock, 1998). The commercial value of propolis is increasing recently, it is used as food additives, cosmetics and over-the-counter preparations (Bankova *et al*., 2014).

1.2. Chemical Content and Structure of Propolis

Propolis originates from plants, in different parts of the world the type of plants might vary regarding the local flora. Bees gather the resinous plant outfit, in various parts of plants such as; lipophilic substances on leaf buds and leaves, mucilages, gums, resines etc. mix with wax to produce final product-propolis (Crane, 1988).

In propolis more than 300 compounds have been determined. Basic groups among these compounds are phenolics, flavonoids, terpenes, lipid and wax materials, beeswax, microelements and other constituents (vitamins, proteins, aminoacids, sugars) (Kedzia and Holderma-Kedzia, 1991; Bankova V, 2005; Geckil *et al*., 2005).

Table 1.2.1. Chemical composition of propolis (Sawicka *et al*. 2012).

Compounds (percentage of content)		
Fatty and aliphatic acids (24-26%)	**Flavonoids (18-20 %)**	**Microelements (0.5 -2 %)**
Butanedioic acid (succinic acid)	Astaxanthin	Aluminium (Al)
Propanoic acid (Propionic acid)	Apigenin	Copper (Cu)
Decanoic acid (Capric acid)	Chrysin	Magnesium (Mg)
Undecanoic acid	Tectochrysin	Zinc (Zn)
Malic acid	Pinpbanksin	Silicon (Si)
D-Arabinoic acid	Squalene	Iron (Fe)
Tartaric acid	Pinostrobin chalcone	Manganase (Mn)
Gluconic acid	Pinocembrin	Tin (Sn)
a-D-Glucopyranuranic acid	Genkwanin	Nickel (Ni)
Octadecanoic acid (Stearic acid)	Galangin	Chrome (Cr)
Haxadecanoic acid	Pilloin	
b-D-Glucoyranuronic acid	Acacetin	
9,12-Octadecanoic acid	Kaemferide	
Tetradecanoic acid	Rhamnocitrin	
Pentadecanoic acid	7,4‟-dimethoxyflavone	
Glutamic acid	5-hydroxy-4‟7-dimethoxyflavone	

2,3,4-Trihydroxy butyric acid	5,7-dimethoxy-3,4‟dihydroxyflavone	
Phophoric acid	3,5-dihydroxy-7,4‟-dimethoxyflavone	
Isoferulic acid	**Sugars (15-18 %)**	**Others (21-27 %)**
	Sorbopyranose	Cyclohexanone
	D-Erytrotetrofuranose	3-methyl,antitricyclo-3-en 10-one
	D-Altrose	Cyclohexane
	D-Glucose	Cyclopentane
	Arabinopyranose	5-n-propyl-1,3 dihydroxybenzene
	d-Arabinose	Butane
	a-D-Galactropyranose	2(3H)-Furane
	Maltose	L-Proline
	a-D-Glucopyranoside	2-Furanacetaldeyde
	D-Fructose	2,5-is-3-phenyl-7-pyrazolopyrimidine
Aromatic acids (5-10 %)		Cliogoinol methyl derivative
Benzoic acid		Fluphenazine
Caffeic acid	**Esters (2-6 %)**	4,8-Propanoborepinpxadiborole
Ferulic acid	Caffeic acid phenethyl ester	1,3,8-Trihydroxy-6-methylanhraquinone
Cinnamic acid	4,3-Acetyloxycaffeate	1-5-oxo-4,4-diphenyl-2-imidazolin-2-yl guanidine
	Cinnamic acid	3,1,2-Azaazoniaboratine /Piperonal
	3,4- dimethoxy-trimethylsilyl ester	3-Cyclohexene
	3-Methoxy-4-cinnamate	1H-Indole
Alcohol and Terpenes (2- 3.3 %)	Cinnamic acid 4 methoxy 3 TMS ester	1H-Indole-3-one
Glycerol		2-Furanacetaldehyde
Erythritol		Guanidine
a-Cedrol	**Vitamins (2-4 %)**	2(3H)Furanone
Xylitol	A, B (1,2), E,C,PP	1,3,8-trihydoxy-6-meyhylanthraquinone

In general, the main components of propolis are fatty, aromatic and aliphatic acids, flavonoids, alcohols, terpenes, sugars and esters (**Table 1.2.1.**). Numerous studies have proved that the variaton in percentages of individual constituents of propolis, with respect to the type of the plants from which the resin is obtained (Daugch *et al*., 2008, Nieva *et al.,* 1999) and the species of bees (Silici and Kutluca, 2005).

In terms of the amount and type of propolis, phenolics comprise the most numerous group of components. Phenolic compounds, in propolis, contain

phenolic acids, phenolic aldehydes, phenols and their esters, ketophenols, coumarins and other composites, that include eugenol, hydroquinone, naphtalene, etc. (Olczyk *et al*., 2007; Kedzia *et al*., 1991; Popova *et al*., 2003).

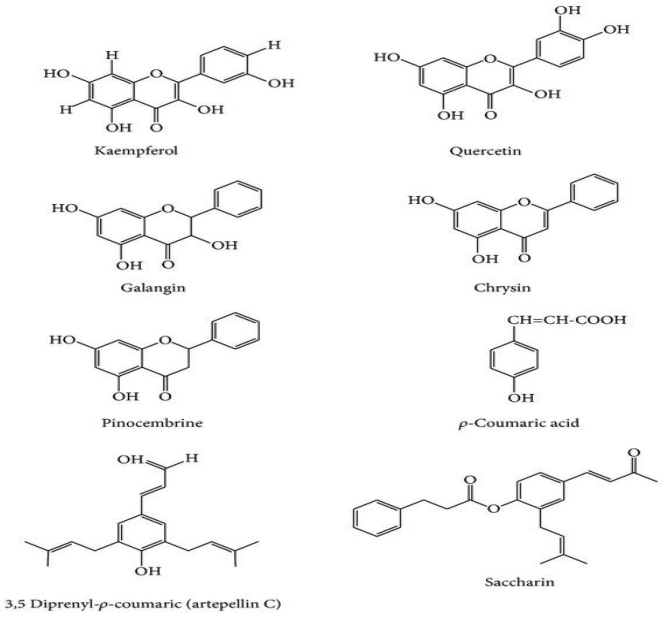

Figure 1.2.1. Some typical flavonoids and phenolics present in propolis.

Phenolic alcohols, in propolis, contain benzyl and cinnamyl alcohol, also coniferyl alcohol, coumaryl alcohol and cyclohexanol. Phenol alcohols constitute esters, which have large numbers in propolis (Kedzia B. 2009). Acetophenone and methylacetophenone are the ketophenols found in propolis. Phenolic aldehydes include vanillin and isovanillin, benzoic, cinnamic, p-coumeric and coniferyl aldehyde are determined (Kedzia B. 1991).

Other important class of chemicals found in propolis are flavonoids. These substances have multiphenol behaviour. Dobrowolski *et al.* (1991) identified 38 flavonoids in propolis. Flavonoids available in propolis are aglycones of

glycosidic compounds found in plants. While gathering propolis, bees secrete β-glucosidase that break down glycosidase of flavonoids to the aglycones and sugars (Kedzia *et al.,* 2006; Sahinler and Kaftanoğlu, 2005).

Ethanolic extract of propolis (EEP) consists of approximately six to nine flavonoids, e.g. tectochrysin, pinocembrin galangin, apigenin, kaempferol (Maciejewicz, 2001). Apart from two fundamental groups of chemicals, phenolic acids and flavonoids, terpenes are found in propolis. Terpenes which are isolated from propolis are monoterpenes, sesquiterpenes and triterpenes. Although triterpenes are squalene and glutinol, monoterpenes are found (Bankova *et al*., 2000; Volpi, 2004; Ellnain-Wojtaszek *et al*., 1992).

Moreover, propolis contains plant waxes or lipid-wax. These type of wax are consist of sterols, fatty acids and their esters, particularly phenolic acids and glycerol esters (Maciejewicz *et al*., 1983).

The total content of protein in EEP amount is on the awarage 2.8%, avaragely (Kedzia, 2009). Free aminoacids are found -17 of them- in lower content (Kedzia, 1991). Propolis have polysaccharides in its composition such as starch, di- and monosaccharides for instance saccharose, gluose, fructose, rhamnose, ribose, talose and gulose (Kedzia *et al.,* 2006). In terms of type and amount, the most valuable components of propolis are polphenols. These substances have strong antioxidative and biological activity. The antioxidative effect of polyphenols change with respect to their structure (Geckil *et al*. 2005; Scheller *et al*. 1990).

1.2.1. Phenolic Acids

Phenolic acids are compounds containing a benzene ring, carboxyl and hydroxyl groups and are found in two different groups; hydroxy cinnamic acids and hydroxybenzoic acids. In general they are not found in free from, carboxyl group of phenolic acids can react with carbohydrates, glycosides, aminoacids and proteins. The hydroxyl group of phenolic acids are highly active and can react

with sugars to form glycosides. The power of the antioxidative activity of these substances depends on the position of hydroxyl groups, as well as the type of substitution on the aromatic ring. (Leja *et al*., 2007; Rice-Evans *et al*., 1996; Budryn and Nebesny, 2006).

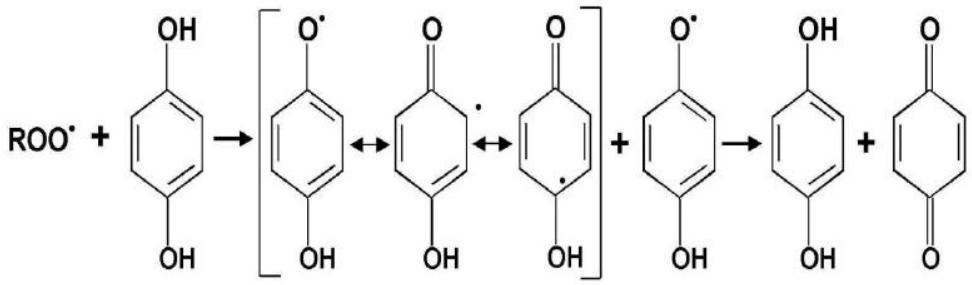

Figure 1.2.1.1 Oxidation of phenolic compounds (Budryn and Nebesny, 2006).

When the hydroxyl groups appear in the position 3 and 5, benzoic acid dihydroxy derivatives display powerful antioxidative activity. Among benzoic acid derivatives, gallic acid, has very good antioxidative properties. Esterification of the carboxyl group in gallic acid decreases its antioxidant abilities (Rice-Evans *et al*., 1996; Budryn and Nebesny, 2006; Silva *et al*., 2002).

According to Rice-Evans *et al*. (1996) antioxidative activity of phenolic acids: caffeic acid > ferulic acid> p-coumaric acid. The antioxidant activity of phenolic acids containing "scavenging" free radicals (superoxide, hydroxyl and hydroxyl superoxide ones), chelating ions of metals (iron, copper), and changing the activity of enzymes by blocking the activity of oxidases (Cos *et al*., 2002).

1.2.2. Flavonoids

Flavonoids (**Figure 1.2.2.1**.) are large group of polyphenols, which changing depend on the structure and properties. They have C5-C3-C6 diphenylpropan

structure and have three Carbon bridge between phenyl groups which bonded with oxygen. The difference between flavonoid groups depending on the number of hydroxyl group, the degree of substituton and the degree of oxidation. Flavonoids are the most important phenolic compounds, there are six different class of flavonoids depending on the structure; antocyanidins, flavonols, flavanes, flovones, catechins, isoflavonoids (Olszewska, 2003; Kohlmünzer, 2003; Makowska and Janezko, 2004).

