ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

INVESTIGATING THE ANTIOXIDANT AND ANTIBACTERIAL PROPERTIES OF PROPOLIS AND ITS POTENTIAL USE IN SOME FOOD PRODUCTS

Ph.D. THESIS

Tuğba ÖZDAL

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(506112505)

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Thesis Advisor: Prof. Dr. Dilek BOYACIOĞLU

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ISTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

PROPOLİSİN ANTİOKSİDAN VE ANTİBAKTERİYEL ÖZELLİKLERİNİN İNCELENMESİ VE GIDA ÜRÜNLERİNDE POTANSİYEL KULLANIMI

Doktora Tezi

Tuğba ÖZDAL
(506112505)

Gıda Mühendisliği Anabilim Dalı

Gıda Mühendisliği Programı

Tez Danışmanı: Prof. Dr. Dilek BOYACIOĞLU

KASIM 2017

Tuğba Özdal, a Ph.D. student of İTU Graduate School of Science Engineering and Technology student ID 506112505, successfully defended the dissertation entitled "**INVESTIGATING THE ANTIOXIDANT AND ANTIBACTERIAL PROPERTIES OF PROPOLIS AND ITS POTENTIAL USE IN SOME FOOD PRODUCTS**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor :	**Prof. Dr. Dilek BOYACIOĞLU** İstabbul Technical University	.....
Jury Members :	**Prof. Dr. Y. Şems YONSEL** Okan University	.....
	Assoc. Prof. Dr. Esra ÇAPANOĞLU GÜVEN İstanbul Technical University	.....
	Assoc. Prof. Dr. Neşe ŞAHİN YEŞİLÇUBUK İstanbul Technical University	.....
	Asst. Prof. Dr. Zeynep TACER CABA İstanbul Aydın University	.....

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Date of Defense : 20 November 2017

To my daughter Zeynep Sare and my husband Mehmet Emrah,

FOREWORD

Propolis is an important bee product receiving renewed attention worldwide because of its beneficial effects, among them, the effective antioxidant, antimicrobial and anticancer activities. The objectives of this thesis are as follows: (1) to investigate the total phenolic and flavonoid contents, total antioxidant capacities and identify individual phenolic compounds of propolis samples collected from all over Turkey, (2) to investigate antiproliferative and proliferative effect of propolis extracts on two different breast cancer cell lines; MDA-MB-231, UACC-3199 and on two normal cell lines; fibroblasts and mouse mesenchymal stem cell lines, and (3) to investigate the effect of addition of propolis extracts on chemical, microbiological and sensory properties of heat-treated sucuk samples produced at two different concentrations of propolis extract reducing the sodium nitrite level. I hope this research will contribute to the limited literature on propolis in Turkey including its antioxidant, antimicrobial and anticarcinogen effects and its usage in food products as a natural food preservative agent lowering the amount of synthetic food preservatives that may cause cancer.

First of all, I would like to express my special thanks and gratitude to my advisor, Prof. Dr. Dilek BOYACIOĞLU who had supervised me to carry out this interesting study for helping me in countless ways to bring me up in the scientific and academic area, constant guidance, motivation and the opportunities she had created for me throughout my study. She not only leads me into an exciting area of scientific research but also educates me with her philosophy of science and life, which makes me realize how essential the characters of patience and persistence are for accomplishing my goals. Being a PhD student under her guidance has been an honor.

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Words cannot describe my gratitude to my mother Mübeccel, my father Cem and my sister Esra who were always very loving and supportive throughout my education life. My special note of appreciation and thanks to my husband Mehmet Emrah ÖZDAL and my daughter Zeynep Sare ÖZDAL for always making me feel very "special" and always motivating me to do my best. I would like to thank my husband for his help, patience for spending weekends from morning till night at the laboratory with me, encouragement and support he provided to me throughout my study. This thesis and further plans would simply be impossible without you. I would like to give this thesis as a gift to my husband and my daughter.

November 2017 Tuğba ÖZDAL

(M.Sc. Food Engineeer)

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ABBREVIATIONS

ABTS	**:** 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
ANOVA	**:** Analysis of Variance
AOAC	**:** Association of Official Analytical Chemists
CAPE	**:** Caffeic Acid Phenyl Ester
CUPRAC	**:** Copper Reducing Antioxidant Capacity
DA	**:** Discriminant Analysis
DC	**:** Direct Current
DPPH	**:** 1,1-diphenyl-2-picrylhydrazyl
DNA	: Deoxyribonucleic Acid
DFS	**:** Disease Free Survival
EEP	**:** Ethanolic Extract of Propolis
ER	**:** Estrogen Receptor
ESMS	**:** Electrospray Mass Spectrometry
FRAP	**:** Ferric Reducing Antioxidant Power
GAE	**:** Gallic Acid Equivalent
GC-MS	**:** Gas Chromatography–Mass Spectrometry
GLM	**:** General Linear Model
HER2	**:** Human Epidermal Growth Factor Receptor 2 Gene
HPLC	**:** High Performance Liquid Chromatography
HPTLC	**:** High Performance Thin Layer Chromatography
IC_{50}	**:** The half maximal inhibitory concentration
LAEP	**:** Lyophilized Aqueous Extract of Propolis
LDA	**:** Linear Discriminant Analysis
MRS Agar	**:** de Man, Rogosa & Sharpe Agar
MSC	**:** Mesenchymal Stem Cells
PC	**:** Principal Component
PCA	**:** Plate Count Agar
PDA	**:** Photodiode Array
QDA	**:** Quadratic Discriminant Analysis
QE	**:** Quercetin Equivalent
RP-HPLC	**:** Reversed Phase High Performance Liquid Chromatography
SPSS	**:** Statistical Package for the Social Sciences
TBARS	**:** Thiobarbauturic Acid Reactive Substances
TBX-agar	**:** Tryptone Bile X-glucuronide Agar
TEAC	**:** Trolox Equivalent Antioxidant Capacity
Trolox	**:** 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
VRBA	**:** Violet Red Bile Agar
XTT	**:** (sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt
3,4-DHPPA	**:** 3,4-dihydroxyphenylpropionic acid
3-HPPA	**:** 3-hydroxyphenylpropionic acid
4-HPPA	**:** 4-hydroxyphenylpropionic acid
3-HBA	**:** 3-hydroxybenzoic acid

4-HBA **:** 4-hydroxybenzoic acid
3,4-DHPAA **:** 3,4-dihydroxyphenylacetic acid
3-HPAA **:** 3-hydroxyphenylacetic acid
4-HPAA **:** 4-hydroxyphenylacetic acid

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INVESTIGATING THE ANTIOXIDANT AND ANTIBACTERIAL PROPERTIES OF PROPOLIS AND ITS POTENTIAL USE IN SOME FOOD PRODUCTS

SUMMARY

Propolis is a resinous bee hive product that has many biological activities. In this study, a total of 54 raw propolis samples were collected from various geographical areas and beekeepers in Turkey in 2013 (32 samples) and 2014 (22 samples). Phenolic compounds were extracted from all samples using 70% ethanol by ultrasound assisted extraction method. Total phenolics and total flavonoids were estimated by Folin-Ciocalteau and aliminium chloride colorimetric method, respectively. The total phenolic contents of ethanolic extracts of propolis (EEP) samples varied between 6.18±0.36 mg GAE/g EEP and 157.25±12.2 mg GAE/g EEP. Their total flavonoid contents were found to be between 10.24 ± 0.33 mg QE / g EEP and 261.61±13.6 mg QE/g EEP. All the samples showed high total phenolic and flavonoid contents.

Total antioxidant capacities of EEP samples collected in 2013 and 2014 from various geographical locations and beekeepers in Turkey were measured using 4 different spectrophotometric methods including 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), cupric reducing antioxidant capacity (CUPRAC) and ferric reducing antioxidant power (FRAP) methods. The highest total antioxidant capacities of samples were found in sample #23 from Istanbul province of Turkey as 1392.27±131.33 μM/g EEP, 1510.64±55.4 μM TE/g EEP, 4220.24±161.75 μM TE/g EEP and 557.49±20.47 μM TE/g EEP measured by DPPH, ABTS, CUPRAC and FRAP methods, respectively. The results show significantly positive high correlations between total phenolic contents, total flavonoid contents, and total antioxidant capacities determined by four different spectrophotometric assays ($p < 0.05$).

Moreover, in this study, the individual phenolic compounds of ethanolic extracts of Turkish propolis were identified using High Performance Liquid Chromatography with Photodiode Array Detection (HPLC-PDA) method. Nine phenolic compounds were identified and quantified in 54 propolis extracts including galangin, chrysin, pinobanksin, pinostrobin, pinocembrin, caffeic acid, ferulic acid, *p*-coumaric acid and *t*-cinnamic acid.

Propolis samples collected in 2013 and 2014 (54 samples) were from 6 different geographical regions of Turkey including 4 samples from Aegean Region, 5 samples from Black Sea Region, 4 samples from Central Anatolia Region 6 samples from East Anatolia Region, 29 samples from Marmara Region and 6 samples from Meditteranean Region. According to their Univariate Analysis of GLM procedure, there were statistically significant differences between total phenolic contents and total flavonoid contents of propolis samples corresponding to different regions ($p < 0.05$), but the differences between total phenolic contents of propolis samples corresponding to 2013 and 2014 were insignificant ($p > 0.05$). In addition, the

differences between total antioxidant capacities of propolis samples according to year and regions were statistically insignificant ($p > 0.05$). There were statistically insignificant differences ($p > 0.05$) between individual phenolic contents of propolis samples collected in 2013 compared to individual phenolic contents of propolis samples collected in 2014. However, statistically significant differences ($p < 0.05$) were observed between individual phenolic contents of propolis samples including chyrisin, galangin, *p*-coumaric acid, pinobanksin, pinocembrin, *t*-cinnamic acid depending on regions.

Moreover, the amounts of total phenolics and flavonoids, phenolic profiles and antioxidant capacities (with 4 different methods) of propolis samples were analyzed using PCA to investigate the differences for the samples collected from 6 different regions of Turkey. The first three principal components (PCs) explained 82.8% of the total variance, where PC1 explained 65.5%, PC2 9.6% and PC3 7.7%, respectively. Additionally, Discriminant Analysis (DA) was used for classifying the propolis based on their sample collection regions. Accordingly, six of the nine phenolic compounds were found to be significant ($p < 0.05$) for the geographical discrimination of propolis samples. Those predictors were subjected to Linear Discriminant Analysis (LDA) if they can be reliable predictors to discrimate the geographical regions of propolis. The results show that there were strong statistical evidence of significant differences between means of geographical regions for all predictors with pinocembrin, *t*-cinnamic acid, *p*-coumaric acid, and chrysin. Furthermore, Quadratic Discriminant Analysis, which is to base the classification not on the combined covariance matrices but on the separate ones, was used by assuming unequal group variances. The first discriminant function (F1) accounted for 71.50% of total variance while the second and third accounted for 14.31% and 8.29%.

In the present research, antiproliferative and proliferative effect of propolis extracts were also investigated on 2 different breast cancer cell lines; MDA-MB-231 and UACC-3199 and on 2 normal cell lines; fibroblasts and mouse mesenchymal stem cell lines. Propolis extract used in this study were a mixture of propolis samples collected from 54 different beekepers of various geographical regions of Turkey in years 2013 and 2014. They were all blended and ground to powder. Ethanolic extract of propolis was prepared using 30% propolis tincture and removing ethanol using rotary evaporator. According to the tetrazolium dye (sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium), XTT, assay results, Turkish propolis sample showed significant antiproliferative effect on MDA-MB-231 and UACC breast cancer cell lines. Interestingly, Turkish propolis sample had proliferative effect on both fibroblasts and mouse mesenchymal stem cells. These results suggest that Turkish propolis can be considered as a potent agent on breast cancer treatment for further investigations.

Furthermore, in this thesis study, heat-treated dry fermented sucuk samples were produced at pilot scale with or without addition of propolis and nitrites to investigate its preservative effects based on its antimicrobial attributes. The sucuk samples were produced with 9 different formulations, as follows: (R) reference sample with 150 ppm nitrite; (A) 150 ppm nitrite and 0.25% propolis extract; (B) 150 ppm nitrite and 0.50% propolis extract (C) 50 ppm nitrite and 0.25% propolis extract; (D) 50 ppm nitrite and 0.50% propolis extract; (E) 75 ppm nitrite and 0.25% propolis extract; (F) 75 ppm nitrite and 0.50% propolis extract; (G) 100 ppm nitrite and 0.25% propolis extract; (H) 100 ppm nitrite and 0.50% propolis extract. Those samples were stored for 200 days. In samples, the physicochemical analyses (pH, moisture content, fat

content, salt content, moisture/protein content, collagen content, color, thiobarbautiric acid reactive substances) and microbiological analyses (total aerobic bacteria, *Eschericia coli*, total coliform, total mold and yeast, *Lactobacillus* spp., *Salmonella* spp. *Listeria monocytogenes*) were performed starting from day zero to the end of storage. Furthermore, sensory analysis was also performed to detect any statistically significant differences in flavor perceptions of panel when propolis was added to the sucuk samples.

The results showed that all samples were acceptable according to their physicochemical characteristics stated in Turkish Food Codex during 200 days of storage time. TBARS values were significantly affected ($p < 0.05$) by the addition of propolis extracts. Besides, increasing the concentration of propolis extract from 0.25% to 0.50% concentration change ($p < 0.05$) the TBARS values. The highest TBARS values were observed at control sample during 200 days of storage. Reference sample has reached to a maximum TBARS value of 1.08±0.012 mg/kg product at the end of 200th day where off-odors were produced above 1 mg/kg product level. Heat-treated sucuks containing propolis extract had significantly lower ($p < 0.05$) TBARS value when compared to the control sample. It was observed that propolis extracts appeared to be more effective on retarding lipid oxidation ($p < 0.05$) than sodium nitrate because of its high antioxidant capacity.

According to the microbiological analysis including total aerobic bacteria, *E. coli*, total coliform, total mold and yeast, *Lactobacillus* spp., *Salmonella* spp. *L. monocytogenes* all samples are considered as acceptable according to the food safety rules. Therefore, it can be stated that propolis is a strong antibacterial agent and can be effective at very small amounts when used in heat-treated dry fermented sucuk formulations to ensure the food safety throughout 200 days of storage time.

Furthermore, a General Lineer Model (GLM) of UNIVARIATE analysis was applied to selected variables where nitrite and propolis are accepted as independent two factors. Such analysis was aimed to understand the effects of those parameters and the presence of any interaction at their various levels on all physicochemical and microbiological analysis. Propolis addition was found to be significant ($p < 0.05$) in physicochemical properties including moisture, fat, collagen contents, TBARS, color (b* value) and microbiological properties of only total mold count ($p < 0.05$). Nitrite concentration was found to be significant ($p < 0.05$) in physicochemical characteristics including fat, protein, collagen contents, TBARS values, color (a* and b* values) and microbiological characteristics of only total mold counts. The differences of physicochemical characteristics including pH, moisture, fat, collagen contents, TBARS values and color characteristics were found to be dependent on interactions between propolis % and nitrite concentration ($p < 0.05$).

Besides, sensory analysis were applied to samples according to duo-trio test procedure to determine whether product differences result from a change of propolis extract content in their formulations and an overall difference exist between samples. As a result of this study it was observed that sample A, sample E, sample F results are below the significance level. It can be concluded that using half of the amount of nitrite than reference sample and addition of propolis as a powerful antimicrobial agent to dry-fermented sucuk formulations result in no statistically significant sensory difference. It would be healthier alternative to use propolis as an antimicrobial agent to decrease the nitrite amount in production of heat-treated dry fermented sucuk.

PROPOLİSİN ANTİOKSİDAN VE ANTİBAKTERİYEL ÖZELLİKLERİNİN İNCELENMESİ VE GIDA ÜRÜNLERİNDE POTANSİYEL KULLANIMI

ÖZET

Propolis, birçok biyolojik aktiviteye sahip reçineli bir arıcılık ürünüdür. Bu tez çalışmasında, Türkiye'nin çeşitli coğrafi bölgelerinden ve arıcılarından toplanmış olan 54 propolis numunesi (32 numune 2013 yılından, 22 numune de 2014 yılından olmak üzere) analiz edilmiştir. Fenolik maddeler %70 etanol kullanılarak ultrasonik banyoda ekstrakte edilmiştir. Toplam fenolik ve toplam flavanoid içerikleri sırasıyla Folin-Ciocalteu ve aluminyum klorür kolorimetrik metodu kullanılarak tespit edilmiştir. Türkiye'nin çeşitli coğrafi bölgelerinden ve arıcılarından toplanmış olan propolis etanolik ekstraktlarının toplam fenolik içerikleri 6.18±0.36 mg GAE/g EEP ve 157.25±12.2 mg GAE/g EEP arasında bulunmaktadır. Aynı örneklerin toplam flavonoid içerikleri de 10.24±0.33 mg QE/g EEP ve 261.61±13.6 mg QE/g EEP olarak bulunmuştur. Bütün örneklerin toplam fenolik ve toplam flavonoid miktarları çok yüksektir.

Türkiye'nin çeşitli bölgelerinden ve arıcılarından 2013 ve 2014 yıllarında toplanmış olan etanolik propolis ekstraktlarının toplam antioksidan kapasiteleri 2,2'-azino-bis(3-etillbenzotiazolin-6-sulfonik asit (ABTS), 2,2-difenil-1-pikrilhidrazil (DPPH), kuprik indirgeyici antioksidan kapasite (CUPRAC) and Demir(III) indirgeyici antioksidan kapasite (FRAP) olmak üzere dört farklı spektrofotometrik yöntemle ölçülmüştür. En yüksek antioksidan kapasiteye sahip olan örnek İstanbul şehrinden örnek 23 olarak belirlenmiş ve DPPH, ABTS, CUPRAC ve FRAP metotları kullanılarak antioksidan kapasitesi sırasıyla 1392.27±131.33 μM/g EEP, 1510.64±55.4 μM TE/g EEP, 4220.24±161.75 μM TE/g EEP ve 557.49±20.47 μM TE/g EEP olarak ölçülmüştür.

Bunun yanı sıra, bu çalışmada, Türkiye'nin çeşitli bölgelerinden ve arıcılarından toplanmış olan propolisin etanolik ekstraktlarının başlıca fenolik bileşikleri fotodiyot dizisi dedektör (PDA) bileşenli yüksek performanslı sıvı kromatografisi (HPLC) kullanılarak tanımlanmıştır. Ellidört propolis ekstraktının galanjin, krisin, pinobanksin, pinostrobin, pinosembrin, kafeik asit, ferulik asit, *p*-kumarik asit ve *t*-sinamik asit olmak üzere dokuz farklı fenolik bileşiği tanımlanmış ve miktarları belirtilmiştir. En yüksek krisin miktarı Türkiye'nin İstanbul şehrinden örnek 26 içerisinde bulunduğu (46.79 ± 9.7 mg/g EEP) ve bu örnekteki en yüksek oranda flavonoidin krisin olduğu tespit edilmiştir. Krisin miktarı, 2013 ve 2014 yıllarında Türkiye'nin çeşitli bölgelerinden toplanmış olan tüm propolis örnekleri içerisinde 1.62±0.37 ve 46.79 ± 9.7 mg/g EPP miktarları aralığında bulunmakta ve bu da Türkiye'den toplanan örnekler arasında krisin içeriği açısından anlamlı farklılıkları açıklamaktadır. Galanjin kavak tipi propolis türünün bir başka önemli flavonoidi olarak İstanbul'da örnek 23'te (31.31 ± 3.92 mg/g EEP) diğer örneklere göre daha yüksek miktarda bulunduğu tespit edilmiştir. Galanjin miktarı, 2013 ve 2014 yıllarında Türkiye'nin çeşitli bölgelerinden toplanmış olan tüm propolis örnekleri içerisinde 0.37±0.06 ve 31.31 ± 3.92 mg/g EPP miktarları arasında bulunmuştur.

Bunun yanında, pinobanksin en yüksek değeri Kocaeli'den örnek 6'da (29.47 ± 0.38 mg/g EEP) bulunmuştur. Türiye'nin Konya şehrinden toplanan propoliste diğer bölgelere kıyasla daha yüksek miktarda pinosembrin bulunmuş (65.94 ± 3.07 mg /g EEP) ve bu örnekteki en yüksek içerikli flavonoid olduğu tespit edilmiştir. Pinostrobin, kavak türü propolislerde yüksek oranda bulunan temel flavonoidlerden bir tanesi olmakla birlikte en yüksek oranda (37.85 ± 0.53 mg/g EEP) İstanbul şehrinde örnek 28'da bulunmuş, fakat bu örnekte pinosembrin (60.3±0.62 mg/g EEP) ve krisinden (41.17±9.9 mg/g EEP) sonra üçüncü en yüksek içerikteki flavonoid bileşeni olduğu tespit edilmiştir. Bütün örnekler arasında en yüksek miktarda (8.84±0.07mg/g EEP) kafeik asit içeren örneğin Kocaeli şehrinden örnek 38 olduğu bulunmuştur. Bunun yanında, en yüksek miktarda ferulik asit de İstanbul şehrinden örnek 26'da (5.19 ± 0.14 mg/g EEP) ölçülmüştür. Propolis örnekleri için tanımlanan diğer bir fenolik asit *p*-kumarik asittir ve en yüksek değer Düzce-Kocaeli şehrinden örnek 20'de (6.62 ± 0.07 mg/g EEP) tanımlanmıştır. Bununla birlikte, diğer propolis örneklerine kıyasla en yüksek miktarda *t*-sinamik asit İstanbul şehrinde örnek 23'te (3.64 ± 1.11 mg/g EEP) bulunmuştur. Genel olarak, bireysel fenolik bileşiklerin miktarları Türkiye'nin Marmara Bölgesi'nden toplanan propolis örneklerinde daha yüksek olduğu ve yüksek antioksidan kapasiteleri ile de pozitif yüksek korelasyonu olduğu tespit edilmiştir.

2013 ve 2014 yıllarında toplanmış olan propolis örnekleri (54 örnek) 4 örnek Ege Bölgesi, 5 örnek Karadeniz Bölgesi, 4 örnek İç Anadolu Bölgesi, 6 örnek Doğu Anadolu Bölgesi, 29 örnek Marmara Bölgesi ve 6 örnek de Akdeniz Bölgesi olmak üzere 6 farklı coğrafi bölgeden alınmıştır. Genel Lineer Model prosedürü Tek Değişken Analizi'ne göre, farklı bölgelerden toplanmış olan propolis örneklerinin toplam fenolik içerikleri ve toplam flavonoid içerikleri arasında istatistiksel olarak anlamlı farklılık bulunmakta ($p < 0.05$), fakat 2013 ve 2014 yılları arasında istatistiksel olarak anlamlı farklılık bulunmamaktadır ($p > 0.05$). Buna rağmen, farklı bölgeler ve farklı yıllar faktör olarak kabul edildiğinde propolis örneklerinin antioksidan kapasiteleri arasındaki farklılıklar istatistiksel olarak anlamlı değildir ($p > 0.05$). Propolis örneklerinin fenolik bileşenleri arasında da 2013 ve 2014 yılları arasında istatistiksel olarak anlamlı farklılık bulunmamaktadır ($p > 0.05$). Buna rağmen, bölgeler faktör olarak dikkate alındığında, propolis örneklerinin fenolik bileşenleri olan krisin, galanjin, *p*-kumarik asit, pinobanksin, pinosembrin, *t*-sinamik asit bölgeler arasında istatistiksel olarak anlamlı farklılık göstermekte, ama diğer fenolik bileşikler olan kafeik asit, ferulik asit ve pinostrobin istatistiksel olarak anlamlı farklılık göstermemektedir.

Bunun yanında, 6 farklı coğrafi bölgeden toplanmış olan propolis örneklerinin toplam fenolik ve flavonoid miktarları, fenolik profilleri ve antioksidan kapasiteleri (4 farklı metotla) arasındaki farklılıklar Temel Bileşen Analizi (TBA) ile analiz edilmiştir. İlk 3 temel bileşen (TB), sırasıyla TB1 %65.5, TB2 %9.6 ve TB3 %7.7 olmak üzere toplam varyansın %82.8'ini açıklamaktadır. Ayrıca, Diskriminant Analizi (DA) propolis örneklerinin toplanmış olduğu coğrafi bölgelerin sınıflandırılması için kullanılmıştır. Sonuç olarak, dokuz fenolik bileşiğin 6 tanesi propolis örneklerinin coğrafi bölgelere göre ayrılması için istatistiksel olarak anlamlı ($p < 0.05$) sonuç vermiştir. Bu fenolik bileşikler, propolisin coğrafi bölgelere göre ayırt edilmesinde güvenilir sonuçlar verip vermediğinin test edilmesi için Lineer Diskriminant Analizine (LDA) tabi tutulmuşlardır. Sonuçlar, pinosembrin, *t*-cinnamik asit, *p*-kumarın asidi ve krisin ile tüm belirleyiciler için coğrafi bölgeler arasında anlamlı farklılıkların bulunduğuna dair güçlü istatistiksel kanıtın olduğunu göstermektedir. Ayrıca, sınıflandırmayı birbirine bağlı kovaryans matrisleri üzerinde

değil de ayrı kovaryans matrisleri üzerine dayandırmak için kullanılan Karesel Diskriminant Analizi, eşit olmayan grup varyansları varsayarak kullanılmıştır. İlk diskriminant fonksiyonu (F1) toplam varyansın %71.50'ini oluştururken, ikinci ve üçüncü diskriminant fonksiyonları %14.31 ve % 8.29'unu oluşturmakta olduğu tespit edilmiştir.

Propolis ekstraktlarının anti-proliferatif ve proliferatif etkileri 2 farklı meme kanser hücre hattı olan MDA-MB-231 ve UACC-3199 üzerinde ve 2 normal hücre hattı olan fibroblastlar ve fare mezankimal kök hücre hattı üzerinde incelenmiştir. Bu çalışmada Türkiye'nin farklı coğrafi bölgelerindeki 54 farklı arıcısından 2013 ve 2014 yıllarında toplanmış olan propolis numuneleri kullanılmıştır. Bütün propolis numuneleri karıştırılarak toz haline getirilmiştir. Propolis etanolik ekstraktı %30 propolis tentürü kullanılarak ve dönen buharlaştırıcı kullanılarak etanol buharlaştırılarak hazırlanmıştır. XTT sonuçlarına göre, Türk propolis numunesi MDA-MB-231 ve UACC meme kanser hücre hattı üzerinde istatistiksel olarak anlamlı anti-proliferatif etki göstermiştir. İlginç olarak, Türk propolis örneğinin hem fibroblastlar hem de fare mezenkimal kök hücreler üzerinde proliferatif etkiye sahip olduğu bulunmuştur. Bu sonuçlar, Türk propolisinin meme kanseri tedavisinde ileri tetkikler için güçlü bir ajan olarak kullanılabileceğini düşündürmektedir.

Bu tez çalışmasında ayrıca ısıl işlem görmüş sucuk örnekleri 9 farklı formülasyonda pilot ölçekli olarak üretilmiştir. Isıl işlem görmüş sucuk formülasyonları : (R) 150 ppm sodyum nitrit içeren referans örnek; (A) 150 ppm sodyum nitrit ve 0.25% propolis ekstraktı içeren örnek; (B) 150 ppm sodyum nitrit ve 0.50% propolis ekstraktı içeren örnek; (C) 50 ppm sodyum nitrit ve 0.25% propolis ekstraktı içeren örnek; (D) 50 ppm sodyum nitrit ve 0.50% propolis ekstraktı içeren örnek; (E) 75 ppm sodyum nitrit ve 0.25% propolis ekstraktı içeren örnek; (F) 75 ppm sodyum nitrit ve 0.50% propolis ekstraktı içeren örnek; (G) 100 ppm sodyum nitrit ve 0.25% propolis ekstraktı içeren örnek; (H) 100 ppm sodyum nitrit ve 0.50% propolis ekstraktı içeren örnek olarak üretilmiştir. Isıl işlem görmüş dokuz farklı formülasyonda sucukların üretimlerinden itibaren 200. depolama gününe kadar fizikokimyasal analizleri (pH, nem içeriği, yağ içeriği, tuz içeriği, nem/protein içeriği, kollajen içeriği, renk, tiobarbitürik asit reaktif maddeler) ve mikrobiyolojik analizleri (toplam aerobik bakteri, *Eschericia coli*, toplam koliform bakteri, toplam maya ve küf, *Lactobacillus* spp., *Salmonella* spp., *Listeria monocytogenes*) yapılmıştır. Bunun yanında, ısıl işlem görmüş sucuklarda propolis ekstraktı içeren sucukların propolis ekstraktı içermeyen referans örneği ile arasında istatistiksel olarak anlamlı farklılığın var olup olmadığı anlaşılması için duyusal analiz gerçekleştirilmiştir.

Depolama süresi boyunca yapılan analizlerin sonucunda bütün örneklerin fizokokimyasal analiz sonuçlarının Türk Gıda Kodeksi'ne uygun değerler içerisinde bulunduğu görülmüştür. TBARS değerlerinin, propolis ekstraktı ilavesine bağlı olarak istatistiksel olarak anlamlı bir şekilde etkilendiği tespit edilmiştir. Ayrıca, propolis ekstraktı konsantrasyonunun %0.25'den %0.50'ye arttırılması da sucuk örneklerinin TBARS değerlerinde istatistiksel olarak anlamlı azalmaya ($p < 0.05$) neden olmaktadır. Kontrol örneği maksimum TBARS değeri olan 1.08±0.012 mg/kg ürün değerine 200. depolama gününde yapılan analiz sonucunda ulaşmış ve istenmeyen koku oluşumunun 1 mg/kg ürün değeri üzerinde oluştuğu saptanmıştır. Propolis ekstraktı içeren ısıl işlem görmüş sucuk ürünlerinin kontrol örneği ile karşılaştırıldığında istatistiksel olarak anlamlı daha düşük sonuçlara ($p < 0.05$) sahip olduğu görülmektedir. Sonuç olarak yüksek antioksidan kapasitesine bağlı olarak

propolis ekstraktının sodyum nitrite göre daha etkili olduğu ($p < 0.05$) tespit edilmiştir.

Toplam aerobik bakteri, *E. coli*, toplam koliform bakteri, toplam maya ve küf, *Lactobacillus* spp., *Salmonella* spp., *L. monocytogenes* mikrobiyolojik analizlerine göre bütün örneklerin depolama süresi boyunca gıda güvenliği kuralları açısından kabul edilebilir olduğu tespit edilmiştir. Bu nedenle, propolisin güçlü bir antibakteriyel ajan olduğu ve 200 gün depolama süresince gıda güvenliğini sağlamak için, ısıl işlem görmüş sucuk formülasyonlarında kullanıldığında çok az miktarlarda dahi etkili olabileceği sonucu çıkartılmıştır.
Bunun yanında, nitrit ve propolisin bağımsız iki faktör olarak kabul edildiği seçilmiş değişkenlere UNIVARIATE analizinin Genel Lineer Modeli (GLM) uygulanmıştır. Bu analiz, bu parametrelerin ve çeşitli fizyokimyasal ve mikrobiyolojik analizler üzerindeki etkileşimlerinin çeşitli düzeylerde etkilerini açıklamayı amaçlamaktadır. Propolis ilavesinin ısıl işlem görmüş fermente sucukların nem, yağ, kollajen miktarları, TBARS değeri, renk (b* değeri) gibi fizikokimyasal özellikleri üzerine ve toplam küf sayısı mikrobiyolojik özelliği üzerine istatistiksel olarak önemli ($p < 0.05$) farklılıklar yarattığı bulunmuştur. Nitrit konsantrasyonun ise, ısıl işlem görmüş fermente sucukların yağ, protein, kollajen içeriği, TBARS değeri, renk (a* ve b* değerleri) gibi fizikokimyasal özellikleri ile toplam küf sayısı mikrobiyolojik özelliği üzerinde istatistiksel olarak anlamlı ($p < 0.05$) etkisi olduğu bulunmuştur. Bunun yanında % propolis ve nitrit konsantrasyonu etkileşimlerinin etkisinin ısıl işlem görmüş sucukların pH, nem, yağ, kollajen içeriği, TBARS değerleri ve renk özellikleri olan fizikokimyasal özellikleri üzerinde istatistiksel olarak anlamlı ($p < 0.05$) sonuçlar verdiği tespit edilmiştir.

Bunun yanında, propolis ekstraktı içeren ısıl işlem görmüş sucuk örneklerinin kontrol örneğinden genel anlamda duyusal farklılıklarının tespit edilmesi amacıyla ikili-üçlü test metodu kullanılarak duyusal analizleri gerçekleştirilmiştir. Bu çalışmanın sonucunda, A, E ve F örneklerinin anlamlılık düzeyinin altında olduğu tespit edilmiştir. Sonuç olarak, ısıl işlem görmüş sucuk ürünlerinde propolis ekstraktı içermeyen ama 150 ppm sodyum nitrit içeren referans örneğinin yerine 75 ppm sodyum nitrit ve güçlü antimikrobiyal ajan olan propolisin (0.25% veya 0.50% konsantrasyonlarda) kullanımının duyusal açıdan istatistiksel olarak anlamlı ($p < 0.05$) bir farklılığa neden olmadığı bulunmuştur. Isıl işlem görmüş sucuk üretiminde sodyum nitrit oranının azaltılarak güçlü antimikrobiyal etkiye sahip propolis ekstraktının kullanılması sağlıklı bir alternatif olacaktır.

1. INTRODUCTION

Propolis is a resinous natural substance produced by honeybees by mixing their own wax with various plant sources they collected and transported to their hive to form strongly adhesive material. It is considered as a glue or the cement for honeybees that they used to seal cracks and block holes in the hive, smooth the internal surface of the hive to minimize moisture loss and to embalm killed insects or invaders (Ghisalberti, 1979). Moreover, propolis provides a healthy environment inside the hive according to its strong antimicrobial activity (Simone-Finstrom and Spivak, 2010).

Propolis has many pharmacological activities such as antioxidant, antibacterial, antiviral, anti-poliovirus; anti-inflammatory, immuno-modulatory, anti-cancer, anti-allergic, hepatoprotective and photoprotective activities (Bolfa et al., 2013; Búfalo et al., 2009; Frozza et al., 2013; Chirumbolo, 2011; Choudhari et al., 2012; Kolankaya et al., 2002; Kouidhi et al., 2010; Schnitzler et al., 2010; Silva et al., 2013. There are more than 300 phenolic compounds identified in propolis from various regions of the world. The chemical composition of propolis varies greatly according to its botanical origin and time of collection, but generally it is composed of 50% resin and vegetable balsams, 30% wax, 10% essential and aromatic oils, 5% pollens, and 5% other substances (Marcucci, 1995).

Propolis from temperate regions and tropical regions show great differences in their individual phenolic contents and in relation with their pharmacological activities. Poplar-type propolis from temperate regions generally rich in flavonoids, aglycone aromatic acids, and their esters (Bankova et al., 2000). However, the individual phenolics of tropical regions mainly consist of prenylated *p*-coumaric acid derivatives, flavonoids, benzophenones, lignans, and terpenes (Popova et al., 2009). Moreover, mixed phenolic profiles can be obtained from Mediterranean countries (Ahn et al., 2007). Pasific propolis is generally consist of prenylated flavanones as major constituents (Chen et al., 2003; Kumazawa et al., 2004). Therefore, the

characterization of Turkish propolis in a 2 years study is needed to provide a basis for determination of its origin.

Recently, some *in vitro*/*in vivo* research studies have presented the anti-cancer activity of propolis from various locations and in some studies bioactive compounds were isolated to understand the effects on tumour and non-tumour cells (Barlak et al., 2011; Chan et al., 2013; Lotfy, 2006; Umthong et al.,2011; Valente et al., 2011; Wu et al., 2011). Considering the results of these studies, it can be concluded that propolis can block specific oncogenic signaling pathways (e.g. β-catenin, c-myc, NF-κB and some intermediary of the PI3K/AKT pathway) resulting in reduced cancer cell proliferation and growth, increasing apoptosis, showing antiangiogenic effects and modulating the tumor microenvironment, more specifically suppressing the invasion and migration (Chan et al., 2013). The results of *in vitro* and *in vivo* studies suggest that propolis possess important cytotoxic properties against cancer cells. There is a very limited data in the literature on the anti-cancer activities of Turkish propolis (Barlak et al., 2011). To our knowledge there is no data on the effects of propolis on MDA-MB-231 breast cancer cell line and UACC-3199 human infiltrating ductal carcinoma of breast, fibroblast and mouse mesenchymal stem cell line C57B/6. If researchers can effectively increase stem cells while preserving the self-renewal capacity, the track to their clinical implementations would become more efficiently possible. Therefore, it is obvious that there is a need for new information about the effect of Turkish propolis on stem cells in addition to various cancer cell lines. Improvement of our scientific knowledge will increase the value of product and contribute Turkish economy and beekeeping sector.

Sucuk is a spicy, dry, fermented sucuk which is popular in Turkey and can also be found in countries located in Balkans, Middle East and Caucasus. It is a sucuk type product manufactured from a mixture of meat (beef, sheep and/or water buffalo meat), fat (beef fat and sheep tail fat), salt, sugar, garlic and spices and seasonings, and this mixture is stuffed into a casing subjected to fermentation and drying processes under certain conditions to reduce the moisture content to less than 50% to produce a semi or dry product (Turkish Food Codex Communique, 2012). During the last three decades, the manufacturing technology of traditional sucuk has been modified, and heat treatment has been added to production steps due to the commercial reasons such as to extend shelf life, to shorten production time and to

reduce production cost although thermal processing is not a part of traditional sucuk processing. The aim of heat-treatment is to eliminate foodborne pathogen bacteria at about 68-70°C for 15-30 mins (Ercoşkun et al., 2010). However, lactic acid bacteria, *Micrococcus* and *Staphylococcus* are also destroyed at the same time. Therefore, insufficient acidification during fermentation of sucuks may result in some quality and safety concerns. Additionally, extreme fermentation conditions (anaerobic environment, high salt concentration, temperature, low pH, etc.), production steps (heat treatment, crushing, drying, etc.) and unsuitable storage conditions have negative influence on viability and growth rate of starter cultures in heat-treated sucuk (Ammor and Mayo, 2007; Kolozyn-Krajewska and Dolatowski, 2012). Preservatives are used in meat and meat products including nitrites and nitrates or their mixture. Nitrite is used for microbiological safety, color properties, flavour development and extending the shelf life of meat products (Honikel, 2008). Nitrites used to control growth of *Clostridium botulinum* (Pegg and Shahidi, 2000) and also *Listeria monocytogenes* (Xi et al., 2012) in meat products. However, there are also serious risks according to the addition of nitrates in meat products including nitrosamine formation from nitrite and secondary amines in acidic medium (Honikel, 2008; Mills, 2014). Nitrosamine formation is affected by the production process, food preparation and human digestive system (Bauer, 2014). Recently, nitrosamines were reported as carcinogenic to humans and classified as group 1 carcinogen compound to humans by the International Agency For Research On Cancer (IARC, 2015). Although nitrates are widely used in meat industry, the addition of these chemical additives must be decreased according their detrimental effects on health. Regarding the reported health concerns of nitrite, consumers are more interested in natural additives that are safer for health without synthetic additives resulting in health-damaging effects (Alahakoon et al., 2015; Jayasena and Jo, 2013). Therefore, the development and application of natural products with high antioxidant and antibacterial capacities in meat products may be necessary and useful to enhance their storage shelf life.

Propolis is also recognized as Generally Recognised as Safe (GRAS) (Burdock, 1998) and this fact makes it an attractive product as a natural preservative in novel food applications, functional foods and nutraceuticals (Fokt et al., 2010). Therefore, propolis can be a good alternative as a natural preservative to meat products

regarding its high antioxidant and antimicrobial capacities. So far, to the best of our knowledge, there have been no investigations on the impact of propolis extract on physicochemical, microbiological and sensory parameters in heat-treated sucuks.

There are three objectives of this thesis; The first aim was to investigate the *in vitro* total phenolic and flavonoid contents, total antioxidant capacities and individual phenolic contents of the 70% ethanol extracts of propolis collected from various regions in Turkey in 2013 and 2014 with a goal of characterizing and defining Turkish propolis as a basis of determination of its origin. The second objective was to investigate the cytotoxic activities of Turkish propolis on human breast cancer cell lines and the proliferative effects on fibroblasts and stem cells. The third and final objective was to investigate the effect of addition of propolis extract on physicochemical, microbiological and sensory properties of heat treated dry fermented sucuk samples during storage period of 200 days for a goal of replacing or reducing the traditional preservative, the nitrites.

2. LITERATURE REVIEW

2.1 Chemical Composition of Propolis

Complex chemical composition of propolis depends on the flora around the hive and time of collection (Bankova et al., 2000, 2005; Marcucci, 1995). However, the factors determining its composition is largely based on the type of bee species and their different choice of resin and plants, the specificity of the flora based on geographical region, variations in the resin composition of plant as well as the presence of plant species (Bankova et al., 2014; Kongkiatpaiboon et al., 2016; Trusheva et al., 2011). For instance, propolis samples from Europe, South America, and Asia have different chemical compositions mostly due to the geographical differences (Ahn et al., 2007).

Propolis contains a variety of chemical compounds reported in several research studies including volatile organic compounds, flavonoid aglycones, phenolic acids and their esters, phenolic aldehydes, alcohols and ketones, sesquiterpenes, quinones, coumarins, steroids, and aminoacids (Ahn et al., 2007; Bankova et al., 2000; Marcucci et al., 2000).

According to Marcucci (1995), main compounds that are identified in propolis resin occur from 3 sources: plant exudates which are collected by honeybees, secreted substances which come from the honeybees' metabolisms, and the other organic materials which are introduced during propolis development. Chemical substances found in propolis occurs from waxes, resins, balsams, aromatic and ethereal oils, pollen, and other organic matter (Marucci, 1995). As shown by Tagliacollo and Orsi (2011) propolis occurs approximately from 50% resin and balsam, 30% wax, 10% essential and aromatic oils, 5% pollen, and 5% impurities. However, analysing propolis to obtain those compounds is difficult due to its complex composition (Marucci, 1995). Propolis standardization is done in the same way as it is done for medicinal plants (Tagliacolla and Orsi, 2011).

The available literature on propolis presents a variation in its composition depending on the region of the World. Yarfani et al. (2011), identified the chemical composition

of south Iranian propolis extract by using gas chromatography–mass spectrometry (GC-MS). They reported the Iranian propolis is composed of phenolic acids (89.8%), sugars (3.2%), and caffeic acid (2.2%), oleic acid (0.8%), hexadecanoic acid (0.5%), cinnamic acid (0.4%), 3-quinoline carboxamide (0.4%), 12β-hydroxypicrasan-3-one (0.3%), octadecanoic acid (0.2%), butanedioic acid (0.2%), ferulic acid (0.2%), levomenol (0.2%), isoferulic acid (0.1%), and unknown components (1.5%) (Yarfani et al., 2011).

Popova et al. (2011), studied on the composition of Maltese propolis. They encountered the typical Mediterranean chemical profile which is rich in diterpene compounds. Thus, they identified 32 individual diterpenes from the 17 samples, 22 of them were present in each sample. They mostly found diterpene acids in all samples, which were isocupressic, communic, pimaric and imbricatoloic acid, together with totarol and 13-epi-torulosal. Also, they found that propolis samples were very rich in sugars and sugar derivatives. Mono- and sesquiterpenyl esters of substituted benzoic acids were identified including two new propolis components which were daucane diterpene esters of hydroxybenzoic acids. Neoabietic acid, manool oxide, 13-epi-torulosol, abietic acid, acetylisocupressic acid, agathadiol, communal, copalol, 13-epi-cupressic acid, dehydroabietic acid, 13,14-dehydrojunicedric acid, 14,15-dinor-13-oxo- 8(17)-labden-19-oic acid, ferruginol, ferruginolon, hydroxydehydroabietic acid, 2-hydroxyferruginol, 6/7-hydroxyferruginol, isoagatholal, junicedric acid, labda-8(17),12,13-triene, 13-epi-manool, sempervirol, 13-epi-torulosol, totarolon, and two unidentified diterpene acids (one of them present in all samples) were identified during the study. Other components identified from Maltese propolis were aliphatic hydroxyacids, aromatic and fatty acids, triterpenes (mainly alcohols of the amyrin type). Moreover, they identified the minor amounts of typical poplar flavonoids (chrysin, pinocembrin chalcone) and poplar caffeic acid esters (pentenyl caffeates) in only one sample (Popova et al., 2011).

Thirugnanasampandan et al. (2012), analysed the chemical composition and bioactive potential of hydroalocoholic extract of propolis collected from India. They used GC- MS, High Performance Thin Layer Chromatography (HPTLC) and High Performance Liquid Chromatography (HPLC) methods to analyse ethanol extract of propolis, and performed *in vitro* antioxidant, anticholinesterase and cytotoxicity

assays. The results showed that propolis samples were containing fatty acids such as 9- octadecenoic acid (3.2%), decanoic acid (2.12%) 9,12 hexadecanoic acid (1.29%), octadecadienoic acid methyl ester (0.49%); alcohols such as 1-tetradecanol (0.89%), octadecanol (0.69%), 1-dotricontanol (0.48%) and 2, 3 epoxy-5, 8-hectadecadien-1-ol (0.6%); quercetin, and cyclopentadiene (Thirugnanasampandan et al., 2012).

According to Bankova et al. (2000) the mineral elements of propolis originated from Macedonia and Cuba are calcium (Ca), magnesium (Mg), manganese (Mn), potassium (K), sodium (Na), iron (Fe), zinc (Zn), and copper (Cu). Also, it has been known that propolis contains vitamins B1, B2, B6, C, D, E and mineral elements silver (Ag), cesium (Cs), mercury (Hg), lanthanum (La), antimony (Sb), aluminium (Al), vanadium (V) and silicon (Si) (Marucci, 1995).

2.2 Phenolic Composition of Propolis

The phenolic composition of propolis is variable and complex depending on the factors such as origin of the sample which is closely related with the flora surrounding the hive, the geographic and climatic characteristics at the site, honey bee species, and botanical and geographical differences. The variations in propolis bioactivity are expected to be associated with the variety of its phenolic constituents (Ahn et al., 2007; Moreno et al., 2000; Silici and Kutluca, 2005). Propolis from temperate regions; also called poplar-type propolis; contains mainly phenolics: flavonoids aglycone, aromatic acids and their esters (Bankova et al., 2000). Propolis from tropical regions contains different phenolics: prenylated *p*-coumaric acid derivatives, flavonoids, benzophenones, lignans, and terpenes (Popova et al., 2009). Propolis is the richest product in bee products for its polyphenolic content as it contains about 10% of flavonoids with 38 different flavonoid structures (Suver, 2008; Haroun, 2006).

Polyphenolic fraction of propolis have much more therapeutic effect than other components of propolis (Erdoğan et al., 2011). The efficiency of extraction highly depends on the method, and the most common is solid-liquid extraction with ethanol, methanol or water. Besides, some other substances can be used for propolis extraction such as oil (Finger et al., 2013). However, for the fractionation purposes, ethanol extract of propolis (EEP) is the most common way used (Marcucci, 1995; Tagliacollo and Orsi, 2011). The ethanolic extraction of propolis (EEP) represents

the richest content of phenolic acids and flavonoid sources (Watanabe et al., 2011). Ethanol extracts of propolis is widely used in antioxidant capsules, throat sprays, toothpaste and cosmetics (Sarıkaya et al., 2009; Coneac et al., 2008).

Biological activity of propolis is associated with the existance of flavonoids. Their biochemical effect can be grouped in four activities: binding to biological polymers, binding to heavy metal ions, catalizing the electron transfer, and the ability to scavange free radicals (Coneac et al., 2008).

Pinocembrin is the most abundant flavonoid in propolis. Pinobanksin and chrysin are the characteristic compounds of propolis (Jaganathan and Mandal, 2009). Some other flavonoids that exist in propolis are galangin, kaempferol, quercetin, tectocricin, acacetin and apigenin (Jaganathan and Mandal, 2009; Suver, 2008). Propolis include some polyphenolic acids such as caffeic acid, benzoic acid, hydroxycinnamic acid, cinnamic acid, coumaric acid, fumaric acid, vanillic acid, hydroxybenzoic acid, and salicylic acid. Flavonoids that have antimicrobial activity are pinocembrin, galangin, pinobanksin, *p*-coumaric acid, caffeic acid (Coneac et al., 2008; Ahn et al., 2007; Kumazawa et al., 2004; Marcucci, 2001). Basim et al. (2006), identified more than 180 compounds from propolis collected from Hatay province in Turkey. They found that propolis samples mainly contained polyphenols (Basim et al., 2006).

Sarıkaya et al. (2009) studied the antioxidant activity and phenolic acid contents of Turkish chestnut honey and poplar type propolis. They have found out that the chestnut propolis has higher antioxidant activity and phenolic acid content than the chestnut honey (Sarikaya et al., 2009). Moreover, there are several studies on the phenolic content and composition of Turkish propolis. Erdogan et al. (2011), performed a study to evaluate antiradical capacities, total phenolic contents and individual phenolic compounds of Turkish poplar type propolis samples collected from different regions of Anatolia; Bingol, Rize, Tekirdag and Van by pressurized liquid extraction. The results of the study showed that major phenolic compounds presented in all samples were gallocatechin, catechin, epicatechin gallate, caffeic acid, chlorogenic acid (ChA), and myricetin (Erdogan et al., 2011). Moreover, Aliyazıcıoglu et al. (2013), researched the phenolic compounds and biological activities of ten different poplar type propolis samples from different regions of Anatolia in Turkey by RP-HPLC method. They identified seventeen different

phenolic components. According to the results of the study, quercetin, benzoic acid, caffeic acid, ferulic acid, and coumaric acid were found in all propolis samples in high amounts, while vanillic acid, chlorogenic acid, epicathecin, rutin, syringic acid, and *o*-coumaric acid were found in very small amounts (Aliyazıcıoglu et al., 2013). Furthermore, Gülçin et al. (2010) measured amounts of caffeic acid (5033.8 mg/kg), ferulic acid (5261.8 mg/kg), syringic acid (12.8 mg/kg), ellagic acid (4202.7 mg/kg), quercetin (3851.4 mg/kg), α-tocopherol (265.5 mg/kg), pyrogallol (45.3 mg/kg), *p*-hydroxybenzoic acid (1370.9 mg/kg), vanillin, *p*-coumaric acid (1908.8 mg/kg), gallic acid (419.1 mg/kg) and ascorbic acid (1098.0 mg/kg) by high performance liquid chromatography and tandem mass spectrometry (LC–MS/MS) in Turkish propolis. Recently, Yesiltas et al. (2015) identified and quantified phenolic compounds including caffeic acid (0.04±0.01 to 3.70 ± 0.08 mg/g), *p*-coumaric acid (0.09±0.03 to 4.53 ± 0.06 mg/g), ferulic acid (0.06 ± 0.01 to 6.27±0.13 mg/g) , quercetin (0.13±0.02 to 0.74 ± 0.32 mg/g), pinobanksin (4.30 ± 0.06 to 38.19 ± 0.77 mg/g), apigenin (0.47 ± 0.05 to 2.89 ± 0.42 mg/g), *t*-cinnamic acid (1.01±0.07 to 15.25 ± 0.28 mg/g), luteolin (0.05 ± 0.01 to 0.19 ± 0.19 mg/g), pinocembrin (5.32±0.11 to 42.37±0.60 mg/g), chrysin (11.35±0.27 to 70.69±2.21 mg/g), galangin (11.89±2.62 to 35.82±3.11 mg/g), kaempferol (0.24±0.20 to 1.29±0.03 mg/g) and pinostrobin (10.85±1.61 to 81.49±2.72 mg/g) in 3 Turkish poplar type propolis samples collected from Istanbul province. They have also found that pinostrobin, chrysin, pinocembrin, pinobanksin and galangin which are the representative flavonoids in propolis were in high quantity in ethanolic extracts of propolis, respectively.

Trusheva et al. (2011) identified 11 compounds from Indonesian poplar type propolis. They found four alk(en)ylresorcinols which were obtained as an inseparable mixture, four prenylflavanones, and three cycloartane-type triterpenes. They identified the alk(en)ylresorcinols as: 5-pentadecylresorcinol, 5-(8'Z, 11'Z-heptadecadienyl)-resorcinol, 5-(11'Z-heptadecenyl)-resorcinol and 5-heptadecylresorcinol. Among them 5-(8' Z,11' Z-Heptadecadienyl)-resorcinol was the most abundant. Three different cycloartane-type triterpenes were isolated and identified as mangiferolic acid, isomangiferolic acid and 27-hydroxyisomangiferolic acid (Trusheva et al., 2011).

Moreover, another study from Asia performed by Shi et al. (2012) who studied 16

Chineese propolis samples and identified and quantified eleven compounds including caffeic acid (1.08±0.00 to 16.68±0.05 mg/g propolis), *p*-coumaric acid (0.92±0.00 to 10.14±0.03), ferulic acid (0.03±0.00 to 4.73±0.01 mg/g propolis), isoferulic acid (1.23±0.00 to 4.98±0.02 mg/g propolis), and 3,4-dimethylcaffeic acid (0.91±0.00 to 10.17±0.02 mg/g propolis), pinobanksin (3.44±0.01 to 22.82±0.03 mg/g), chrysin (5.63±0.02 to 44.40±0.36 mg/g propolis), pinocembrin (13.90±0.23 to 46.01±0.39 mg/g propolis), galangin (7.09±0.05 to 26.26±0.28 mg/g propolis), pinobanksin 3-acetate (9.92±0.10 to 46.75±0.41 mg/g propolis), and caffeic acid phenylethyl ester (0.73±0.00 to 7.99±0.02 mg/g propolis) using a UHPLC method. In another study of Chineese poplar type propolis, Yang et al. (2011) identified four main flavonoids as pinocembrin, pinobanksin, chrysin and galangin.

There are also many studies performed in European propolis. Mihai et al. (2012) studied total phenolic and flavonoid contents of 20 poplar type propolis from eleven different regions of Romania. They have found out that the propolis extracts from different origins showed major differences in the content of total flavonoids (ranging from 2.4% to 16.4%) and the total polyphenols (ranging between 23.3% and 63.2%). For instance, according to the results of Kustiawan et al. (2014), propolis collected in temperate regions contains many flavonoids and phenolic acid esters, such as pinocembrin, pinobanksin, galangin, chrysin and caffeic acid phenyl ester (CAPE) which are the main bioactive compounds. Moreover, Dudonné et al. (2011) characterized the antioxidant properties of an aqueous Romanian poplar bud (*Populus nigra*) extract. They observed a high total phenolic content, and moderate antioxidant properties. The main phenolic compounds were identified as phenolic acids (caffeic, *p*-coumaric, ferulic, isoferulic, di-*O*-methylcaffeic and cinnamic acids), salicylates (salicin) and flavonoid aglycons (pinobanksin 5-methyl ether, pinobanksin and pinocembrin). Caffeic and *p*-coumaric acids were identified as the major antioxidant components (Dudonné et al., 2011).

Furthermore, Barbaric et al. (2011), identified the chemical composition of propolis samples collected from Balkans (Croatia, Bosnia and Hercegovina, and Macedonia) by using HPLC method. The results showed that propolis samples have phenolic acids (ferulic acid, *p*-coumaric acid, caffeic acid) and flavonoids (tectochrysin, galangin, pinocembrin, pinocembrin-7-methylether, chrysin, apigenin, kaempferol, quercetin). All samples contained tectochrysin in ranges between 0.1988 mg/g and

1.2004 mg/g. Also, the samples contained flavonoid in ranges from 0.3706 mg/g to 47.4879 mg/g (Barbaric et al., 2011).

Additionally, Falcao et al. (2010), performed a study with EEPs collected from Portugal. They identified the phenolic components of the Portuguese propolis by using HPLC and electrospray mass spectrometry (ESMS) methods. They identified 37 phenolic compounds which included not only typical phenolic acids and flavonoids found in propolis from temperate zones, but also some compounds in which its occurrence have never been referred to in the literature before. Four of the new phenolic compounds were methylated and/or esterified or hydroxylated derivatives of common poplar flavonoids. The flavonoids of the Portuguese propolis were mainly described as dihydroflavonols, flavones, flavanones, and flavonols mainly. Caffeic acid and five esters of caffeic acid (the caffeic acid isoprenyl ester its isomer, the caffeic acid benzyl ester, the caffeic acid phenylethyl ester and the caffeic acid cinnamyl ester), 3,4-dihydroxy vinylbenzene, *p*- coumaric acid and its esthers (*p*-coumaric acid methyl ester and *p*-coumaric acid isoprenyl ester), ferulic and isoferulic acids were found in the Portuguese propolis sample. Also, a methylated derivative of this phenolic acid, the 3,4-dimethyl-caffeic acid was identified (Falcao et al., 2010).

Besides, Aliboni et al. (2011), studied a new analytical methodology based on GC-MS for the quantitative analysis of two allergenic esters (benzyl salicylate and benzyl cinnamate) dissociated from Italian propolis samples. They identified that chemical composition of the propolis samples. The major components of the propolis samples were caffeic acid, *p*-coumaric acid, ferulic acid, quercetin, apigenin, naringenin, kaempferol, chrysin, pinocembrin, phenylethyl caffeate, and galangin. Also, they identified benzyl salicylate, benzyl cinnamate, hexyl salicylate and hexyl cinnamate in Italian propolis (Aliboni et al., 2011).

Moreover, Lagouri et al. (2014) analyzed propolis collected from the Greek mainland (West Macedonia) and the Greek island, Rhodes using HPLC and their results showed that West Macedonia propolis contained the highest amount of phenolic compounds: phenolic acids (caffeic acid, caffeic acid phenethylester, ferulic acid, *p*-coumaric acid) and flavonoids (quercetin, galangin, luteolin, apigenin). Caffeic acid (0.639–4.172 mg/g propolis) and galangin (1.317–8.551 mg/g propolis) were found to be the predominant phenolic compounds in these propolis extracts.

In South America, there are studies performed on the identification and quantification of phenolic compounds of red propolis. Araújo et al. (2016) identified the chemical composition of red propolis from southeast Brazil as benzoic acid, dihydrocinnamic acid, coumaric acid, caffeic acid, prenyl-*p*-coumaric acid, flavonoids, artepillin C, trihydroxymethoxy flavonon, tetrahydroxy flavonon, and triterpenes. Also, Agüero et al., (2011), investigated propolis samples from Argentina, and found many beneficial compounds including the flavanones 7-hydroxy-8-methoxyflavanone, pinocembrin, and pinobanksin, the flavones chrysin and tectochrysin, and the flavonol galangin.

Besides, Tosi et al. (2007), identified the components of Argentinian propolis during the study of antibacterial effects of Argentinian propolis. They used EEP to identify its composition and found that the extracts had 32.31% total soluble compounds which are 2.1% coumaric and syringic acids, 5.16% quercetin, 0.47% apigenine, 8.15% galangine, 7.2% caffeic acid and chrysine, and 9.23% unidentified phenolics compounds (Tosi et al., 2007).

Moreover, Frozza et al. (2013) evaluated phenolic substances present in the Brazilian red propolis. They identified 6-acetyl-2,2-dimethyl-3-hydroxychroman, 2-hydroxy-4-methoxychalcon, liquiritigenin, formononetin, medicarpin, biochanin A, retusapurpurin B and hesperetin7- rhamnoglucoside as the major compounds of the red propolis tested (Frozza et al., 2013).

Additionally, Castro et al. (2014), performed a study to identify phenolic compounds of dry and wax free ethanolic extracts of propolis samples collected from the Santiago of Chile using HPLC-MS/MS method. They identified 30 different phenolic compounds including caffeic acid, caffeic acid phenylethyl ester (CAPE), caffeic acid benzyl ester, quercetin, kaempheride, pinobanksin, pinobanksin ester, ermanin, ethoxy benzoic acid, ferulic acid, galangin-5-methyl ether, vanillin and two coumarins; the 7,8-dihydroxy-6- methoxycoumarin and esculetin. The results showed that CAPE and quercetin were flavonoids which have the best antioxidant activity. Pinobanksin was the only phenol present in the six samples of propolis. The authors suggested that pinobanksin may be a good candidate for the standardization of propolis ethanolic extracts as it is present in all propolis samples (Castro et al., 2014).

Besides, Lotti et al. (2010), investigated the chemical components of a red-type Mexican propolis. They isolated three new compounds from the propolis samples which were 1-(3',4'-dihydroxy-2'-methoxyphenyl)-3-(phenyl)propane, (Z)-1-(2'-methoxy-4',5'-dihydroxyphenyl)-2-(3 phenyl) propene and 3-hydroxy-5,6-dimethoxyflavan. They also isolated seven known flavanones [which were (-)-7-hydro- xyflavanone and (þ)-pinocembrin, the isoflavans (-)-mucro- nulatol, (-)-arizonicanol A, and (þ)-vestitol, and the pterocarpans (-)-melilotocarpan A and (-)-melilotocarpan D], isoflavans (which were mucronulatol, arizonicanol A and vestitol) and pterocarpans (Lotti et al., 2010).

Another study performed in Africa by Piccinelli et al. (2013) who identified the chemical composition of propolis samples collected from north Algeria by chromatographic and spectroscopic analyses. They extracted the propolis samples by methanol. They found that the main constituents were caffeate esters and flavonoids in the propolis samples. Some of the samples contained mainly diterpenes which were labdane and clerodane diterpenes, and a polymethoxyflavonol. They observed that some of the propolis samples were rich in polyphenols. Three caffeic acid derivatives (3-methyl-3-butenyl (E)- caffeate, 2-methyl-2-butenyl (E)-caffeate, and phenethyl (E)-caffeate), two flavones (chrysin and apigenin), two flavonols (kaempferol and galangin), and three flavanonols (pinobanksin, pinobanksin 3-acetate, and pinobanksin 3-(E)-caffeate) were identified as main constituents of some of the propolis samples. The other samples contained nine diterpenes (cupressic acid, isocupressic acid, imbricatoloic acid, torulosal, isoagathotal, torulosol, agathadiol, cistadiol, and 18-hydroxy-cis-clerodan-3-ene-15-oic acid), and together with myricetin 3,7,4′,5′-tetramethyl ether (Piccinelli et al., 2013).

In Australia, Tran et al. (2012), identified major flavonoid components of propolis. The major flavonoid components were containing 2',3',4'-trimethoxychalcone, 2'-hydroxy-3',4'-dimethoxychalcone, 2',4'-dihydroxy-3'-methoxychalcone, 5,7-dihydroxy-2,3-dihydrofl- avonol 3-acetate (pinobanksin 3-acetate) and 5,7-dihydroxy-6-methoxy-2,3-dihydroflavonol 3-acetate, and an unknown substance (Tran et al., 2012).

Propolis is a complex mixture of bioactive substances which has no approved health claims provided in the Regulation (EU) No 432/2012 (Regulation (EU), No.

432/2012). In the EU Register of Nutrition and Health Claims, there are eight non-authorised health claims for propolis. For each of these, a non-compliance with the Regulation has been pronounced as on the basis of scientific evidence assessed such food has been deemed not sufficiently characterised to be claiming an effect on a health condition and the claim could not therefore be substantiated. Only nutritional claims such as "containing propolis" can be displayed on the product label but the quantity of propolis contained in the product is obligatory. The non-authorised claims are the following:

- Antioxidants can protect from free radicals and help in case of food intake deficiency or increased amount of nutrients (ID 3797)
- Helps increase the antioxidative capacity of the body (ID 1243)
- Helps natural defences (ID 1248)
- Helps physiological blood fluidity (ID 3526)
- Helps to maintain healthy liver function, supporting digestion and body purification (ID 3527)
- Increases the physiological resistance of the organism in case of severe ambiance conditions (ID 3798)
- Promotes upper respiratory tract health (ID 1242)
- Supports the immune system and the body's defence (ID 3799).

In order to solve this problem, there are now new trends in standardizing statements on the amount of propolis in terms of some of its bioactive components performed at national levels. These studies should be gathered together in European Union to achieve standardization of propolis. As a result of the literature search so far, galangin can be suggested to be one of the flavonoids present in propolis in temperate regions and numerous extracts can be standardised based on its total content. The final solution is still not clear as propolis with different geographical origin contain different biologically active components.

Turkish propolis have a high content of flavonoids rather than phenolic acids. According to the literature so far, it is now clear that it has higher content of flavonoids when compared to Brazilian red type propolis and also many poplar type propolis around the world. The most abundant flavonoids in Turkish propolis was found to be pinocembrin, pinobanksin, galangin, pinobanksin and chyrisin. The

comparison of these bioactive flavonoids between regions may give an important result of standardization of Turkish propolis.

2.3 Antioxidant Activity of Propolis

There are many methods for the determination of the antioxidant capacities of honey products cited in the literature. Single antioxidant capacity measurement method cannot examine all the antioxidant compounds as all methods have their own advantages or disadvantages (Capanoglu et al., 2008). These methods vary greatly according to the generated radicals, the reaction time and end-point of detection. Although ABTS (2,2'-azino-bis(3 ethylbenzothiazoline-6-sulphonic acid) and DPPH (2, 2-diphenyl-1-picrylhydrazyl) methods work with the same principle, the results vary according to their response to antioxidants under certain conditions. There are also variability in radical formation and solubility in different solvent systems (Arnao, 2000; Apak et al., 2007). Results of the antioxidant activity measurement methods cannot be evaluated satisfactorily using a single method because of the great variability observed in the results. In order to evaluate the antioxidant activity correctly, using several test methods for measuring antioxidant capacities is highly recommended (Capanoglu et al., 2010).

Biological effects of propolis can be associated with its pronounced antioxidant activity. The general principles, recent applications, as well as strengths and limitations of the methods most widely used to determine antioxidant activity were reviewed recently by Karadag et al. (2009). Various assays have been employed to determine propolis antioxidant activity. Table 2.1 illustrates various studies from different regions of the world on determination of total phenolic contents, total flavonoid contents and total antioxidant capacities measured by different methods.

Table 2.1 : Total phenolic, flavonoid contents and antioxidant capacity of propolis from different regions.

Variety Region	Extraction Method	Total Phenolic Content (Folin- Ciocalteau method)	Total Flavonoid Content ($AlCl_3$ method)	Total Antioxidant Capacity Measurement Methods	Total Antioxidant Capacity	Sample Number	References
Turkey	MeOH	Min. (115.49±2.04) Max. (210.33±2.04) (mg GAE/g propolis)	-	FRAP (µM Trolox/g)	Min. (182.12±2.25) Max. (325.47±3.12)	10	Aliyazıcıoglu et al., 2013
Turkey	MeOH:H_2O:HCl (70:25:5 v/v) included 0.1 g of tBHQ	Range: 500-570 mg GAE/g	-	DPPH (mg TEAC/g) ABTS (mg TEAC/g)	Min. 409.6 ± 2.5 Max. 503.7 ± 1.6 Min. 237.7 ± 1.8 Max. 285.3 ± 7.8	4	Erdoğan et al., 2011
Turkey	H_20	124.3 µg (GAE)/g	8.15 (µg QE/g propolis)	(IC_{50}) values for trolox (µg/mL) $DPPH^{\cdot}$ Scavenging $ABTS^{\cdot+}$ scavenging $DMPD^{\cdot+}$scavenging 0_2 scavenging H_2O_2 scavenging Fe^{2+} chelating	 11.45 6.11 12.45 20.92 13.26 21.61	1	Gülçin et al., 2010
Turkey	95% EtOH	PR1: 313±9.48 PR2: 476±4.78 (mg GAE/g propolis)	-	FRAP (1,000 mM Trolox equivalent/g sample) CUPRAC (1,000 mM Trolox equivalent/g sample) DPPH (IC_{50} (mg/mL)	PR1: 327.76± 3.16 PR2: 659.88± 6.35 PR1: 0.692±0.108 PR2: 1.410±0.120 PR1: 5.0±0.1 PR2: 5.2±0.2	2 (inside hive:PR1, entrance of hive:PR2)	Sarıkaya et al., 2009
Turkey	70% EtOH	Min. 143.15±15 Max. 380±19 (mg GAE/g)	Min. 206±40 Max. 705±70 (mg QE/g)	ABTS CUPRAC DPPH FRAP	74±44 to 671±56 575±31 to 1433±124 135±47 to 454±13 123±11 to 362±43 (mg TE/g)	4	Yesiltaş et al., 2015

Table 2.1 (continued): Total phenolic, flavonoid contents and antioxidant capacity of propolis from different regions.

Variety Region	Extraction Method	Total Phenolic Content (Folin- Ciocalteau method)	Total Flavonoid Content ($AlCl_3$ method)	Total Antioxidant Capacity Measurement Methods	Total Antioxidant Capacity	Sample Number	References
China	EtOH	Min. 42.9 ± 0.8 Max. 302 ± 4.3 (mg/g of Ethanolic extract of propolis (EEP))	Min. 8.3 ± 3.7 Max. 188 ± 6.6 (mg/g of EEP)	β-carotene bleaching (%) DPPH(%) ABTS(%)	over 60% over 70% over 10%	20	Ahn et al., 2007
China	MeOH	Min. 87.11 ± 0.96 Max. 257.93 ± 2.32 (mg GAE/g propolis)	Min. 105.25 ± 1.06 Max. 351.25 ± 5.30 (mg QE/g propolis)	DPPH (IC_{50}) (µg/mL) ABTS (IC_{50}) (µg/mL) FRAP (mmol TE/g) HOSC (mmol TE/g) ORAC (mmol TE/g)	Min. 21.79 ± 0.24 Max. 173.38 ± 1.19 Min. 21.45 ± 0.07 Max. 152.80 ± 0.71 Min. 0.20 ± 0.01 Max. 1.72 ± 0.05 Min. 1.83 ± 0.04 Max. 5.00 ± 0.20 Min. 2.56 ± 0.11 Max. 7.63 ± 0.24	15	Shi et al., 2012
China	Water	Min. 10.05 mg GAE/g Max. 377.25 mg GAE/g	Min. 3.47 mg QE/g Max. 15.33 mg QE/g	-	-	26	Guo et al., 2011
Korea	EtOH	Min. 120.0±3.5 Max. 212.±7.4	Min. 48.2±2.1 Max. 77.9±1.7	DPPH (%) (100µg/mL) B-carotene bleaching (100µg/mL)	Min. About 80% Max. Above %90 Min. About 60% Max. Above %90	5	Choi et al., 2006
India	Aqueous (AAP) EtOH: H_2O (80:20 v/v) (EEP)	269.10±0.17 159.10±0.26 (mg GAE/g propolis)	25.50±0.36 57.25±0.34 (mg QE/g propolis)	FRAP DPPH (IC_{50}) Cyclic Voltammery	EEP>AAP 0.05 mg/ml 0.07 mg/ml 87 ± 5 mg/g 136 ± 7 mg/g	1	Laskar et al., 2010

Table 2.1 (continued): Total phenolic, flavonoid contents and antioxidant capacity of propolis from different regions.