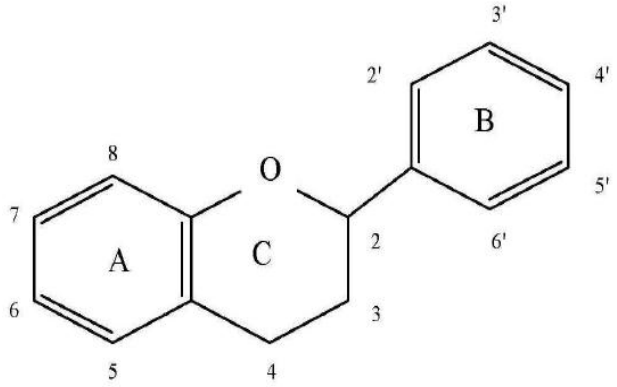

Figure 1.2.2.1. Basic structure of flavonoids (Heim *et al*., 2002).

The antioxidative properties are also decreased by the presence of methoxhyl groups in the C-3 position of a flavonoid, probably as a result of steric hindrance (Heim *et al*., 2002; Ostrowska and Skrzydleska, 2005; Dugas *et al*., 2000). The most powerful antioxidative properties are display by flavon-3-ols (e.g –quercetin, myrycetin, morin), flavan-3-ols-catechins (epicatechin gallate, epigallocatechin gallate, epigallocatechin, catechin) antocyanidins (cyanidin) (Rice-Evans *et al*., 1996; Heim *et al.,* 2002).

1.3. The Biological Properties of Polyphenols

The antioxidative activity of poylphenols is their most admired feature. A broad range of biological activity of them to on the human body results from their antioxidative activity (Gulcin, 2012; Harborne and Williams, 2000). Polyphenols are essential exogenous antioxidants. When enter the body with food, they are absorbed as unchanged, or they are metabolized via hydroxylation, methylation, sulfation and glucuronidation. The bioavailability of polyphenols depends on the kind of food consumed. Although proteins, when bind to them, decrease absorption, alcohol increases it. Ingested polyphenols are mainly utilized by the intestinal bacterial flora (Makowska and Janeczko, 2004; Majewska and Czeczot 2009; Ostrowska and Skrzydlewska. 2005).

The mechanism of the antioxidative activity of polyphenols are as followings (Makowska and Janeczko 2004; Majewska and Czeczot, 2009; Pietta, 2000; Silva *et al*., 2002):

-Inhibiting the activity of some enzymes and so inhibiting the formation of the reactive oxygen species (ROS),

-Chelating ions of metals in the process of free radical scavenging,

-Scavenging reactive oxygen species (ROS), hence inferring with the cascade of reactions of lipid peroxidation.

1.4. Antimicrobial Activity of Propolis

It has been proven that the chemical variations in propolis with different origins lead to different biological activities (Bankova *et al*., 2014). Several scientists have proved the effect of propolis volatiles against various kinds of microorganisms. Bacteria include Gram-positive bacteria such as *Staphylococcus aureus, Bacillus subtilis, Bacillus cereus, Sarcina lutea, Streptococcus pyogenes, Steptococcus mutans, Streptococcus faecalis* (Petri *et al*., 1988; Kujumgiev *et al*.,

1999; Bankova *et al*., 1998; Melliou *et al*., 2007; Oliveira and Murga, 2010; Simionatto *et al*., 2012; Hames-Kocabas *et al*., 2013; Damianova *et al.,* 1999) also Gram-negative bacteria: *Escherichia coli, Enterobacter cloacae, Klesiella pneumonie, Pseudomonas aeruginosa* (Petri *et al.,* 1988; Melliou *et al*., 2007; Oliveira and Murga, 2010; Simionatto *et al*., 2012; Hames-Kocabas *et al.,* 2013; Damianova *et al*., 1999).

Several authors accept that alcoholic extracts of propolis are either have no activity or relatively low activity against Gram-negative bacteria on the other hand propolis essential oils have reasonable activity against both Gram-positive and Gram-negative bacteria (Bankova *et al*., 2014). Propolis volatiles are effective also against non-pathogenic fungi and fungal human pathogens *Aspergillus niger, Saccharomyces cerevisae, Candida albicans, Candida tropicalis, Candida glabrata*, *Cladosporium cladosporioides, Cladosporium sphaerospernum* (Perti *et al.,* 1988; Melliou *et al.,* 2007; Simionatto *et al*., 2012; Hames-Kocabas *et al*., 2013). Propolis shows synergetic behaviour with different antituberculotic drugs (streptomycin, rifamycin, isoniazid) and antibiotics (e.g. chloramphenicol, gentamicin, vancomycin, tetracycline, clindamycin, netilmicin) (Krol *et al*.,1993). Propolis may also show synergistic effects with antimicrobial drugs (Oksuz *et al.,* 2005). The antibacterial impact of propolis may be the result of the synergistic behaviour of the many compounds found in propolis. Pinocembrin acts an antibacterial against *Streptococcus spp*. Apigenin inhibits bacterial glycosyltransferase enzyme. P-coumaric acid, artepillin C and 3-phenyl-4-dihyrocinnamyllocinnamic acid are impressive against *Helicobacter pylori* (Marcucci, 1995).

Experimental studies on the therapeutic impact of propolis to bacteria cultivating from burn wounds, demonstrated that inhibit the development pathogens and higher effectiveness than 1% silver sulfadiazine (SSD) (Kabala-Dzik *et al*., 2003). Propolis has an inhibitory impact on *Staphylococcus aureus* strain which cause nosocomial infections having high antibiotic resistance which exists after the use of chemotherapeutics (Wojtyczka *et al*., 2013).

1.5. Anti-inflammatory Effect of Propolis

Phenolic acids and flavonoids have anti-inflammartory activity because of their antioxidative properties (Yao *et al*., 2004; Bogdanov, 2013). There is a correlation between reducing the synthesis of prostaglandin E2 (PGE2), thromboxane A2, leukotriene B4, NO (nitric oxide II) and anti-inflammatory activity (Tapas *et al*., 2008; Rosa *et al*., 2001; Iravani and Zolfaghari, 2011).

Because of high content of polyphenol groups, propolis display anti-inflammatory action on both acute and chronic inflammation (Borelli *et al*., 2002). Caffeic acid phenethyl ester (CAPE) is a valuable inhibitor of the enzymes of arachidonic acid metabolic pathway and has an effect on cyclooxygenase activity leading to curing of anemia (Mirzoeva *et al.,* 1996; Rossi *et al*., 2002).

Chrysin, kaempferol, quercetin and galangin, in the content of propolis, have effects on mRNA synthesis, but quercetin has the highest activity among them (Bloǹska *et al*., 2004). It was shown that propolis has the same anti-inflammatory activity with non-steroidal anti-inflammatory drugs and it does not cause any side effects (Reis *et al.,* 2000). Park and Kahng (1999) found that propolis display anti-inflammatory activity because of prostaglandin inhibiton on rats according to the arthritis model. By means of macrophage activation and the inhibition of NO (nitric oxide) propolis extracts create non-specific immunological response (Orsi *et al*., 2000).

Aqueous extract of propolis has inhibitory impact on cell migration, this can promote the control of the inflammation process without affecting the cell repair because of the high amount caffeic acid (Moura *et al*., 2011). Propolis extract decline the level of IL-6, TNF-α and CRP, this derive the rising in the activity of hepatic enzymes, bilirubin levels and lipid parameters in the action of inflammation and toxic liver deterioration (Ahmed *et al*., 2012).

1.6. Anticarcinogenic Effects

In the content of propolis there are lots of biologically active materials such as caffeic acid, caffeic acid phenyl ester, artepillin C, quercetin, naringenin, resveratrol, genistein and galangin that are promoting the stimulation of cell proliferation or apoptosis (Diaz-Carballo *et al*., 2008). Flavonoids consumed with with food has a certain effect on cell proliferation, differentiation and apoptosis of cancer cells particularly on gastrointestinal type of cancer cells as a result of direct relationship with ingesta. Flavonoids which are in the content of propolis have significant cytotoxic effect on breast cancer and papillomaous urinary tract tumors (Benkovic *et al,*. 2008). Flavonoids in propolis block the proliferation of different kinds of cancer cells, specifically monocytic and lymphatic leukemia (Lugli *et al*., 2009).

The anticancerogenic action involves in the blockage of tyrosine kinase C that takes part in the growth and proliferation of cancer cells (Sánchez *et al*., 2008). Some flavonoids, present in propolis, block cyclin D1 during the cell cycle in cancer cells such as genistein, querctin, kaempferol, luteolin, chrysin and apigenin (Zhang *et al*., 2009). Galangin, genistein, naringenin and resveratrol show antiproliferative effect on breast cancer estrogen receptor (Qin *et al*., 2009). Animal experiments show that flavonoids that are present in propolis stop the development of lung cancer and oral cavity cancer, skin, esophagus, stomach, colorectal, prostate and breast cancer (Orsolić *et al.,* 2007).

In 1998 Kimoto *et al*. established that artepilin C acts cytostatic and cytotoxic effect on cancer cells over immunostimulation via activating macrophages, by stimulating their phagocyctic activity. It is proven that hydroxycinnamic acids-ferulic and caffeic acid – inhibit the growing of cancer cells and appearance of mutagenic nitrosamines (Slavin *et al.,* 2000).

1.7. Antiatherogenic Effects

Flavonoids prevent blood platelets aggregation, this lipid oxidation based on the possibility of the initiation of chain reaction by free radicals. Aside from the decreasing activity on free radicals and preventive effect on aggregation, flavonoids have the ability to bond with blood platelets (Kuźnicki, 2006; Nijveldti, 2001). Propolis has regulatory response on lipid and lipoprotein metabolism and has a direct impact on decreasing the cholesterol levels and the production of the triglyceride in the liver of rats (Li *et al*., 2012; Fuliang *et al.,* 2005). It was noted that mice with deactivated LDL receptor which treated with propolis, the triglyceride level decreased (Daleprane *et al*., 2012). Recent studies show that they act as anti-atherosclerosis chemicals. The major factor on the development of atherosclerotic lessions is the aggregation of blood platelets. Propolis, contains CAPE, is an inhibitor for the aggregation of blood platelets (Chen *et al*., 2007).

1.8. Cardiovascular System Effects

Polyphenolic substances, especially flavonoids, balance and restore blood vessels, so they could help the avoidance of bleeding, varicellate veins and atherosclerosis (Makowska and Janeczko, 2004). Polyphenols also have advantageous impact on coronary circulation and have hypotensive influence (Kohlmünzer, 2003). Researchers indicate that quercetin, troxerutin and rutin block the blood platelet aggregation more efficiently than acetylsalicyclic acid in a same dose. Flavonoids have profitable impact on endothelium of vessels and cardiac muscle by way of inhibiting xanthine oxidase and this led to the lowering the production of superoxide and hydroxide radicals during ischaemia (Heim, 2002; Yao *et al*., 2004; Nijveldt *et al*., 2001).