Variety Region	Extraction Method	Total Phenolic Content (Folin- Ciocalteau method)	Total Flavonoid Content ($AlCl_3$ method)	Total Antioxidant Capacity Measurement Methods	Total Antioxidant Capacity	Sample Number	References
India	EtOH–H_2O 70% (v/v)	-	-	DPPH (IC_{50}) (µg/mL) HOSC (IC_{50}) (µg/mL) TBARS(IC_{50}) (µg/mL)	16.20 34.33 55.56	1	Thirugnana sampandan et al., 2012
Serbia	MeOH	Min. 1.45 ± 0.02%g GAE Max. 5.31 ± 0.05%g GAE	-	DC polarograpghy DPPH	Min. 1.71 ± 0.11 µL Max. 8.00 ± 0.18 µL Min. 0.093 ± 0.004% Max. 0.346 ± 0.006%	5	Potkonjak et al., 2012
West Macedonia and Rhodos	MeOH 80% MeOH aqueous	77.56±1.71 and 127.79±2.66 21.8±0.62 and 179.99±3.43 2.33±0.51 and 58.53±1.51 (mg GAE/g propolis)	54.51±1.5 and18.39±0.58 88.26±1.58 and 5.96±0.58 5.05±0.76 and 0.25±0.02 (mg QE/g propolis)	DPPH(IC_{50}) (mg/ml) FRAP(IC_{50}) (mg/ml)	0.181 and1.247 0.138 and 1.527 0.218 and 1.557 0.00997 and 0.258 0.0065 and 0.189 0.0394 and 0.169	2	Lagouri et al., 2014
Greece and Cyprus	EtOH: H_2O (80:20 v/v)	Min. 80.2±3.2 Max. 338.5±13.2 (mg GAE/g)	-	DPPH (mmol Trolox/g) FRAP (mmol AAE/g)	Min. 0.33 ± 0.03 Max. 1.11 ± 0.07 Min. 2.14 ± 0.11 Max. 3.35 ± 0.27	12	Kalogeropoulos et al., 2009
Portugal	MeOH	Min. 31.2 ± 0.7 mg/g of GAE Max. 302 ± 4.3 mg/g of GAE	-	DPPH EC_{50} (mg/mL) FRAP EC_{50} (mg/mL)	Min. 0.006 ± 0.003 Max. 0.052 ± 0.003 Min. 0.009 ± 0.001 Max. 0.055 ± 0.001	2	Moreira et al., 2008

Table 2.1 (continued): Total phenolic, flavonoid contents and antioxidant capacity of propolis from different regions.

Variety Region	Extraction Method	Total Phenolic Content (Folin- Ciocalteau method)	Total Flavonoid Content ($AlCl_3$ method)	Total Antioxidant Capacity Measurement Methods	Total Antioxidant Capacity	Sample Number	References
France	Water	29.2±13.1 mg GAE/g	20.5±1.2 mg QE/g	DPPH	1731±28	6	Boisard et al., 2014
	95% EtOH	253.6±5.1 mg GAE/g	67.0±2.6 mg QE/g	(μmole TE/g)	1605±26		
	70% EtOH	246.3±10.6 mg GAE/g	69.3±3.7 mg QE/g		1650±149		
	MeOH	238.6±13.3 mg GAE/g	66.4±4.9 mg QE/g		1386±171		
	DCM	273.5±6.8 mg GAE/g	78.6±2.7 mg QE/g		1437±105		
	Mixed Solvents	281.0±7.1 mg GAE/g	80.0±1.2 mg QE/g		1964±124		
Brazil	EtOH–H_2O 70% (v/v)	151.55 ± 1.95 mg GAE/g propolis	-	DPPH (IC_{50}) (μg/ml)	270.13 ± 24.77	1	Frozza et al., 2013
Brazil	EtOH	Min. 0.50±0.01 Max. 3.90±0.02 (mg GAE/g)	Min. 0.06±0.01 Max. 0.65±0.01 (g of QE/g)	DPPH (%)	Min. 4.2% Max.87.5%	49	Da Silva et al., 2006
Argentina	MeOH	Min. 25.7±1.6 Max. 39.3±0.9 (g of GAE/100 g of MeOH extract of propolis (MEP))	Min. 6.6±0.5 Max. 13.3±0.7 (g of QE/100 g of MEP)	%DPPH (10 μg/mL)	Min. 46.6±0.6 Max. 89.5±2.5	10	Lima et al., 2009
Argentina	EtOH	Min. 14.3±1.0 Max. 348.1±2.8 (mg GAE/g propolis)	Min. 0.0±3.3 Max. 255.9±8.5 (mg QE/g propolis)	DPPH	Average over %50 radical scavenging activity	17	Kumazava et al., 2010
Argentina	EtOH	Min. 92 Max. 170 (mg GAE/g propolis)	Min. 6 Max. 18 (mg QE/g propolis)	DPPH (%) β-carotene bleaching (%)	Min. 42.1 Max. 64.1 Min. 49.4 Max. 66.7	6	Chaillou and Nazareno, 2009
Argentina	EtOH	-	Min. 13.3±0.90 Max. 42.6±0.80	DPPH (%) (20 μg/ml)	Min. 20.0±1.1 Max. 67.5±1.5	11	Moreno et al., 2000

Aliyazıcıoglu et al. (2013) studied the total phenolic contents and total antioxidant capacities with FRAP method of 10 propolis samples collected from different regions of Anatolia in Turkey including northern Kastamonu, southern Kastamonu, northern Eskişehir, southern Eskişehir, Ereğli, Zonguldak, İzmir, Erzurum, Sinop and Artvin provinces. Total phenolic content of methanolic propolis extracts were found to be 115–210 mg gallic acid/g of methanolic propolis extract by using Folin-Ciocalteu method. The total antioxidant capacities of propolis samples were in a range of 182.12±2.25 to 325.47±3.12 μM Trolox/g propolis. The results may justify that propolis can be used as a source of natural antioxidants (Aliyazıcıoglu et al., 2013). Moreover, Erdoğan et al. (2011) collected four propolis samples from different important honey producing locations of Anatolia: Bingol, Rize, Tekirdag and Van, evaluated for their antiradical capacity and total phenolic contents. Antiradical tests showed that all propolis samples have superior antiradical capacities up to 500 mg Trolox equivalent activity per gram of extract (Erdoğan et al., 2011). Besides, Gülçin et al. (2010) determined the antioxidant properties of lyophilized aqueous extract of propolis (LAEP) from Erzurum province of Turkey and correlated the values with total levels of polyphenolic compounds. In order to estimate the capacity of LAEP to act as antioxidants, they studied its DPPH, ABTS, N,N-dimethyl-*p*-phenylenediamine radicals ($DMPD^{\cdot+}$), superoxide anion radicals (O_2) oxidant activity, ferric ions (Fe^{3+}) and cupric ions (Cu^{2+}) reducing ability, ferrous ions (Fe^{2+}) chelating activity. The IC_{50} values were found to be 11.45, 6.11, 12.45, 20.92, 13.26, 21.61 for $DPPH^{\cdot}$ scavenging, $ABTS^{\cdot+}$ scavenging, $DMPD^{\cdot+}$ scavenging, O_2 scavenging, H_2O_2 scavenging and Fe^{2+} chelating activities, respectively. LAEP was found to be an effective antioxidant in several *in vitro* assays including total antioxidant activity, ferric ions (Fe^{3+}) and cupric ions (Cu^{2+}) reducing ability, DPPH, ABTS, DMPD and O_2 radical scavenging, H_2O_2 scavenging and metal chelating activities (Gülçin et al., 2010). Besides, Sarıkaya et al. (2009) studied antioxidant activity of 2 chestnut honey samples (H1 and H2) and 2 samples of Turkish propolis (Pr1 and Pr2) by three different methods, DPPH, FRAP, and CUPRAC (cupric reducing antioxidant capacity). Total phenolic contents were determined by using Folin–Ciocalteu reagent as gallic acid equivalent (GAE). The antioxidant activities were compared with standard antioxidants such as catechin, BHT and Trolox. The antioxidant activities of all the samples were found high and related to the sample concentrations. The ethanolic propolis extracts showed the highest antioxidant

activity. They have found 313±9.48 and 476±4.78 mg/g sample total phenolic content for Pr1 and Pr2, respectively. They have found the highest total antioxidant capacity in Pr2 as 659.88±6.35 mg/g, 1.410±0.12 μM TE/g sample, 5.2±0.2 μg/ml using FRAP, CUPRAC and DPPH methods, respectively. The researchers found out that the total antioxidant capacity of the chestnut propolis samples were higher than the same-floral honey samples. They also observed that collection procedure of the propolis is important for the analyses. Pr2, from the entrance of beehives, showed better antioxidant activity than the Pr1, obtained from the insides (Sarıkaya et al., 2009). Recently, Yesiltas et al. (2015) measured the antioxidant capacities of 3 propolis samples collected from Istanbul, Turkey. According to their results, Turkish propolis antioxidant capacities were ranged from 74±44 to 671±56 mg TE/g measured by ABTS method, 575±31 to 1433±124 mg TE/g measured by CUPRAC method, 135±47 mg TE/g to 454±13 mg TE/g measured by DPPH method and 123±11 to 362±43 measured by FRAP method.

Ahn et al. (2007) examined the antioxidant activity of propolis collected from various areas of China: Heilongjiang, Neimongol, Hebei, Shandong, Shanxi, Gansu, Henan, Hubei, Sichuan, Hunan, Yunnan and Hainan. Ethanol extracts of propolis (EEP) were prepared and evaluated for their antioxidant activities by β-carotene bleaching, DPPH and ABTS assays. All propolis samples except that from Yunnan had relatively strong antioxidant activity. Majority of their samples showed high antioxidant activity, over 60%, according to β-carotene bleaching assay. Most of their samples showed radical scavenging activity over 70% in DPPH assay. Samples except Yunnan sample showed ABTS radical cation percentage inhibition over 10% (Ahn et al., 2007). Another study from China by Shi et al. (2012) compared total phenolic and flavonoid contents and antioxidant capacity of fifteen propolis samples collected from different regions of China with 5 different methods: DPPH, ABTS, FRAP, HOSC (hydroxyl radical scavenging capacity), and ORAC (oxygen radical absorbing capacity). The propolis samples significantly differed in their total phenolic and total flavonoid contents. Additionally, the propolis samples differed in their DPPH, ABTS, hydroxyl, and peroxide radical scavenging capacities and ferric reducing abilities. Propolis collected from various locations may differ in total phenolic and total flavonoid contents and radical scavenging activities (Shi et al., 2012). Guo et al. (2011) also investigated the total phenolic and flavonoid contents of

the water extract of propolis collected from 26 locations in China. They have observed total phenolic content and total flavonoid content in a range of 10.05 – 377.25 mg GAE/g propolis and 3.47 – 15.33 mg QE / g propolis (Guo et al., 2011). Besides another study from Asia reported by Choi et al. (2006) who studied biological activities of different poplar type propolis extracts in Korea and compared their quality with those obtained from Brazilian red propolis. Total polyphenol and flavonoid contents of propolis extracts from Yeosu and Cheorwon, showed higher values (in the range of 160.6±2.4 and 212.7±7.4 for total polyphenolics and 48.2±2.1 to 77.9±1.7 mg/g extract for flavonoids) than those of Brazil samples that have total polyphenol content of 120.0±3.5 mg/g extract and flavonoids of 51.9±1.2 mg/g extract. Also the samples showed strong antioxidant activities based on the linoleic acid peroxidation and DPPH free radical-scavenging activity methods. However, the extract from Brazil had less active antioxidant activity on linoleic acid peroxidation and DPPH free radical-scavenging activity of less than 70% than other extracts. The authors concluded that DPPH free radical-scavenging activity may be related to antioxidant activity of linoleic acid peroxidation (Choi et al., 2006). Furthermore, Laskar et al. (2010) reported the total phenolic contents, total flavonoid contents and antioxidant capacity of the aqueous and ethanol extracts of Indian propolis (AEP and EEP, respectively). The antioxidant activity was measured by reducing power and DPPH radical scavenging activity in addition to cyclic voltammetry as the electrochemical assay. In all these assays, AEP showed significantly greater activity over EEP; which is in contradiction with the previous reports of propolis from other countries. This may be due to its higher polyphenol content. Hence aqueous extract may well be a substitute of organic solvent extracts of propolis (Laskar et al., 2010). Another study performed using Indian propolis was performed by Thirugnanasampandan et al. (2012) who studied total antioxidant capacities of Indian propolis with 3 different methods: DPPH, hydroxyl radical scavenging activity (HOSC) and TBARS (thiobarbituric acid reactive species). It was reported that the highest antioxidant capacity results were obtained with TBARS assay. The IC_{50} values were found as 16.20 µg/ml, 34.33 µg/ml and 55.56 µg/ml for DPPH, HOSC and TBARS, respectively (Thirugnanasampandan et al., 2012).

There are also many studies on antioxidant capacity evaluation of European propolis. In the study of Potkonjak et al. (2012), antioxidant activity of commercial propolis

extracts available on Serbian market, was determined by direct current (DC) polarography and DPPH radical scavenging activity. Antioxidant activity was expressed in H_2O_2 equivalent in DC polarography method (1.71±0.11 - 8.00±0.18 μL). DPPH radical scavenging activity ranged from 0.093±0.004% to 0.346±0.006%. Total phenolic content of propolis extracts (PE) with superior antioxidant activity was 5.31±0.05%g GAE, while the extract with the lowest activity contained 1.45±0.02% GAE. Besides, Lagouri et al. (2014) studied to determine the total phenols, total flavonoids, and major phenolic compounds in the polar (methanol, 80% methanol, and aqueous) extracts of propolis collected from the Greek mainland (West Macedonia) and the Greek island, Rhodes. The antioxidant properties of the propolis extracts were evaluated by using ABTS and DPPH methods. The results showed that propolis from West Macedonia was found to be the strongest radical scavenger and ferric reducing agent (mean IC_{50} 0.179 and 0.009 mg/ml, respectively). The results obtained showed that the methanol and 80% methanol propolis extracts from both geographical regions (West Macedonia, Rhodes) showed higher radical scavenging and reducing activities than the aqueous extracts. (Lagouri et al., 2014). Furthermore, Kalogeropoulos et al. (2009) determined *in vitro* antioxidant activity of twelve propolis ethanolic extracts (EEP) from mainland Greece, Greek islands, and east Cyprus. Greek and Cyprus propolis ethanolic extracts were shown to be very rich in bioactive compounds, possessing antioxidant activities. The propolis extracts studied exhibited significant activity towards scavenging DPPH radicals, ranging from 0.33±0.03 to 1.11±0.07 mmol Trolox equivalents/g propolis ethanolic extract. The propolis ethanolic extracts reducing power values measured using FRAP assay ranged between 2.14±0.11 and 3.35±0.27 mmol ascorbic acid equivalent/ g propolis ethanolic extract (Kalogeropoulos et al., 2009). In the study of Moreira et al. (2008) total phenols content and antioxidant activity were studied in Portuguese propolis samples from Bornes and Fundão regions. Total phenols content was determined by colorimetric assay and their amount was of 329 mg/g of GAE in Bornes sample and 151 mg/g of GAE in Fundão propolis. The antioxidant capacity of propolis extracts was assessed through the scavenging effects on and FRAP assays. A concentration-dependent antioxidative capacity was verified in DPPH and reducing power assays. Low values of EC_{50} on DPPH scavenging assay were obtained for Bornes and Fundão propolis (of 6.22 μg/mL and 52.00 μg/mL, respectively). For reducing power the values were

9.00 µg/mL, for Bornes propolis, and 55.00 µg/mL, for Fundão propolis. It was suggested that the high activity of propolis from Bornes could be related with their different pollen composition (Moreira et al., 2008). Another study from France by Boisard et al. (2014) observed the total phenolic and flavonoid contents of 6 batch of poplar-type propolis samples collected in 2010 and 2011 in apiaries originating from different regions of France. They have found the total phenolic and flavonoid contents in a range of 238.6±13.3 mg GAE/g- 292.1±13. mg GAE/g and 20.5mg QE/g -80.0±1.2 mg QE/g measured using 6 different solvent systems including water, 95% EtOH, 70% EtOH, MeOH, DCM and mixed solvents (a mixture of DCM, MeOH and H_2O (31/19/4). They have observed the antioxidant capacities measured using DPPH method in a range of 1386±26 µmole QE/g to 1605 µmole QE/g (Boisard et al., 2014).

Red propolis from Brazil is another type of propolis having different phenolic compounds and antioxidant properties from poplar type propolis and there are many studies on literature from south America, especially Brazil and Argentina. Frozza et al. (2013) studied polyphenol content and total antioxidant capacity of Brazilian red propolis with DPPH method. They have found IC_{50} value of 270.13±24.77 for Brazilian propolis sample. Their results also indicate that propolis is an excellent sources of antioxidant agent (Frozza et al., 2013). Besides, Da Silva et al. (2006) assayed ethanolic commercial Brazilian propolis extracts for their ability to scavenge DPPH radicals. These activities were correlated with their total phenolic and flavonoid levels. In one group of extracts there was a strong linear relationship between total phenol contents and the measured activities, while in the other group this relationship was weaker. They have found radical scavenging activity ranged from 4.2 to 87.5% (Da Silva et al., 2006). Furthermore, Lima et al. (2009) reported total phenolic, total flavonoids, and free radical scavenging capacity by DPPH bleaching method in Argentina propolis. Methanolic propolis extracts (MEP) showed total phenolic contents ranging from 25.7 to 39.3 g of gallic acid equivalents/100 g of MEP, whereas flavonoids ranged from 6.6 to 13.3 g of quercetin equivalents per 100 g of MEP. Free radical scavenging activity, measured as percent DPPH bleaching, ranged from 46.6 to 89.5 at 10 µg/mL. A direct relationship of DPPH free radical scavenging activity with TP or with compounds 1-6 was not found, showing the need of further evaluation on the origin of free radical activity in propolis samples (Lima

et al., 2009). Additionally, Kumazawa et al. (2010) examined the radical-scavenging activity of 17 samples of propolis from the following regions of Argentina: Mendoza, Rio Negro, La Pampa, and Entre Rios. Ethanol extracts of propolis (EEP) were prepared and their DPPH radical-scavenging activities were evaluated. Almost all Argentine propolis tested, except propolis from La Pampa, had similar radical-scavenging activities, total phenolic and flavonoid contents. Most of the samples showed potent free radical-scavenging activity over 50% and the highest total phenolic and flavonoid contents were 348.1±2.8 mg/g of EEP and 255.9±8.5 mg/g of EEP (Kumazawa et al., 2010). Moreover, Chaillou and Nazareno (2009) studied antiradical and antioxidant activities of propolis from 30 different localities in Argentina and correlated the values with total levels of polyphenolic compounds and flavonoids. Analyzed samples presented a noticeable variability in their antioxidant and antiradical activities, although, linear relationships were found between them and also between polyphenol and flavonoid total levels. Total phenolic and flavonoid contents, total antioxidant capacities and total antiradical capacities were in the range of 42 – 253 mg/g, 2 - 22 mg/g, 28.8 – 84.7% and 5.8 – 89.9%, respectively (Chaillou and Nazareno, 2009). Additionally, Moreno et al. (2000) studied on ethanolic extracts of propolis from different regions of Argentina. The extracts were analysed for the determination of total flavonoid content (from 13.3 to 42.6 mg/g of propolis). All of them contained high total flavonoid content. It was also observed that all samples of ethanolic extracts of propolis showed free radical-scavenging activity in a range of 20.0±1.1 and 67.5±1.5 % of scavenging of the radical DPPH (Moreno et al., 2000).

According to the literature reviewed, propolis collected from various locations of different locations of continents, even the same country may differ in total phenolic and total flavonoid contents and antioxidant capacities. Moreover, antioxidant capacities of the same propolis sample may change according to the difference in extraction methods and antioxidant capacity measurement methods.

2.4 Antimicrobial Activity of Propolis

The antimicrobial activity of propolis covering antibacterial, antifungal and antiviral attributes has been known by human for a long time (Agüero et al., 2011; Bankova et al., 2000). Due to those properties of propolis has an anti-inflammatory effect on

many micro-organisms (Bankova et al., 2000; Kartal et al., 2003; Kongkiatpaiboon et al., 2016; Mihai et al., 2012; Silva et al., 2012; Trusheva et al., 2011).

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The antibacterial activity of propolis has been attributed to the presence of phenolic compounds, especially flavonoids, phenolic acids, and their esters (Kujumgiev et al., 1999). Isoflavonoids are important antimicrobial components of red propolis, especially concerning their activity against *Candida albicans* (Trusheva et al., 2006). Pterocarpans possessing a *cis*-6a,11a-dihydro-6H-benzofuro[3,3-c]chromene skeleton constitute the second largest group of natural isoflavonoids, and had been gaining considerable interest due to their wide range of biological effects. Many of them are phytoalexins possessing high antifungal and antibacterial activity, and several of them have been reported to inhibit HIV- 1 reverse transcriptase and cytopathic effect on HIV-1 in cell cultures (Engler et al., 1996). Chalcones represent an important group of natural compounds with a variety of biological activities, including antibacterial and antifungal (Nowakowska et al., 2008).

In vitro antimicrobial activity of propolis on bacterial strains has attracted many researchers all around the world. Sforcin et al. (2000) showed that the growth of Gram-positive bacteria *Staphylococcus aureus* was inhibited by low propolis concentrations, whereas Gram-negative bacteria were less susceptible. Similarly, Velazquez et al. (2007), performed a study to evaluate the antibacterial and free-radical scavenging (FRS) activities of propolis collected from three different locations of Sonoran Desert in northern Mexico by using the broth microdilution method and the DPPH (1,1-diphenyl-2-picrylhydracyl) assay. They extracted propolis samples with methanol, and evaluated the antibacterial activity of propolis samples against *S. aureus (ATCC 6538P), Enterococcus faecalis (ATCC 29212), L. monocytogenes (ATCC 7644), Escherichia coli* (ATCC 25922) *and Pseudomonas aeruginosa* (ATCC 27853) (Velazquez et al., 2007). The data revealed that the propolis samples showed antibacterial activity only against Gram-positive bacteria species. The samples showed the highest antibacterial activity against

Staphylococcus aureus. Moreover, CAPE isolated from the propolis samples, showed high growth-inhibitory activity against Gram-positive bacteria, especially against *S. aureus* (Velazquez et al., 2007). In order to utilize the antimicrobial effects of propolis, Bulman et al. (2011) studied propolis as an alternative approach to antibiotics against pathogen bacteria species *E.coli*, *Chromobacterium violaceum* and *Pseudomonas aeruginosa*. They identified differences in the propolis samples by physiochemical profiles and absorption spectra and concluded that, anti-pathogenic compounds from propolis can be used for the further development of therapeutics against many pathogenic bacteria (Bulman et al., 2011). A possible synergism between propolis and antibiotics (ciprofloxacin and norfloxacin) on their actions of DNA and the metabolism (cotrimoxazole) against the bacteria *Salmonella Typhi* has been studied by Orsi et al. (2012). They investigated the synergism by using the minimal inhibitory concentration for propolis and antibiotics. According to the results, Brazilian and Bulgarian propolis showed high antibacterial activity, although did not show any synergistic effects with the antibiotics tested (Orsi et al., 2012). More evidence about the antibacterial effects of propolis has been demonstrated by Inui et al. (2012) who isolated three new prenylated flavonoids, namely, solophenols B (1), C (2), and D (3), as well as a new prenylated stilbene, solomonin (4) and 17 known compounds from propolis collected from the Solomon Islands. They tested these new compounds and several known compounds for antibacterial activity against *S. aureus*, *Bacillus subtilis*, and *Pseudomonas aeruginosa*. Most of them exhibited potent antibacterial activity (Inui et al., 2012). Similar to those researches, Miorin et al. (2003), tested the antibacterial activity of honey and propolis collected from Brazil (Parana and Minas Gerais regions), against *Staphylococcus aureus*, and investigated the chemical composition of propolis and honey samples. The results showed that propolis samples had higher antibacterial activity against *S. aureus* when compared with honey (Miorin et al., 2003). Moreover, Righi et al. (2011) evaluated the antimicrobial activity against both Gram-positive (*Bacillus subtilis, Enterococcus faecalis* and *Streptococcus pyogenes*) and Gram-negative (*Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhimurium* and *E.coli)* bacteria and the fungi *C. albicans*. Methanol extract inhibited the growth of all tested microorganisms. It is reported that propolis is active against Gram-positive bacteria, showing a limited activity against Gram-negative ones (Righi et al., 2011). Kim and Chung (2011) who evaluated the effects of Korean propolis against foodborne pathogens and spores

of *Bacillus cereus* and investigated the antimicrobial activity against *B. cereus* structure by transmission electron microscopy (TEM) also supported those findings. The antimicrobial effects of the Korean propolis were tested against foodborne pathogens including Gram-positive (*B. cereus, L. monocytogenes* and *Staphylococcus aureus*) and Gram-negative (*Salmonella typhimurium, E. coli* and *Pseudomonas fluorescence*) bacteria by agar diffusion assay. Gram-positive bacteria were more sensitive than were Gram-negative bacteria. Against spores of *B. cereus*, propolis inhibited germination of spores up to 30 hours, compared to control at higher concentration than vegetative cells yet acted sporostatically. The bactericidal and sporostatic action of propolis were dependent on the concentration of propolis used and treatment time. Electron microscopic investigation of propolis-treated *B. cereus* revealed substantial structural damage at the cellular level and irreversible cell membrane rupture at a number of locations with the apparent leakage of intracellular contents (Kim and Chung, 2011). In the study of Pepeljnjak and Kosalec (2004), they examined the antimicrobial activity of three propolis ethanol extracts (EEP) for various Gram-negative and Gram-positive bacterial species, including multiple-resistant *Staphylococcus aureus*, *Enterococcus spp*. and *Pseudomonas aeruginosa* strains. EEP had a good bactericidal activity against Gram-positive species, and all multiple-resistant bacterial strains tested were sensitive to EEP. Antimicrobial-guided separation of flavonoid aglycones (bioassay in situ on thin-layer chromatogram) showed that galangin (3,5,7-trihydroxyflavone) is one compound in EEP with bactericidal activity and they found its activity both against *S. aureus*, *Enterococcus spp.* and *P. aeruginosa* strains (Pepeljnjak and Kosalec, 2004). Yang et al. (2006) who investigated the susceptibility of *L. monocytogenes* to the ethanol extract of propolis (EEP) proposed that the activity depends on EEP concentration, incubation temperature, pH, and cell age. The results of this study revealed that EEP at a dosage of 7.5 µg/mLor higher exerted a bactericidal effect on *L. monocytogenes*. *L. monocytogenes* was most susceptible to EEP at 37 °C followed by 25 and 4 °C. At acid pH values, cells of the test organism were more sensitive to EEP than at neutral pH, while most resistant at alkaline pH values. Cell age was also found to affect the susceptibility of *L. monocytogenes* to EEP. Cells in the mid-exponential phase showed the highest susceptibility, followed by cells in the late exponential phase and stationary phase. EEP caused cell leakage of the test organism (Yang et al., 2006). In the study of Tosi et al. (2007), fifteen propolis from Argentine in 20% w/w ethanolic

extracts were tested upon *E. coli* ATCC 25922 by agar diffusion and plate culture methods. By relating the zone of growth inhibition with extracts concentration, a linear response was obtained. On the propolis samples tested, a single value of the minimum inhibitory concentration could not be established. Those values were strongly dependent on propolis composition and botanical origin. The propolis extracts tested, may successfully inhibit the *E. coli* development *in vitro*, and consequently suggested to be useful as natural food preserver (Tosi et al., 2007).

Interestingly, findings of Seidel et al. (2008) who studied *in vitro* screening of EEP (n=40), collected from a wide range of countries within the tropical, subtropical and temperate zones presented a relationship between the antimicrobial activity and the climate conditions of the regions. They compared their activity against a range of Gram-positive and Gram-negative bacteria using a broth microdilution assay. The results obtained exhibited that propolis extracts were mostly active against Gram-positive bacteria. The samples were subjected to principal component analysis (PCA) in order to model their activity against Gram-positive microorganisms. Three distinct clusters were distinguished in the PCA mapping based on MIC values, categorizing samples with strong (MIC range 3.9–31.25 mg/L), moderate (MIC range 31.25≥500 mg/L) and weak antibacterial activity or inactivity (MIC ≥ 500 mg/L only). It is hypothesized that for samples of tropical provenance differences in the activity profiles may depend on the climatic characteristics of the collection sites. High antibacterial activity was observed for samples from locations characterized by a wet-tropical rainforest-type climate (Seidel et al., 2008).

The antimicrobial effects of Turkish propolis also attracted researchers. Kartal et al., (2003) investigated the antimicrobial activity of propolis samples come from Kazan and Marmaris regions in Turkey by using disc diffusion method. They tested the antimicrobial activity by using four different percentage version of ethanolic extracts of the samples against seven Gram-positive, four Gram-negative bacteria and a fungus culture which were *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212), *Bacillus subtilis* (ATCC 6633), *Corynebacterium diphtheriae* (RSKK 632), *Streptococcus pneumoniae*, and *Streptococcus pyogenes* (Gram-positive bacteria), and *Pseudomonas aeruginosa* (ATCC 27853), *E. coli* (ATCC 25922), *Klebsiella pneumoniae* (RSKK 256), *Branhamella catarrhalis* (Gram-negative bacteria). As a

result, they found that caffeic acid and its esters are the major contribution of antimicrobial activity. Morover, they found that propolis samples have antimicrobial effects against *S. aureus*, *S. epidermidis*, *B. subtillis*, *C. diphtheriae*, *B. catarrhalis*, and *C. albicans*, however they have not any antimicrobial effects on *S. pyogens*, *P. aeruginosa*, *E. coli*, *K. pneumoniae*, and *E. faecalis* (Kartal et al., 2003). Moreover, Uzel et al. (2005) evaluated the antimicrobial activity of four different Anatolian propolis samples on different groups of microorganisms including some oral pathogens and comparison between their chemical compositions. Ethanol extracts of propolis (EEP) were prepared from four different Anatolian propolis samples and examined whether EEP inhibit the growth of the test microorganisms or not. Although propolis samples were collected from different regions of Anatolia all showed significant antimicrobial activity against the Gram-positive bacteria and yeasts. Propolis can prevent dental caries since it demonstrated significant antimicrobial activity against the microorganisms such as *Streptococcus mutans*, *Streptococcus sobrinus* and *C. albicans*, which involves in oral diseases (Uzel et al., 2005). In addition, Basim et al. (2006) investigated the *in vitro* antibacterial activities of Turkish pollen and propolis extracts against 13 different species of agricultural bacterial pathogens including *Agrobacterium tumefaciens*, *A. vitis*, *Clavibacter michiganensis* subsp. *michiganensis*, *Erwinia amylovora*, *E. carotovora* pv. *carotovora*, *Pseudomonas corrugata*, *P. savastanoi* pv. *savastanoi*, *P. syringae* pv. *phaseolicola*, *P. syringae* pv. *syringae*, *P. syringae* pv. *tomato*, *Ralstonia solanacearum*, *Xanthomonas campestris* pv. *campestris* and *X. axonopodis* pv. *vesicatoria*. These pathogens cause crown gall, bacterial speck, leaf spot, halo blight, olive knot, stem pith necrosis and bacterial wilting diseases on monocotyledon and dicotyledon plants, tomato, tomato and pepper, bean, olive, tomato, tomato and eggplant plants, respectively. *P. syringae* pv. *phaseolicola* was the most sensitive one to 1/10 concentration of propolis extract, and the sensitivity of the bacteria followed the sequence *P. syringae* pv. *phaseolicola* > *P. savastanoi* pv. *savastanoi*, *P. corrugata*, R. s*olanacearum* > *E. carotovora* pv. *carotovora*, *P. syringae* pv. *syringae*, *E. amylovora*, *A. tumefaciens*, *A. vitis*, *C. michiganensis* subsp. *michiganensis*, *P. syringae* pv. *tomato*, *X. campestris* pv. *campestris*, *X. axonopodis* pv. *vesicatoria*. The least active concentrations towards the tested bacteria were 1/100 of the pollen extract and 1/1000 of the propolis extract (Basim et al., 2006). Data from Silici and Kutluca (2005) provides information about the differences in

antimicrobial activity of bee races. They investigated antibacterial activity of three types of propolis collected three different races of *Apis mellifera* bee in the same apiary. The antimicrobial activity against *Staphylococcus aureus, E. coli, Pseudomonas aeruginosa* and *Candida albicans* was evaluated. Ethanolic extracts of propolis samples showed high antibacterial activity against Gram-positive cocci (*Staphylococcus aureus*), but had a weak activity against Gram-negative bacteria (*E. coli* and *Pseudomonas aeruginosa*) and yeast (*C. albicans*). Propolis sample collected by *Apis mellifera caucasica* showed a higher antibacterial activity than collected by *Apis mellifera anatolica* and *Apis mellifera carnica*. The experiments revealed that there could be minor differences in antibacterial activity of propolis extracts depending on the different races of honeybee (Silici and Kutluca, 2005).

Furthermore, antibacterial activity of propolis due to its significant activity against oral pathogens, such as *Streptococcus mutans, Streptococcus sobrinus* and *C. albicans* has been frequently accounted for its preventive effect on dental caries (Duarte et al., 2006; Leitão et al., 2004; Ishida et al., 2011). Likewise, the antibacterial effects of propolis supporting oral health has been demonstrated in many researches. The bacteria *Enterococcus faecalis* is naturally a part of oral flora (Awawdeh et al., 2009). Also, *E. faecalis* is present in small numbers in uninstrumented infected root canals, and is a big problem in root canal therapy (Awawdeh et al., 2009). Therefore, the researchers performed an *ex vivo* study, to investigate the antibacterial activity of Jordanian propolis in intracanal medicament against *E. faecalis* by using infected dentine models, and to compare antibacterial efficacy of propolis and non-setting calcium hydroxide paste. They used dentine discs (7-mm length) cut from extracted human teeth. Their data showed that propolis samples were significantly more effective than non-setting calcium hydroxide against *E. faecalis* after a short-term application. Consequently, their study revealed that propolis is very effective as intracanal medicament in inhibition of *E. faecalis ex vivo* (Awawdeh et al., 2009).

The antibacterial activity of propolis has also been shown on the growth of phytopathogenic bacteria by Ordónez et al. (2011) in samples collected from North of Argentina. Their results showed that propolis provided strong antibacterial activity against *Erwinia carotovora spp carotovora* CECT 225, *Pseudomonas syringae pvar tomato* CECT 126, *Pseudomonas corrugata* CECT 124 and *Xanthomonas campestris*

pvar vesicatoria CECT 792. The researchers also identified the most active components of propolis, and found that chalcone was more active than the EEP. Furthermore, propolis samples were sprayed on tomato fruits infected with *P. syringae*, and found that the process reduced the severity of the disease. The authors suggested that Argentinian propolis extracts diluted with water may be used for the management of post harvest diseases of fruits (Ordónez et al., 2011).

The antibacterial effects of propolis was also investigated on the health of bee colonies in hive. Mihai et al. (2012), tested (*in vitro*) the antibacterial effects of ethanolic extracts of propolis from different regions of Romania on the bacterial pathogen *Paenibacillus larvae* to understand the basics of honeybee colony health to know which compounds have the strongest antibiotic capacity. According to the research, colonies treated with propolis extracts showed a significant decrease in the number of *P. larvae* spores/g of honey. They found that the propolis extracts showed differences in the contents of total flavonoids and the total polyphenols which influences the strength of antibacterial effect. In addition, it was reported that there was a significant interaction between the flavonoids and the observed antibacterial effect (Mihai et al., 2012).

It has been also demonstrated that propolis is an effective ingredient in animal feeds against growth of pathogenic bacteria by Kačániová et al. (2012) who studied the effect of propolis extracts on the microbial colonization of chicken gastrointestinal tract *in vivo*. The propolis was administered to both feed mixtures in various amounts except of the control group. The addition of 150 mg propolis to 1 kg of feed was included in the first experimental group, the addition of 450 mg/kg in the second experimental group, the addition of 600 mg/kg the third experimental group and 800 mg/kg in the fourth experimental group. The highest count of faecal enterococci was found in the third group. The highest count of lactobacilli was detected in the fourth experimental group and number of *Enterobacteriaceae* genera count was found in control group. All gram-positive and gram-negative bacterias *in vitro* are susceptible to propolis (Kačániová et al., 2012).

Based on the available literature on the antimicrobial activity of propolis it might be concluded that those findings may show variations depending on the original source of the microorganism utilized in each research. For example, the data presented by Silva et al. (2011) comparing the antimicrobial activity of propolis on Gram-positive

and Gram-negative bacteria and yeasts showed differences between the isolated microorganisms from different biological fluids and reference microorganisms. Concerning the antimicrobial activity, *C. albicans* was the most resistant and *S. aureus* the most sensitive. The reference microorganisms were found to be more sensitive than the ones isolated from biological fluids (Silva et al., 2011).

In the view of the available literature it is obvious that propolis is also effective in preventing the growth of fungi and yeast. In the study of Mohammadzadeh et al. (2007), antibacterial and antifungal activities of Iranian propolis ethanolic extract were examined on Gram-positive (*S. aureus*; *S. epidermidis*; *B. subtilis*) and Gram-negative (*E. coli*; *P. aeruginosa*) and fungal strains (*C. albicans*; *A. niger*). The ethanolic extract of propolis inhibited the growth of all examined microorganisms including bacteria and fungi with the highest antimicrobial activity against Gram-positive bacteria such as *S. aureus* and *S. epidermidis* (Mohammadzadeh et al., 2007). Likewise, De Souza et al. (2013) isolated fungi present on propolis samples, identified and tested for the production of antimicrobial metabolites. Twenty-two fungal isolates were obtained, some of which were identified as *Alternaria alternata, Aspergillus flavus, Bipolaris hawaiiensis, Fusarium merismoides, Lasiodiplodia theobromae, Penicillium citrinum, Penicillium crustosum, Penicillium janthinellum, Penicillium purpurogenum, Pestalotiopsis palustris, Tetracoccosporium paxianum* and *Trichoderma koningii.* Those fungi were grown in liquid media to obtain crude extracts that were evaluated for their antibiotic activity against pathogenic bacteria, yeast and *Cladosporium cladosporioides* and *A. flavus*. The most active extract was obtained from *L. theobromae*. Some extracts showed to be more active than the positive control in the inhibition of *S. aureus* and *L. monocytogenes*. The researchers proposed that propolis is a promising source of fungi, which produces active agents against relevant food poisoning bacteria and crop-associated fungi (De Souza et al., 2013). The same effect of propolis at nano-sizes has been been demonstrated by Afrouzan et al. (2012) who investigated its antibacterial and antifungal activity against *Staphylocuccus aureus* and *C. albicans*. They used agar well diffusion method to evaluate the antimicrobial activity and prepared nanopropolis by milling media method at a size under the 100 nanometers. The results showed that there were significant differences between the inhibition values of *S. aureus* and *C. albicans* of propolis and nanopropolis samples. The highest inhibition was obtained

by nanopropolis against *S. aureus*. The results of the study supports that propolis in the form of nanoparticles have a potential to be used efficiently in the control of bacterial and fungal diseases (Afrouzan et al., 2012). More data provided by Agüero et al. (2011) supported the potential of propolis to treat fungal diseases. The main antifungal compounds were found to be 3'methyl-nordihydroguaiaretic acid (MNDGA) 1, nordihydroguaiaretic acid (NDGA) 2 and a NDGA derivative 3, showing strong activity against *Trichophyton mentagrophytes*, *T. rubrum* and *Microsporum gypseum* (Agüero et al., 2011). Finger et al. (2013) who studied the antifungal activity of canola oil extract of propolis (ODEP) *in vitro* against *C. albicans* strains also demonstrated that kaempferide, isosakuranetin and dihydrokaempferide has a potential activity against *C. albicans* (Finger et al., 2013). The study proved that some fractions from ODEP produced better inhibition of *C. albicans* than the propolis extract itself (Finger et al., 2013). In addition to these, Yang et al. (2011) identified antifungal components against *Penicillium italicum* from Chinese propolis. The results showed propolis ethanolic extracts and its fractions had strong antifungal activity against *P. italicum*. Band I, showed the strongest antifungal activity and completely inhibited the growth of *P. italicum* at 200 mg/L with bioactive components identified as pinobanksin, pinocembrine, chrysin and galangin. This study exhibited Chinese propolis and its main flavonoids was potential natural alternatives for the control of citrus blue mould caused by *P. italicum* (Yang et al., 2011).

In addition to those findings, Quiroga et al. (2006) showed the contribution of the propolis to agriculture. They studied *in vitro,* the fungitoxic effect of ethanolic propolis extracts (EEP) come from different places against *Xylophagous fungi* species which were *Ganoderma applanatum* IEV 017, *Lenzites elegans* IEV 012, *Pycnoporus sanguineus* IEV 006 and *Schizophyllum commune* IEV 009, and phytopathogenic fungi species which were *Aspergillus niger* (from citrus), *Fusarium sp.* (isolated 20 from Glycine max), *Fusarium sp.* (isolate 21 from *Manguifera indica*), *Fusarium sp.* (isolate 22 from G. max), *Macrophomina sp.* (isolated from G. max), *Penicillium notatum*, *Trichoderma spp.* and *Phomopsis sp.* (from G. max). They performed the study to determine the antimycotic and cytotoxic activities of partially purified propolis extract. The results showed that partially purified propolis extract inhibited fungal growth. The researchers recommended that propolis can be a

suitable source for antifungal products, and the partially purified propolis extract and its isolated compounds, (pinocembrin and galangin) have the capacity to be used as antifungals in agrochemicals without giving any harm to the equilibrium of agro-ecosystems, to reduce economic costs causing by the ilnesses came with fungi (Quiroga et al., 2006). Similarly, Araujo et al. (2016) investigated the effects of both propolis and geopropolis (stingless bee) on *Pythium insidiosum*, a fungus-like organism which is the causative agent of pythiosis, a life-threatening disease of the subcutaneous tissue transmitted by the contact with contaminated water and zoospores constitute its infective form, that affects mostly horses, dogs and humans. They used propolis produced by Africanized honeybees, and geopropolis produced by *M. fasciculata* against *Pythium insidiosum* isolates. Their data showed that propolis was more effective than geopropolis in inhibiting *P. insidiosum*, possibly due to the chemical composition of propolis which has more active compounds than geopropolis (Araujo et al., 2016).

Antiviral activity of propolis has been shown by the study of Schnitzler et al. (2010) who investigated aqueous and ethanol extracts of propolis for their antiviral activity *in vitro*. Both propolis extracts exhibited high levels of antiviral activity against HSV-1 in viral suspension tests and plaque formation was significantly reduced by >98%. The extracts were added at different times during the viral infection cycle in order to determine the mode of antiviral action of propolis. Both propolis extracts exhibited high anti-HSV- 1 activity when the viruses were pretreated with these drugs prior to infection. Galangin and chrysin displayed some antiviral activity among the analysed compounds. However, the extracts containing many different components showed significantly higher antiherpetic effects as well as higher selectivity indices than single isolated constituents. Propolis extracts suggested to be suitable for topical application against herpes infection (Schnitzler et al., 2010).

In general, there are limited information about the effects of propolis on protozoa. According to Mirzoeva et al. (1997), ethanol extracts of propolis are effective against protozoa. Salamao et al. (2004) investigated the chemical composition of ethanol extracts from a Brazilian (Et-Bra) and a Bulgarian (Et-Blg) propolis, and their activity against the protozoan *Trypanosoma cruzi*, several fungi and bacteria species. Microbiological activity was assayed *in vitro* against *T. cruzi*, *C. albicans*, *Sporothrix schenckii*, *Paracoccidioides brasiliensis*, *Neisseria meningitidis*, *Streptococcus*

pneumoniae and *Staphylococcus aureus*. Et-Bra and Et-Blg, although with totally distinct compositions, were active against *T. cruzi* and the three species of fungi. Et-Blg was more effective than Et-Bra against bacteria, particularly *N. meningitidis* and *S. pneumoniae*. Although with different classes of components, both propolis extracts showed microbicidal activity. For the bactericidal activity it was possible to establish a positive correlation with the high content of flavonoids of the Bulgarian extract (Salamao et al., 2004). Additionally, Soufy et al. (2017) researched the effects of Egyptian propolis *in vivo* (on *cryptosporidiosis* infected immunosuppressed rats) on *cryptosporidiosis* which is an infectious disease caused by *cryptosporidium* a protozoan parasite. *Cryptosporidiosis* has limited treatments, and it may couse some complications such as immune deficiency syndrome, chronic diarrhea (Soufy et al., 2017). Aim of that study was to evaluate the effect of ethanol extracted propolis (EEP) and water extracted propolis (WEP) in comparison with a drug agains cryptosporidiosis called nitazoxanide, and they observed that the both Egyptian propolis extracts have an anti-protozoal activity on *cryptosporidiosis* in rats (Soufy et al., 2017).

According to the available literature, it can be concluded that propolis is an important antimicrobial, antifungal, antivirus and antiprotozoal agent. There may be differences in antimicrobial activity of propolis extracts depending on several factors such as different races of honeybee, geographical location of propolis, flora around the hive, environmental and climate conditions. Most of the available data reports that propolis has a better antibacterial activity on Gram-positive bacteria than Gram-negative bacteria. These reports should be considered when propolis is determined to be used as a natural antibacterial agent. There is a significant interaction between the flavonoid content and observed antimicrobial effect. According to literature, caffeic acid and esters are considered as the major contribution of antimicrobial activity Turkish propolis samples, whereas pinobanksin, pinocembrin, chrysin and galangin was considered as the major flavonoids in Chineese propolis samples (Kartal et al., 2003; Yang et al., 2011). Therefore, the content of individual phenolics have a major impact on antimicrobial properties of propolis.

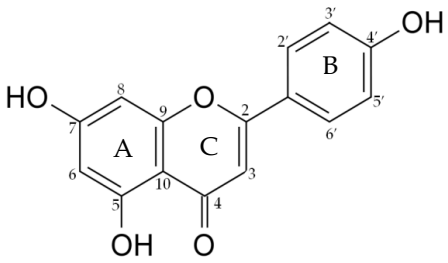
2.5 Metabolism and Bioavailability of Phenolics

Phenolics are mostly found bound to sugars and organic acids that can be grouped into flavonoids and non-flavonoids. Figures 2.1 and Figure 2.2 exhibit the structure of flavonoid-type phenolics and nonflavonoid-types, respectively. Flavonoid-type phenolics have a primary structure existing of two benzene rings, A and B, connected through a heterogeneous pyrone C ring. In contrast, nonflavonoid-type phenolics have a more diverse group of compounds from the simplest C6-C1 benzoic acids to more complicated C6-C2-C6 stilbenes, C6-C3-C3-C6 lignans and gallotannins, hydrolyzable tannins and ellagitannins (Selma et al., 2009). The available literature presented in this section particularly focuses on metabolic activities and biodegradation of phenolic compounds available in propolis.

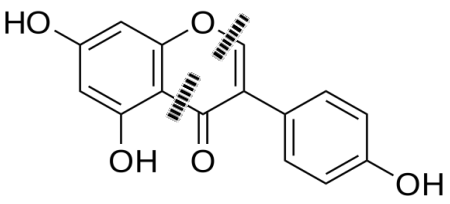
2.5.1 Flavonoid-type phenolics

2.5.1.1 Flavonols

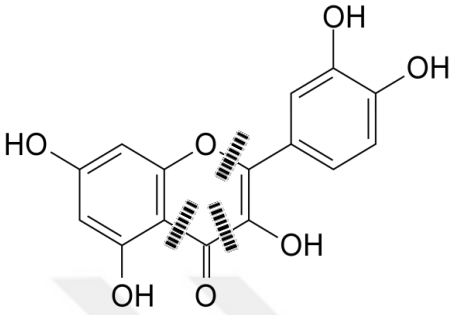
Flavonols contain a 3-hydroxyflavone base (3-hydroxy-2-phenylchromen-4-one) and have a planar ring system (Figure 2.1). They are differentiated by their hydroxy modification at distinct positions of the phenol residue. Foods particularly enriched in flavonols are onions, broccoli, tea, apples, and red wine (Hollman and Katan, 1999). Flavonols are extensively hydrolyzed into their metabolite-derivative products by gut microbiota at the A and B rings as a result of the C ring cleavage (Winter et al., 1989, Winter et al., 1991). Accordingly, quercetin provides 2-(3,4-dihydroxyphenyl) acetic acid, 2-(3-hydroxyphenyl) acetic acid, and 3,4-dihydroxybenzoic acid from the B ring, while phloroglucinol, 3-(3,4-dihydroxyphenyl) propionic acid, and 3-(3-hydroxyphenyl) propionic acid are formed from the A ring. Additional phenolic-metabolites such as 3-methoxy-4-hydroxy-benzoic acid (vanillic acid), 2,4,6-trihydroxybenzoic acid, 2-(3,4-dihydroxyphenyl)ethanol, 3,4-dihydroxybenzaldehyde, 3-(3,4-dihydroxyphenyl) benzoic acid methyl ester, and 3-(*m* or *p*-hydroxyphenyl) propionic acid have also been recognized. The types of phenolic compounds produced are affected by the mechanism of B ring hydroxylation. Myricetin trihydroxylation produces 2-(3,5-dihydroxyphenyl) acetic acid, 2-(3-hydroxyphenyl) acetic acid, and 2-(3,4,5-trihydroxyphenyl) acetic acid. Moreover, kaempferol (which has a 4′-hydroxy-ring B), releases only 2-(4-hydroxyphenyl) acetic acid. In summary, flavonols are biotransformed by C ring fission, and later by dehydroxylation reactions in the intestine (Rechner et al., 2004).



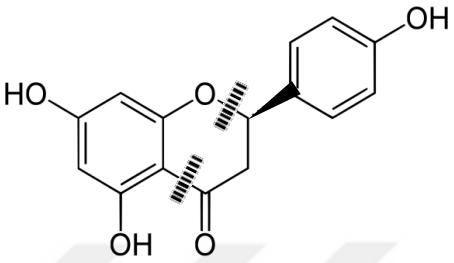
FLAVONES



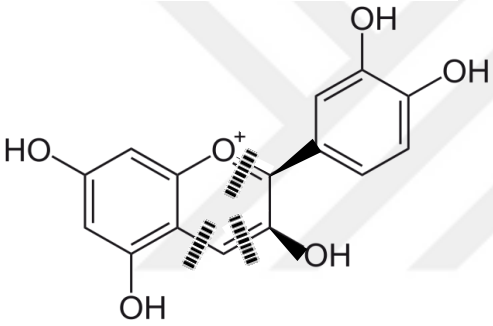
ISOFLAVONES (genistein)



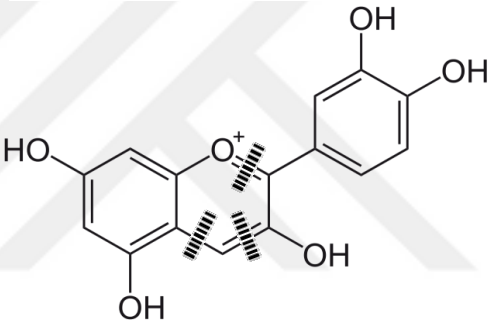
FLAVONOLS (quercetin)



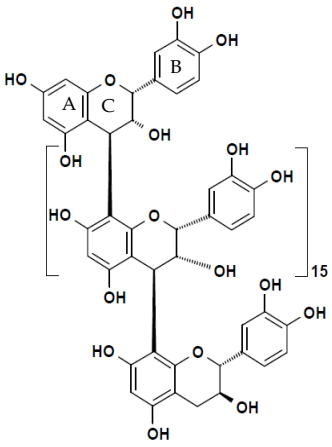
FLAVANONES (naringenin)



FLAVAN-3-OLS (epicatechin)



ANTHOCYANINS (cyanidin)



CONDENSED TANNINS (epicatechin$_{16}$-(4 → 8)-catechin)

Figure 2.1 : Microbiota heterocyclic C ring cleavage of flavonoids; (|||) positions of the potential C-ring cleavages (Selma et al., 2009).

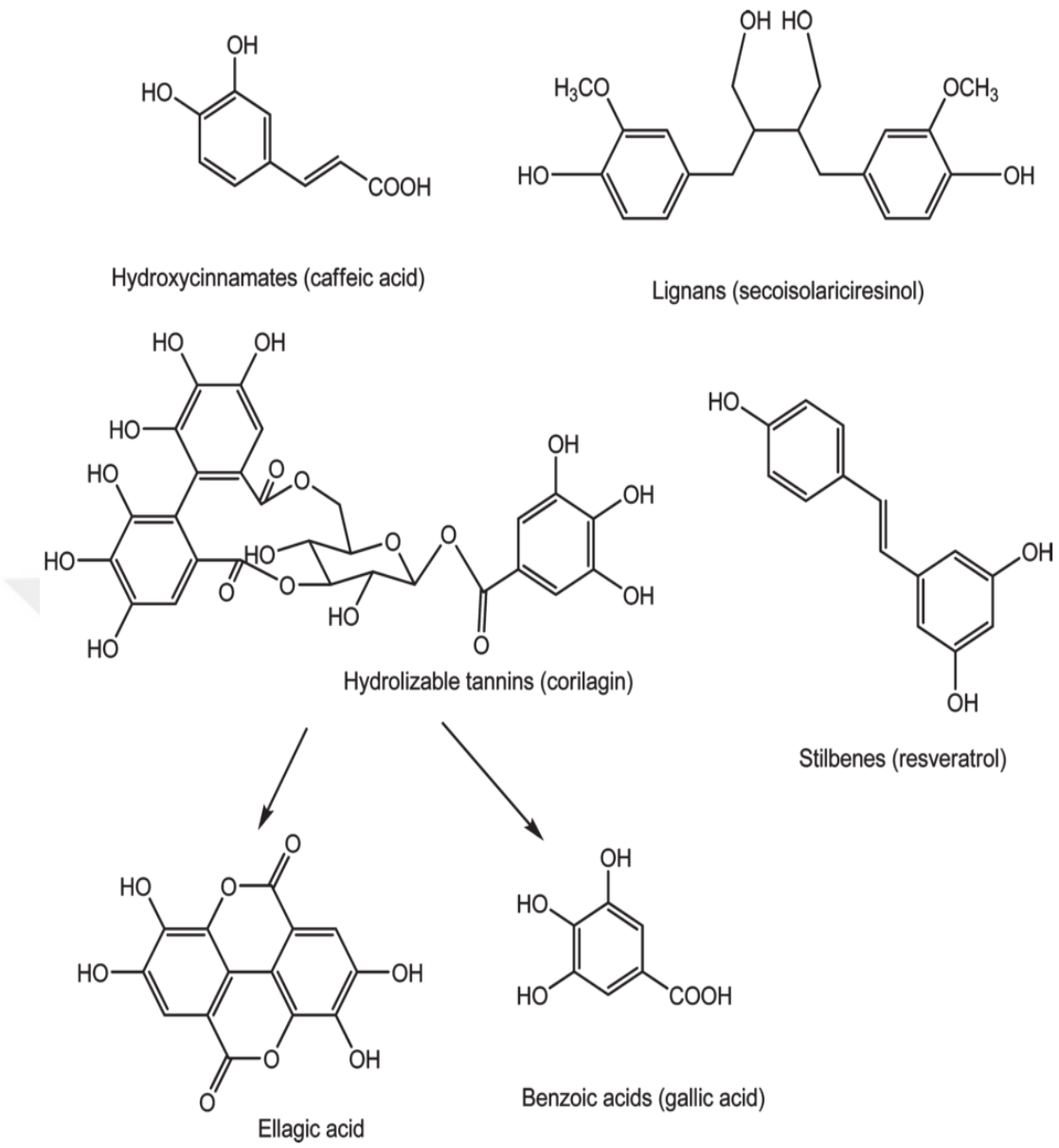


Figure 2.2 : Nonflavonoid-type phenolics that are metabolized by the gut microbiota (Selma et al., 2009).

2.5.1.2 Flavones and Flavanones

Flavones are flavonoids that have a basic structure consisting of a 2-phenyl-benzo-γ-pyrone skeleton formed by two phenyl rings (A and B) linked with a heterocyclic pyrone ring (C) (Figure 2.1). Flavanones have a 2,3-dihydro-2-phenylchromen-4-one structure. (Figure 2.1). The pyran rings of flavanones are nonlinear because they contain saturated C2–C3 bonds. They can form linkages with estrogen receptors. Furthermore, they do not contain double bonds between C2 andC3, contrary to isoflavones (Figure 2.1). Citrus fruits such as lemon, grapefruit, and orange are the most important dietary sources of flavanones (Toma's-Barbera'n and Clifford, 2000).

In comparison, flavanones have a higher bioavailability compared to flavonols and flavan-3-ols. This could be explained in part by less degradation by gut microbiota and greater accessibility for absorption in the intestine. Flavanones occur as glycosides, usually rutinosides (6-*O*-α-L-rhamnosyl-D-glucosides) and neohesperidosides (2-*O*-α-L-rhamnosyl-D-glucosides) at the 7th position (Robards et al., 1997). The degradation pathways of flavanone glycosides such as naringin are similar to flavonols. The first reaction is a deglycosylation to form naringenin, which then turns into phloroglucinol and 3-(3,4-dihydroxyphenyl) propionic acid by cleavage of the C ring (Rechner et al., 2004).

2.5.1.3 Flavone C-Glycosides

Most dietary flavonoids exist in their *O*-glycosidic forms. However, *C*-glycosylated flavonoids, especially flavones, are widespread in a variety of plants (Xiao, 2017). In most cases, flavone *O*-glycosides are hydrolyzed by digestive enzymes or degraded by gut bacteria to their aglycones in the intestine to be reduced and conjugated to form *O*-glucuronides and *O*-sulfates in the liver (Xiao and Höger, 2015; Xiao et al., 2016). However, flavone *C*-monoglucosides exhibit different metabolism pathways compared to flavone *C*-multiglycosides (Xiao et al., 2016) (Figure 2.3). Flavone *C*-monoglucosides such as orientin, vitexin, homoorientin, and isovitexin are observed to be poorly absorbed in the gastrointestinal tract of rats and, consequently, were able to reach the colon (Zhang et al., 2007). Once in the colon, flavone *C*-monoglucosides are deglycosylated and degraded to smaller metabolites, such as phloroglucinol, hydrocaffeic acid, and phloretic acid, by human gut bacteria, yielding very few metabolites in the urine and blood. Flavone *C*-multiglycosides are absorbed intact in the intestine and are minimally changed in the liver. Afterwards, they are returned to the gut by enterohepatic recirculation (Xiao et al., 2016).

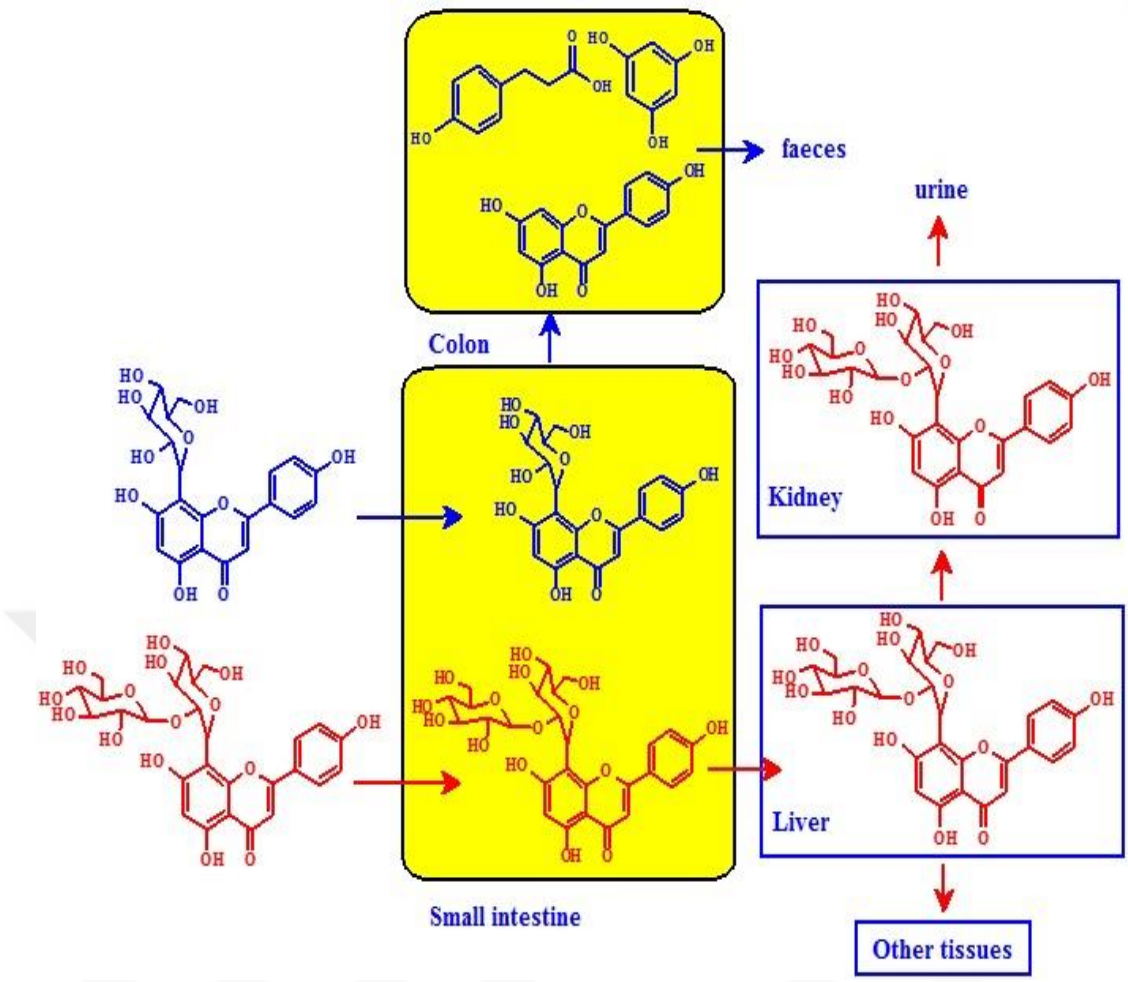


Figure 2.3 : Absorption and metabolism of flavone C-monoglycosides (blue arrow) and C-multiglycosides (red arrow) (Xiao et al., 2016).

2.5.1.4 Isoflavones

Isoflavones have a planar basic ring system with the benzenoid B ring attached to C3, differentiating it from the other flavonoids. Isoflavonoids are nonsteroidal and estrogen-like due to their chemical structure. As they are able to bind to estrogen receptors (ERα and ERβ), they are classified as natural selective estrogen receptor modulators (SERMs) (Messina, 2000; Sarkar and Li, 2002; Setchell et al., 2002; Linford and Dorsa, 2002; Cornwell et al., 2004; Yuan et al., 2007). Isoflavones are in the form of water-soluble glycosides and they cannot be absorbed completely through the enterocyte. This is a consequence of their high hydrophilicity and molecular weight. Soy is one of the food products rich in health-promoting isoflavonoids (Yuan et al., 2007). Soy products contain 12 identified isoflovone compounds: three aglycones (genistein, daidzein, glycitein); three glucosides; three acetyl ester glucosides; and three malonyl ester glucosides. Isoflavones can be

biotransformed by β-glucosidase from gut microbiota into their aglycones for increased bioavailability (Linford and Dorsa, 2002; Setchell et al., 2002]. The aglycones can be both absorbed completely or metabolized by gut microbiota (Zubik and Meydani, 2003). Aglycones are also biotransformed into their metabolites, namely daidzein to *O*-demethylangolensin (*O*-DMA) and equol, and genistein to *p*-ethylphenol and 4-hydroxyphenyl-2-propionic acid. Equol has higher antioxidant activity than soy isoflavones due to its nonplanar structure which is more flexible to conformational modification. Though, due to the absence of specific constituents of the gut microbiota, only about one-third of the human population can metabolize daidzein into equol. Other observed microbial metabolites of daidzein are 2-dehydro-*O*-DMA, dihydrodaidzein, tetrahydrodaidzein, 6-hydroxydaidzein, 8-hydroxydaidzein, and 3′-hydroxydaidzein, and 3-(4 hydroxyphenyl) benzopyran-4,7-diol. They are biotransformed by deglucosylation, reduction, C ring fission, and hydroxylation reactions (Yuan et al., 2007). Soybean products contain less than 10% glycitein, but it comprises about half of the isoflavone mass in soy germ. It is demethoxylated into 6,7,4′-trihydroxyisoflavone *in vitro* by *Eubacterium limosum* (Hur and Rafii, 2000). Metabolites of glycitein have been isolated and characterized as dihydroglycitein, 6-*O*-methyl-equol, 5′-*O*-methyl-*O*-desmethylangolensin, and dihydro-6,7,4′-trihydroxyisoflavone (Simons et al., 2005; Heinonen et al., 2003).

2.5.1.5 Flavanols

Simple flavanols such as (+)-catechin (C), (−)-epicatechin (EC), epigallocatechin, their gallate esters, and polymeric procyanidins from dimers and polymers collectively named condensed tannins are all classified as flavanols. They are the primary contributors to dietary phenolics and are found mostly in fruits, tea, wine and chocolate. Similar to flavanones and flavonols, flavanols such as catechin, epigallocatechin and epicatechin have a B ring attached to C2, but they do not have a carbonyl group in their C4 position or double bonds between C2 and C3. They are not found in a planar conformation as flavanones are (Selma et al., 2009).

Flavanols are promptly metabolized to several *O*-sulfated, *O*-glucuronidated, and *O*-methylated forms by phase II enzymes (Kuhnle et al., 2000; Vaidyanathan and Walle, 2002; Garcıá-Ramirez et al., 2006; Hackman et al., 2008). The main phenolic-metabolites of catechin and epicatechin are 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 3-(3-hydroxyphenyl)propionic acid, and 3- hydroxyhippuric acid, 5-(3′-hydroxyphenyl)-

γ-valerolactone (Deprez et al., 2000; Rios et al., 2003; Rechner et al., 2004;). Tzounis et al. (2011) found out that when (−)-epicatechin or (+)-catechin were incubated with intestinal bacteria, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 5-phenyl-γ-valerolactone, and 3-phenylpropionic acid were formed. For the biotransformation of these metabolites to proceed, initial conversion to (+)-epicatechin from (+)-catechin is essential. The metabolites pyrogallol, 3-(3-hydroxyphenyl) propionic acid, 5-(3,4-dihydroxyphenyl) valeric acid, 5-(3-hydroxyphenyl) valeric acid, 3-(3,4-dihydroxyphenyl) propionic acid, 3-(3-methoxyphenyl) valeric acid, and 2,3-dihydroxyphenoxyl 3-(3′,4′-dihydroxyphenyl) propionic acid are obtained after *in vitro* incubation of epicatechin with the human intestinal microbial flora. Dietary condensed tannins are biotransformed into benzoic, phenylacetic, phenylpropionic, and phenyllactic acid derivatives, with phloroglucinol, 5-(3′-hydroxyphenyl)-γ-valerolactone, and 1-(3-hydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol being produced (Meselhy et al., 1997). In another study, condensed tannins produced 3-(4-hydroxyphenyl) propionic acid, 3-phenylpropionic acid, 2-(3-hydroxyphenyl)acetic acid, 2-(4-hydroxyphenyl) acetic acid, 5-(3-hydroxyphenyl)valeric acid, and 3-(3-hydroxyphenyl) propionic acid by way of the gut microbiota (Deprez et al., 2000).

The dry weight of green tea consists of 30%–42% catechin. Major catechins are (+)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epicatechin (EC), in which EGCG is the most abundant, and may account for 50%–80% of the total catechins in tea (Sng et al., 2011). The metabolism of tea catechins by gut microbiota has been studied extensively, demonstrating that the absorption of catechins in the small intestine is relatively low. As a result, the majority of tea catechins are biotransformed by gut microbiota, followed by absorption in the bloodstream or excretion in the feces.

Takagaki and Nanjo (2013) identified several new metabolites of (+)-C or (−)-EC biotransformed by rat intestinal microbiota and they revised the suggested metabolic pathway of these compounds (Takagaki and Nanjo, 2013). They have observed four different strains of human intestinal bacteria that have the ability to biotransform (+)-C and (−)-EC.

Wang et al. (2001) reported that *Eubacterium* sp. strain SDG-2 biotransformed (+)-C to 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol. However, the bacteria biotransformed (−)-EC into two compounds, 1-(3′,4′-dihydroxyphenyl)-

3-(2″,4″,6″-trihydroxyphenyl) propan-2-ol and 1-(3′-hydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl) propan-2-ol. Thus, *Eubacterium* sp. strain SDG-2 has the ability of *p*-dehydroxylation in the B ring of (−)-EC but not in (+)-C (Wang et al., 2001).

Kutschera *et al.* (2011) revealed that both (+)-C and (−)-EC could be biotransformed to 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol by *Eggerthella lenta* rK3. However, the conversion of (+)-C progressed five times faster than that of (−)-EC. *Flavonifractor plautii* aK2 further converted 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol to δ-(3′,4′-dihydroxyphenyl)-γ-valerolactone and δ-(3′,4′-dihydroxyphenyl)-γ-valeric acid (Kutschera et al., 2011).