Propolis is used in the avoidance of circulatory system disease by its active compound flavonoids for example quercetin, kaempferol and rhamnetin which inhibit the transport of calcium over cell membranes to the cytoplasm, that led to vessel expansion and lowers the blood pressure (Konishi, 2005). Propolis have defensive activity against cardiovascular system diseases because of its antihypertensive action on rats (Yoko *et al*., 2004). Cardioprotective action of propolis was obtained in the case of cardiomyopathy in mice, the test animals were treated with intraperitoneal administration of propolis. Intensification was proved via both biochemical parameters and histological scene (Chopra *et al.*, 1995).

1.9. Estrogenic Effects

Flavonoids and isoflavonoids have structural similarity with endogenic sex hormones, having this information scientist have great interest with the estrogenic activity of propolis. Flavonoids show affinity to both ER-α estrogen receptors, located on breast, endometrium and ovaries, and ER-β receptors; on brain, blood vessels, lungs and bones. It is shown that propolis act estrogenic effect via activating estrogen receptors. Jung *et al.* (2010) established that caffeic acid phenethyl ester (CAPE), responsible for the estrogenic effect of propolis and CAPE tends to show more affinity to ER-β receptor than ER-α. CAPE is a selective agonist of ER-β also a probable mediator of the ER-β.

1.10. Antidiabetic Effects

Type-2 diabetes mellitus (noninsulin-dependent diabetes mellitus)(NIDDM) one of the most important diseases in the modern world and its level is getting increase every year, so scientists are looking for new and natural ways for both preventing and treating this illness. Therfore, some experiments have been done and antidiabetic effects of flavonoids was demonstrated.

Epicathechin, one of the components of propolis, stimulates insulin secretion by raising the level of cAMP in β cells located in Langerhans islets. Epigallacatechin 3-gallate (EGCG) shows hypoglycemic activity via blocking the synthesis of glucose in the liver (Jachak, 2002).

Flavonoids valuable natural substances because they prevent the fast rises in blood sugar in serum and also they can prevent the development of complications due to NIDDM. It has been explained that quercetin protect diabetics from the progress of cataracts by inhibiting aldose reductase, which takes part in the production of sorbitol deposition (Sanderson *et al*., 1999).It was demonstrated, by Fuliang *et al*. (2005) , that the administration of propolis extract decrease the level of glucose, fructosamine, malonic aldehyde, nitric oxide synthase, total cholesterol and LDL by decreasing the lipid peroxidation.

1.11. Anti-HIV Effect

It was proven that flavonoids have antiviral effect and they are deliberated as potential anti-HIV agents. Flavonoids behave not only reverse-transcriptase (enzyme necessary for the development of HIV) inhibitors, but also RNA-directed DNA polymerase inhibitors (Ng *et al*., 1997).Viral Vpr protein, that is important for the proliferation of the HIV virus and activates integrase and proteinase, inhibited by quercetin (Veljkovic *et al.,* 2007). It is proven that moronic acid, in Brazilian propolis show anti-HIV activity (Ito *et al*., 2001). Propolis is expected to be used for AIDS treatment in future.

1.12. Aim & Scope of Study

The composition of propolis, its physico-chemical properties, biological activities and therapeutic uses are changing depending on the vegetation where the hives are placed, the climate and the variety of queen.

So far Ankara (Mamak) propolis was not studied and characterized in literature. In this study, it was aimed to investigate the chemical composition and bioactivity Ankara (Mamak) propolis for its antioxidant, antimicrobial and cytotoxic activities. Also total phenolic and flavonoid contents are sought to provide preliminary data for future studies.

CHAPTER 2

MATERIALS AND METHODS

2.1 Sample Collection

In this study the propolis samples were obtained from a local beekeeeper Ankara Mamak (Kıbrıs village) province in August 2014. This village has an altitude of 1124 meter and accepted as the first degree archeological site by Ministry of Culture in 1995. It has a special plant flora that contains around 1000 different plant types, some of them are endemic.

2.2. Extraction

64 grams of propolis were cut into small pieces and frozen at -20^{o}C. After that propolis was grinded and mixed with 70% ethanol with the ratio of 1: 20. The solution was shaken for 10 days in the dark, then it was filtered and the filter cake was mixed with 70% ethanol again for additional two days. The solution was filtered and it was kept overnight in the refrigerator for wax removal. Then the solution was filtered again through Whatman filter No.1 and let evaporated for removal of the solvent. The filtrate lyophilized for 48 hours. Extract was loaded into eppendorf tubes and stored at – 20 ^{o}C (Trusheva *et al.,* 2007). The extraction yield was calculated as follows:

Percentage extraction (w / w) % = [mass of raw propolis (in solution) / mass of total propolis] X 100

2.3. DPPH Scavenging Activity

DPPH• (1,1-diphenyl-2-picrylhydrazyl) is a free radical which is stable in its radical form. It can catch an electron or hydrogen to evolve a stable diamagnetic molecule. It has a purple color in its radical form, when it accept an electron or hydrogen radical its color turns into yellow and form diphenylpicryl hydrazine depending on the antioxidant or a radical capacity of species. The decrease in the concentration of DPPH• is a measure in the degree of antioxidant activity at 517 nm (Blois, 1958).

Experiments in this study were repeated 3 times in duplicates.

1.4 mL of 0.05 mg/ mL DPPH solution in ethanol and 0.1 mL of extract were mixed vigorously. After 20 minute the absorbance was determined at 517 nm. Extract solution was used as control. The radical scavenging activity was calculated according to the following equation:

RSA (Radical Scavenging Activity) % = $[(A_0-A_1)/A_0] * 100$ %

A_0: the absorbance of the DPPH solution with ethanol

A_1: the absorbance of the DPPH solution with the extract concentrations in ethanol.

The RSA% vs final concentrations of the extracts (mg / mL) was plotted and IC_{50} (half-maximal inhibitory concentration) values were calculated.

2.4. Trolox Equivalent Antioxidant Capacity (TEAC)

Trolox Equivalent Antioxidant Capacity was determined by using ABTS (2,2‟-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) method according to Arnao *et al.* (2001) and Re *et al*. (1999) with some modifications. Powder form of $ABTS^+$ was dissolved in ethanol to form 7 mM $ABTS^+$ and 2.5 mM potassium persulfate

($K_2S_2O_8$) was added into the final solution and kept in the dark at room temperature for 16 hours before use.

ABTS solution was diluted with 60 mL ethanol to 1 mL ABTS solution until the absorbance balanced at 1.1 ± 0.02 at 734 nanometer. Then, different concentrations of propolis extract (2.5 µg/ mL to 10 µg/ mL) was mixed with 1 mL of $ABTS^+$ solution. After that, the mixture kept 6 minutes in the dark and then the absorbance was monitored at 734 nm. To calculate the trolox equivalent capacity (TEAC) values, trolox used as control.

The experiments were repeated three times, in duplicates.

Percent inhibition was calculated as;

Inhibition % = $[(A_0-A_1)/A_0]$ x 100

Where $\mathbf{A_0}$ is the absorbance of the ABTS solution and ethanol,

$\mathbf{A_1}$ is the absorbance of the ABTS with the extract.

TEAC values were calculated by dividing the slope of extract concentration to percent inhibition graph to that of trolox standard curve. The results were divided by dilution factor (60) (dilution of stock solution).

2.5. Determination of Total Phenolic Content (I)

To determine the total phenolic content of propolis, Folin-Ciocalteu method was modified according to Singleton and Rossi (1965). Gallic acid (0.05-0.3 mg / mL) was used as standard to obtain gallic acid standard curve. At first 0.1 mL standard / or extract (in ethanol) and 2 mL of 2% Na_2CO_3 were mixed, 3 minutes later 0.1 mL of 50% Folin-Ciocalteu was added and shaken vigorously. The mixture was kept in the dark, at room temperature for 30 minutes. Then, the absorbance was monitored at 750 nm by spectrophotometer. Results were calculated as miligram per gram of gallic acid equivalents (GAE). All steps were carried out three times

in duplicates and the results were expressed as average with the standard deviations.

mg GAE / g extract = [(Absorbance of sample- Absorbance of blank))- Absorbance of blank / slope x Dilution Factor

2.6. Determination of Total Flavonoid Content (II)

To determine the total flavonoid content of propolis aluminum chloride colorimetric assay was applied with some modifications (Marinova *et al*. 2005). At the beginning of the experiment, 1 mL of extract or standard solution was mixed with 4 mL distilled water and 0.3 mL of 5% $NaNO_2$ and kept for five minutes, after that 0.3 mL of 10% $AlCl_3$ was added to the mixture and waited for one more minute, then 2 mL of 1M NaOH was added and the total volume of mixture was brought to 10 mL with the addition of distilled water. The absorbance monitored at 510 nm. To determine the total flavonoid content quercetin was used as standart. Experiments were run three times with duplicates.

mg QE / g dry extract mass = [(Absorbance of sample – Absorbance of blank)- Absorbance of blank / slope x Dilution Factor

2.7. Microbial Inhibition Studies

2.7.1 Culturing of microorganisms and Antimicrobial Activity Assays

Mueller Hinton broth (23 g) and Mueller Hinton agar (38 g) media were prepared with 1L of dH20. Medium were autoclaved at 121^0 C for 15 minutes. Bacterial species assayed were to cultivate Escherichia coli and Staphylococcus aureus, respectively.

The antimicrobial assay was performed with Kirby-Bauer disk diffusion susceptibility test. The cultures were inoculated into 4 mL Mueller Hinton broth

by the aid of a sterile plastic loop and incubated at 37ºC for 18 h. After incubation, they were diluted in the range of 1:100 with sterile bi-distilled water and 100 µL (10^7 -10^8 cells/ mL) of the cell suspension spread over Mueller-Hinton agar plates. The 6 mm paper disks containing (10 mg / mL) 20 µLpropolis were placed onto inoculated agar surfaces and incubated at 37ºC for 18 h. Tetracycline (10 mcg), Amikacin (30 mcg) and ethanol were used as positive control; distilled water used as negative control. When the incubation was completed, the zone diameters around the discs measured and compared with the break points of Clinical Laboratory Standards Institute (CLSI) and ranked as susceptible (S), intermediate (I), and resistant (R).

2.7.2. Culturing of Fungal Species and Antifungal activity Assay

Malt Extract agar media was prepared as 48.0 g/ L with d H_2O and sterilized at 121 °C for 15 minutes. *Candida albicans* (0.5x 10^6 cells/ mL) cultivated on Malt Extract agar plates. The 20 µL propolis extract containing discs (20 mg / mL) and antibiotic discs were located on the inoculated plates. Incubation was carried for 36-48 hours at 25° C. Amikacin (0.30 mg/ mL) and ethanol used were as positive control and sterile distilled water was used as negative control. When the incubation was completed, the zone diameters around the discs measured and compared with the break points of Clinical Laboratory Standards Institute (CLSI) and ranked as susceptible (S), intermediate (I), and resistant (R).

2.8. Determination of MIC value

Minimum inhibitory concentrations (MICs) are described as the lowest concentration of an antimicrobial agent which inhibits the visible growth of a microorganism after overnight inoculation. MICs are used by diagnostic laboratories mainly to confirm antibiotic resistance.

To find out minimum inhibition concentration of propolis, Kirby-Bauer (CLSI, 2002) disc diffusion assay was performed. Different concentrations of propolis extracts (64 mg / mL to 1 mg / mL) with serial dilution were loaded on sterile blank discs. Then, these discs were located on the agar plates with newly subcultured microorganisms and incubated at 37°C for 24 hours. After incubation period the zones of inhibition were observed and the first disc which had no inhibition zone around is accepted as MIC value against the specific bacterial strain assayed.