Epigallocatechin (EGC) can be biotransformed by *Eubacterium* sp. strain SDG-2 into 1-(3′,5′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl) propan-2-ol [85]. Moreover, it was observed that 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone can be formed as an additional major metabolite of EGC (Vaidyanathan and Walle, 2002; Hackman et al., 2008).

Furthermore, it was reported that some phenolic acids were produced such as 3,4-dihydroxyphenylpropionic acid (3,4-DHPPA), 3- hydroxyphenylpropionic acid (3-HPPA), 4- hydroxyphenylpropionic acid (4-HPPA), 3,4- dihydroxyphenylacetic acid (3,4-DHPAA), 3- hydroxyphenylacetic acid (3-HPAA), 3- hydroxybenzoic acid (3-HBA), 4- hydroxybenzoic acid (4-HBA) and phloroglucinol (Van't Slot and Humpf, 2009; Roowi et al., 2010; Schantz et al., 2010). Meng et al. (2002) identified δ-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone, δ-(3′,4′-dihydroxyphenyl)-γ-valerolactone, and δ-(3′,5′-dihydroxyphenyl)-γ-valerolactone in the urine after EGC consumption (Meng et al, 2002). Upon consumption of 200 mg of pure ECG, δ-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone, δ-(3′,4′-dihydroxyphenyl)-γ-valerolactone, and δ-(3′,5′-dihydroxyphenyl)-γ-valerolactone were identified in the urine (Meng et al., 2002).

Furthermore, (−)-EGCG is the 3-*O*-gallate product of (−)-EGC, which is the major catechin derivative found in tea. Van't Slot and Humpf (2009) reported that (+)-GCG and (−)-EGCG were degraded by the intestinal microbiota of pig cecum, and biotransformed into (+)-GC and (−)-EGC, respectively (Van't Slot and Humpf, 2009). This degradation is also observed by human and rat intestinal microbiota in *in vitro* models of the colon (Schantz et al., 2010; Van't Slot and Humpf, 2009; Meng et

al., 2002; Takagaki and Nanjo, 2010). In addition, Takagaki and Nanjo (2010) analyzed the metabolism and biotransformation of (−)-EGCG by rat intestinal bacteria and proposed three metabolic pathways. In the first step, (−)-EGCG is hydrolyzed into (−)-EGC and gallic acid. Then, EGC is biotransformed into 1-(3′,4′,5′-trihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl) propan-2-ol by reductive opening among the first and second positions of EGC. Subsequently, 1-(3′,4′,5′-trihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl) propan-2-ol is biotransformed into 1-(3′,5′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol by dehydroxylation of 1-(3′,4′,5′-trihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl) propan-2-ol at the 4′ position. Moreover, as a moor degradation pathway of EGCG metabolism, 5-(3′,5′-dihydroxyphenyl)-γ-valeric acid is formed as the main metabolite by ring fission of the phloroglucinol moiety of the metabolite 1-(3′,5′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl) propan-2-ol. This is the major pathway of EGCG metabolism. Simultaneously, just after the ring fission, the 5-(3′,5′-dihydroxyphenyl)-γ–valerolactone metabolite may be formed according to the lactonization of 5-(3′,5′-dihydroxyphenyl)-γ-valeric acid and its small part is biotransformed into 3,5-dihydroxyphenyl-propionic acid. In addition, an insignificant amount of 4′-dehydroxylated metabolite is biotransformed from EGC that is not further metabolized by the gut microbiota (Takagaki and Nanjo, 2010).

There are also *in vivo* studies on the metabolism of EGCG explaining the likely metabolic pathway as in *in vitro* studies. *In vivo* studies on the biotransformation of EGCG after oral administration to rats revealed that EGCG was relocated into the cecum and large intestine, and then underwent degradation by intestinal bacteria to 5-(3′,5′-dihydroxyphenyl)- γ-valerolactone with EGC as an intermediate product (Kohri et al., 2001a; Kohri et al 2001b]. An abundant quantity of 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone is absorbed in the body by going through glucuronidation in the intestinal mucosa or liver, transformed to its glucuronidated metabolites which enter blood circulation. Next they are distributed to various tissues and excreted in the urine (Meng et al., 2002; Kohri et al., 2001a; Kohri et al., 2001b). *In vivo* and *in vitro* biotransformations of flavan-3-ols are given in detail with their degradation products in Table 2.2.

Table 2.2 : *In vitro* and *in vivo* biotransformation of flavan-3-ols by gut microbiota.

Compound	Metabolite	Model (*in vivo*/*in vitro*) and References
(+)-C or (−)-EC	1-(4′-hydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol	Rat *in vitro* (Takagaki and Nanjo, 2013)
	1-(3′-hydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol	Rat *in vitro* (Takagaki and Nanjo, 2013)
	1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol	Rat *in vitro* (Takagaki and Nanjo, 2013);
	5-(3′-hydroxyphenyl)pentanoic acid	Rat *in vitro* (Takagaki and Nanjo, 2013);
	5-(3′,4′-dihydroxyphenyl)-4-oxo-valeric acid	Rat *in vitro* (Takagaki and Nanjo, 2013);
	5-(3′-hydroxyphenyl)-4-oxo-valeric acid	Rat *in vitro* (Takagaki and Nanjo, 2013);
	5-[(3′,4′-dihydroxyphenyl)methyl]oxolan-2-one	Rat *in vitro* (Takagaki and Nanjo, 2013); Human *in vitro* (Roowi et al.,2010); Human *in vivo* (Meng et al., 2002)
	5-[(3′-hydroxyphenyl)methyl)oxolan-2-one	Rat *in vitro* (Takagaki and Nanjo, 2013); Human *in vitro* (Schantz et al., 2010)
	5-(3′,4′-dihydroxyphenyl)-pentanoic acid	Rat *in vitro* (Takagaki and Nanjo, 2013); Human *in vitro* (Roowi et al.,2010)
	3,4-DHPPA	Rat *in vitro* (Takagaki and Nanjo, 2013); Pig *in vitro* (Van't Slot and Humpf, 2009).
	3-HPPA	Rat *in vitro* (Takagaki and Nanjo, 2013); Human *in vitro* (Roowi et al., 2010)
	4-HPAA	Pig *in vitro* (Van't Slot and Humpf, 2009)
	3-HBA	Pig *in vitro* (Van't Slot and Humpf, 2009)
	4-HBA	Pig *in vitro* (Van't Slot and Humpf, 2009)
	Phloroglucinol	Pig *in vitro* (Van't Slot and Humpf, 2009)
	5-[(3′,4′,5′-trihydroxyphenyl)methyl]oxolan-2-one	Human *in vitro* [88]
(+)-GC or (−)EGC	1-(3′,5′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol	Human *in vitro* (Roowi et al., 2010; Schantz et al., 2010)
	5-[(3′,4′,5′-trihydroxyphenyl)methyl]oxolan-2-one	Human *in vitro* (Roowi et al., 2010; (Schantz et al., 2010)
	4-HPAA	Human *in vitro* (Roowi et al., 2010; Pig *in vitro* (Van't Slot and Humpf, 2009)
	Phloroglucinol	Pig *in vitro* (Van't Slot and Humpf, 2009)
	3,4-DHPPA	Pig *in vitro* (Van't Slot and Humpf, 2009)
	3-HPPA	Pig *in vitro* (Van't Slot and Humpf, 2009)
	3-HBA	Pig *in vitro* (Van't Slot and Humpf, 2009)
	4-HBA	Pig *in vitro* (Van't Slot and Humpf, 2009)
(−)-EGC	5-[(3′,4′,5′-trihydroxyphenyl)methyl]oxolan-2-one	Human *in vivo* (Meng et al., 2002)
	5-[(3′,4′-dihydroxyphenyl)methyl]oxolan-2-one	Human *in vivo* (Meng et al., 2002)
	5-[(3′,5′-dihydroxyphenyl)methyl)]oxolan-2-one	Human *in vivo* (Meng et al., 2002)
(−)-ECG	EC	Rat *in vivo* (Kohri et al., 2001a)
	Gallic acid	Rat *in vivo* (Kohri et al., 2001a)
	Pyrogallol	Rat *in vivo* [(Kohri et al., 2001a)
	1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol	Rat *in vivo* (Kohri et al., 2001a)
	5-[(3′,4′-dihydroxyphenyl)methyl]oxolan-2-one	Rat *in vivo* (Kohri et al., 2001a)
	5-[(3′-hydroxyphenyl)methyl)]oxolan-2-one	Rat *in vivo* (Kohri et al., 2001a)
	5-(3′,4′-dihydroxyphenyl)pentanoic acid	Rat *in vivo* (Kohri et al., 2001a)
	3-HPPA	Rat *in vivo* (Kohri et al., 2001a)
	(E)-3-(3-hydroxyphenyl)-acrylic acid	Rat *in vivo* (Kohri et al., 2001a)
	EGC	Rat *in vivo* (Kohri et al., 2001a)

Table 2.2 (continued): *In vitro* and *in vivo* biotransformation of flavan-3-ols by gut microbiota.

Compound	Metabolite	Model (*in vivo*/*in vitro*) and References
(+)-GCG or (−)-EGCG	EGC	Rat *in vitro* [91]; Human *in vitro* (Roowi et al., 2010; Schantz et al., 2010); Pig *in vitro* (Van't Slot and Humpf, 2009)
	Gallic acid	Rat *in vitro* (Takagaki and Nanjo, 2010); Human *in vitro* (Roowi et al.,2010; Schantz et al., 2010); Pig *in vitro* (Van't Slot and Humpf, 2009)
	5-[(3′,4′,5′-trihydroxyphenyl)methyl]oxolan-2-one	Rat *in vitro* (Takagaki and Nanjo, 2010); Pig *in vitro* (Van't Slot and Humpf, 2009)
	1-(3′,4′,5′-trihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol	Rat *in vitro* (Takagaki and Nanjo, 2010)
	1-(3′,5′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol	Rat *in vitro* (Takagaki and Nanjo, 2010)
	5-(3′,5′,- dihydroxyphenyl) pentanoic acid	Rat *in vitro* (Takagaki and Nanjo, 2010)
	5-(3′,4′,5′-trihydroxyphenyl) pentanoic acid	Rat *in vitro* (Takagaki and Nanjo, 2010)
	5-(3′-hydroxyphenyl)-pentanoic acid	Rat *in vitro* (Takagaki and Nanjo, 2010)
	5-[(3′,5′-dihydroxyphenyl)methyl)]oxolan-2-one	Rat *in vitro* (Takagaki and Nanjo, 2010)
	3,5-DHPPA	Rat *in vitro* (Takagaki and Nanjo, 2010)
	5-[(3′,4′,5′-trihydroxyphenyl)methyl]oxolan-2-one	Human *in vitro* (Roowi et al., 2010).
	Pyrogallol	Human *in vitro* (Roowi et al., 2010)
	Pyrocatechol	Human *in vitro* (Roowi et al., 2010)
	4-HPAA	Human *in vitro* (Roowi et al., 2010)
(−)-EGCG	EGC	Rat *in vivo* (Kohri et al., 2001a; Kohri et al., 2001b)
	Gallic acid	Rat *in vivo* (Kohri et al., 2001a; Kohri et al., 2001b)
	1-(3′,4′,5′-trihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol	Rat *in vivo* (Kohri et al., 2001a; Kohri et al., 2001b)
	1-(3′,5′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol	Rat *in vivo* (Kohri et al., 2001a; Kohri et al., 2001b)
	5-[(3′,4′,5′-trihydroxyphenyl)methyl]oxolan-2-one	Rat *in vivo* (Kohri et al., 2001a; Kohri et al., 2001b)
	5-[(3′,5′-dihydroxyphenyl)methyl)]oxolan-2-one	Rat *in vivo* (Kohri et al., 2001a; Kohri et al., 2001b)
	5-[(3′,4′-dihydroxyphenyl)methyl]oxolan-2-one	Rat *in vivo* (Kohri et al., 2001a; Kohri et al., 2001b)

2.5.1.6 Anthocyanins

Anthocyanins belong to a large group of secondary plant metabolites collectively known as flavonoids (Fleschhut et al., 2006). They are responsible for the red and blue pigmentation of many fruits and vegetables (Keppler and Humpf, 2005). The health benefits of anthocyanins have been demonstrated in several *in vivo* and *in vitro* studies (Wang et al., 1999; Hou, 2003; Katsube et al., 2003). However, the low bioavailability of anthocyanins is a clear obstacle in achieving desired beneficial effects (Xiao and Högger, 2014).

Several studies on the intestinal absorption of anthocyanins have been reported. The anthocyanins delphinidin 3-*O*-rutinoside, cyanidin 3-*O*-rutinoside, delphinidin 3-*O*-glucoside, and cyanidin 3-*O*-glucoside were found to be directly absorbed and excreted as the glycosylated forms in both rats and human subjects (Matsumoto et al., 2001). A number of studies showed that the proportion of total anthocyanins absorbed and subsequently excreted in urine was far below 1% (Vitaglione et al., 2007). By collecting ileostomy effluent after the consumption of anthocyanin-rich blueberries, Kahle *et al.* (2006) determined that up to 85% of blueberry anthocyanins reach the colon under physiological conditions (Kahle et al., 2006). However, about 69% of the anthocyanins disappeared from the gastrointestinal tract within 4 h after food ingestion (Aura et al., 2005; El Mohsen et al., 2006). As demonstrated by *in vitro* fermentation of anthocyanins seeded by a fecal community obtained from rats, cyanidin-3-glucoside and cyanidin-3-rutinoside extracted from wild mulberry were completely degraded within 10 hours (Hassimotto et al., 2008). It is thus likely that intestinal microbiota contribute to the biotransformation and the metabolism of anthocyanins (Hanske et al., 2013).

Table 2.3 : The expected B ring fragments for the common anthocyanidins. Reproduced from the original source (Williamson and Clifford, 2010).

Anthocyanidin	Initial B-Ring Fragmentation Product
Pelargonidin	4-Hydroxybenzoic acid
Cyanidin	Protocatechuic acid
Delphinidin	Gallic acid
Peonidin	Vanillic acid
Petunidin	3-Methoxy-4,5-dihydroxybenzoic acid
Malvidin	Syringic acid

Fleschhut *et al.* (2006) incubated an anthocyanin-rich extract from red radish with human fecal suspension *in vitro* and demonstrated that the first step of the bacterial hydrolysis of anthocyanins (*i.e.*, monoglucosides, diglucosides as well as acylated anthocyanins) involves the cleavage of the sugar moiety leading to the formation of the anthocyanin aglycon. The activities of two bacterial enzymes in particular, α,L-rhamnosidase and β,D-glucosidase, may be responsible for the deglycosylation of anthocyanins (Fleschhut et al., 2006). Due to the high instability of the liberated

aglycones at the pH of various locations in the intestine, they might spontaneously change to form quinoid bases, which further break down into a phenolic acid and an aldehyde via an α-diketone intermediate (Figure 2.4). Therefore, the major degradatory pathway of this process is the formation of the phenolic acid descending from the B ring of the anthocyanin skeleton (Table 2.3) (Fleschhut et al., 2006; Keppler and Humpf, 2005).

Many studies have shown that protocatechuic acid is one of the most likely major degradation products of anthocyanins. In a human study, it was identified that protocatechuic acid was the major metabolite of cyanidin-*O*-glucosides, accounting for almost 73% of ingested cyanidin-*O*-glucosides (Vitaglione et al., 2007).

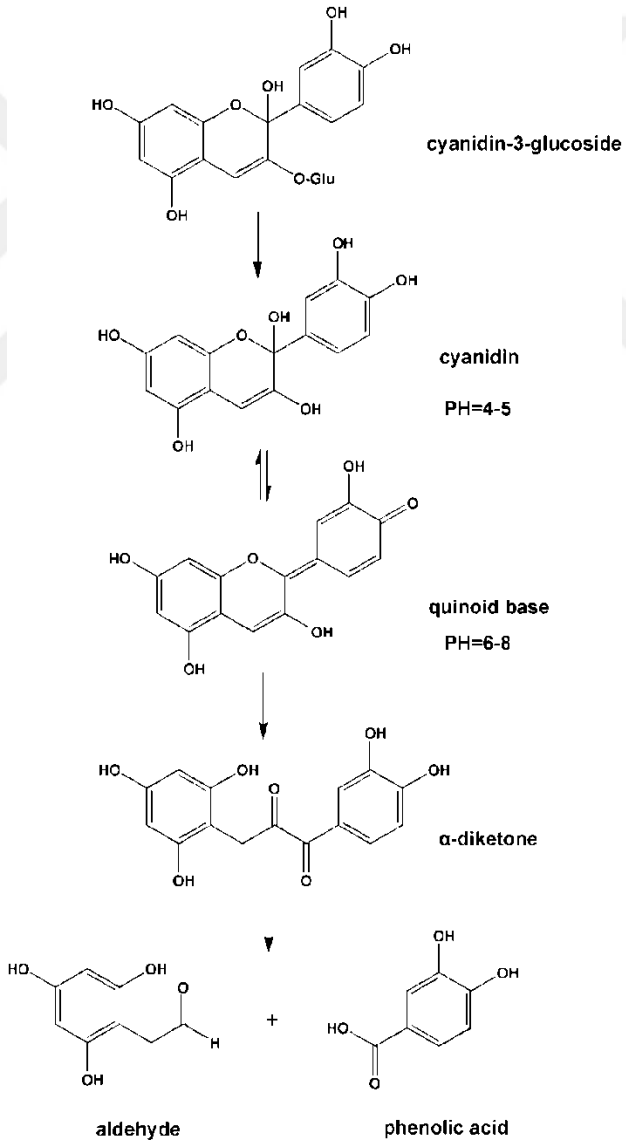


Figure 2.4 : pH-dependent structural changes and degradation of cyanidin-3-glucoside. Reproduced from the original source (Fleschhut et al., 2006).

After consumption of black raspberries in a porcine model, the phenolic acid profile in the gastrointestinal tract indicated that protocatechuic acid was the major metabolic derivative, followed by *p*-coumaric acid, caffeic acid, ferulic acid and 3-hydroxybenzoic acid (Wu et al., 2009). In addition, a high level of protocatechuic acid was also identified in rat plasma after the oral intake of cyanidin 3-*O*-β-D-glucoside (Tsuda et al., 1999). Cyanidin 3-*O*-β-D-glucoside was reported to metabolize to protocatechuic acid *via* cyanidine when incubated with fecal microbiota in an *in vitro* model. This resulted in a more potent anti-scratching behavioral effect than the parent cyanidin 3-*O*-β-D-glucoside in mice, thus suggesting the biological activity of anthocyanins *in vivo* may increase due to their metabolites, such as phenolic acids (Han et al., 2009).

2.5.2 Nonflavonoid-type phenolics

2.5.2.1 Phenolic acids

Phenolic acids are found in many foods, and in high concentrations in whole grains, wine and berries. Cereals contain mostly the hydroxycinnamate ferulic acid as a phenolic acid. It is found bound to arabinoxylans by ester bonds in rye. Therefore, esterases (e.g., cinnamoyl estarase) are required during digestion or by the gut microbiota to improve its bioaccessibility (Andreasen et al., 2001a; Rondini et al., 2004). As a result of this hydrolysis, unbound phenolic acids can be absorbed through the gastrointestinal barrier and enter peripheral circulation (Andreasen et al., 2001b).

Moreover, *in vitro* hydroxycinnamic acid conversions by gut microbiota can be observed. In a study conducted by Gonthier and colleagues (2006), caffeic acid and its esters, caftaric acid and chlorogenic acid, were used as a substrate in a human colon model and phenolic acids were transformed into 3-(3′-hydroxyphenyl)propionic acid and minor quantities of benzoic acid. The side-chain shortening of phenylpropionic acid arises through β-oxidation (Gonthier et al., 2006). Caffeic acid can also be decarboxylated and metabolized into 4-ethylcatechol by gut microbiota (Peppercorn and Goldman, 1971).

Ferulic acid dimers are other important phenolic acids released from cereals (Andreasen et al., 2000). Two ferulic acids can be bound to each other either through 8-*O*-4- or 5-5-linkages. In the study of Braune et al. (2009), dehydrodiferulic acid 8-

O-4- and 5-5-derivatives were incubated with human fecal microbiota. The 8-*O*-4 derivative was shown to degrade temporarily to monomeric ferulic acid, which was then biotransformed into 3-(3′,4′-dihydroxyphenyl) propionic acid, 3′,4′-dihydroxyphenyl acetic acid, 3-phenylpropionic acid, and benzoic acid. An alternative pathway to benzoic acid is related to the metabolism of 3-(4′-hydroxy-3′-methoxyphenyl)pyruvic acid. Contrarily, the 5-5-diferulate derivatives were exposed only to demethylation and/or side-chain reductions (Braune et al., 2009).

2.5.2.2 Stilbenes

Stilbenoids are very typical polyphenols in our diets, which mainly present in red grapes, wines, cranberries, strawberries, and peanuts [Xie and Bolling, 2015; Mulat et al., 2014). Resveratrol and its derivatives are the most important dietary stilbenoids associated with many benefits for human health (Folmer et al., 2014; Fu et al., 2015; Liu et al., 2014). *trans*-Resveratrol is metabolized by human gut microbiota to dihydroresveratrol, 3,4′-dihydroxy-trans-stilbene and 3,4′-dihydroxybibenzyl (lunularin) *in vivo* (Bode et al., 2013) *trans*-Piceid, the 3-*O*-β-D-glucoside of resveratrol, is metabolized to resveratrol, dihydropiceid and dihydroresveratrol by gut microbiota (Wang et al., 2011).

2.5.2.3 Lignans

Lignans consist of pinoresinol, matairesinol, secoisolariciresinol, isolariciresinol, syringaresinol and lariciresinol diphenolic compounds with a 1,4-diarylbutane structure (Cassidy et al., 2000). They are mostly found in fruits and vegetables, tea, cereal products, and coffee (Touillaud et al., 2007). If consumed, they are potentially metabolized by microbial processes (Scalbert et al., 2005; Aura et al., 2006; Possemiers et al., 2007; Rowland et al., 2003). Lignans are considered phytoestrogens because of their estrogen agonist or antagonist properties (Scalbert et al., 2005). The biological activity of lignans is associated with their activation into enterolactone and enterodiol, which are mammalian phytoestrogens (Bowey et al., 2003; Eeckhaut et al., 2008; Wang et al., 2000). The bioavailability of lignans is directly associated with intestinal bacterial metabolism. These biotransformation reactions require demethylation and dehydroxylation (Blaut et al., 2007).

2.6 Possible Role in Cancer Prevention and Treatment

Cancer is a serious worldwide health problem increasing due to the growth and ageing of the population, prevalence of risk factors, such as smoking, being overweight, physical inactivity (Lefranc et al., 2017). The World Health Organization (WHO) considers that, in the next decade, there will be 70% increase in the cancer incidence in developing nations which could not access the required cancer medicines but can reach the foods have got therapeutic effects on cancer (Lefanc et al., 2017).

Cancerous tissue is highly heterogeneous, and this may make current chemotherapy agents ineffective (Lefanc et al., 2017). Many dietary compounds can prevent, or decrease greatly the initiation of cancerous tissues (El Rahman, 2010). These bioactive natural products may help to cure some types of cancer, such as prostate and breast cancer (Premratanachai and Chanchao, 2014). Therefore, cancer chemotherapy drugs include over 70% of anticancer compounds which are natural substances derived from natural products (Watanabe et al., 2011). Natural anticancer compounds contribute the multiplication of healthy cells (El Rahman, 2010).

According to many researches done both *in vitro* and *in vivo*, about the therapeutic effects of propolis on cancer cells, it is proved that propolis has therapeutic effects against different type of tumour cells (Watanabe et al., 2011). In the literature, it is mentioned that propolis has anticancer, antitumour and immunomodular effects (Bankova et al., 2014; Ebeid et al., 2016; El-Rahman-2010; Kongkiatpaiboon et al., 2016; Premratanachai and Chanchao, 2014; Watanabe et al., 2011). Moreover, *in vitro* assays proved that propolis and its isolated compounds have cytotoxic activity, which means cell killing effects, on various cell lines derived from human malignant tumours (Lefranc et al., 2017; Ribeiro et al., 2015; Watanabe et al., 2011).

Current literature proves that propolis has the ability to induce apoptosis, a natural mechanism to regulate cell death by which cells undergo death to control cell proliferation or in response to DNA damage (Premratanachai and Chanchao, 2014; Watanabe et al., 2011). There are two main pathways of apoptosis; the first one is induced by an external signal stimulated by TNF receptors, TNF- related apoptosis-inducing ligand (TRAIL)-R1 and TRAIL-R2, and the second pathway is mediated by mitochondria and pro-apoptotic proteins, including cytochrome (Premratanachai and

Chanchao, 2014). Some studies showed that bee product peptides induce apoptotic cell death in vitro in many human cancer cell lines, including renal, lung, liver, prostate, bladder and lymphoid cancer cells (Premratanachai and Chanchao, 2014). Moreover, some of the other studies showed that propolis induce apoptosis in human melanoma cells, and inhibits the *in vitro* growth of MCF-7 breast cancer cells (Premratanachai and Chanchao, 2014). Also, it is known that the mechanism of propolis-induced apoptosis is independent from the type of cancer cells, however, it is dependent on the concentration of the propolis extract (Premratanachai and Chanchao, 2014).

Chemo-therapy and radio-therapy are generally used for the treatment of cancer (Premratanachai and Chanchao, 2014). Radio-therapy is used directly on the cancerous tissue area to kill cancerous cells, however, using radiation causes toxic effects on normal tissues (Ebeid et al., 2016). In fact, both chemo- and radio-therapies harm healthy cells during the therapy process, and cause undesirable side effects that limit the treatment and its effectiveness (Premratanachai and Chanchao, 2014). Moreover, in some cases, chemo- and radio- therapies may kill the patient faster than the cancer would do in its usual cource (Premratanachai and Chanchao, 2014). Radio-protectors, oftenly antioxidants, are used to reduce the radiation damage on healthy cells, which cleans free radicals produced during the radio-therapy (Ebeid et al., 2016). This encourages people to make researches about the radioprotective effects of natural substances, such as propolis, to overcome harmful side effects of radio-therapy (Ebeid et al., 2016). Furthermore, it is known that polyphenols play an important role in cancer chemoprevention (Watanabe et al., 2011).

Ebeid et al. (2016) studied the radio-protective effect of propolis in breast cancer (BC) patients undergoing radiotherapy. They applied the study on 135 subjects divided into three main groups which are, group 1 (healthy females as a control group), group 2 (45 chemotherapy received breast cancer patients followed by radiation therapy), and group 3 (45 chemotherapy received breast cancer patients followed by radiation therapy and propolis supplements). The results showed that propolis have the ability to reduce significantly the radiation induced DNA damage in the group 3. They observed that radiotherapy significantly down regulate RRM2 protein but the results became still significantly higher than normal control value.

On the other hand, the propolis supplementation given during radiotherapy significantly caused down regulation of RRM2 level and became within the normal control level. Hematological parameters including hemoglobin concentration, white blood cells and platelets counts decreased after radiotherapy treatment, while the parameters increased and became within the normal control level after the propolis supplemented radiotherapy. They showed that supplementation of propolis with radiotherapy provides protection against DNA damage caused by ionizing radiation in BC patients' leukocytes and inhibits RRM2 overexpression. Also, they observed that propolis had beneficial effects on the serum antioxidant capacity (Ebeid et al., 2016).

Studies performed about the antitoumor effect of propolis do not show any side-effects, and this situation provides potential use of propolis in the the development of new antitumour medicines (Murtaza et al., 2015; Watanabe et al., 2011).

Antitumour effect of propolis is due to its rich phenolic sources (Premratanachai and Chanchao, 2014; Watanabe et al., 2011). Some compounds isolated from propolis have been investigated if they are responsible for the main curative action on tumour cells (Watanabe et al., 2011). Recent studies have suggested that the astaxanthin and flavonoids in propolis can protect an adenergic cell line derived from a human neuroblastomic bone marrow from beta-amyloid induced apoptotic death. Polyphenolic compounds such as caffeic acid, CAPE and quercitin have anticarcinogenic activity which can inhibit cancer cell growth. According to many studies, CAPE and chrysin have important therapeutic effects on cancer (Premratanachai and Chanchao, 2014).

Caffeic acid phenethyl ester (CAPE) provides strong antitumor effects in oral, neck and tongue cancer cells (Premratanachai and Chanchao, 2014). Biological studies showed that CAPE-induced oxidative stress may affect the radiosensitivity and proliferation of medulloblastoma cells. It is known that CAPE inhibited the growth of human medulloblastoma cells (Watanabe et al., 2011). Also, biological studies revealed the activity of CAPE against tumor invasion, metastasis, proliferation, and apoptosis in different cancers such as human pancreatic and colon cancer (Murtaza et al., 2015). It is proved that CAPE generally inhibits the activated nuclear factor-kB (NF-kB) via suppressing their binding with DNA (Murtaza et al., 2015).

Chrysin (5,7-dihydroxyflavone) is a natural and biologically active flavonoid compound found in propolis (Watanabe et al., 2011). Chrysin, is found at higher concentrations in propolis and has significant biological and pharmacological properties including antioxidant and anti-inflammatory and anticancer effects. Chrysin affects the apoptotic process in many cancer cell lines, especially leukemia (Premratanachai and Chanchao, 2014). As it was mentioned in Watanabe et al. (2011), results of a study showed a significant decrease in human telomerase reverse transcriptase expression levels due to chrysin in the cancer cells of leukaemia patients treated with Turkish propolis. Also, it is mentioned in the literature that chrysin induced cell death in human and murine melanoma cells (Watanabe et al., 2011).

Quercetin, provides anti-inflammatory effects (Lefanc et al., 2017). Many studies have proved that the quercetin induces the pro-apoptotic effects in various cancer cell lines. Quercetin kills cancer cells which are resistant to pro-apoptotic stimuli, and induces non-apoptotic-related cell death pathways in cancer cells (Lefanc et al., 2017). Moreover, according to available data, it is known that quercetin provides the strongest cytotoxic effect on all leukemia cell lines (Watanabe et al., 2011).

Propolin G isolated from propolis, has a broad spectrum of biological activities. Nymphaeol C. and Propolin G can efficiently induce apoptosis in brain cancer cell lines (glioma and glio- blastoma) according to some studies. Furthermore, mucronulatol is one of the cytotoxic substances present in propolis. Mucronulatol provides cytotoxicity in cancer cell lines (Watanabe et al., 2011). Also, according to the literature, artepillin C isolated from propolis, induce cytotoxicity of carcinomas and malignant melanoma cells by apoptosis (Premratanachai and Chanchao, 2014). Mitomycin-C also shows antitoumor effects (Watanabe et al., 2011).

Cavalcante et al. (2011), studied the antitumoral activity of the flavonoid compounds of a hydroalcoholic extract of green propolis (EPV) on chemically induced epithelial dysplasias in rat tongues. The results showed that the EPV yield was 41% and the flavonoid yield was 0.95±0.44%. They proved that EPV plays an important protective role against chemically-induced carcinogenesis on the tongue in rats, and this protection was directly related with the EPV's concentration (Cavalcante et al., 2011).

Ribeiro et al. (2015), investigated the effect of oral administration of hydroalcoholic extract of Brazilian red propolis (HERP) on DMBA-induced oral squamous cell carcinomas in rodents. They identified the chemical composition of the HERP by HPLC method as propyl gallate, daidzein, catechin, epicatechin, formononetin and biochanin A. The results showed that HERP at doses of 50 and 100 mg/ kg inhibited 40% of oral squamous cell carcinoma growth due to its chemical composition. The results also showed that HERP had chemopreventive activity on the progress of DMBA-induced epithelial dysplasia to aggressive type oral squamous cell carcinoma in labial carcinogenesis (Ribeiro et al., 2015).

Carvalho et al. (2011), performed a study to evaluate *in vivo* and *in vitro* the antitumoural activity of a propolis extract. They performed hematological, biochemical, histopathological and morphological analyses on treated animals to evaluate the toxicological effect of propolis samples. Their data revealed that all propolis extracts showed an *in vivo* antitumour activity in the experimental model with a moderate toxicity effect. The oil extract of propolis was as effective as the ethanolic extract of propolis. Both inhibited the tumour growth. *In vitro* assays showed that the extract of propolis with canola oil (ODEP) showed a moderate cytotoxicity against leukemia (HL-60), melanoma (MDA-MB-435) and glioblastoma (SF-295) cancer cells, which is a better result than the result of EEP. There was a significant decrease of the tumour weight in all extracts tested (Carvalho et al., 2011).

Frozza et al. (2013), studied the antioxidant and antitumor activity of the red propolis from the northeast of Brazil. Cytotoxic activity was determined for human laryngeal epidermoid carcinoma cell (Hep-2), human cervical adenocarcinoma (HeLa) and human normal epithelial embryonic kidney (Hek-293). Survival analysis for non-tumor cell line showed higher IC_{50} compared to tumor cell lines. Their results showed that the Brazilian red propolis can inhibit cancer cell growth due to its natural antioxidant and antitumor composition (Frozza et al., 2013).

El-Rahman (2010), performed a study to investigate the effects of topical application of propolis extract, rosemary extract and a mixture of both extracts together on 12-O-tetradecanoylphorbol 13-acetate-induced tumor promotion in mice. The propolis extract (75% ethanolic extract), rosemary extract and a mixture of both extracts were applied on mice. The results showed a decrease in the number of skin tumors per

mouse by 27, 39, 71%, and a decrease in the number of mice with tumors by 22, 31, 75% for propolis, rosemary and both together respectively. The inhibited the tumor size per mouse by 46, 62, 72% for propolis, rosemary and both together respectively. According to the results of the study, it was observed that the combination of the biological activities of propolis and rosemary has the highest inhibitory effect on carcinogenesis. It was proved that they both have antioxidant and cytotoxic activities (El-Rahman, 2010).

Kustiawan et al. (2014), performed a study to investigate the cytotoxic activity of propolis extracts, bee pollen and honey collected from four different stingless bee species (*T. incisa, Timia apicalis, Trigona fusco-balteata* and *Trigona fuscibasis*), against five human cancer cell lines which are HepG2, SW620, ChaGo-I, KATO-III and BT474. They extracted all samples with methanol. They used four bioactive compounds isolated from propolis (apigenin, CAPE, kaempferol and naringenin) and two chemotherapeutic drugs (doxorubicin and 5-fluorouracil) to evaluate the sensitivity of the cell lines. The results showed that the extracts of propolis and honey had higher cytotoxic activities against cancer cell lines. They observed that the cytotoxic activity was dependent on the extraction solvent, bee species and cell line. During the experiment, propolis samples produced by *T. incisa* and *Trigona fusco-balteata* showed an *in vitro* cytotoxic activity against the four human cancer cell lines. The extracts of propolis samples produced by *T. incisa* and *Timia apicalis* showed the highest and lowest cytotoxic activity, respectively. Among the compounds of propolis doxorubicin was the most cytotoxic in general. However the ChaGo-I cell line was sensitive to kaempferol at 10 μg/mL and KATO-III was sensitive to kaempferol and apigenin at 10 μg/mL. And they observed that all compounds were effective against the BT474 cell line (Kustiawan et al., 2014).

Valente et al. (2011), studied the biological properties of Portuguese propolis on human renal cancer cell growth. The antioxidant potential of propolis collected from Portugal was evaluated by their ability to inhibit the 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced oxidative hemolysis and lipid peroxidation in human erythrocytes. According to the results, Portuguese propolis showed a strong protection on the erythrocyte membrane from hemolysis. Moreover, human erythrocytes treated with propolis extracts, showed a decrease in levels of malondialdehyde, a breakdown product of lipid peroxidation. In addition, they

observed that propolis extracts revealed their anticancer properties on human renal cell carcinoma. Also, propolis extracts provide a selective toxicity against malignant cells compared to normal cells. The results have proved that Portuguese propolis is a good source of therapeutic natural antioxidants and chemopreventive agents (Valente et al., 2011).

Banskota et al. (2000), evalueted free radical scavenging activity, cytotoxicity and hepatoprotective activity, of MeOH and water extracts of nine different propolis samples collected from Brazil, Peru, Netherlands and China. Their results showed that MeOH extracts of all propolis samples provided stronger cytotoxicity than the water extracts against murine colon 26-L5 carcinoma and human HT-1080 fibrosarcoma cells (Banskota et al., 2000).

In conclusion, according to the studies performed about the anticancer effects of propolis, it is apparent that it has therapeutic effects on many cancers such as human carcinoma, colon cancer, prostate cancer, melanoma, bladder cancer, pancreatic cancer, fibrosarcoma, colon adenocarcinoma, renal cancer, blood related cancers, breast cancer, brain cancer (glioma and glio- blastoma), lung carcinoma etc (Lefanc et al., 2017; Murtaza et al., 2015; Premratanachai and Chanchao, 2014; Watanabe et al., 2011).

2.7 Potential Use of Propolis in Food Industry

Recently, there is a growing interest in some food industries to find natural additives has increased the efforts both in obtaining bioactive compounds from natural raw materials and in developing stable and functional derivative products. The antimicrobial and antioxidant properties of propolis are interesting for the food industry because of its potential effect in the retardation of lipid oxidation and its positive effect on food product stability and shelf-life. According to Mendiola et al. (2010), propolis has potential as a natural food additive and as a functional food ingredient.

However, the application of propolis in food is still limited because it is soluble in alcohol and has a strong taste and aroma. Spray-drying might be an alternative method that could reduce these problems. Pharmaceutical and food industries are using this technique to protect products from environmental conditions, extend

product shelf life and mask unpleasant flavours (Re, 1998; Re, 2006; Favaro-Trindade et al., 2010).

Silva et al. (2011) spray-dried propolis without a carrier agent, but unsatisfactory results were obtained because of the low yield of the spray-drying process, the physical instability of the powder and the reduced dispersibility of the powder in water.

Bruschi et al. (2003) produced propolis microparticles through spray-drying using gelatine as a carrier agent, and they achieved products with entrapment efficiency of 41%. The spray-drying technique maintained the antimicrobial activity of propolis, therefore suggesting that it can be a promising process for developing an intermediary or eventual propolis dosage form without ethanol or a strong, unpleasant taste. However, the use of gelatine originating from bovine has been reduced in recent years in some countries due the risk of bovine spongiform encephalopathy. Therefore, alternative carriers are needed.

According to Silva et al. (2013) gum Arabic and octenyl succinic anhydride (OSA) starch could act as alternative carrier agents to obtain spray-dried propolis products because they have emulsifying properties that could improve the dispersibility of propolis in water. The researchers obtained propolis in a powder, alcohol-free, water-dispersed and shelf-stable form by spray-drying with gum Arabic and octenyl succinic anhydride (OSA) starch as carriers in two different weight ratios (1:4 and 1:6). Spray-dried propolis samples were evaluated for morphology, moisture, water activity, water dispersibility, hygroscopicity, particle size, particle distribution, entrapping efficiency, stability, isotherms and antioxidant properties. This process preserved the antioxidant activity of propolis and also allowed propolis to be obtained in a powder form, which was stable during storage at room temperature, had low hygroscopicity and was highly dispersible in cold water. They suggested that this technology could increase the use of propolis in various industrial applications, such as an antimicrobial and as an antioxidant in food (Silva et al., 2013).

Propolis has potential to be used as a natural food additive by food industry. Due to the disadvantages of solubility in alcohol and sharp flavor, microencapsulation may be an another alternative for reducing these problems. Nori et al. (2011) studied to encapsulate propolis extract by complex coacervation using isolated soy protein and

pectin as encapsulant agents. The coacervation was studied as a function of pH (5.0, 4.5, 4.0 and 3.5) and the concentration of encapsulants and core (2.5 and 5.0 g/100 mL). Samples obtained at pH 4.0 in both concentrations were lyophilized and analyzed for hygroscopicity, encapsulation efficiency, particle size, morphology, thermal behavior, stability of phenolic and flavonoids during storage, as well as antioxidant and antimicrobial activities. They suggested that it is possible to encapsulate propolis extract by complex coacervation and to obtain it in the form of powder, alcohol-free, stable, with antioxidant property, antimicrobial activity against *Staphylococcus aureus* and with the possibility of controlled release in foods (Nori et al., 2011).

Aly and Elewa (2007) used propolis during ripening of Ras cheese to test its antifungal effect against *Aspergillus versicolor* ATCC 12996 as well as biosynthesis of sterigmatocystin. They used different concentrations of propolis: 250, 500 and 1000 part per million (ppm) on the cheese surface. It was reported that mould growth and toxin production were completely inhibited at the highest concentration 1000 ppm, while the lower concentrations exhibited definite fungistatic activity during 90 days of ripening. Control sample cheese demonstrated that the amount of sterigmatocystin produced was proportional to the growth of *Asp. versicolor* during three months of ripening. It could be concluded that propolis concentration of 1000 ppm could prevent mould growth and sterigmatocystin production in Ras cheese. It was found important to use propolis in cheese manufacture according to economic and health concerns (Aly and Elewa, 2007).

Gregory et al. (2008) examined the efficacy of vapors from polar and non-polar sub-fractions of propolis on microbial and oxidation control during rice (*Oryza sativa*, hinohikari var.) storage. The sub-fractions (absolute ethanol, methylene chloride, hexane extracts: AEPEV, MCPEV and HEPEV, respectively) were infused in synthetic adsorbents and their volatiles released during 6 months of storage. The propolis sub-fractions controlled microbial load in rice storage. Bacterial colonization remaining on milled rice following treatment with the propolis sub-fractions was limited to Gram-positive strains. The volatiles from sub-fraction prepared by absolute ethanol extraction (AEPEV) were the most effective in fat acidity control. This sub-fraction limited oxidation of milled rice lipids at 30°C to the same level as that of the control grain stored at 5°C within 2 months.Compositional

analysis revealed that of the propolis sub-fractions tested AEPE had the highest amount of caffeic acid and caffeic acid phenethyl ester (CAPE). These compounds being antioxidants therefore explained the strongest antioxidant efficacy in AEPE (Gregory et al., 2008).

Özdemir et al. (2010) determined the effect of propolis on the storage life of Star Ruby grapefruit. Fruits were dipped in ethanol-extracted propolis (EEP) in various concentrations (1%, 5%, and 10%) immediately after harvest and then stored at 8 °C and 90% relative humidity for 6 months. The effects of propolis on the incidence of physiological disorders and fungal decay, and some fruit quality characteristics (weight loss, fruit juice content, total soluble solids, titratable acidity, juice pH, percent of fruits with green calyx, and skin color) were assessed at monthly intervals during the storage period. Treatment with 5% EEP was effective in preventing fungal decay. The percentage of weight loss was significantly higher in the control fruits (6.36%-7.83%) than in those treated with 5% EEP (5.71%) and 10% EEP (4.95%) at the end of the storage period. Star Ruby grapefruit treated with 5% EEP was successfully stored at 8 °C for 5 months (Özdemir et al., 2010).

Mascheroni et al. (2010) designed antimicrobial/antioxidant release system for food packaging applications by incorporation of propolis into polylactic acid (PLA) film. They suggested that the incorporation of propolis in the biopolymer PLA correctly modified could provide a possible delivery system for food packaging applications (Mascheroni et al., 2010).

Han and Park (2002) treated cured ground pork meat samples with (1) 0.4% w/w ethanol extracted propolis (EEP 0.4%), (2) 0.6% w/w water-extracted propolis (WEP 0.6%), (3) 0.8% w/w dried residue of ethanol-extracted propolis (DREEP 0.8%) and (4) 0.1% w/w potassium sorbate (PS 0.1%) and produced sausages. Thiobarbituric acid-reactive substances (TBARS) were measured in the sausages to assess the effects of storage at 5, 10, and 20°C after 0, 2, and 4 weeks. After 4 weeks of storage at 5°C the sausages treated with EEP 0.4%, DREEP 0.8%, WEP 0.6% and PS 0.1% yielded TBARS values which were 50, 59, 35, and 91% respectively of the control value. It was investigated that the curing process will confconclutrol microbiological spoilage, as will potassium sorbate (Han and Park, 2002).

2.8 Effects of Nitrate/Nitrate Addition to Cured Meat Products

Fermentation and drying of meat products have been used for centuries to prevent spoilage at room temperature. In order to produce rapid and shelf-stable dry fermented sucuks there are so many different meat formulations with spices and process parameters have been used. Regardless all differences in formulations, preservatives, like nitrite (KNO_2/E249 and $NaNO_2$/E250) and indirectly nitrate ($NaNO_3$/E251 and KNO_3/E252), are generally used to avoid the growth of putrefactive and pathogenic bacteria, like *C. botulinum*. Moreover, nitrate have been used for processing adjuvant as it promotes the color formation, delays lipid oxidation, and gives the product a typical cured meat flavor.

Despite the given advantages, nitrite can induce methaemoglobinemia. Because of this reason, addition of nitrite and nitrate in meat products is restricted to 150 mg/kg for each additive (Official Journal of the EU, 2006). Nitrite can also be converted to the nitrosating agent NO^+ which can react with biogenic amines to form carcinogenic N-nitrosamines (Honikel, 2008).

Biogenic amines are formed by decarboxylation of free amino acids. They are basic nitrogenous compounds that occur in dry fermented products in high concentrations since their accumulation is mainly related to the action of decarboxylase-positive bacteria and meat enzymes during the fermentation and ripening. Biogenic amine formation rate is effected by other intrinsic and extrinsic factors, such as salt content and drying parameters during the sucuk processing (Suzzi and Gardini, 2003). The wide range of product formulations and hygienic practices is resulted in a great variety of types and amounts of biogenic amines. In view of food safety, high concentrations of biogenic amines must be avoided. It is reported that, histamine (HIS) and tyramine (TYR) may induce migraines and hypertensive crises in sensitive individuals (Latorre-Moratella et al., 2008). Moreover, putrescine (PUT) and cadaverine (CAD) are known to intensify the adverse effects of HIS and TYR as they compete for some of the mechanisms involved in their detoxification (Bardocz, 1995). In addition to the direct toxicological effects, it was reported that the biogenic amines (PUT and CAD) and the natural polyamines spermine (SPM) and spermidine (SPD) can be precursors of the carcinogenic N-nitrosamines during the heating of meat products (Drabik-Markiewicz et al., 2011). These amines undergo deamination and cyclization to secondary amines before reacting with the nitrosating agent (NO^+).

In this way CAD can be converted to N-nitrosopiperidine (NPIP), while PUT, SPM and SPD can form N-nitrosopyrrolidine (NPYR) (Hernández-Jover et al., 1997).

The concern about the presence of N-nitrosamines in meat products were started in the late 1960s, early 1970s, when high concentrations of N-nitrosodimethylamine (NDMA) and NPYR were detected in fried bacon. The amount of added nitrite was limited and the use of nitrite-scavenging anti-oxidants like ascorbate was introduced in the meat production to avoid this contamination (Gray and Dugan, 1975). However, in the period 1980–1986, meat and meat products contributed for approximately 80% to the total volatile N-nitrosamine intake of Swedish food products (Österdahl, 1988). In the same period, a survey of several foods present on the Italian market proved that all of the four analyzed salami samples, were contaminated with various amounts of N-nitrosamines, e.g. NDMA (0.59–7.76 μg/kg), N-nitrosodimethylamine (NDEA, nd−4.04 μg/kg), N-nitrosodibutylamine (NDBA, 0.73–50.12 μg/kg) and NPIP (nd−0.38 μg/kg) (Gavinelli et al., 1988). More recently lower, N-nitrosamine levels in several meat products have been reported (Domanska and Kowalski, 2003; Yurchenko and Mölder, 2007; De Mey et al., 2014). However, there is a growing and current interest in the meat sector replacing those dangerous additives due to the increase in consumer demands. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are usually preferred by food industries. However, in long-term consumption of these synthetic antioxidants, carcinogenic effects may occur (Botterweck et al., 2000). Therefore, the search for natural antioxidants to reduce the nitrite levels has increased dramatically. In recent literature there are several studies about using natural antioxidants (seeweed, acid whey and mustard seed, grape pomace, parsley extract powder) to reduce nitrite levels in sucuks (Wojciak et al., 2014; Riazi et al., 2016; Sellimi et al., 2017; Riel et al., 2017).

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

For the determination of total phenolic, total flavonoid and total antioxidant capacity analysis, gallic acid (≥98%), quercetin (≥95%), ethanol (≥99.8%), Folin-Ciocalteu phenol reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), neocupraine (Nc), glacial acetic acid (CH_3CO_2H), Iron (III) chloride hexahydrate ($FeCl_3$,$6H_2O$), 2,4,6-tripyridyl-s-triazine (TPTZ), hydrochloric acid (HCl) from Sigma-Aldrich Chemie GmbH (Steinheim, Germany); methanol (≥99.9%), formic acid (≥98%), sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), sodium acetate trihydrate ($CH_3COONa.3H_2O$), sodium hydroxide (NaOH), potassium persulfate ($K_2S_2O_8$), dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), copper (II) chloride ($CuCl_2$) and ammonium acetate (NH_4Ac) from Merck KGaA (Darmstadt, Germany); 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and aluminum chloride ($AlCl_3$) from Fluka Chemie (Buchs, Switzerland); potassium chloride (KCl) from Riedel-de Haen Laborchemikalien GmbH (Hanover, Germany) and 2,2'-azinobis(3-ethylbenzo-thiazoline-6-sulphonic acid) diammonium salt (ABTS) from Applichem GmbH (Darmstadt, Germany) were purchased.

The following standards and reagents were used for the quantification of phenolic compounds: caffeic acid, galangin, pinobanksin, pinocembrin, pinostrobin, chyrisin, trifluoroacetic acid and acetonitrile from Sigma-Aldrich (Steinheim, Germany); *p*-coumaric acid, ferulic acid and *t*-cinnamic acid from Fluka (Buchs, Switzerland). For all analysis, water was distilled and purified with the water purification system (TKA GenPure; TKA, Niederelbert, Germany).

All cell lines were obtained from ATCC® (Wesel, Germany). For culturing the cells, the media Roswell Park Memorial Institute Dulbecco′s modified Eagle′s medium (DMEM) and DMEM/F12 were purchased from Wisent Bioproducts (Quebec, Canada). The media supplementary materials fetal bovine serum (FBS), penicillin–

streptomycin (100 U/ml), L-glutamine (1 mM) and 25 μM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were also supplemented from Wisent Bioproducts (Quebec, Canada). Cell viability test was performed via XTT assay which was purchased from Xenometrix (Allschwil, Switzerland).

For microbiological analysis, Violet Red Blue Agar (VRBA), Plate Count Agar (PCA), Dichloran Rose Bengal Agar (DRBC) were purchased from Merck (Darmstadt, Germany), deMan-Ragosa-Sharpe Medium (MRS), TBX Agar were purchased from Oxoid (Basingstoke, United Kingdom). Half-fraser medium, half-fraser broth, VIDAS ® *L. monocytogenes* II (Ref. 30 704) were purchased from bioMérieux (Marcy l'Etoile, France) for *L. monocytogenes* analysis and *Salmonella* Xpress broth 2 (SX2), VİDAS were purchased from bioMérieux (Marcy l'Etoile, France) and selective agar (XLD and SS) (Biokar Diagnostics, Beauvais, France) for Salmonella spp. analyses.

3.1.2 Propolis Samples

Propolis samples were provided by SBS Scientific Bio Solutions Inc., İstanbul. Heat-treated dry fermented sucuk formulations were produced at the pilot plan of Namet Gıda San. ve Tic. A.Ş., İstanbul. All beef meat and fat used in heat-treated dry fermented sucuk formulations were provided by Namet Food Inc., İstanbul.

Propolis samples produced by honey-bees (*Apis mellifera* L.) were provided by SBS Scientific Bio Solutions Inc. (Istanbul, Turkey) including samples from 54 different regions of Turkey, directly from the beekeepers. They are used separately for the spectrophotometric analysis and phenolic profile identification using HPLC/PDA, but used as a mix in cell viability analysis and in the formulations of heat-treated dry fermented sucuk. They are mixed as three different batches as a representative sample of Turkish propolis for cell viability analysis.

To study the heat treated dry fermented sucuk formulations a single batch of propolis was obtained by mixing selected samples with high amounts of phenolics and flavonoids. All propolis samples were ground to fine powder using a precooled grinder (IKA A11 Basic, IKA-Werke GmbH & Co. KG, Germany). Samples were protected from light and frozen at -20°C until the extraction.

The geographical areas and beekepers of Turkey where samples were collected are presented in Figure 3.1 and further detailed in Appendix A, Table A.1.

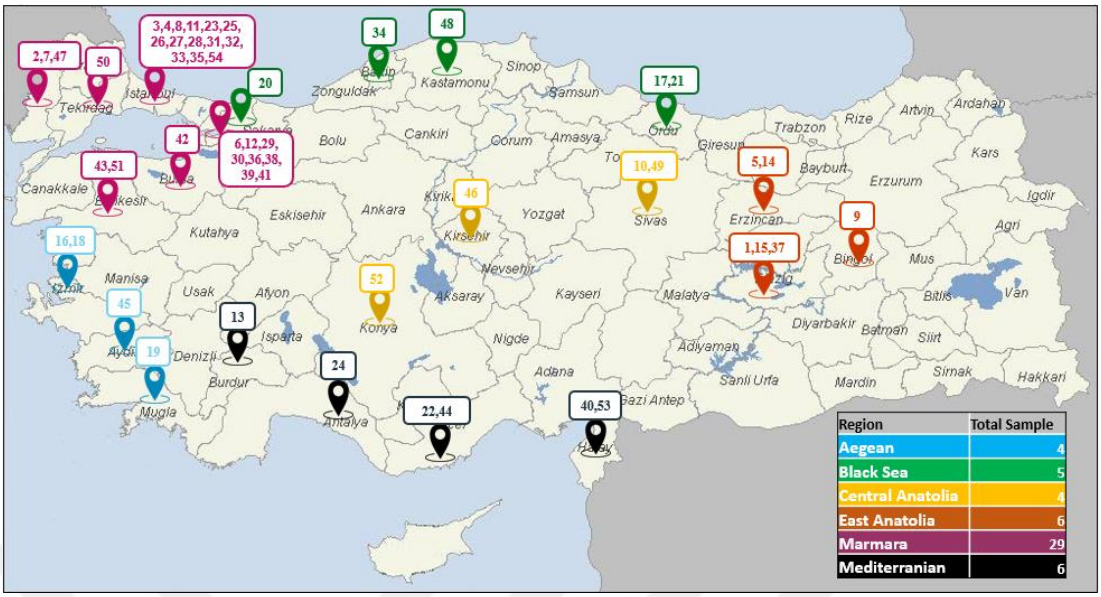


Figure 3.1: Propolis samples collected from various geographical areas and beekepers of Turkey.

3.2 Methods

3.2.1 Spectrophotometric Methods

3.2.1.1 Extract preparation

The propolis ethanolic extracts were prepared in a similar manner to that carried out by Trusheva et al. (2007) with slight modifications. Ultrasound-assisted extraction was performed in an ultrasonic cleaning bath (USC900TH type, 45 kHz, 200 W, VWR International, Germany) with a volume of 12.3 l (internal dimensions: 300 x 240 x 200 mm). Working frequency was fixed at 45 kHz. 1 ±0.001 g propolis samples were placed in 15 ml centrifuge tubes. 10 ml of 70% ethanol were added on each tube and sonicated at room temperature for 30 minutes. The temperature was controlled in water bath. The phenolic and flavonoid content and antioxidant capacities of the extracts were closely dependent of the solvent used for the extraction. Extraction method was choosen according to the study of Jug et al. (2014) who proved that ultrasound assisted extraction is a very efficient extraction method resulting in comparable extraction yield and total phenolic and flavonoid contents and total antioxidant capacities, with a significant advantage in term of shorter extraction time (30 min) and lower temperatures of the process.

After the extraction, the flask was removed from the bath and centrifuged at 5000 g for 15 mins at 4 ˚C and supernatants were collected. These extracts were placed at -20˚C freezer overnight for the precipitation of wax and then filtered to the method described by Castro et al. (2014). This procedure was repeated until there was no precipitation of wax. Proper dilutions were prepared before spectrophotometric analysis.

3.2.1.2 Analysis of total phenolics

The total phenolic content measured colorimetrically as described by Velioglu et al. (2006). Ten-fold diluted Folin-Ciocalteu's Phenol Reagent with milli-Q water, 7.5% Na_2CO_3 dissolved in milli-Q water and catechin stock solutions were prepared before analysis. Folin-Ciocalteu's phenol reagent should be kept in dark. A 100 µl diluted phenolic extract, a 1.5 ml of ten-fold diluted Folin-Ciocalteu's Phenol Reagent, 1.2 ml of 7.5% sodium carbonate solution were mixed in test tubes and all the tubes were vortexed well. Gallic acid was used as standard. Absorbances were measured at 765 nm after waiting for 90 minutes at room temperature in dark using Optima SP-3000 nano spectrophotometer (Tokyo, Japan). All analyses were repeated for each sample extract three times. The content of phenolics was expressed as mg gallic acid equivalent (GAE)/g propolis. The calibration curve is shown in Appendix B, Figure B.1.

3.2.1.3 Analysis of total flavonoids

The total flavonoid content was measured colorimetrically as described by Kim et al. (2003) with slight modifications. Prior to analysis 5% sodium nitrite ($NaNO_2$), 10% aluminium chloride hexahydrate ($AlCl_3.6H_2O$), 1 M sodium hydroxide (NaOH) and quercetin stock solution (1 mg/ml) were prepared. A 0.25 ml of diluted sample was mixed with 1.25 ml of distilled water 75 µl of 5% $NaNO_2$. 6 minutes later, 150 µl of 10% $AlCl_3.6H_2O$ was added. 5 mins later, 0.5 ml of 1 M of NaOH were added. The total volume was adjusted to 2.5 ml with distilled water and vortexed. Quercetin was used as standard, diluted from the same quercetin stock solution. Absorbances were read at 510 nm using Optima SP-3000 nano spectrophotometer. All analyses were repeated for three times. The content of flavonoids was expressed as mg quercetin equivalent (QE) / g propolis. The calibration curve is shown in Appendix B, Figure B.2.

3.2.1.4 Analysis of total antioxidant capacity

There are many methods used in the literature to determine the antioxidant capacities of honey products. Single antioxidant capacity measurement method cannot examine all the antioxidant compounds as all methods have their own advantages or disadvantages (Capanoglu et al., 2008). Therefore, the total antioxidant capacity was estimated by four different assays; The DPPH, ABTS, CUPRAC and FRAP. In each antioxidant capacity assay, all the analyses were repeated for three times using Optima SP-3000 nano spectrophotometer and the results were expressed as mg trolox equivalent (TE)/g propolis.

ABTS assay

The ABTS (2,2- azinobis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) assay was performed according to Miller and Rice-Evans (1997) with some slight modifications. ABTS and potassium persulfate solutions were mixed and kept at room temperature in the dark for overnight. ABTS stock solution was diluted in 50 mM potassium phosphate buffer (pH 8.0) to an absorbance of 0.90 (±0.05) at 734 nm to prepare the ABTS-working solution. Then, 100 µL of sample extract was mixed with 1 mL of ABTS-working solution and the absorbance was measured at 734 nm exactly 1 min after initial mixing at 734 nm. The calibration curve is shown in Appendix B, Figure B.3.

DPPH assay

The DPPH assay was carried out as described by Kumaran and Karunakaran (2006) and Rai et al. (2006) with slight modifications (Kumaran and Karunakaran, 2006; Rai et al., 2006). Prior to analysis 0.1 mM of DPPH solution was prepared in methanol and kept in dark. 100 µl of diluted sample extracts were mixed with 2 ml of 1 mM DPPH in methanol. They were vortexed for 10 seconds and stored in the dark at room temperature. The absorbances were measured at 517 nm against methanol after 30 minutes. The calibration curve is shown in Appendix B, Figure B.4.

CUPRAC assay

The CUPRAC assay was carried out as described by Apak et al. (2004) with few optimised modifications. Prior to analysis copper (II) chloride solution at a concentration of 10^{-2} M, ammonium acetate (NH_4Ac) buffer at pH 7.0 and neocuproine (Nc) solution in ethanol at a concentration of $7.5x10^{-3}$ M were prepared. Neocuproine solution in ethanol at a concentration of $7.5x10^{-3}$ M was prepared daily.

1 ml of copper (II) chloride solution, 1 ml of neocuproine solution, 1 ml of ammonium acetate buffer, 100 µl of sample extract or standard extracts for calibration curve and 1 ml of Milli-Q water were mixed, respectively. The absorbances were measured at 450 nm after 30 minutes incubation time. The calibration curve is shown in Appendix B, Figure B.5.

FRAP assay

Antioxidant capacity was measured colorimetrically as described by Benzie and Strain (1996), Deighton et al. (2000), and Tavarini et al. (2007) with few modifications. Prior to analysis 300 mM sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$) solution at pH 3.6, 20 mM ferric chloride hexahydrate ($FeCl_3.6H_2O$) solution, 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution were prepared. Ferric chloride hexahydrate should be prepared fresh immediately prior to analysis. TPTZ solution and sodium acetate solution should be stored in dark glass bottles at room temperature. FRAP reagent was prepared by combining 10x volume of sodium acetate solution with 1x volume of TPTZ solution and 1x volume of sodium chloride solution. FRAP reagent must be prepared fresh immediately prior to procedure and is stable for at least 2 hours at room temperature. After preparing the FRAP reagent, 900 µl of FRAP reagent and 100 µl of diluted sample extract / standard Trolox were mixed in test tubes and vortexed quickly for 20 seconds. The absorbances were measured at 593 nm after 4 minutes following the addition of FRAP reagent. The calibration curve is shown in Appendix B, Figure B.6.

3.2.2 Analysis of phenolic profiles of propolis using HPLC/PDA

Extracts of propolis and standards dissolved in methanol filtered through a 0.45-µm membrane filter and injected into Waters 2695 HPLC system with PDA (Waters 2996) detector. Stationary phase was Supercosil® (Sigma-Aldrich, St. Louis, MO, USA) LC-18 column (25 × 4.6 mm, 5 µm), and the mobile phase consisted of solvent A (Milli-Q water with 0.1 % (v/v) trifluoroacetic acid (TFA) and solvent B (acetonitrile with 0.1 % (v/v) TFA). The flow rate was 1.2 ml/min. Separation of phenolics in the extracts was conducted in a 60 min run. A linear gradient was used as 75% solvent A and 25% solvent B at 0 min, 70% solvent A and 30% solvent B at 10 min, 60% solvent A and 40% solvent B at 40 min, 40% solvent A and 60% solvent B at 50 min, and at 55 min returned to the initial conditions (Ahn et al., 2007; Bino et al., 2005). The spectrum acquisition range was between 210 and 530 nm.

Flavonols were detected at 360 nm and phenolic acids at 312 nm. Identification was achieved based on the retention times and characteristic UV spectra, and quantification was done by external standard curves. Calibration curves for standards including chyrsin, galangin, pinostrobin, pinobanksin, pinocembrin, caffeic acid, *p*-coumaric acid, ferulic acid and *t*-cinnamic acid were shown in Appendix B, Figure B.7 - B.15, respectively.

3.2.3 Analysis of effects on cancer cells

3.2.3.1 Cell culture

MDA-MB-231 breast cancer cells and primary dermal fibroblasts (PCS-201-012) were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin–streptomycin, 1 mM L-glutamine and 25 μM HEPES at 37 °C in a humidified 5% CO_2 incubator. UACC-3199 human infiltrating ductal carcinoma of breast, strain C57B/6 mouse mesenchymal stem cell line (bone marrow derived) and fibroblasts were cultured in DMEM/F12 media with 10% FBS, 100 U/ml penicillin–streptomycin, 1 mM L-glutamine and 25 μM HEPES at 37 °C in a humidified 5% CO_2 incubator (Thermo Scientific, USA) (Thirugnanasampandan et al., 2012).

3.2.3.2 Proliferation assays (*XTT*)

Cells were counted using Trypan Blue stain via neubauer counting chamber and light microscopy. Cells were seeded in 96-well plates (NEST®, USA) and there were 10^4 cells in each well. After cells are confluent, extracted propolis samples were added to wells. Tested concentrations for each cell line and each measuring time gap were; 0 μg/μl, 50 μg/μl, 100 μg/μl, 150 μg/μl, 200 μg/μl. All experiments were done with biological replicates for each measuring parameter.

After adding propolis extract, cell viability was tested at 24 h, 48 h and 72 h (Thirugnanasampandan et al., 2012). Cell viability was tested using Tetrazolium XTT assay according to supplier's instructions. Spectrophotometric measurement was performed with Varioskan™ Flash Multimode Reader (Thermo Ficher Scientific, Waltham, MA USA).

3.2.4 Analysis of propolis added heat treated dry fermented sucuks

3.2.4.1 Preparation of alcohol free propolis concentrate

Propolis were collected from 54 different beekepers of various geographical regions of Turkey in years 2013 and 2014. They were all blended and ground to powder.

Propolis concentrate was prepared using 30% propolis tincture and removing ethanol with a rotary evaporator.

1500 gr of propolis blend was ground to powder. Then, 892 ml (700 g) of 70% ethanol was added on 300 g of propolis. Totally 4460 ml of 70% EtOH was added on 1500 g of propolis. Propolis was shaken through 2 days using shakers and it is filtered after 2 days. The filtrate was evaporated using rotary evaporator. The remaining propolis was again added 70% EtOH and shaken through 24 hours and filtered and evaporated using rotary evaporator. This procedure again applied once again for 24 hours. Ethanol was evaporated. All impurities and waxes were removed from EtOH and 958 g of propolis concentrate extract was prepared at the end of this procedure.

3.2.4.2 Production of heat treated dry fermented sucuk

Dry fermented sucuks were prepared using nine different formulations. Each formulations were prepared as 20 kg in Namet Food Inc. R&D pilot plant. These formulations are listed according to their codes mentioned in Table 3.1.

Table 3.1 : Heat treated dry fermented sucuk formulations.

Heat-treated dry-fermented sucuk codes	Propolis	Sodium Nitrite
R	-	150 ppm
A	0.25%	150 ppm
B	0.50%	150 ppm
C	0.25%	50 ppm
D	0.50%	50 ppm
E	0.25%	75 ppm
F	0.50%	75 ppm
G	0.25%	100 ppm
H	0.50%	100 ppm

The flow chart of heat-treated fermented sucuk production is presented in Figure 3.2 At the beginning, 50% of frozen and 50% of fresh beef meat were weighed. The maximum fat content of this meat aimed to be less than 35%. Meat size was decreased to 13 mm using mincers. Then it is mixed with spices and other

ingredients (nitrite and propolis concentrates) according to the formulations given in Table 3.1. Each formulation was minced to 2.5 mm particle size and filled into collagen casings that are filled with 285-290 g of sucuks. They were fermented for 18-24 hours at 24˚C, until pH decreases below 5.2. Following fermentation, sucuks were heat-treated until their core temperature reaches to 68˚C. The samples were then vacuum packed and stored at 0-4 ˚C for 200 days for physicochemical, microbiological and sensory analysis. The commercially shelf life of heat treated sucuks is totally 180 days.

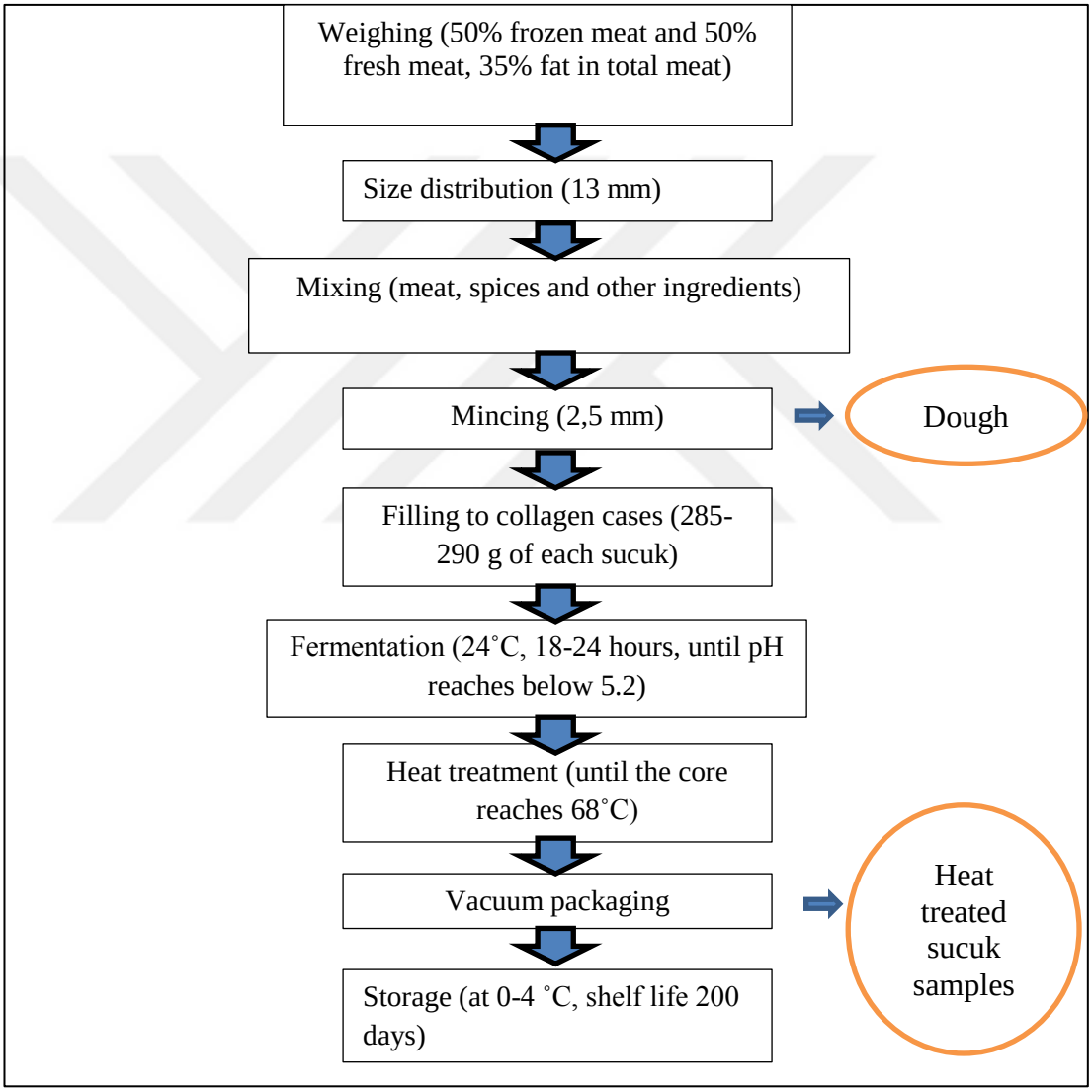


Figure 3.2 : Production flow chart of heat-treated dry fermented sucuk.