2.9 Determination Minimum Bacteriocidal Concentrations

Minimum Bacteriocidal Concentrations (MBCs) are defined as the lowest concentration of an antimicrobial agent which prevents the growth of a microorganism after subcultured on antibiotic-free media. The MBC is determined using series of steps, based on Minimum Inhibitory Concentration. Pure culture of microorganisms were grown overnight diluted in growth-supporting broth (Mueller Hinton broth) to a concentration between $1x10^5$ and $1x10^6$ CFU / mL. Further 1:1 dilutions of propolis extract were prepared in test tubes (64 mg / mL to 1 mg / mL). All dilutions of propolis extract inoculated with equal volumes of the specified microorganism. Positive controls (Tetracyclin, amikacin, Chloramphenicol and ethanol) and negative control (distilled water) tubes were prepared for every test organism to demonstrate microbial growth over the course of the incubation period. An aliquot of the positive control was plated and used to establish baseline concentration of the organism used. Turbidity indicated growth of microorganism; MIC and MBC values were determined with respect to turbidity but due to the chemical nature of propolis directly affected the turbidity.

When the concentration of propolis increases so does the turbidity. Therefore, it was impossible to find MIC and MBC values visually or spectrophotometric measurement. Therefore, to determine MBC value, the media that contained different concentrations of EEP and selected microorganisms incubated at

specified incubation time and temperature depending on the type of organism. After incubation period the media was spreaded on to plates (MHB) and incubated. The MBC was defined as the lowest concentration of the agent that demonstrated a pre-determined reduction in mg / mL when compared with the MIC results (**Figure 2.9.1**).

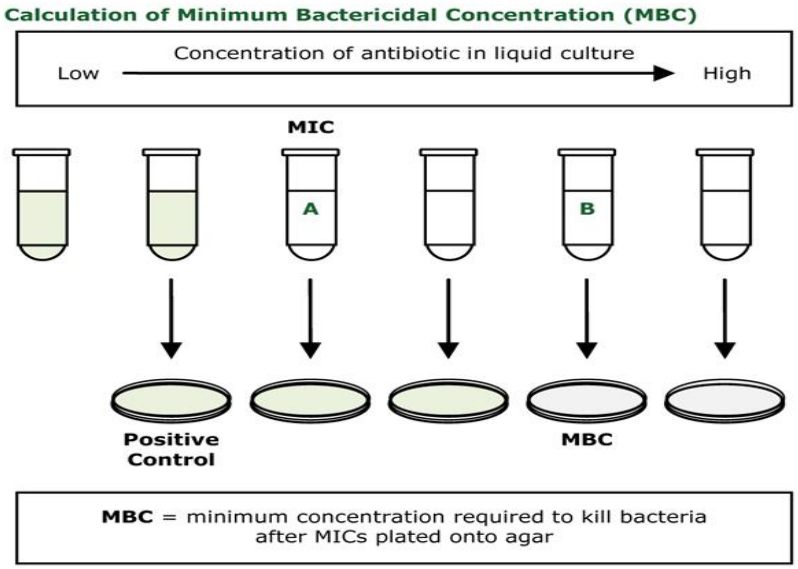

Figure 2.9.1. Schematic summary of MBC assay

2.10. Heat stability control

To check the effect of heat on propolis, 40 mg / mL propolis extract was grinded and mixed with 70% ethanol. After vortexing the solution was put into 2 test tubes and one of them was heated to 60 °C in incubator and the other was heated to 120 °C in the autoclave. Disc diffusion, DDPH, ABTS, total flavonoid content, total phenolic content assays were performed to compare with the non heat-treated samples.

2.11. Cell culture studies

HT-29 and Caco-2 cells were obtained from Foot and Mouth Diseases Institute from Ankara Turkey, were used in the experiments..

2.11.1 Cell culture growth conditions

HT-29 and Caco-2 cells were maintained in DMEM supplemented with 10% (w / w) FBS (fetal bovine serum) 1% (w / w) complete medium. Cells were incubated at 37° C under 5% CO_2 with humidified incubator. Cells were cultured in sterile T-75 tissue culture flasks.

2.11.2. Subculturing

Cells were subcultured when the cell confluency reaches to 90% in the flask. The medium was removed and washed with 1XPBS to remove the residual medium, then Trypsin-EDTA added and incubated at 37 °C for 2 minutes. To see cells were detach, the flask was observed under light microscope. When cells were reached their standard morphology, 2 mL medium was added to stop trypsinization and pipetted to detach all cells then incubated at 37 °C and replaced with fresh culture medium every 2-3 days during experiment.

2.11.3 Cell stock Preparation

The medium was removed and the cell was trypsinized. Centrifugation was carried at 800 rpm for 5 minutes then the medium was aspired. 3 mL of 10%

DMSO were added and the cells were transferred into 1 mL cryovials. The vials were kept frozen at -80°C.

2.11.4. Cell thawing

Frozen cell stock was placed into water bath at 37 °C for 1-2 minutes. Then centrifugation was done at 800 rpm for 5 minutes. Then the supernatant was aspired to remove DMSO and 1 mL medium was added onto the remaining pellet and homogenized with pipetting. Transferred into T-25 flask and 4 mL fresh medium was added and incubated at 37 °C, CO_2 humidified incubator as the starter cell.

2.11.5. Cell viability assay

The cell viability assay MTT was performed according to Dahl *et al.* (2006) and Holst-Hansen and Brünner (2000). HT-29 and Caco-2 cells were grown $2x10^4$-$5x10^4$ cells/mL in cell suspension. Then the cell suspension transferred into 96 well-plates that each well contains 100µL / well and incubated at 37 °C in 5% CO_2 humidified incubator for 24 hours.

After the incubation period the medium was aspired and the 100 µL media which contains different concentrations of propolis (0.01µg / mL to 100µg / mL) was added on to the cells suspension. As a control only fresh medium was used. Then, plates were incubated at 37 °C in 5% CO_2 humidified incubator for 24, 48 and 72 hours.

At the end of incubation periods the medium was aspired and to each well new medium 100µL / well, 10% FBS and 130µL / well MTT dye was added, then further inoculated in similar conditions for 4 hours. After 4 hours the formazan

crystals were checked under inverted microscope. To dissolve the formazan crystals 100 µL isopropyl alcohol was added to each well without aspirating medium. Resulting colorimetric changes were measured at 570 nm by spectrophotometer. 100 µL medium and 130 µL MTT solution were used as control. All experiments were repeated three times.

2.12. Mass Spectrometry

Agilent 1200 HPLC in-line with Agilent 6460 LCMSMS mass spectrometer unit (Central Laboratory, METU) was used in the identification of phenolic masses in propolis. Phenol mix was applied to Agilent system Zorbax SB-C18 (1.21x 50mm 1.8 µ) reversed phase column. Phenolics were baffled by a gradient buffer A for LC/MS/MS to 60% B for LC/MS TOF with 0.3 mL/min flow rate and 13 minutes run time. The system was connected with ESI unit, which supplies ionization. Data analysis were performed manually on Agilent MassHunter Qualitative Analysis system.

2.13. Statistical Analysis

Statistical analysis was performed for cell viability assay. The data represented as mean and error bars represent SD (n =3). The analysis was performed on GraphPad Prism Software (GraphPad Prism Software Inc., USA) and Two-way ANOVA test were applied.

CHAPTER 3

RESULTS AND DISCUSSION

In this research, raw propolis sample was analyzed to determine the potential antioxidant, antimicrobial and cytotoxic activity and also for identifiying its phenolic profile. Antioxidant activity of propolis was determined by DPPH and ABTS methods. The total phenolic and flavonoid contents were identified by gallic acid and quercetin standard equivalence. In addition, to check MIC and MBC values on microorganisms Kirby-Bauer disc diffusion method and serial dilution method were performed. MTT assay was used to understand the effect of propolis on cell viability and amounts of phenolics in propolis measured by Mass Spectrometry.

3.1. Yield of Extraction

There are different factors that affect the yield of extraction such as type of solvent, temperature, technique (maceration, sonication, Soxhlet extraction or Supercritical fluid extract). In this research the extraction method was maceration and the solvent was ethanol.To calculate the extraction yield, the sample was grinded before and after the extraction process. The yield obtained was 20.30 ±0.01 % (w / w).

To obtain the extract of propolis many different types of solvents and methods have been used and researchers concluded that although the yield of maceration is lower than soxhlet extraction, maceration is found to be more convenient because it was quick (Paviana *et al*., 2013). Also they concluded that 70 % ethanolic

extract of propolis has more phenolics and biological activity compared water exctract, methanol, isoprophyl alcohol and ethyl acetate (Mahani *et al.,* 2012). The solubility of propolis depending on the solvent used is as follows:

Ethanol › Methanol › isopropyl alcohol › ethanol › water

Khacha-Ananda *et al*. (2013) extracted propolis with 70 % ethanol and calculated the yield of extract as 18.1 % (w / w). In another study, the yield of 70 % ethanol extract of propolis was reported as 18.63 % (w / w) (Mahani *et al.* 2012).

3.2. Antioxidant Activity of Propolis

Antioxidant activity of propolis extract was evaluated by DPPH (2.2-diphenyl-1-picrylhydrazyl) method. For different concentrations of propolis, the antioxidant activity was tested by spectrophotometric analysis. Measuring the ability of propolis extract to scavenge stable DPPH radical and the antioxidant activity was calculated and compared to α-tocopherol, which is vitamin E that shows strong antioxidant activity, as an antioxidant standard (**Table 3.2.1**).

Table 3.2.1. Antioxidant activity of propolis and α – Tocopherol via DPPH .

	Antioxidant Activity	**Maximum RSA %**
Sample	IC_{50} mg / mL ± SD	RSA % ± SD
Propolis Extract	4. 101 ± 0.014	96.0 ± 0.1
α-tocopherol	0.306 ± 0.086	97.0 ± 0.1

Each measurement was repeated three times and the average of those repetation were used in calculations. For the calculation of % RSA the formula given below is used.

Radical Scavenging Activity (%) = $(A_0\text{-}A_1) / A_0*100$

Where $\mathbf{A_0}$ is the absorbance of blank (contain only ethanol), $\mathbf{A_1}$ is the absorbance of propolis extract at 517 nm. (Ghasemi *et al*., 2009).