3.2.4.3 Chemical Analysis

The amounts of moisture, fat, salt, collagen, and free fatty acids and pH measurements were performed in samples every 15 days for the first 2 months and every 30 days for the following 2 months. The samples were tested during the last

50 days of storage period at decreasing intervals of 20, 10, 10 & 10 days. In addition ratios of moisture/protein and fat/protein were also calculated. All analysis were performed in triplicate and results were given as mean ± standart deviation.

pH value

Heat-treated dry fermented sucuks of 10 g were homogenized in Waring blender in 100 ml distilled water for determination of pH using a pH meter (Mettler Toledo, MP220).

Moisture content

Moisture content of traditional sucuk types was analyzed according to the method described by AOAC (1990). 5 g of sucuk sample was placed into hot air oven at 125 ± 2°C for 3 hours. Moisture content was also analysed using FoodScan Meat Analyser (FOSS, Denmark).

Fat content

Fat analysis were performed using AOAC FOSS SOXTEC 2055 app. Notes (AOAC, 1997) and FoodScan Meat Analyser (FOSS, Denmark).

Salt content

Salt analysis was performed according to TS 1747-1 standard (TS 1747-1 ISO 1841-1., 1999) and FoodScan Meat Analyser (FOSS, Denmark).

Protein content

Protein Content was analysed according to AOAC FOSS KJELTEC 8100 app. notes (AOAC, 1997) and FoodScan Meat Analyser (FOSS, Denmark).

Moisture/Protein ratio

Moisture/protein content ratio was calculated after moisture and protein values were determined.

Fat / Protein ratio

Fat/protein content ratio was calculated after fat and protein values were determined.

Collagen content

Collagen content was analysed on FoodScan Meat Analyser (FOSS, Denmark).

Lipid oxidation

Formation of malonaldehyde was analysed by Thiobarbauturic Acid Reactive Substances (TBARS) assay based on measuring absorbance at 538 nm in a spectrophotometer as described by Bozkurt and Bayram (2006).

Color analysis

Following cutting sucuk samples the color of their surface was measured using an automatic colorimeter (Konica Minolta, model CR-400, Mississauga, ON, Canada) at 0^{th}, 90^{th} and 200^{th} days of storage. The color values were recorded as L*, a*, and b* values.

3.2.4.4 Microbiological Analysis

The prepared sucuk samples were analysed for total aerobic bacteria, *E. coli*, total coliform bacteria, total mold and yeast and *Lactobacillus* spp. at every 15 days for the first 2 months and every 30 days for the following 2 months. The samples were tested during the last 50 days of storage period at decreasing intervals of 20, 10, 10 & 10 days. Pathogenic bacteria, *L. monocytogenes* and *Salmonella* spp. were analysed only at day zero and the end of storage priod. All tests were performed in triplicate and results were given as mean ± standart deviation of logarithm colony forming unit/gram (log CFU/g).

Total aerobic bacteria

Total bacteria were counted using the spread plate method on aerobic plate count agar (Merck, Darmstadt, Germany). The Petri dishes were incubated at 30 ˚C up to 72 h (Ertas, 2010). Bacterial counts were expressed as colony-forming units per gram of sample (cfu/g) and were transformed into logarithms (TS ISO 4832, 2010).

Eschericia coli

Total *E. coli* were analysed according to ISO 16649-2; in short 1 ml of sample dilutions were placed into petri dishes and mixed with 15 ml of molten TBX-agar (Oxoid), solidified plates were incubated for 24 h at 44 C ± 1.0 ^{o}C aerobically. The remaining initial peptone water sample dilution was enriched by incubation for 24 h at 37 ^{o}C ± 1.0 ^{o}C, aerobically (TS ISO 16649-2, 2012). Bacterial counts were expressed as colony-forming units per gram of sample (cfu/g) and were transformed into logarithms.

Total coliform bacteria

Coliform bacteria count was carried out using the pour plate method using violet red bile agar (VRBA) (Merck, Darmstadt, Germany) and incubated at 30 ˚C for 24 h. The purple-red colonies that are 0.5 mm or larger in diameter and surrounded by zone of precipitated bile acids are counted. To confirm that the colonies are coliforms, representative colonies were transferred to tubes containing 10 ml of Brillant Green Bile Broth with Durham tubes. The tubes were examined for 24 to 48 hours for gas production (TS ISO 4832, 2010). Bacterial counts were expressed as colony-forming units per gram of sample (cfu/g) and were transformed into logarithms.

Total mould and yeast

Total mould and yeast values were counted using the spread plate method on Violet Red Bile Agar (Merck, Darmstadt, Germany) and incubated at 25 ˚C for 2–5 days (Erkmen, 2000). Bacterial counts were expressed as colony-forming units per gram of sample (cfu/g) and were transformed into logarithms (TS 6580, 1989). Total mould and yeast counts were expressed as colony-forming units per gram of sample (cfu/g) and were transformed into logarithms.

***Lactobacillus spp*. analysis**

Total lactic acid bacteria values were observed using the spread plate method on de Man, Rogosa & Sharpe (MRS) Agar (Merck, Darmstadt, Germany). Petri dishes were incubated at 30 ˚C for 48–72 h (Erkmen, 2000). Bacterial counts were expressed as colony-forming units per gram of sample (cfu/g) and were transformed into logarithms.

***Salmonella* spp. Analysis**

The detection of Salmonella spp. by alternative VIDAS Easy Salmonella (bioMérieux, SA Marcy l'Étoile, France) technique, was performed in four steps: primary enrichment, selective enrichment, VIDAS and confirmation (Cheung and Kam, 2012). Twenty five gram of heat-treated dry fermented sucuk samples was homogenized in 225 ml of Buffered Peptone Water (Biokar Diagnostics, Beauvais, France). The mixture was incubated at 37 °C for 24 h. A volume of 100 µl of media was transferred to 10 ml Salmonella Xpress broth 2 (SX2) (bioMérieux SA, Marcy l'Étoile, France) and incubated at 41.5 °C for 24 h. One milliliter of the culture was

heated for 15 min at 100 °C then cooled at room temperature. A volume of 0.5 ml SX2 broth was transferred into a SLM array and deposited in the VIDAS (bioMérieux SA, Marcy l'Étoile, France). Confirmation of positive samples was performed by isolation of *Salmonella* from unheated SX2 broth on selective agar (XLD and SS) (Biokar Diagnostics, Beauvais, France) and identification by urease test followed by API 20E system test (bioMérieux SA, Marcy l'Étoile, France) according to ISO 6579, 2002 reference technique (TS EN ISO 6579, 2005).

***Listeria monocytogenes* analysis**

Samples analyzed for the presence of *L. monocytogenes* were homogenized in half-Fraser broth (bioMérieux, Marcy l'Etoile, France) and incubated at 30ºC for 24 h (ISO 11290-1, 2005). The second enrichment step was carried out by inoculating a volume of 0.1 ml from the first enrichment up to 10 ml of Fraser broth (bioMérieux) in tubes and incubated at 37 C for 24 h. The enriched broths were distributed in 10 ml aliquots that were immediately analyzed by mini-VIDAS (ISO 11290-2, 2005).

3.2.4.5 Sensory Analysis

Sensory analysis of the nine different formulations of heat-treated dry fermented sucuk samples was carried out in a sensory analysis laboratory. Duo-trio test procedure is applied to determine overall differences exist between samples. Samples were presented to the panelists using a fixed reference technique, where only 150 ppm nitrite added sample was the reference. The procedure of this method involved presenting an R labeled reference sample first which was followed by giving two coded samples, one of which matches the reference sample. The subjects then were asked which coded sample was different from the reference (Meilgaard et al., 2007). The number of different replies were counted and referred to Appendix C, Table C.1 for interpretation (TS ISO-10399, 2002).

Sensory panels were composed of 24 trained panelists, who have joined to sensory panels several times, from Department of Food Engineering, Istanbul Technical University. All of the heat-treated dry fermented sucuk samples were divided to about 20 mm diameter and 20 ± 0.5 mm thickness pieces and grilled with a grill (Tefal Family Flavour Grill Black Edition TG8000, France) for 1 min on each sides of sucuk samples.

Nine different types of heat-treated dry fermented sucuk samples were prepared according to duo-trio test procedure, immediately served to panelists on a plastic white color plate and the panelists were instructed to taste samples starting from left to right. Samples were labeled with three-digit random numbers and the reference sample was coded as 'R'. Samples were evaluated in 2 subsequent sessions in 2 days giving a total of 24 results as follows; each session was carried out with 12 subjects as "R" is the reference with sample combinations of RRA or RAR. In order to avoid carryover effects panelists were instructed to have a piece of bread, drink warm water and wait for 1 min between tasting each sample.

The heat- treated dry fermented sucuk samples in sensory panel were: (R) reference sample with 150 ppm nitrite; (A) 150 ppm nitrite and 0.25% propolis extract; (B) 150 ppm nitrite and 0.50% propolis extract (C) 50 ppm nitrite and 0.25% propolis extract; (D) 50 ppm nitrite and 0.50% propolis extract; (E) 75 ppm nitrite and 0.25% propolis extract; (F) 75 ppm nitrite and 0.50% propolis extract; (G) 100 ppm nitrite and 0.25% propolis extract; (H) 100 ppm nitrite and 0.50% propolis extract. Panel settings and sample codes were detailed in Table 3.2.

Table 3.2 : Sensory analysis panel settings and sample codes. [1, 2]

Test #	Sample Combination	Panel#	Sucuk Sample	Random Codes of Samples		Total Panelist Number
				Sucuk Sample	Reference	
1	R-A-R	1	A	874	912	12
2	R-R-A	2	A	171	334	12
3	R-B-R	1	B	966	504	12
4	R-B-A	2	B	232	470	12
5	R-C-R	1	C	332	554	12
6	R-C-A	2	C	699	814	12
7	R-D-R	1	D	358	203	12
8	R-D-A	2	D	963	504	12
9	R-E-R	1	E	110	380	12
10	R-E-A	2	E	677	273	12
11	R-F-R	1	F	607	753	12
12	R-F-A	2	F	386	158	12
13	R-G-R	1	G	388	938	12
14	R-G-A	2	G	172	895	12
15	R-H-R	1	H	892	660	12
16	R-H-A	2	H	344	672	12

[1] Each sample (A, or B, or C, or D, or E, or F, or G, or H) tested for a total of 24 panelists.
[2] Sample presentation involves the combinations of reference sample (R; 150 ppm nitrite added sample) ; Propolis added sample (A, or B, or C, or D, or E, or F, or G, or H) and ; Coded Reference sample which is shown as R in the sample combinations the table

3.2.5 Statistical Analysis

Data representing the contents of phenolics and flavonoids, the antioxidant capacities, and the individual antioxidant compounds of collected samples were subjected to a number of statistical analysis using SPSS software (version 24.0; SPSS Inc., Chicago, IL, USA). Firstly, Univariate Analysis of Variance of General Linear Model (GLM) procedure was applied to analyze the differences between those values obtained from each province by grouping them into their corresponding geographic regions. Accordingly, the regions and years of sample collection were accepted as two factors without an interaction in the model, and multiple comparisons of the mean values were achieved with Duncan's method.

Furthermore, multivariate analysis was applied to all collected propolis samples (54) to understand a more simplified view of the relationship among the variables of total phenolics, total flavonoids, antioxidant activity, and individual phenolic structures. Principal component analysis (PCA) and linear discriminant analysis (LDA) were performed on data using SPSS software program (SPSS, 2016). Pearson correlation factor analysis using principal components as the method of factor extraction (PCA) was applied to examine relationships between the variables, visualize the data structure and identify important variables. It was carried out on a 162×15 data matrix, where 162 is the number of propolis samples (54 samples with 3 measurements of each variable) and 15 is the number of variables tested. The missing values were deleted in pairwise, and Promax rotation (as the variables are correlated to each other) with Kaizer Normalization was applied to data. Quadratic Discriminant Analysis (QDA) was applied to explore the possibility of classification of propolis samples according to regions using the XLSTAT 20.0 statistical software (Trial Version of 19.5 of Microsoft Excel). Both original and cross validation methods were used to test the prediction of classification ability.

One way ANOVA analysis was also performed (SPSS 24.0; Chicago, IL, USA) on the changes of physicochemical and microbiological attributes as a function of either storage time during shelf life of heat-treated dry fermented sucuks or different formulations of heat-treated dry fermented sucuks to determine significant differences ($p < 0.05$) by using the Duncan's new multiple range test (Montgomery & Runger, 2014). Furthermore, a General Lineer Model (GLM) of UNIVARIATE analysis was applied to selected variables where nitrite and propolis are accepted as

independent two factors to evaluate their individual or interaction effects on microbial growth and chemical attributes.

In proliferation assays, data was expressed as mean ± SD (Standard Deviation). Statistical analysis involved the Bonferroni post tests and two-way ANOVA with GrapPad Prism 5. Differences were considered statistically significant at $p < 0.05$. In addition, Pearson's correlation analysis between antioxidant capacity, total phenolic content and total flavonoid content of ethanolic extract of propolis samples collected in year 2013 and 2014 were performed and correlation coefficients were calculated using SPSS software (version 24.0; SPSS Inc., Chicago, IL, USA). All statistical analyses tables were presented in Appendix E.

4. RESULTS AND DISCUSSION

The results of this research is presented in three parts. In the first part of this study, data obtained from 32 and 22 propolis samples collected from various regions of Turkey in 2013 and 2014, respectively, show total phenolic and total flavonoid contents and also for their total antioxidant capacities using four different analytical methods. Furthermore, their phenolic profiles were also determined using HPLC/PDA method in addition to analysis of selected physicochemical characteristics and pH values. Then, detected significant differences in individual compounds according to the regions was investigated to elucidate how powerful they were to discriminate regions through advanced statistical analysis.

The second part of the study presents the antiproliferative effects of Turkish propolis extracts presents tested on two different breast cancer cell lines; MDA-MB-231 (ER-) and UACC-3199 (c-erbB-2 positive). Moreover, proliferative effects of Turkish propolis were tested on normal fibroblasts and C57B/6 mouse bone marrow derived MSCs.

Third part of the study demonstrates the effect of propolis when it is included in the formulation of heat-treated dry fermented sucuk produced at a pilot plant. Many tests were carried out to observe the influence of propolis on physicochemical, microbiological, and sensory qualities of sucuk samples. The physicochemical tests included pH value, moisture, fat, salt and collagen contents, moisture/protein ratio, fat/protein ratio, color, and lipid oxidation. The microbiological analysis involved enamurations of total aerobic bacteria, *E. coli*, total coliform bacteria, total yeast, total molds, *Lactobacillus* spp., *Salmonella* spp. Moreover, a duo-trio test was performed to detect overall sensory differences of heat-treated dry fermented sucuk samples that contain propolis from the traditional ones which contains only nitrite as a preservative.

4.1 Antioxidant Properties of Turkish Propolis

Six different spectrophotometric analyses were performed on ethanolic extracts of 54 propolis samples collected from various regions of Turkey in 2013 and 2014.

4.1.1 Total phenolic and flavonoid contents

Total phenolic and flavonoid contents of ethanolic extracts of propolis samples collected in 2013 and 2014 from each regions (54 samples) were given in Appendix D, Table D.1 and Table D.2, respectively. Propolis samples collected in 2013 and 2014 were from 6 different geographical regions of Turkey including 4 samples from Aegean Region, 5 samples from Black Sea Region, 4 samples from Central Anatolia Region 6 samples from East Anatolia Region, 29 samples from Marmara Region and 6 samples from Meditteranean Region (Figure 3.1).

The total phenolic contents of EEP samples collected in 2013 were varied between 10±0.63 mg GAE/g EEP and 157.25±12.20 mg GAE/g EEP. The total flavonoid contents of EEP samples collected in 2013 were varied between 16.66±0.93 mg QE/g EEP and 261.61±13.60 mg QE / g EEP. The lowest total phenolic and total flavonoid content was observed in sample 19 from Muğla province of Turkey and the highest total phenolic and flavonoid content was observed in EEP sample 28 collected from Istanbul province of Turkey.

The total phenolic contents of EEP samples collected in 2014 were varied between 6.18±0.36 mg GAE/g EEP and 154.89±4.34 mg GAE/g EEP. The total flavonoid contents of EEP samples collected in 2014 were varied between 10.24±0.33 mg QE/g EEP and 257.55±8.41 mg QE/g EEP. The lowest total phenolic and total flavonoid contents were observed in sample 46 from Kırşehir province of Turkey and the highest total phenolic and flavonoid contents were observed in EEP sample 38 collected from Kocaeli province of Turkey. All the samples except sample 46 showed high total phenolic and flavonoid contents.

According to their Univariate Analysis of GLM procedure, there were statistically significant differences between total phenolic contents of propolis samples corresponding to different regions ($p < 0.05$), but the differences between total phenolic contents of propolis samples corresponding to 2013 and 2014 were insignificant ($p > 0.05$) as shown in Appendix E, Table E.1.

Table 4.1 : Total phenolic, total flavonoid and total antioxidant capacity values of propolis samples collected in 2013 & 2014. [1, 2, 3]

Year	Region No	N	Total Phenolic Content (mg GAE/g EEP)	Total Flavonoid Content (mg QE/g EEP)	Total Antioxidant Capacity (μmole TE/g EEP)			
					DPPH	ABTS	CUPRAC	FRAP
2013	1	1	10.00 ± .	16.66 ± .	83.75 ± .	91.11 ± .	256.59 ± .	33.90 ± .
	2	2	104.69 ± 62.71	174.13 ± 104.33	380.18 ± 119.67	408.44 ± 130.38	1150.60 ± 367.96	151.80 ± 48.32
	3	2	86.84 ± 1.56	144.31 ± 2.70	798.78 136.28	877.72 141.82	2426.30 397.94	320.64 ± 52.38
	4	3	37.62 ± 15.91	62.48 ± 26.47	273.97 ± 113.37	273.88 ± 111.06	834.04 ± 347.52	110.22 ± 45.91
	5	21	93.07 ± 38.12	154.75 ± 63.42	625.64 ± 372.21	655.65 ± 380.42	1921.83 ± 1095.76	253.34 ± 145.23
	6	3	41.90 ± 33.31	69.82 ± 55.10	365.35 ± 336.27	395.17 ± 368.25	1061.21 ± 1032.21	146.02 ± 133.97
	Total	32	80.81 ± 42.22	134.38 ± 70.19	546.82 ± 354.92	576.23 ± 368.34	1670.46 ± 1061.06	220.86 ± 139.94
2014	1	3	46.22 ± 23.83	76.90 ± 39.60	318.68 ± 165.43	343.49 ± 182.44	957.37 ± 493.78	126.56 ± 65.36
	2	3	77.01 ± 38.90	128.15 ± 64.78	280.81 ± 222.51	292.84 ± 224.54	849.79 ± 675.84	112.31 ± 89.31
	3	2	34.14 ± 39.53	56.77 ± 65.80	474.07 ± 66.22	587.87 ± 180.45	1659.18 ± 501.01	219.18 ± 66.16
	4	3	48.72 ± 30.46	81.04 ± 50.68	435.90 ± 324.67	487.98 ± 362.08	1174.94 ± 946.48	155.35 ± 125.27
	5	8	63.01 ± 47.33	104.77 ± 78.68	447.32 ± 355.06	481.10 ± 378.63	1350.88 ± 1069.99	178.47 ± 141.33
	6	3	30.26 ± 27.13	50.28 ± 45.13	256.17 ± 310.69	282.38 ± 344.81	777.64 ± 946.88	102.75 ± 125.12
	Total	22	53.59 ± 37.62	89.13 ± 62.56	381.88 ± 275.56	420.21 ± 302.88	1154.76 ± 842.09	152.59 ± 111.27
TOTAL	1	4	37.16 ± 26.58c	61.84 ± 44.19c	259.95 ± 179.01a	280.40 ± 195.22a	782.18 ± 534.15a	103.40 ± 70.67a
	2	5	88.08 ± 44.38a	146.54 ± 73.85a	320.56 ± 176.91a	339.08 ± 182.94a	970.11 ± 537.93a	128.11 ± 70.99a
	3	4	60.49 ± 38.05ab	100.54 ± 63.25ab	636.42 ± 206.88a	732.79 ± 213.45a	2042.74 ± 576.73a	269.91 ± 76.19a
	4	6	43.17 ± 22.57ab	71.76 ± 37.57ab	354.94 ± 234.89a	380.93 ± 266.70a	1004.49 ± 664.46a	132.78 ± 87.93a
	5	29	84.77 ± 42.25ab	140.96 ± 70.26ab	576.45 ± 370.21a	607.50 ± 381.47a	1764.33 ± 1100.59a	232.69 ± 145.66a
	6	6	36.08 ± 27.91c	60.05 ± 46.30c	310.76 ± 295.67a	338.78 ± 324.99a	919.43 ± 899.41a	124.39 ± 118.34a
	Total	54	69.72 ± 42.25	115.94 ± 70.26	479.62 ± 332.35	512.66 ± 348.84	1460.36 ± 1002.45	193.05 ± 132.31

[1] Data represent mean values ± standard deviation of three measurements obtained from each sample collected from provinces of which were grouped into relevant geographical regions.

[2] Provinces of each geographic regions follow as: (1) Aegean region - Aydin, Izmir, Mugla; (2) Black Sea region - Bartin, Duzce, Kastamonu, Ordu; (3) Central Anatolia region – Kirsehir, Konya, Sivas ;(4) East Anatolia region provinces – Bingol, Elazig, Erzincan; (5) Marmara region – Balikesir, Bursa, Edirne, İstanbul, Kocaeli, Tekirdağ; (6) Mediterranean region - Antalya, Burdur, Hatay, Mersin.

[3] Different letters in the columns represent statistically significant differences ($p < 0.05$).

Besides, the total phenolic content of Black Sea Region was found to be different compared to Aegean and Meditterranean Regions. However, there were no statistically significant differences between total phenolic contents of Aegean and Mediterranean Regions ($p > 0.05$). Additionally, the differences between the total phenolic contents of Black Sea, Central Anatolia, East Anatolia and Marmara were statistically insignificant ($p > 0.05$). Statistically significant differences between total phenolic contents of propolis samples between regions were given in Table 4.1 and statistical analysis results were shown in Appendix E, Table E.2.

According to their Univariate Analysis of GLM procedure, there were statistically significant differences between total flavonoid contents of propolis samples corresponding to different regions ($p < 0.05$), but the differences between total flavonoid contents of propolis samples corresponding to 2013 and 2014 were insignificant ($p > 0.05$) as shown in Appendix E, Table E.1. According to the Duncan's method of multiple comparisons between total flavonoid contents of propolis from different regions, it was found that there were statistically significant differences between total flavonoid contents of propolis from Marmara region and 3 regions including Aegean, Black Sea and Mediterranian Regions ($p < 0.05$). Besides, the total flavonoid content of Black Sea Region was found to be different compared to Aegean and Meditterranean Regions. However, there were no statistically significant differences between total flavonoid contents of Aegean and Mediterranean Regions ($p > 0.05$). Additionally, the differences between the total flavonoid contents of Black Sea, Central Anatolia, East Anatolia and Marmara were statistically insignificant ($p > 0.05$). Statistically significant differences between total flavonoid contents of propolis samples between regions were given in Table 4.1 and statistical analysis results were shown in Appendix E, Table E.1.

The results of our study were compared to literature starting from studies on total phenolic and flavonoid contents of Turkish propolis and continue with Asian, European, South American propolis studies shown in Table 2.1. When we compare our results with Aliyazicioglu et al. (2013) who studied 10 poplar-type Turkish propolis samples, only 9 of our samples were in the total phenolic content ranges of 115–210 mg gallic acid/g of methanolic propolis extract. Other samples in our study contain less total phenolic contents than their findings. Moreover, Erdoğan et al. (2011) also found higher total phenolic contents (in a range of 500-590 mg GAE/g

propolis) for four different propolis samples collected from Turkey. However, Gülçin et al. (2010) found lower total phenolic content in their lyophilized aqueous extract of propolis from Erzurum and all of our sample total phenolic contents were higher than their findings. Furthermore, Sarıkaya et al. (2009) found higher values for total phenolic content. Moreover, Yeşiltaş et al. (2015) reported total phenolic contents of Turkish propolis close to our findings, but they also observed higher values as 380±19 mg GAE/g total phenolic content was observed in Çatalca (Istanbul) sample. Their values for total flavonoid contents were also in accordance with the results of seven samples of our study. They have also found higher results for total flavonoid content.

Our findings are in accordance with the results of Ahn et al. (2007) who measured the total phenolic contents of 20 samples of poplar-type Chineese propolis. We have found higher results of total flavonoid contents in six samples in our study including sample #3, #6, #20, #23, #28 and #36 and these samples are all from İstanbul and Kocaeli provinces located in the Marmara Region of Turkey. The total phenolic and total flavonoid content results of our samples are also in accordance with the findings of Shi et al. (2012) who studied 15 samples of poplar-type Chineese propolis. Moreover, although the total phenolic contents of our samples are in accordance with Guo et al. (2011), our results for total flavonoid content is much higher than their results. Choi et al. (2006) also found close results to our findings in their poplar-type Korean propolis samples. We have observed higher total flavonoid contents in 36 of our samples than their maximum total flavonoid content including sample. Laskar et al. (2010) studied the total phenolic and flavonoid contents of aqueous and ethanolic extracts of Indian propolis. Their total flavonoid contents are also lower than our results which may be related with geographical variations and different extraction procedures.

When we compare total phenolic contents of our Turkish propolis samples, Potkonjak et al. (2012) who studied 5 different Serbian propolis, found lower results than our samples. Our highest result found in sample #28 (157.25±12.2 mg GAE/g EEP) exceeds by about three times their maximum total phenolic content including sample. Moreover, Lagouri et al. (2014) determined the total phenolic content and total flavonoid content in the polar (methanol, 80% methanol, and aqueous) extracts of propolis collected from the Greek mainland (West Macedonia) and the Greek

island, Rhodes. Propolis from West Macedonia had the highest concentration of total phenols and total flavonoids (105.36 and 49.27 mg/g). In particular, the 80% methanol extract from West Macedonia showed the highest amount (179.99 and 88.26 mg/g), while the aqueous extract from Rhodes showed the lowest (2.33 and 0.25 mg/g). Our total phenolic and total flavonoid results are higher than their results and it may be because of the difference in geographical locations and also variations in extraction solvent and method. Our total phenolic content results are also in the range of the findings of Kalogeropoulos et al. (2009) who studied 12 poplar-type propolis samples from Greece and Cyprus and also with Moreira et al. (2008) who studied 2 poplar-type propolis samples from Portugal. Dias et al. (2013)'s findings were also close to our results for total phenolic content which was reported to be in the range of 111±13 to 280±20 mg GAE/g dry propolis samples from Portugal. Moreover, Boisard et al. (2014) observed the total phenolic and flavonoid contents of 6 batch of poplar-type propolis samples collected in 2010 and 2011 in apiaries originating from different regions of France. They have found the total phenolic and flavonoid contents in a range of 238.6±13.3 mg GAE/g- 292.1±13. mg GAE/g and 20.5mg QE/g -80.0±1.2 mg QE/g. Those results are higher than our highest value (157.25±12.2 mg GAE/g EEP) for total phenolic content. However, we have found about 3 times higher result of total flavonoid content (261.61±13.6 mg QE / g EEP) than their highest value.

When we compare our results with the Argentinian poplar-type propolis and Brazilian red-type propolis samples, Turkish propolis samples have higher total phenolic and flavonoid contents (Da Silva et al., 2009; Lima et al., 2009). It is apparent that type of propolis and geographical differences affect the total flavonoid content in great amounts. However, in some studies total phenolic contents of Argentinian propolis samples have accordance with our findings, but lower values for total flavonoid contents as seen in Table 2.1 (Chaillou and Nazereno, 2009; Kumazawa et al., 2010). There is a great difference especially for total flavonoid contents as our highest result is about 15 times higher than the highest result obtained from the study of Chaillou and Nazereno (2009) for total flavonoid contents of propolis samples in Argentina. It is also similar with the results of Moreno et al. (2000) who observed the total flavonoid contents of 12 red propolis samples from

Argentina as their values varied from 13.3±0.90 mg QE/g propolis to 42.6±0.80 mg/g propolis, which are significantly lower than our results.

4.1.2 Total Antioxidant Capacities

Total antioxidant capacities of EEP samples collected in 2013 and 2014 and measured using 4 different spectrophotometric methods of DPPH, ABTS, CUPRAC and FRAP were given in Appendix D, Table D.1 and Table D.2, respectively.

The highest total antioxidant capacities of samples collected in 2013 was obtained in sample 23 from Istanbul province of Turkey, by all methods as 1392.27±131.33 µM/g EEP, 1510.64±55.4 µM TE/g EEP, 4220.24±161.75 µM TE/g EEP and 557.49±20.47 µM TE/g EEP measured by DPPH, ABTS, CUPRAC, and FRAP methods, respectively. The lowest total antioxidant capacities of samples collected in 2013 was obtained in sample 13 from Burdur province of Turkey, by all spectrophotometric methods as 73.35±15.23µM/g EEP, 76.63±2.82µM TE/g EEP, 223.5±8.64µM TE/g EEP and 29.54±1.03 µM TE/g EEP measured by DPPH, ABTS, CUPRAC, and FRAP methods, respectively.

The highest total antioxidant capacities of samples collected in 2014 was obtained in sample 38 from Kocaeli province of Turkey, by all methods as 1152.12±118.01µM/g EEP, 1222.7±38.69 µM TE/g EEP, 3475.78±113.96 µM TE/g EEP and 459.22±14.29µM TE/g EEP measured by DPPH, ABTS, CUPRAC, and FRAP methods, respectively. The lowest antioxidant capacity observed in sample #44 from Mersin province of Turkey. Sample #44 has a total antioxidant capacity of 26.15±0.92 µM/g EEP, 28.39±0.36 µM TE/g EEP, 79.29±1.11 µM TE/g EEP and 10.48±0.13 µM TE/g EEP measured by DPPH, ABTS, CUPRAC and FRAP methods, respectively.

According to their Univariate Analysis of GLM procedure, the differences between total antioxidant capacities of propolis samples according to year and region factors were statistically insignificant ($p > 0.05$). The statistically insignificant differences were shown as same letters in Table 4.1 and statistical analysis results were shown in Appendix E, Table E.1.

Aliyazicioglu et al. (2007) investigated *in vitro* antioxidant activity of ten different methanolic propolis extracts from various Anatolian areas from Turkey using FRAP method. Their FRAP values of propolis methanolic extracts were in the range of

182.12±2.25 to 325.47±3.12 μM Trolox / g propolis. Our results are in accordance with their findings, but we have observed some statistically significant higher results in samples #4, #23, #28, #29 and #38 which have 440.96±8.5, 557.49±20.47, 527.76±25.93, 400.64±9.85and 459.22±14.29 μM Trolox / g propolis, respectively. These samples are all collected from Marmara Region of Turkey, sample #4, #23 and #28 from Istanbul province and sample #29 and #38 from Kocaeli province. Moreover, Erdoğan et al. (2011) evaluated the antioxidant capacities of propolis samples from important honey producing locations of Anatolia including Bingol, Rize, Tekirdağ, and Van using ABTS and DPPH methods. They have found highest antioxidant capacity value in Rize sample 503.7±1.6 mg TE/g (2012.28±6.39 μmole TE/g) and 285.3±7.8 mg TE/g (1139.77±31.16 μmole TE/g) measured by DPPH and ABTS methods, respectively, and our highest results were close to their results. It may be suggested that the samples from Marmara region of Turkey may have higher total antioxidant capacities compared to other samples from Anatolian region. The composition of propolis depends on the source of the various trees used by honeybees. The antioxidant activity of propolis is due to its enrichment of polyphenols such as phenolic acids and flavonoids. Hence, total antioxidant capacity may vary greatly according to the phenolic compounds from different areas (Zabaiou et al., 2017). Furthermore, Sarıkaya et al. (2009) observed 327.76±3.16 μM TEAC/g from the Turkish propolis sample collected from inside the hive which is close to our findings for sample #3 (345.33±4.36 μM TEAC/g) and sample #52 (357.68±8.76 μM TEAC/g) measured with the same FRAP method. However, their sample collected from inside the hive have higher total antioxidant capacity than our samples (659.88± 6.35 μM TEAC/g) and our maximum total antioxidant capacity measured by FRAP method was about 18% lower than their result for the sample collected inside the hive. There is a great difference between CUPRAC results of this study and our results (Appendix D, Table D.1 and D.2). We have found greatly higher results for total antioxidant capacity measured by CUPRAC method for our propolis samples. Besides, recently Yeşiltaş et al. (2015) observed the highest total antioxidant capacity using CUPRAC method among other 3 spectrophotometric methods including ABTS, DPPH and FRAP and their results are in accordance with our results. However, they have found higher results for propolis sample collected from Çatalca than our maximum results. The maximum antioxidant capacity was measured in our sample #23 as 348.47 TE mg/g, 378.09 mg TE/g, 1056.28 mg TE/g and 139.54 mg

TE/g propolis, respectively using DPPH, ABTS, CUPRAC and FRAP methods. Their maximum values exceeds about 77%, 43%, 35% and 160% higher than our maximum results.

Boisard et al. (2014) observed the total antioxidant capacities of 6 batch of poplar-type propolis samples collected in 2010 and 2011 in apiaries originating from different regions of France using DPPH method. They have found the highest total antioxidant capacity as 1650±149 μmole TE/g when 70% ethanol was used as extraction solvent. The highest antioxidant capacity was measured in sample #23 in our study as 1392.27±131.33 μM/g EEP, which is very close to their results. Furthermore, Kalogeropoulos et al. (2009) determined the antioxidant activity of twelve propolis ethanolic extracts (PEE) from mainland Greece, Greek islands, and east Cyprus. They have observed the antioxidant capacities in a range from 0.45±0.02 mmole TE/g propolis to 1.11±0.07 mmole TE/g propolis. Our results are in accordance with their results with some exceptions.

The results of our research also showed that among the different assays used for measuring total antioxidant capacities of EEP samples, CUPRAC method gave the highest values, while the lowest values were reached by the FRAP method (Appendix D, Table D.1 and Table D.2).

4.1.3 Correlations between total phenolics, flavonoids and antioxidant capacities of propolis samples

The correlations between the total phenolic contents, total flavonoid contents and total antioxidant capacities according to ABTS, DPPH, CUPRAC and FRAP methods of EEP were investigated. The Pearson's correlation coefficients of all EEP samples for total antioxidant capacities, total phenolics, and flavonoids are represented in Table 4.2.

Table 4.2 : Correlations between antioxidant capacities, total phenolics and flavonoids of ethanolic extracts of propolis samples.