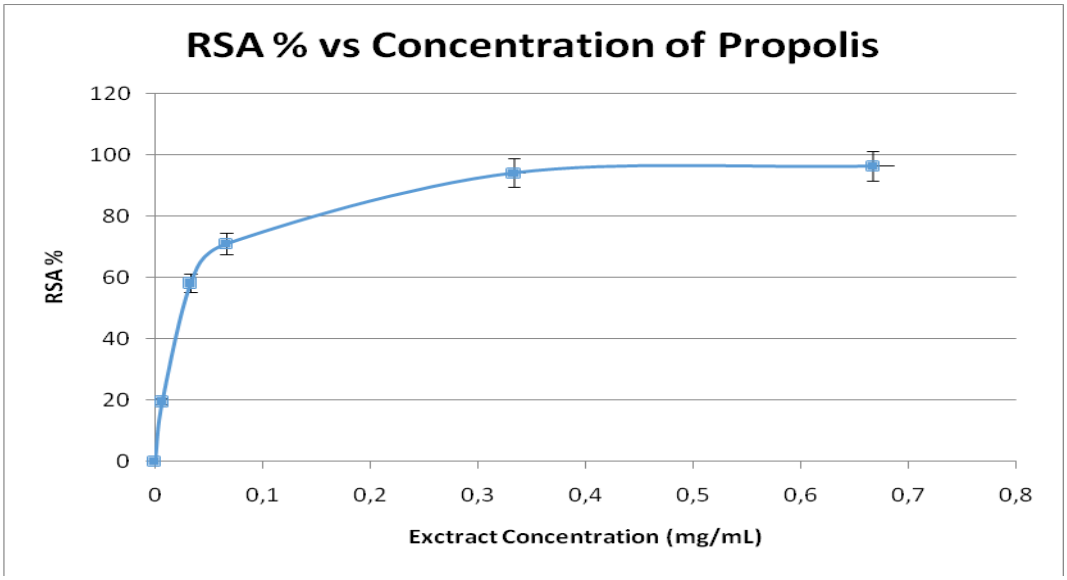

Figure 3.2.1. **%RSA of different concentrations of propolis. (n= 3).**

Figure (3.2.1.) shows an incerasing percent of radical scavenging activity in a dose dependent manner. IC_{50} value of propolis extract was calculated as 4,101± 0,014 mg / mL.

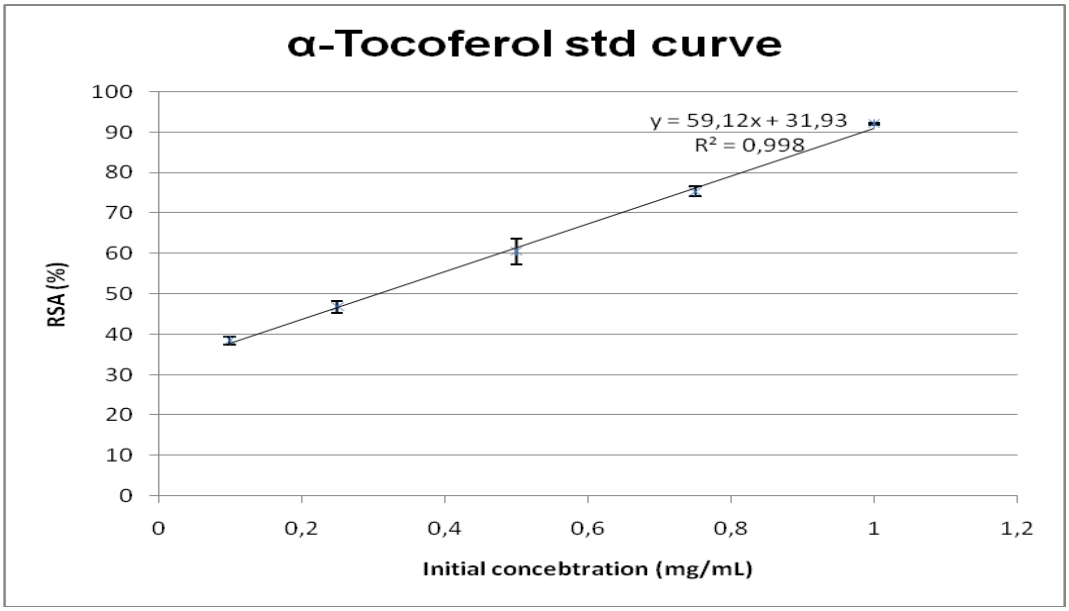

Figure 3.2.2. RSA % of α-Tocopherol versus concentration (n= 3).

Antioxidant activity was calculated from **Figure 3.2.2.** as follows :

Antioxidant activity = (mg α-Tocopherol/g extract x 1,000) / (IC_{50} of α-Tocopherol).

Propolis has the antioxidant activity of 1,26± 0,01 mg α- Tocopherol / g extract.

There is a relation between the IC_{50} value and antioxidant activity , lower IC_{50} value express the higher antioxidant activity. From our results 3 mg / mL α-Tocopherol has 97% inhibition where as 10 mg / mL ethanolic extract of propolis has 96 % inhibiton on DPPH. It is obvious that α-Tocopherol has 3 times more antioxidant activity than propolis of Ankara.

Literature indicates a higher RSA % activity for Ankara propolis than China propolis (Mok-Ryeon Ahn *et al.,* 2006). This is possibly due to the variation between geographic properties such as plant flora which affects the chemical composition and biological activity of propolis (Kujumgiev *et al*., 1999).

3.3. Trolox Equivalent Antioxidant Capacity (TEAC)

To check the Trolox Equivalent Antioxidant Capacity, ABTS method was performed as previously described in Chaper 2. Trolox is used as the standard to calculate percent radical scavenging activity of trolox (**Figure 3.2.3**) in different concentrations.

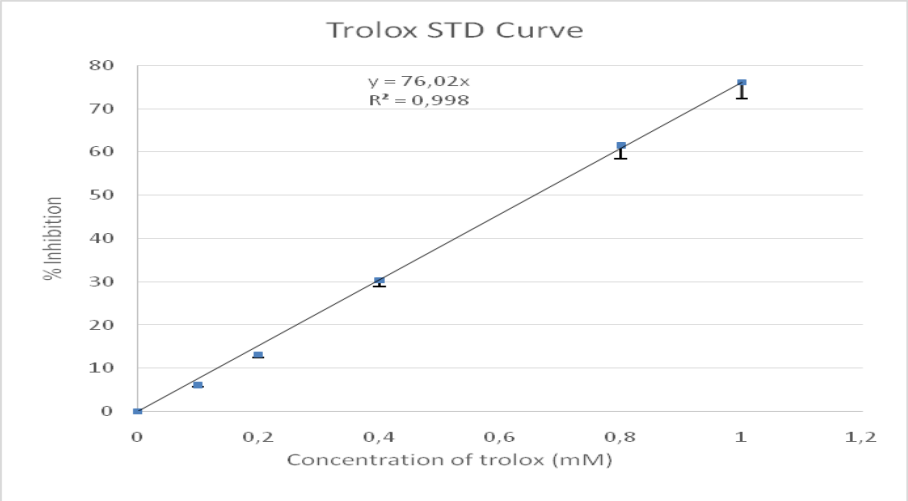

Figure 3.3.1. Calibration curve prepared by Trolox standart solution in ethanol (n=3).

Table 3.3.1. Trolox equivalent antioxidant capacity (TEAC) of Ankara propolis

Concentration (µg / mL)	**TEAC value (mmol TE / g extract ± SD)**
10	2.970 ± 0.086
20	0.148 ± 0.043
30	0.099 ± 0.002
40	0.074 ± 0.021
50	0.060 ± 0.001

Trolox equivalent anioxidant capacity was calculated as 2.970 ± 0.072 mmol TE / g extract. In a study, the TEAC value of ethanol extract of propolis from Brazil was obtained 1.22 ± 0.14 mmol TE / g extract (Skaba *et al*., 2013). This may be as a result of the plant flora around the hive, season that propolis obtained.

3.4. Quantitation of Total Phenolic Content

To determine the total phenolic content of EEP Folin-Ciocalteu"s method was performed. The experiment was done as mentioned earlier in the method part . Gallic acid was used as a standard to obtain gallic acid calibration curve. By using y= 81,36x and R2 = 0,998 (**Figure 3.4.1.)**, gallic acid equivalent in propolis was calculated as 10. 712 ± 0.007 mg GAE / mg extract.

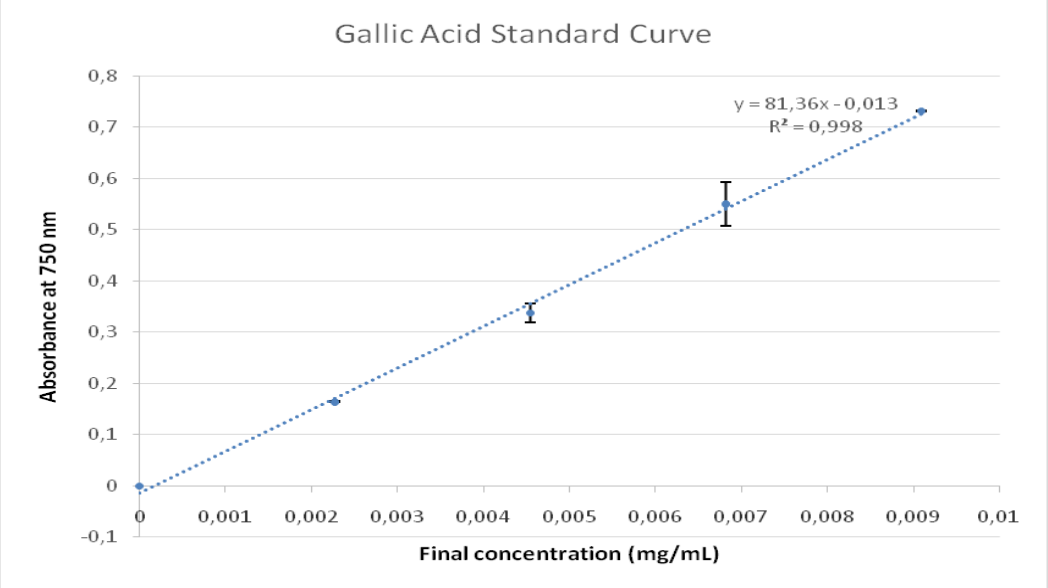

Figure 3.4.1. Gallic acid calibration curve (n= 3).

Table 3.4.1. Total phenolic content of propolis in gallic acid equivalents.

Concentration (mg / mL)	**Total Phenolic Content (mg GAE / mg extract ± SD)**
0.25	4.218 ± 0.004
0.5	7.878 ± 0.007
1.0	13.333 ± 0.013
1.5	17.511 ± 0.005

When compared this result (**Table 3.4.1.**) with previous studies; Khacka-Ananda *et al*. (2013) found 17,17 ± 2.19 to 18,27 ± 3.30 mg GAE / g extract (propolis from Phayao province, Thailand), close GAE are obtained. According to Kumazawa *et al.* (2004) total phenolic content of China propolis (from Hubei province) has 298.0 ± 8.7 mg GAE / g extract. This result is so interesting that it shows content and activity of propolis variable according to the geographic location. Although Thailand and China have nearly the same geographic properties the total phenolic profile of these countries are noticably different from each other. On the other hand Leandro- Moreira *et al*.(2008) also obtained 151.00 to 329.00 mg GAE / g extract for propolis from Portugal that were also quite high.

3.5. Total Flavonoid Content of Propolis

Total flavonoid content of Ankara propolis was checked by aluminium chloride calorimetric assay also as mentioned previously in chapter 2. Quercetin is used as standart to obtain quercetin calibration curve (**Figure 3.5.1**).

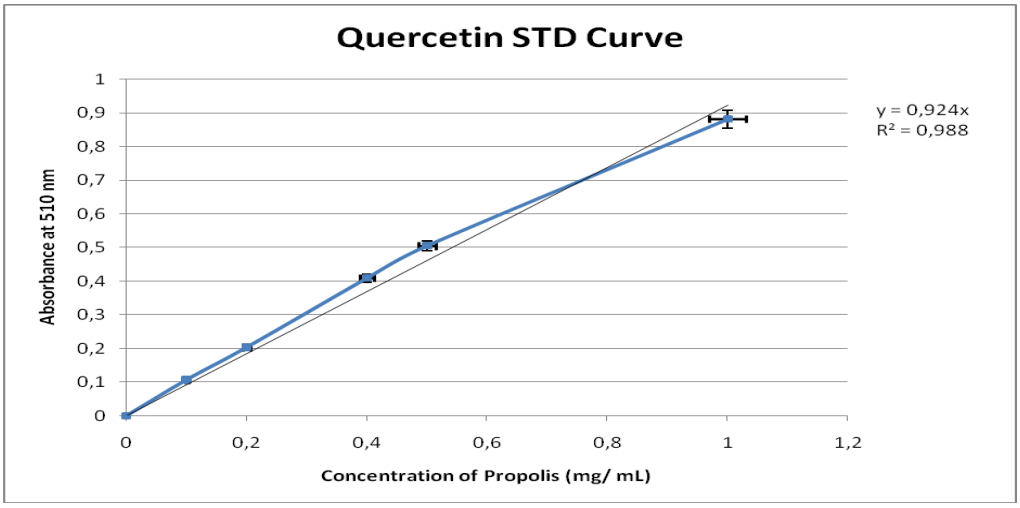

Figure3.5.1. Quercetin calibration curve (n =3).

Table 3.5.1. Total flavonoid content of Propolis extract in quercetin equivalents

Concentration (mg / mL)	**Total Flavonoid Content (mg QE / mg extract ± SD)**
0.25	0.223 ± 0.005
0.5	0.437 ± 0.001
1.0	1.127 ± 0.045
1.5	1.187 ± 0.003

The quercetin equivalent amount in propolis was calculated as 0.742 ± 0.014 mg QE / mg extract (**Table 3.5.1**). In a study, the total flavonoid content of Sonoran EEP (Northwest Mexico) was reported as 57.8 ± 3.6 mg QE / g extract (Valezquez *et al.,* 2007). In an other study, total flavonoid content for different Brazilian propolis were reported between 2.5 to 176 mg QE / g extract (Cottica *et al.,* 2011).

3.6. Antimicrobial Activity of Propolis

As reported by many researchers, antioxidants mainly phenolics in propolis have antimicrobial activity on broad range microorganisms. To study antimicrobial activity of Ankara propolis, *Staphylococcus aureus*, *Escheria coli* and one type of fungal strain of *Candida albicans* were studied.

Table 3.6.1. Antibacterial and Antifungal activity of Propolis (n =3).

Microbial Strain	**Zone of Inhibition (mm)**				
	Propolis extract	Antibiotic	Distilled Water	Ethanol	Antibiotic discs
E. coli	NA	25.0	NA	NA	TE 30
S. aureus	13.3	23.0	NA	NA	AMC 30
C. albicans	55.0	52.0	NA	12.3	AMC 30

(NA: No Activity, TE: Tetracyclin, AMC: Amikacin).

The antibacterial activity of propolis extract was observed only on *S. aureus.* The most effective activity was observed on *C. albicans*, this antifungal activity was even higher than amikacin (**Table 3.6.1**.).

In one study from Turkey, researchers checked the antimicrobial activity of propolis from Bingöl province, the ethanol extract of propolis did not show zone of inhibiton on *E. coli* on the other hand Chloroform extracted propolis showed 33.0 mm zone of inhibiton. In same study it was found that EEP showed 23.0 mm zone of inhibition and 16.0 mm on *C. albicans* (Aksoy & Dığrak, 2006).

Another study, EEP from Muş province from Turkey, showed 18.0 mm zone of inhibition on *E. coli*, 20.0 mm on S.aureus and no zone of inhibition on *C. albicans* were determined (Alan *et al*., 2014).

To check the effect of medium type on efficiency of propolis, different types of medium were used. Results were as follows (**Table 3.6.2**).

Table 3.6.2 . The effect of propolis on microorganims on different medium.

	Baird parker	Mueller hinton	Nutrient	Mac conkey
E.coli	-	-	-	-
S.aureus	+	++	++	-
C.albicans	+	++	++	+
Amikacin	++	++	++	++
Tetracyclin	++	++	++	++

(-) No Activity, (+) Intermediate, (++) Sensitive.

3.7. Determination of MIC Value of Propolis

Minimum inhibitory concentrations were determined again with Kirby-bauer disc diffusion method. Results were in **Table 3.7.1.**

Table 3.7.1. Minimum Inhibitory Concentrations of Propolis on microorganisms

Type of Microorganism	**MIC value** (mg / mL)
E. coli	16.0
S. aureus	8.0
C. albicans	4.0

,The MIC values of propolis on microorganisms were found as 16.0 mg/ mL for *E. coli*, 8.0 mg / mL for *S.aureus* and 4.0 mg / mL for *C. albicans*. As the data show the most sensitive microorganism was *C. albicans* to propolis and the most resistant one was *E. coli*, in this study.

In a study, scientists collected propolis from Brazil and they investigated the MIC value, for *S.aureus* 3.1 mg / mL and for *C. albicans* 3.1 mg/ mL but the MIC value for *E. coli* could not determined (Compos *et al*., 2014).

In another study, it was foud that ethanol extract of propolis from Turkey (Kayseri), the MIC value for *E. coli* was 1024.0 mg / mL, for *S. aureus* 64.0 mg / mL and for *C. albicans* 128.0 mg / mL (Gunduz- Kaya *et al.,* 2012).

3.8. Determination of MBC of Propolis

To determine MBC value, in the media that contained different concentrations of EEP, selected microorganisms were incubated at specified incubation time and temperature depending on the type of organism. After incubation period the media were spreaded on to the plates (MHB or Malt extract agar) and incubated. The MBC was defined as the lowest concentration of the agent that demonstrated a pre-determined reduction in mg /mL when compared with the MIC results.

Table 3.8.1.Minimum Bacteriocidal Concentrations of Propolis on microorganisms (n=3).

Type of Microorganisms	**MBC value** (mg / mL)
E.coli	64.0
S.aureus	32.0
C. albicans	16.0

After the MIC values for microorganisms were determined, the MBC values 64.0 mg / mL for *E. coli,* 32.0 mg / mL for *S. aureus* and 16.0 mg / mL for *C. albicans* were found (**Table 3.8.1**).

Suleman *et al*. (2015) worked wih ethanol extract of South African propolis and the MBC values were determined as 1563.0 µg / mL for *S. aureus*, 1563.0 µg / mL for *E. Coli* and for *C. albicans* 781.0 µg / mL.

3.9. Heat Stability of Propolis

To check the effect of heat on propolis in terms of the antioxidant activity, total phenolic and flavonoid content and antimicrobial activity were investigated. 40 mg / mL of propolis extract mixed with % 70 ethanol and put into 2 test tubes, one of the tube was heated up to 60 °C and other up to 120 °C.

Table3.9.1. Comparison of antioxidant activity, total phenolic and total flavonoid content of heat-treated propolis samples (n= 3).

Propolis sample treated	**Antioxidant Activity** IC_{50} ± SD	**Maximum RSA %** RSA%±SD	**TEAC** (mmol TE / g extract ± SD)	**Total phenolic content** mg GAE / mg extract ± SD	**Total Flavonoid content** (mg QE / mg extract ± SD
60 °C	8.31±0.01	43.0±1.08	0.521±0.063	4.199±0.003	0.224±0.019
120 °C	10.06±0.09	30.0±0.183	2.003±0.140	2.573±0.006	0.097±0.047
Non-treated	4.101±0.014	96.0±1.090	0.290±0.072	10.712± 0.007	0.742± 0.014

When compared to heat-treated samples, 120 °C treated one showed lower antioxidant activity, total phenolic and flavonoid content than that of 60 °C treated one. Heat treatment decreased the activity of propolis, this may be the result of denaturation of phenolics with heat or heat may decrease the stability of active content of propolis.

When the antimicrobial activities of heat-treated sample were checked, there was no effect of those sample on microorganisms, only *S.aureus* had 1.00 ± 0.08 mm zone of inhibition with 60 °C treated propolis. The MIC and MBC values could not be obtained.

3.10. Effect of Ankara Propolis on Cell Viability

Many researchers concluded that propolis have cytotoxic effect on different cancer cell lines by its active components such as phenolics and antioxidants. In this study, in order to investigate the cytotoxic activity of propolis Caco-2 and HT29 cell lines were used and knowledge of EEP led us to think inhibition of cell

proliferation and decrease the cell viability should occur due to induction of apoptosis. The Caco-2 cell line is a continous cell line of heterogenous human epithelial colorectal adenocarcinoma and the HT-29 is a human colorectal adenocarcinoma cell line with epithelial morphology. The experiments were done as mentioned in chapter 2, previously.

3.10. 1. The Morphological Analysis by Light Microscopy

In order to observe the effects of propolis in cell viability on both Caco-2 and HT-29 cell lines, morphological changes were observed under inverted light microscope. (**Figure 3.10.1.1** and **Figure 3.10.1.2**).

The control group of cells were at the normal morphology at 24th hour and at 48th and 72th hours the cells gradually aged and swelled. 100 μg / mL EEP was highly toxic; there was no alive cells, the cells swelled, burst. The granulation and vacuolization amount were high. When the concentration of propolis has got lower, the number of live cells increased, the membrane structure of cells attained normal morphology, on the other hand vacuolization and granulation numbers decreased in a dose dependent manner. At the lowest dose (0.01 μg / mL) the general morphology of cells were similar to control groups. The granulation, vacuolization and swelling of cells could be seen on cell lines normally but the numbers show the toxicity levels. By this information it could be said that the 100 μg / mL propolis showed the highest toxicity on each cell line but the level of toxicity decreased when the concentration decreased.

Treated **Control**

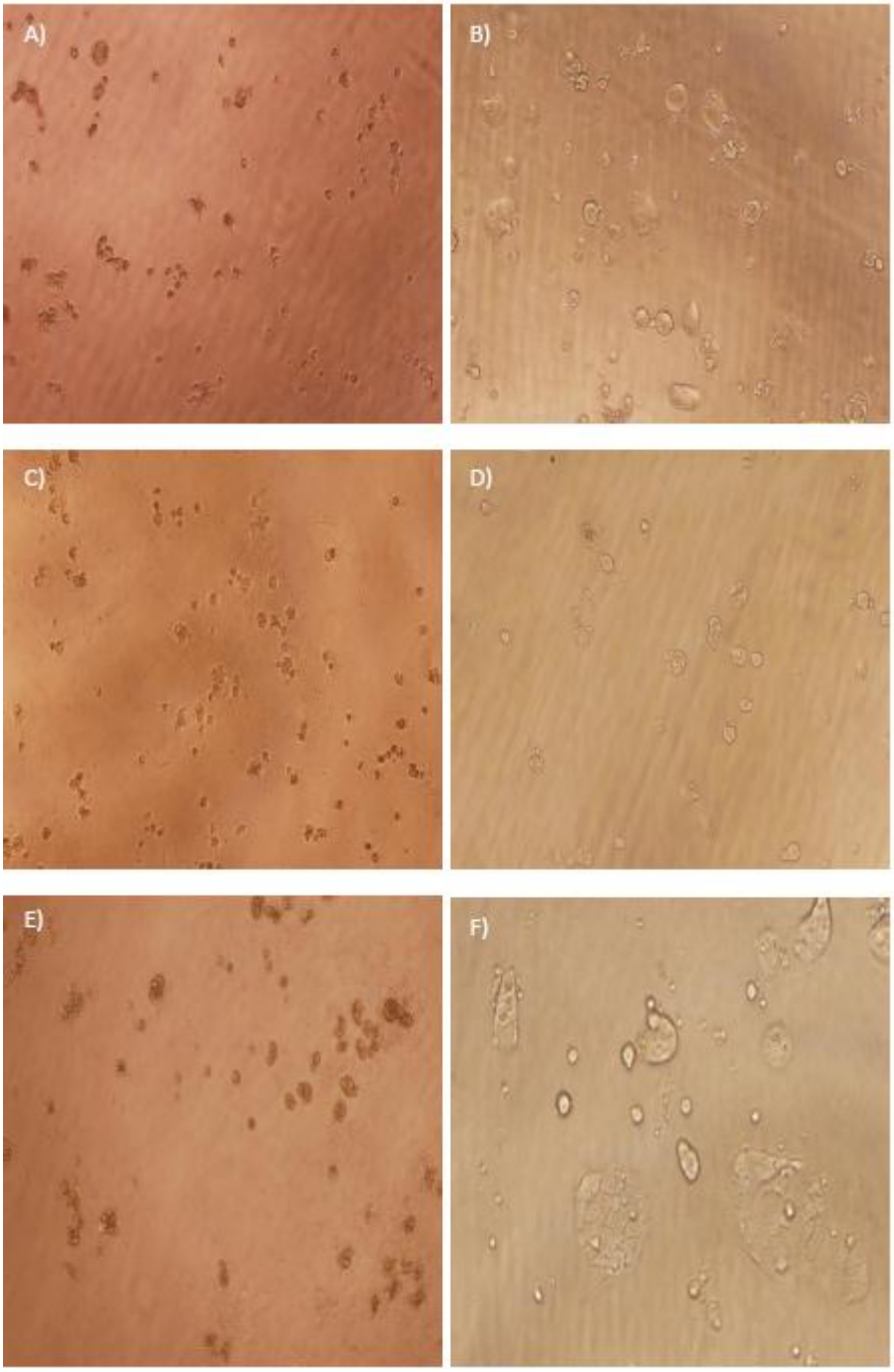

Figure 3.10.1. 1. The effect of propolis on Caco-2 cell lines (100 µg / mL)

Where figures A, C, E are treated cells with propolis at 24th 48th and 72th hours, Figures B, D, F are the control groups at 24th, 48th and 72th hours.

Treated **Control**

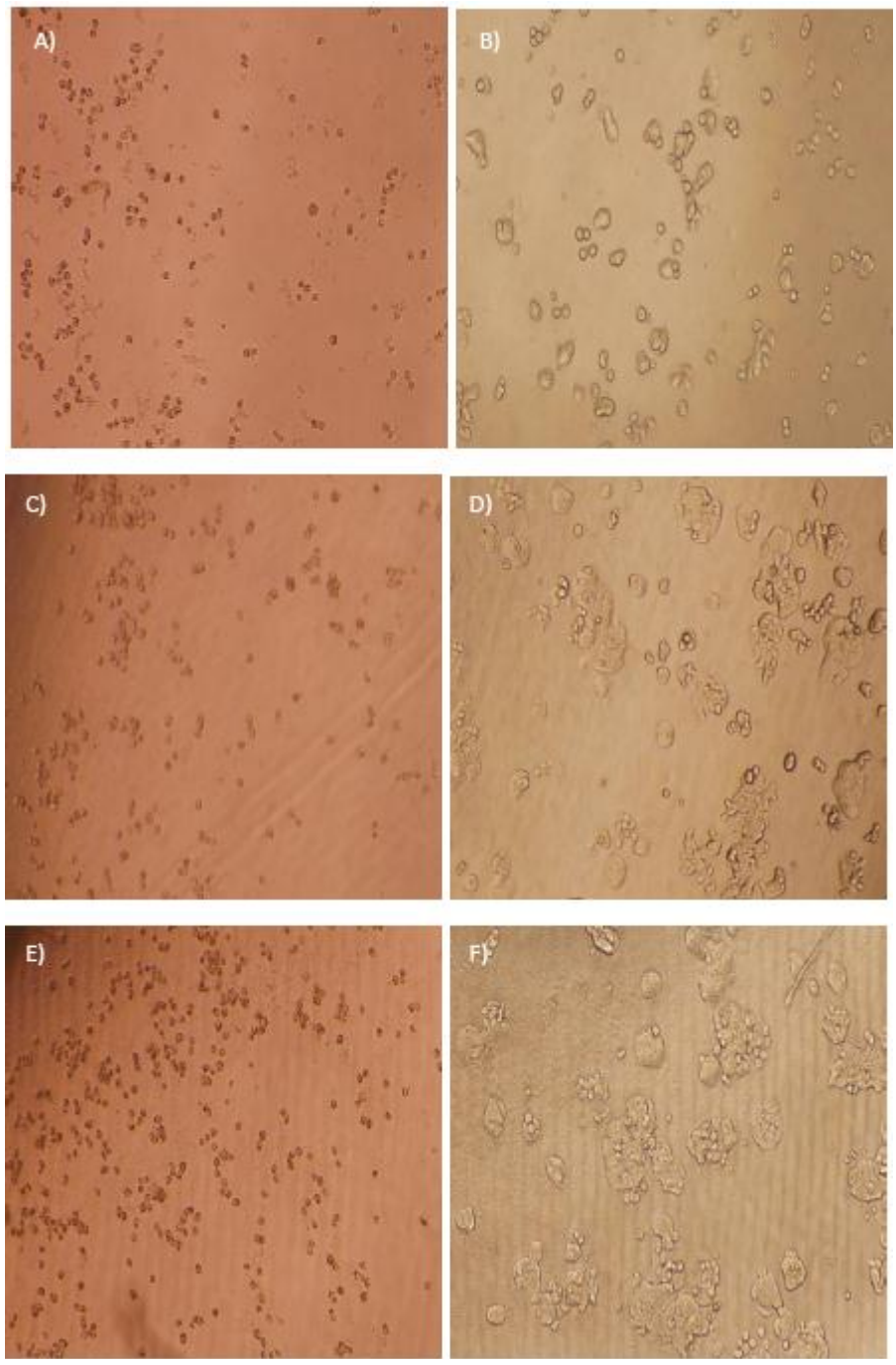

Figure 3.10.1.2. The effect of propolis on HT-29 cell lines (100 µg / mL)

Where figures A, C, E treated cells with propolis at 24th 48th and 72th hours, Figures B,D,F are control groups at 24th,48th and 72th hours.

3.10.1 MTT Results

The MTT assay was performed as explained in Chapter 2. According to Figure 3.10.1.1 on Caco-2 cell lines, propolis showed toxic effect with depending on time and dose. At 24 th hour 100 µg / mL was highly toxic on Caco-2 cell line on the other hand the number of cells increased at 48 th hour the reason may be the survived cells proliferated on the 72 th hour since the number decreased again. There were no significant changes in the number of cells at the concentrations of 2.5 µg / mL, 1 µg / mL, 0.1 µg / mL, 0.01µg / mL treated wells with time time.

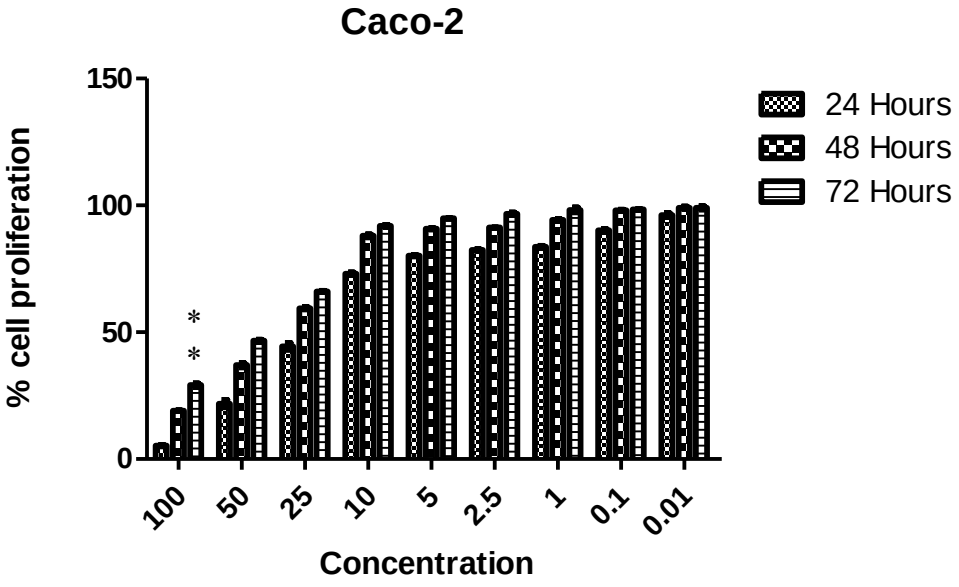

Figure 3.10.1.1 Cell proliferation vs concentration of propolis (µg / mL).

According to the **Figure** (3.10.1.2) 100µg / mL propolis was toxic at 24th hour on HT-29 cell line but there was no significant change at 48th and 72th hours in number of cells. The number of cells at 48th hour for 50µg / ml higher than both 24th and 72th hours. The reason may be the survival of proliferated cells.

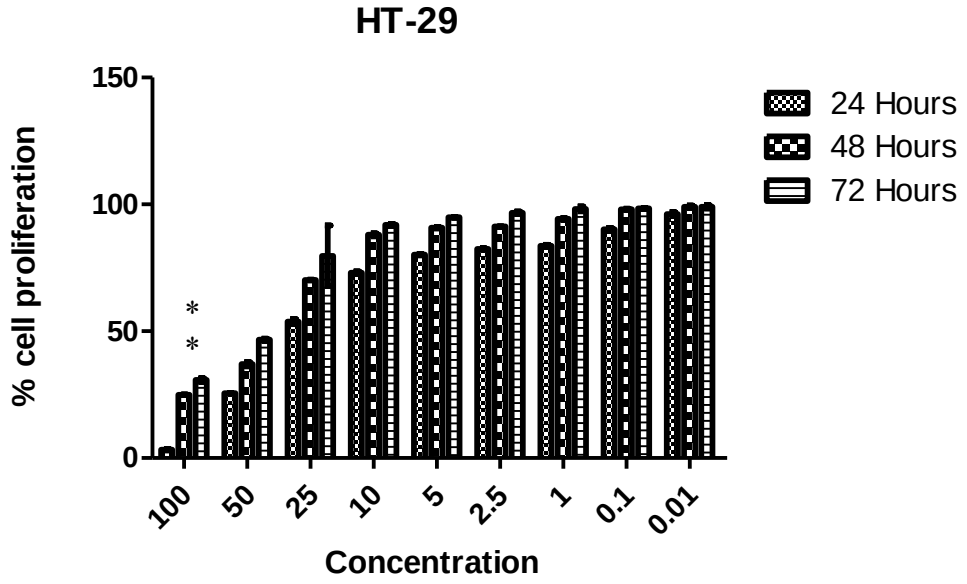

Figure 3.10.1.2 Cell proliferation vs concentration of propolis (µg / mL).

Calhelha et al. (2014) found that EPP from Portugal show cytotoxicity on MCF7 (breast adenocarcinoma), NCI-H460(non-small cell lung carcinoma), HCT15 (colon carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma) cell line in dose dependent manner.

In a study, researchers obtained that ethanol extract of Indian propolis had cytotoxic and apoptotic effect on MCF-7(human breast cancer), HT-29 (human colon adenocarcinoma), Caco-2(human epithelial colorectal adenocarcinoma) and B16F1(murine melanoma) cell line in the dose of 250 µg / mL (Choudhari *et al*., 2013).

In literature researchers had proven that propolis show cytotoxic effect on various cancer cell lines and they concluded that the active components of propolis such as caffeic acid phenetyl ester (CAPE) and chrysin influence the apoptotic process especially in leukemia by activation of caspases, suppression of anti-apoptotic proteins (McEleny *et al*., 2004; Bulavin *et al*., 1999).

3.11. Mass Spectrometry Results

Mass spectrometry was performed as explained in Chapter 2. Phenolic acids; vanillic acid, caffeic acid and p-coumeric acid contents were determined. Amount of vanillic acid was found to be 0. 4875 ± 0. 0014 ppm, caffeic acid 223.50 ± 0. 44 ppm and 341.06 ± 1.43 ppm p-coumeric acid calculated according to **Figure 3.11.1**. The result indicates a high concentration of the p-coumeric acid in Ankara propolis explains the high antimicrobial and high cytotoxic effects of Ankara propolis, according to literature.

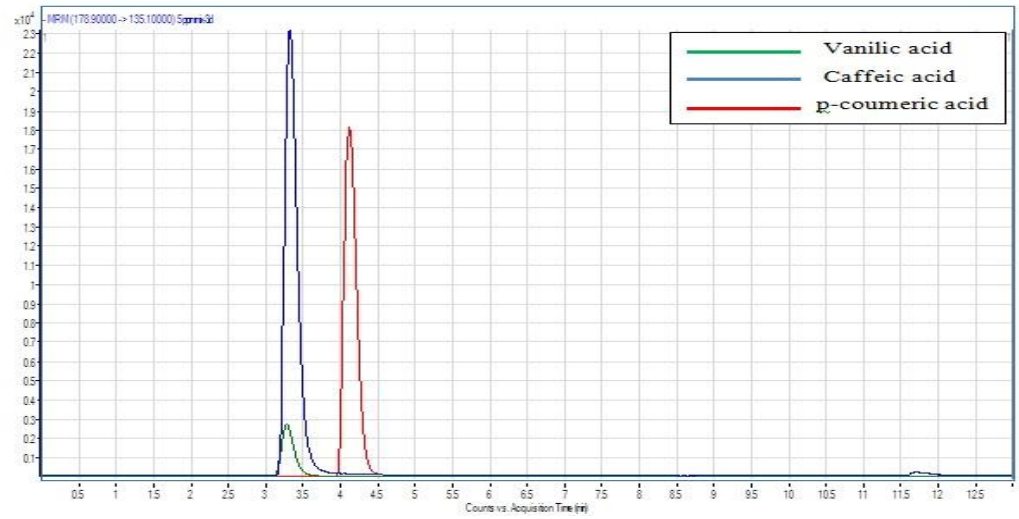

Figure 3. 11.1 LC/MS Chromatogram of Ankara propolis for vanillic, caffeic and p-coumeric acids.

Table 3.11.1 Ionization parameters of phenolics.

Compound Name	Precursor Ion(m/z)	Product Ion	Polarity
Caffeic acid	178.9	135.1	Negative
Vanillic acid	166.9	151.6 – 107.7	Negative
p-coumeric acid	163	119.1	Negative

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

In this study, propolis obtained from Ankara (Mamak) province of Turkey is studied for its antioxidant, antimicrobial and its cell proliferation characteristics.

From the study, it is found that ethanol extract of Ankara propolis has high content of phenolics and flavonoids supported by high Radical Scavenging activity. Although the location of hive is the same there could be variation in the composition and percentages of individual components of propolis with respect to years and seasons.

It was observed that ethanolic extract of propolis (EEP), from Ankara, has significant anti-fungal activity on selected fungus, has susceptible effect on gram-positive microorganism on the other hand there was no effect on gram- negative one this may the result of the high content of lipopolysaccharides on this kind of microorganism.

The HPLC studies were in-line with LC/MS, used for identification of phenolic masses in Ankara propolis. Most abundant phenolic was p-coumeric acid (341.06 ± 1.43 ppm) when compared to the caffeic acid (223.50 ± 0.44 ppm) and vanillic acid (0.4875 ± 0.0014 ppm).

Ethanol extract of Ankara propolis was found to induce cell proliferation significantly on HT-29 and Caco-2 cells at 100 μg / mL dose

Heat-treatment of propolis decreased the Radical Scavenging activity against DPPH and ABTS radicals, together with antimicrobial activity.

Among the most significant chemical compounds comprising Ankara propolis are phenolic acids and flavonoids. These compounds display high antioxidative

activity. But it still needs to establish the mechanism of action both on bacterial / fungal strains and cell lines. In consideration of the high antioxidative activity of Ankara propolis new therapeutic possibilities connected with this natural bee product are being actively sought.

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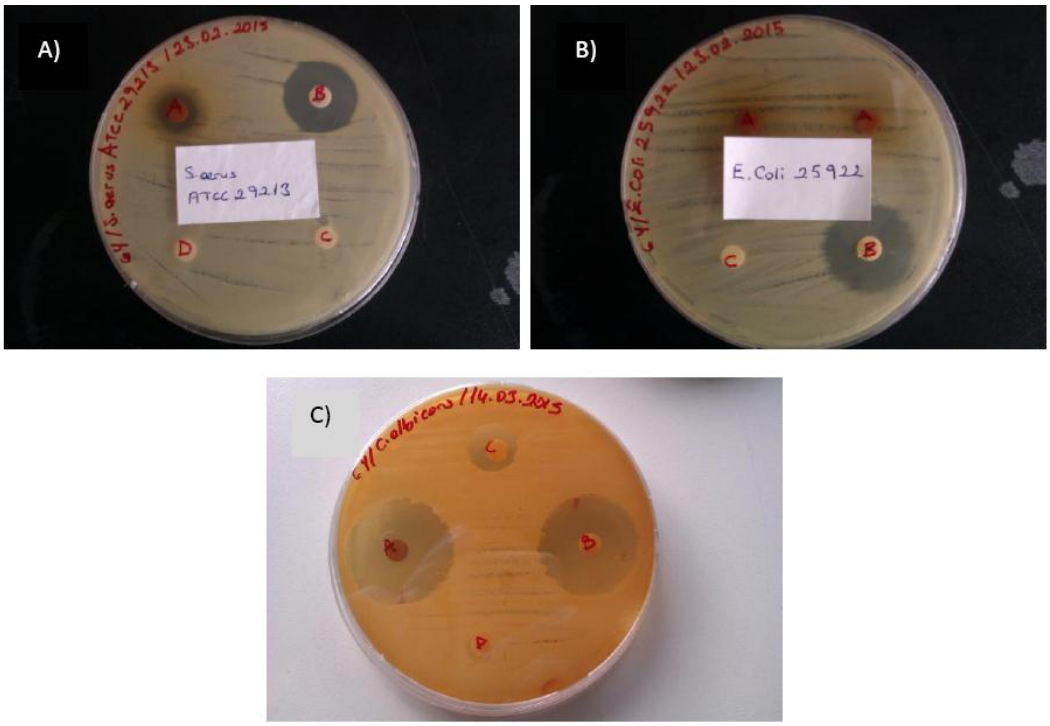
APPENDICES

A. CHEMICALS AND SUPPLIER INFORMATION

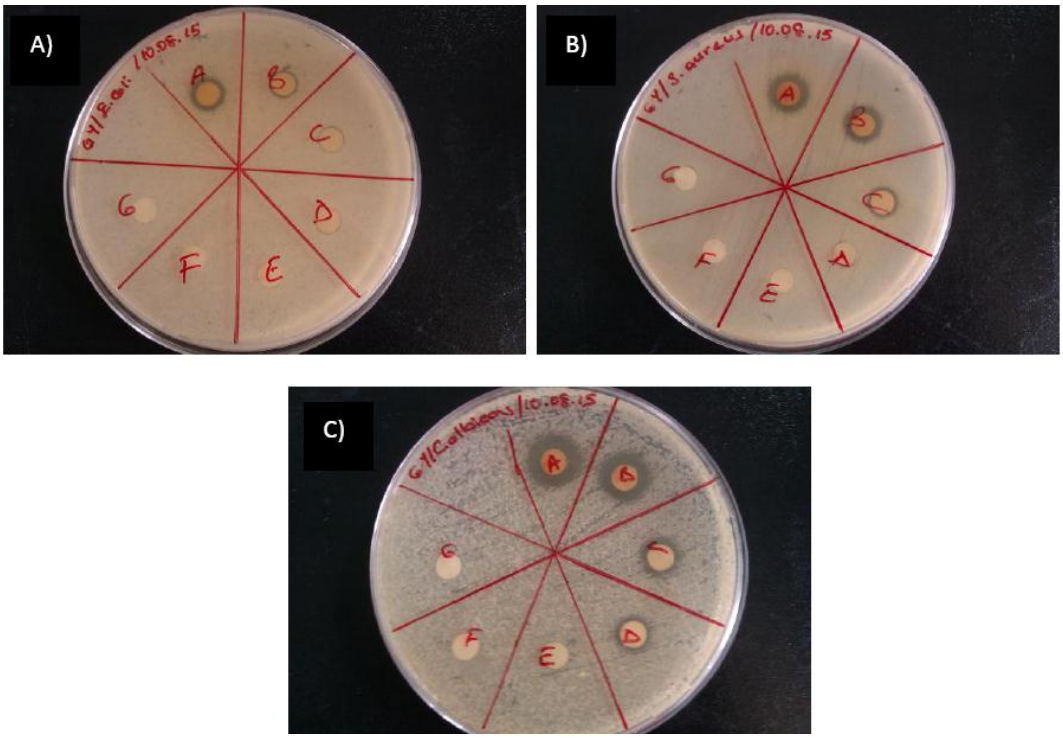
Table A.1 Chemicals and supplier information

Chemical	Supplier
DPPH	Sigma Aldrich
ABTS	Sigma Aldrich
Potassium persulfate	Sigma Aldrich
Trolox	Merck
Gallic acid	Sigma Aldrich
Quercetin	Sigma Aldrich
Mueller Hinton Agar	Merck
Mueller Hinton Broth	Merck
Malt Extract Agar	Merck
Aluminium chloride	Merck
Sodium Nitrate	Sigma Adrich
Sodium Hydroxide	Sigma Aldrich
Folin-Ciocalteau	Merck
Amikacin	Merck
Tetracyclin	Sigma Aldrich
DMEM	Biochrome
FBS	Gibco
Trypsin-EDTA	Sigma Aldrich
DMSO	Biochrome
Ethanol (HPLC grade)	Merck

B. ANTIMICROBIAL ACTIVITY OF PROPOLIS



Appendix B.1. Antimicrobial activity of propolis



Appendix B.2. The MIC values of propolis on microorganism (A: 64.0 mg / mL, B: 32.0 mg / mL, C: 16.0 mg / mL, D: 8.0 mg / mL, E: 4.0 mg /mL, F: 2.0 mg / mL, G: 1.0 mg / mL)