		Total phenolics	Total flavonoids	DPPH	ABTS	CUPRAC	FRAP
Total phenolics	Pearson Correlation	1	.993**	.784**	.754**	.790**	.791**
	Sig. (2-tailed)		.000	.000	.000	.000	.000
	N	162	162	162	162	162	162
Total flavonoids	Pearson Correlation	.993**	1	.776**	.758**	.791**	.795**
	Sig. (2-tailed)	.000		.000	.000	.000	.000
	N	162	162	162	162	162	162
DPPH	Pearson Correlation	.784**	.776**	1	.956**	.978**	.977**
	Sig. (2-tailed)	.000	.000		.000	.000	.000
	N	162	162	162	162	162	162
ABTS	Pearson Correlation	.754**	.758**	.956**	1	.973**	.974**
	Sig. (2-tailed)	.000	.000	.000		.000	.000
	N	162	162	162	162	162	162
CUPRAC	Pearson Correlation	.790**	.791**	.978**	.973**	1	.998**
	Sig. (2-tailed)	.000	.000	.000	.000		.000
	N	162	162	162	162	162	162
FRAP	Pearson Correlation	.791**	.795**	.977**	.974**	.998**	1
	Sig. (2-tailed)	.000	.000	.000	.000	.000	
	N	162	162	162	162	162	162

**. Correlation is significant at the 0.01 level (2-tailed).

Significantly positive high correlations were observed between total phenolic contents, total flavonoid contents and total antioxidant capacities using four different spectrophotometric assays. The highest correlation coefficient between antioxidant capacities and total phenolic content were observed with the results of FRAP (*r = 0.790*) and CUPRAC (*r = 0.791*) methods whereas the lowest value (*r* = 0.754) with ABTS method (Table 4.2). The relationship between flavonoid contents and antioxidant capacities was observed to be highest with FRAP method (*r = 0.795*) which was followed by CUPRAC method (*r = 0.791*). Similar to the relationship of phenolics and antioxidant capacities, ABTS method yielded the lowest coefficient of correlation for flavonoids (*r = 0.758*).

The results of each antioxidant capacity determination methods also revealed significant correlation coefficient values in the range 0.956-0.998. According to Table 4.2, the methods of CUPRAC and FRAP, which are based on the reducing power of copper and iron atoms (Apak et al., 2004; Benzie and Strain, 1996), presented the highest relationship for the determination of antioxidant power of propolis.

Based on the observed values of total phenolic contents, total flavonoid contents and antioxidant capacities of EEP samples, it is obvious that these values are highly correlated. These results were also in agreement with Ahn et al. (2007) who found positive correlations between total antioxidant capacity measured by DPPH method and total phenolic contents (r = 0.762) for ethanolic extracts of propolis samples collected in various areas of China. However, the correlation of total antioxidant capacity measured by ABTS method and total phenolic content (r = 0.459) is lower than those observed values in this study.

Aliyazicioglu et al. (2013) also observed a high positive correlation ($r = 0.8544$) between the phenolic content and FRAP values of the propolis samples. This positive correlation has been similarly reported in several investigations (Silva et al., 2006; Küçük et al., 2007; Socha et al., 2009). Moreover, Kumazawa et al. (2010) reported high correlation coefficient ($r = 0.9149$) between DPPH free radical-scavenging activity and total polyphenol content in propolis. Dias et al. (2012) also found a high positive correlation between total flavonoids and total phenolics ($r = 0.94$). Sarıkaya et al. (2007) have also found positive correlations between two antioxidant capacity analysis methods of FRAP and CUPRAC. They have also found high positive correlations between total phenolic content of Turkish propolis with antioxidant capacities measured with FRAP and CUPRAC methods.

4.2 Phenolic Profile of Propolis

The major components of EEP samples were identified by HPLC analysis using PDA detector. The chemical structures of compounds identified were given in Table 4.3. The amounts of individual antioxidative compounds as mean values representing each of six regions are presented in Table 4.4.

Table 4.3: Chemical structures of phenolic compounds of propolis samples identified using HPLC/PDA method.

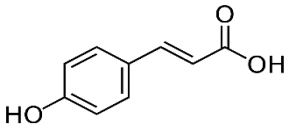
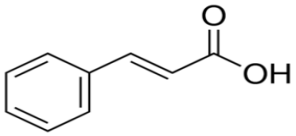
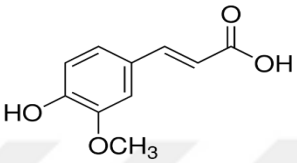
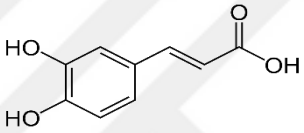
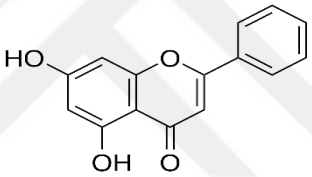
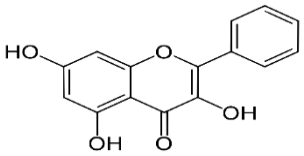
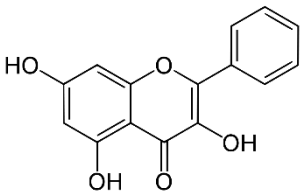
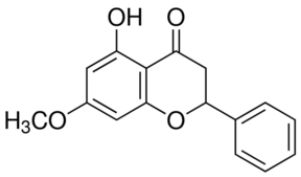
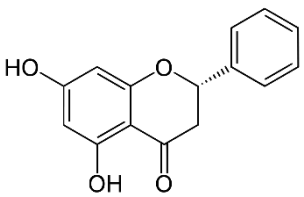
Name of the compound identified	Chemical Structure
p-coumaric acid	
t-cinnamic acid	
Ferulic acid	
Caffeic acid	
Chrysin	
Galangin	
Pinobanksin	
Pinostrobin	
Pinocembrin	

Table 4.4: Phenolic profiles of propolis samples collected in 2013 & 2014. [1, 2, 3]

Year	Region No	N	Caffeic Acid mg/g EEP	Chrysin mg/g EEP	Ferulic acid mg/g EEP	Galangin mg/g EEP	*p*-Coumaric acid mg/g EEP	Pinobanksin mg/g EEP	Pinocembrin mg/g EEP	Pinostrobin mg/g EEP	*t*-Cinnamic acid mg/g EEP
2013	1	1	0.31 ± .	2.49 ± .	0.03 ± .	0.52 ± .	0.13 ± .	0.68 ± .	8.02 ± .	1.68 ± .	0.11 ± .
	2	2	1.85 ± 0.6	26.66 ± 2.12	0.7 ± 0.16	8.7 ± 5.54	3.8 ± 4	21.37 ± 21.43	43.42 ± 1.17	9.9 ± 1.63	0.55 ± 0.37
	3	2	2.61 1.1	22.27 2.26	0.93 0.81	4.2 1.57	1.06 0.27	10.76 0.14	61.44 6.36	12.76 0.46	1.36 0.06
	4	3	0.94 ± 0.44	18.95 ± 6.04	0.1 ± 0.06	13.62 ± 7.61	0.43 ± 0.28	4.03 ± 2.19	8.87 ± 4.1	4.93 ± 3.3	0.35 ± 0.36
	5	21	3.78 ± 2.1	26.78 ± 8.33	1.43 ± 1.45	8.16 ± 6.27	2 ± 1.63	9.85 ± 5.98	45.66 ± 9.41	16.51 ± 9.17	1.48 ± 0.83
	6	3	1.88 ± 1.59	16.49 ± 11.06	0.64 ± 0.49	6.18 ± 5.78	0.69 ± 0.55	4.16 ± 3.23	33.17 ± 26.38	9.58 ± 8.27	0.56 ± 0.42
	Total	32	3.03 ± 2.08	24.03 ± 9.17	1.11 ± 1.28	8.03 ± 6.21	1.72 ± 1.72	9.26 ± 7.5	40.71 ± 16.95	13.66 ± 8.92	1.18 ± 0.83
2014	1	3	2.04 ± 0.36	22.53 ± 7.45	0.86 ± 0.59	4 ± 1.67	0.57 ± 0.32	5.35 ± 2.01	36.89 ± 11.27	8.7 ± 3.7	0.69 ± 0.17
	2	3	3.73 ± 2.55	26.64 ± 5.52	1.7 ± 0.52	5.16 ± 2.88	2.08 ± 1.22	8.26 ± 3.28	44.44 ± 12.34	13.28 ± 8.51	1.53 ± 1.15
	3	2	1.69 ± 2.11	12.69 ± 13.36	0.08 ± 0.08	2.91 ± 2.65	0.36 ± 0.43	4.46 ± 5.46	32.35 ± 35.56	6.74 ± 6.94	0.47 ± 0.56
	4	3	1.44 ± 1.01	17.33 ± 7.51	0.65 ± 0.7	9.82 ± 9.54	0.49 ± 0.04	5.27 ± 3.94	31.84 ± 23.89	7.42 ± 4.95	0.28 ± 0.11
	5	8	2.96 ± 2.69	21.97 ± 9.55	0.97 ± 0.8	5.36 ± 3.62	0.97 ± 0.85	6.95 ± 4.99	40.95 ± 13.76	13.56 ± 10.11	1.19 ± 0.97
	6	3	1.25 ± 1.43	10.64 ± 8.88	0.04 ± 0.03	5.32 ± 7.87	0.29 ± 0.3	2.13 ± 2.26	15.11 ± 10.31	8.03 ± 7.2	0.18 ± 0.22
	Total	22	2.38 ± 2.08	19.66 ± 9.34	0.8 ± 0.76	5.53 ± 4.9	0.85 ± 0.85	5.8 ± 4.08	35.32 ± 17.31	10.65 ± 7.78	0.84 ± 0.84
TOTAL	1	4	1.61 ± 0.92a	17.52 ± 11.72ab	0.65 ± 0.63a	3.13 ± 2.21c	0.46 ± 0.35c	4.19 ± 2.86c	29.67 ± 17.12ab	6.95 ± 4.63a	0.55 ± 0.32ab
	2	5	2.98 ± 2.1a	26.65 ± 4.04a	1.3 ± 0.67a	6.57 ± 3.95ab	2.77 ± 2.37a	13.5 ± 13.1a	44.03 ± 8.77a	11.92 ± 6.35a	1.14 ± 0.99ab
	3	4	2.15 ± 1.47a	17.48 ± 9.58ab	0.5 ± 0.68a	3.55 ± 1.93c	0.71 ± 0.5c	7.61 ± 4.81ab	46.89 ± 26.78a	9.75 ± 5.31a	0.91 ± 0.61ab
	4	6	1.19 ± 0.75a	18.14 ± 6.16ab	0.38 ± 0.54a	11.72 ± 7.99a	0.46 ± 0.18c	4.65 ± 2.93c	20.35 ± 19.83c	6.17 ± 4a	0.32 ± 0.24c
	5	29	3.56 ± 2.26a	25.45 ± 8.78a	1.3 ± 1.31a	7.38 ± 5.74ab	1.71 ± 1.52ab	9.05 ± 5.79ab	44.36 ± 10.73a	15.7 ± 9.35a	1.4 ± 0.86a
	6	6	1.57 ± 1.4a	13.57 ± 9.53c	0.34 ± 0.45a	5.75 ± 6.19ab	0.49 ± 0.45c	3.14 ± 2.73c	24.14 ± 20.46c	8.81 ± 6.99a	0.37 ± 0.37c
	Total	54	2.77 ± 2.09	22.25 ± 9.4	0.98 ± 1.1	7.01 ± 5.79	1.37 ± 1.49	7.85 ± 6.51	38.51 ± 17.14	12.43 ± 8.53	1.04 ± 0.84

[1] Data represent mean values ± standard deviation of three measurements obtained from each sample collected from provinces of which were grouped into relevant geographical regions.

[2] Provinces of each geographic regions follow as: (1) Aegean region - Aydin, Izmir, Mugla; (2) Black Sea region - Bartin, Duzce, Kastamonu, Ordu; (3) Central Anatolia region – Kirsehir, Konya, Sivas ;(4) East Anatolia region provinces – Bingol, Elazig, Erzincan; (5) Marmara region – Balikesir, Bursa, Edirne, İstanbul, Kocaeli, Tekirdağ; (6) Mediterranean region - Antalya, Burdur, Hatay, Mersin.

The mean ± standard deviation of individual phenolic compounds of EEP samples collected from various geographical regions and beekeepers of Turkey in 2013 can be listed as pinocembrin (40.71±5.12 mg/g EEP), chrysin (24.03±2.88 mg/g EEP), pinostrobin (13.66±1.06 mg/g EEP), galangin (8.03±0.94 mg/g EEP), pinobanksin (9.26±1.00 mg/g EEP), caffeic acid (3.03±0.23 mg/g EEP), *p*-coumaric acid (1.72±0.04 mg/g EEP), *t*-cinnamic acid (1.18±0.06 mg/g EEP) and ferulic acid (1.11±0.23 mg/g EEP) in a descending order.

Pinocembrin is a major flavonoid in poplar-type propolis and the highest content was found in sample #52 (65.94±3.07 mg/g EEP) from Konya province of Turkey. The amount of pinocembrin in all propolis samples collected in 2013 varied from 4.16±0.09 to 65.94±3.07 mg/g EEP. Another major flavonoid was found to be chrysin in Turkish propolis (2.49±0.01 to 46.79±9.7 mg/g EEP) with a highest value in sample #26 from İstanbul (46.79±9.70 mg/g EEP). Pinostrobin, another flavonoid found in poplar-type propolis ranged between 1.68±0.24 mg/g EEP and 37.85 ± 0.53 mg/g EEP. Sample #28 from Istanbul represented the highest amount of pinostrobin (37.85±0.53 mg/g EEP) in addition to high levels of pinocembrin (60.3±0.62 mg/g EEP) and chrysin (41.17±9.90 mg/g EEP) in this sample. Besides, galangin is another major flavonoid in poplar-type propolis and the highest content was found is sample #23 from Istanbul (31.31 ± 3.92 mg/g EEP) compared to other samples. The amount of galangin in all propolis samples collected in 2013 varied from 0.52±0.01 to 31.31±3.92 mg/g EPP. Additionally, another major flavonoid was found to be pinobanksin in Turkish propolis (0.68±0.01 mg/g EEP - 29.47±0.38 mg/g EEP) with a highest value in sample #6 from Kocaeli (29.47±0.38 mg/g EEP). The great variations in individual phenolic contents between propolis samples explain the great variations among propolis samples collected from different geographical areas and beekepers of Turkey.

There are also minor phenolic acids identified in Turkish propolis samples collected in 2013 including caffeic acid (0.31±0.00 - 7.61±0.08 mg/g EEP), *p*-coumaric acid (0.07±0 - 6.62±0.07 mg/g EEP), *t*-cinnamic acid (0.11±0 - 3.64±1.11mg/g EEP) and ferulic acid (0.03±0.00 - 5.19±0.14 mg/g EEP). The results of the quantitative analysis of all EEP samples collected in 2013 are shown in Appendix D, Table D.3. Values are expressed as means ± standard deviations of triplicate analysis of each sample.

The mean ± standard deviation of individual phenolic compounds of EEP samples collected from various geographical regions and beekeepers of Turkey in 2014 can be listed as pinocembrin (35.32±2.00 mg/g EEP), chrysin (19.66±1.22 mg/g EEP), pinostrobin (10.65±2.17 mg/g EEP), pinobanksin (5.80±0.22 mg/g EEP), galangin (5.53±0.76 mg/g EEP),caffeic acid (2.38±0.65 mg/g EEP), *p*-coumaric acid (0.85±0.02 mg/g EEP), *t*-cinnamic acid (0.84±0.03 mg/g EEP) and ferulic acid (0.80±0.30 mg/g EEP) in a descending order.

Pinocembrin is a major flavonoid in poplar-type propolis and the highest content was found in sample #37 (58.07±5.11 mg/g EEP) from Elazığ province of Turkey. The amount of pinocembrin in all propolis samples collected in 2014 varied from 3.23±0.40 to 58.07±5.11 mg/g EEP. Another major flavonoid was found to be chrysin in Turkish propolis (1.62±0.37 to 33.86±1.69 mg/g EEP) with a highest value in sample #11 from İstanbul (33.86±1.69 mg/g EEP). Pinostrobin, another flavonoid found in poplar-type propolis ranged between 1.23±0.12 mg/g EEP and 30.34±0.88 mg/g EEP. Sample #38 from Kocaeli represented the highest amount of pinostrobin (30.34±0.88 mg/g EEP) in addition to high levels of pinocembrin (54.24±1.06mg/g EEP) and chrysin (30.87±1.95 mg/g EEP) in this sample. Besides, galangin is another major flavonoid in poplar-type propolis and the highest content was found is sample #15 from Elazığ (19.79±0.27 mg/g EEP) compared to other samples. The amount of galangin in all propolis samples collected in 2014 varied from 0.37±0.06 to 19.79±0.27 mg/g EPP. Additionally, another major flavonoid was found to be pinobanksin in Turkish propolis (0.39±0.00 mg/g EEP - 16.97±0.27 mg/g EEP) with a highest value in sample #39 from Kocaeli (16.97±0.27 mg/g EEP).

There are also minor phenolic acids identified in Turkish propolis samples collected in 2014 including caffeic acid (0.19±0.00 - 8.84±0.07 mg/g EEP), *p*-coumaric acid (0.04±0.00 - 2.97±0.00 mg/g EEP), *t*-cinnamic acid (0.02±0.00 - 3.27±0.15 mg/g EEP) and ferulic acid (0.02±0.00 - 2.32±0.01 mg/g EEP). The results of the quantitative analysis of all EEP samples collected in 2014 are shown in Appendix D, Table D.4. Values are expressed as means ± standard deviations of triplicate analysis of each sample.

The mean ± standard deviation of individual phenolic compounds of all 54 EEP samples collected from various geographical regions and beekeepers of Turkey in 2013 and 2014 can be listed as pinocembrin (38.51±3.81 mg/g EEP), chrysin

(22.25±2.19 mg/g EEP), pinostrobin (12.43±1.53 mg/g EEP), pinobanksin (7.85±0.67 mg/g EEP), galangin (7.01±0.86 mg/g EEP), caffeic acid (2.67±0.14 mg/g EEP), *p*-coumaric acid (1.34±0.03 mg/g EEP), *t*-cinnamic acid (1.02±0.05 mg/g EEP) and ferulic acid (0.99±0.25 mg/g EEP) in a descending order.

Besides, the mean ± standard deviation of minor phenolic compounds which are individual phenolic acids of all 54 EEP samples collected from various geographical regions and beekeepers of Turkey in 2013 and 2014 can be listed as caffeic acid (2.77±0.14 mg/g EEP), *p*-coumaric acid (1.37±0.03 mg/g EEP), *t*-cinnamic acid (1.04±0.05 mg/g EEP) and ferulic acid (0.98±0.25 mg/g EEP). Table 4.5 shows the minimum, maximum and mean ± standard deviation values of all propolis samples collected from different geographical areas and beekepers of Turkey in 2013 and 2014.

Table 4.5 : Flavonoids and phenolic acid contents of all propolis samples – minimum, maximum and mean±standart deviations.

Compounds	Propolis (mg/g)		
	Minimum	Maximum	Mean±std. dev.
Pinocembrin	3.23±0.40	65.94±3.07	38.51±3.81
Chrysin	1.62±0.37	46.79±9.7	22.25±2.19
Pinostrobin	1.23±0.12	37.85 ± 0.53	12.43±1.53
Pinobanksin	0.39±0.00	29.47 ± 0.38	7.85±0.67
Galangin	0.37±0.06	31.31±3.92	7.01±0.86
Caffeic acid	0.19±0.00	8.84±0.07	2.77±0.14
p-coumaric acid	0.05±0.00	6.62±0.07	1.37±0.03
t-cinnamic acid	0.07±0.00	3.64±1.11	1.04±0.05
Ferulic acid	0.02±0.00	5.19±0.14	0.98±0.25

According to their Univariate Analysis of GLM procedure, individual phenolic contents of propolis samples showed no difference accoding to the years (Appendix E, Table E.2). However, only chyrisin, galangin, *p*-coumaric acid, pinobanksin, pinocembrin, and *t*-cinnamic acid presented significant differences according to the regions. Duncan's method of multiple comparisons between chyrisin contents of propolis samples from 6 different regions showed statistically significant differences ($p < 0.05$). Chyrisin contents of propolis obtained from Marmara region and Black

Sea regions were the highest and significantly different from the other regions. Galangin contents of samples also differentiated according to the regions, the Black Sea region with the lowest mean value while East Anatolian region the highest.

Similarly, pinobanksin contents of propolis samples differentiated Marmara region from 3 other regions including Aegean, East Anatolia and Mediterranean regions. In addition, pinobanksin content of propolis samples from Black Sea region also was different from 3 other regions including Aegean, East Anatolia and Mediterranean regions.

According to Table 4.4 and Appendix E, Table E.2 there were also statistically significant differences ($p < 0.05$) of pinocembrin contents of propolis samples from Aegean region compared to East Anatolia and Mediterranean regions.

When data was analysed considering regions as a factor, two phenolic acid contents of propolis samples including *t*-cinnamic acid and *p*-coumaric acid also showed differences (Table 4.4). The *t*-cinnamic acid contents of propolis samples from Aegean region compared to East Anatolia and Mediterranean regions were different. Similarly, *t*-cinnamic acid contents of propolis samples from Black Sea, Central Anatolia and Marmara regions differentiated from East Anatolia and Mediterranean regions. Another phenolic acid that showed significant differences ($p < 0.05$) between six regions was *p*-coumaric acid (Table 4.4 and Appendix E, Table E.2). Based on *p*-coumaric acid content, Black Sea and Marmara regions differed from Aegean, Central Anatolia and East Anatolia regions.

As a result of these comparisons it is apparent that Turkish propolis showed statistically significant differences ($p < 0.05$) in their individual flavonoids including chyrisin, galangin, pinobanksin and pinocembrin and individual phenolic acids including *p*-coumaric acid and *t*-cinnamic acids between regions. The differences of individual phenolic contents between regions may have been resulted from the climate and surrounding flora of the hive.

When we compare our results with other studies on Turkish propolis, Yesiltas et al. (2015) have found similar results with our findings with some exceptions. We have found higher values for pinocembrin in our samples for pinocembrin as their highest value was 42.37 ± 0.60 mg/g propolis. They have found highest value of chyrisin as 70.69±2.21 which is 33% higher than our maximum result. They have also found

higher value of pinostrobin in their samples. We have found very similar results of pinobanksin and galangin to their findings. When we compare the phenolic acids, we have found higher results for caffeic acid and *p*-coumaric acid, but lower results for *t*-cinnamic acid and ferulic acid.

Moreover, Aliyazicioglu et al. (2012) have observed caffeic acid in methanolic extracts of propolis samples from 1.26 mg/g propolis to 4.66 mg / g propolis. Some of our results are in accordance with their results for caffeic acid, but we have found higher results in our samples. Sample #38 collected from Kocaeli has the highest amount of caffeic acid (8.84± 0.07 mg/g EEP) which is about 2 times higher than their highest result for caffeic acid in their samples. They have observed *p*-coumaric acid in methanolic extracts of propolis samples from 0.7 to 4.58 mg/g propolis. Some of our results are in accordance with their results for *p*-coumaric acid, but in our study the highest value was reached in sample 20 collected from Düzce/Kocaeli in 2013 (6.62±0.07 mg/g EEP). They have observed ferulic acid in methanolic extracts of propolis samples from 0.2 to 5.74 mg/g propolis. In our study, the highest result observed for ferulic acid was 5.19±0.14 mg/g from sample #26 collected from Istanbul in 2013. Our results for ferulic acid are very close to their results for ferulic acid. They have observed *t*-cinnamic acid in methanolic extracts of propolis samples in a range of 0.03 - 1.08 mg/g propolis. Some of our results are in accordance with their findings. However, in our study, we have found higher results for *t*-cinnamic acid as sample #23 had a *t*-cinnamic acid content of 3.64±1.11 mg/g EEP and sample 38 had 3.27±0.15 mg/g *t*-cinnamic acid content which is about 3 times higher than their highest result for *t*-cinnamic acid content. It can be suggested that extraction solvent may have an important role in higher results of our study. Hydro-alcoholic solvent prepared using ethanol showed the highest results of phenolic extraction for propolis and it is reported as a optimum extraction solvent in many studies in literature (Mouhoubi-Tafinine et al., 2016; Miguel et al. 2010). It can be also suggested that the variotions can be resulted from different geographical locations even all the samples are collected from Turkey. Even collected in the same geographical region, propolis may differ qualitatively and quantitatively between apiaries and even inside the same apiary from one hive to another one (DeCastro et al., 2011).

As we compare our results for Chinese propolis, our results for chrysin observed to be between 1.62±0.37 – 46.79±9.7 mg/g EEP represent a higher range value than Chinese propolis (3.9- 19.5 mg/g EEP) by Ahn et al. (2007). Moreover, our results for pinocembrin observed to be between 3.23±0.40 - 65.94±3.07 mg/g EEP also represent a higher range value than Chinese propolis (3.9 - 35.7 mg/g EEP) in the same study. Another major flavonoid galangin is also found to be in a higher range in our samples (0.37±0.06 - 31.31±3.92) than their given range between 3.8 - 12.8 mg/g EEP. The caffeic acid content was found to be between 3.3 and 24.6 mg/g of EEP. Some of our findings (0.19±0.00 – 8.84±0.07 mg/g EEP) are in accordance with their results. The *p*-coumaric acid content in Chinese propolis samples were found to be 2.3 - 42.3 mg/g EEP. However, most of their samples were between the range of 2.3 – 7.5 mg/g EEP. Our results for *p*-coumaric acid were very similar to their findings. Moreover, another study on Chinese poplar type propolis by Guo et al. (2011) have shown that phenolic compounds in Chinese propolis samples were in range of 0.70 – 2.14 mg/g pinocembrin, 0.10-3.60 mg/g chrysin, 1.09 – 33.94 mg/g caffeic acid, 0.46 -77.71 mg/g *p*-coumaric acid, 0.83 – 10.03 mg/g ferulic acid. Their results for major flavonoid including pinocembrin and chrysin were in a lower range compared to our results, but phenolic acids found in their samples were found in a higher range. Furthermore, in a recent study of Jin et al. (2017) on Chinese poplar type propolis, they have observed 18.18 mg/g chrysin, 7.971 mg/g galangin, 7.36 mg/g pinobanksin, 7.17 mg/g pinocembrin, 1.83 mg/g *p*-coumaric acid, 8.02 mg/g caffeic acid, 1.89 mg/g ferulic acid. Their results for flavonoids including galangin, pinobanksin and pinocembrin are lower than our results for Turkish propolis. According to these comparisons between Turkish and Chinese poplar type propolis, it can be suggested that Turkish propolis samples generally have more flavonoid contents including galangin, pinocembrin and chrysin than Chinese propolis samples. It is suggested that the variations may be mainly resulted from the differences of geographical locations.

Dudonne et al. (2011) have found lower results according to their analysis on Romanian poplar buds for pinobanksin (9.8±1.0 mg/g) and pinocembrin (1.0±0.1 mg/g), but higher results for caffeic acid (17.9±0.7 mg/g) and *p*-coumaric acid (18.8±0.8 mg/g). Their results for ferulic acid content (2.4±0.1 mg/g) is in our range of samples. The difference is that poplar buds contain mostly phenolic acids, but

Turkish propolis contain mostly flavonoids including galangin, chrysin, pinobanksin, pinocembrin and pinostrobin.

When we compare our results with poplar type propolis from Europe, Gregoris et al. (2011) have found lower result for Italian poplar type propolis for pinocembrin (45.6 mg/g EEP) from our given range values, although it is the highest amount of polyphenol in their sample. We have also found pinocembrin as a highest content of polyphenol in our samples similar to their findings. We have found higher results for the phenolic compounds they have observed in our samples. Our highest result for chrysin was 46.79±9.7 mg/g EEP (sample #26), followed by galangin 31.31±3.92 mg/g EEP (sample #23), pinocembrin 65.94±3.07 mg/g EEP (sample #52) which are higher than their findings for 41.4 mg/g chrysin, 17.3 mg/g galangin, 9.9 mg/g caffeic acid in Italian poplar type propolis sample.

Moreover, Barbari´c et al. (2011) have found the most abundant flavonoid as galangin in Craotian poplar type propolis samples in ranges from none to 47.4879 mg/g. They didn't observe galangin in four samples and 14 samples are in the range from 0.1257 mg/g to 7.7154 mg/g which are very close to the galangin content in most of the samples in our study. The highest values they have observed was is sample #9 and sample #13, 47.4879 and 34.8201 mg/g raw propolis. In our study, the highest result of galangin content was observed in sample #23 as 31.31±3.92 mg/g EEP and only 2 of their samples exceeds our highest value. Besides, one of the most important flavonoid found in poplar-type propolis named pinocembrin was not found in four samples and the other samples had pinocembrin content in a range from 0.0664 mg/g to 7.6651 mg/g raw propolis. When pinocembrin content of Turkish propolis samples are compared to their results for propolis from Croatia our range (3.23±0.40 - 65.94±3.07 mg/g EEP) is higher than their findings. Our highest result for pinocembrin was observed in sample #52 (65.94±3.07 mg/g EEP) and it is about 9 times higher than their maximum amount of pinocembrin content including sample. Besides, they didn't observe chrysin in 12 of their samples and other samples had a chrysin content in a range from 0.7844 mg/g to 4.1356 mg/g raw propolis. Furthermore, chyrsin range of our samples (1.62±0.37 - 46.79±9.7) were higher than their results. Our highest result for chrysin content was observed in sample #27 as 46.79±9.7 mg/g propolis which is about 11 times higher than their maximum result for chrysin content. Their results for ferulic acid and *p*-coumaric acid also varied as

0.0017 mg/g – 0.9590 mg/g and 0.0023 mg/g – 0.1562 mg/g whereas our results were varied in a higher range as 0.02±0.00 – 5.19±0.14 mg ferulic acid /g EEP and 0.05±0.00 – 6.62±0.07 mg *p*-coumaric acid /g EEP. We have observed higher range of flavonoids including pinocembrin and chrysin and phenolic acids including ferulic acid and *p*-coumaric acid. The identified differences from phenolic compound contents may vary according to the differences mainly in geographical locations and also method of extraction and extraction solvent.

Additionally, we have also found higher range of flavonoids and phenolic acids in our Turkish propolis samples compared to results of French poplar-type propolis by Boisard et al. (2014). According to their results, they have found 6.0±0.1 mg/g caffeic acid, 11.1±0.1 mg/g *p*-coumaric acid, 4.4±0.1 mg/g ferulic acid, 33.4±0.2 mg/g pinocembrin, 23.5±0.2 mg/g chrysin, 20.7±0.3 mg/g galangin and 12.9±0.3 mg/g pinostrobin. We have found similar results with their findings as shown in Table D.3 and D.4, but our range for caffeic acid was 0.19±0.00 – 8.84±0.07 mg/g, for ferulic acid 0.02±0.00 - 5.19±0.14 mg/g ferulic acid, for pinocembrin 3.23±0.40 - 65.94±3.07 mg/g, for chyrisin 1.62±0.37 – 46.79±9.7 mg/g and for galangin 0.37±0.06 – 31.31±3.92 mg/g and for pinostrobin 1.23±0.12 – 37.85±0.53 mg/g.

Furthermore, we have also found higher range of galangin (0.37±0.06 – 31.31±3.92 mg/g EEP) in our Turkish propolis samples than findings of Lagouri et al. (2014) who studied the extracts of propolis collected from the Greek mainland (West Macedonia) and the Greek island, Rhodes. They have found a lower range of galangin (1.31±0.13 – 8.55±0.64 mg/g propolis) in their samples. They have also observed lower range values than our results for phenolic acids including caffeic acid (0.64±0.01 – 4.17±0.27 mg/g propolis) and ferulic acid (0.53±0.02 – 1.41 ± 0.02 mg/g propolis).

Brazilian-type (Baccharis-type) propolis is known to be rich in *p*-coumaric acid derivatives, while European-type (poplar-type) propolis rich in flavonoids (Fujimoto et al., 2001). When the results of Park et al. (2004) representing the small amounts of flavonoids such as pinobanksin (1.66 mg/g propolis) and chrysin (1.86 mg/g propolis) in Brazilian propolis (Park et al., 2004) are compared with our findings, it is apparent that Turkish propolis is richer in flavonoids addressing a poplar-type. We have found pinobanksin and chrysin in a range of 0.39±0.00 - 29.47±0.38 mg/g and 1.62±0.37 - 46.79±9.7, respectively.

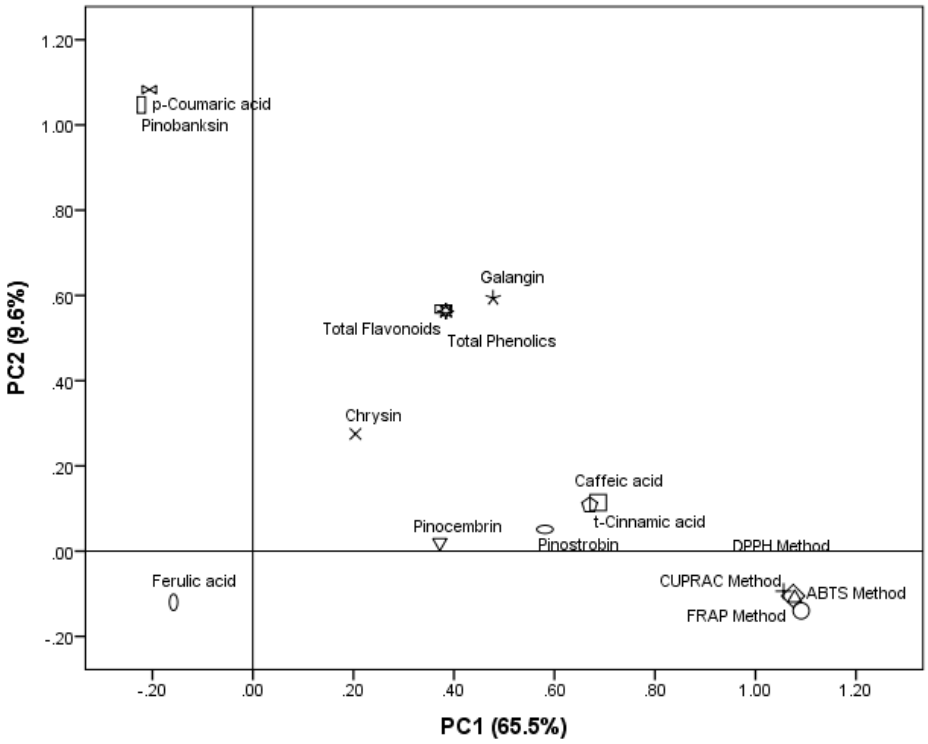
Moreover, the phenolic compounds of poplar-type propolis collected from Argentina (Chaillou and Nazerano, 2009) with high levels of pinocembrin ranging from 0.208±0.003 to 88±1 mg / g are similar to our results (3.23±0.4 mg/g – 65.94±3.07). More data from Argentina propolis present higher amounts of flavonoids including pinobanksin (3.5-27.9 mg/g EEP), pinocembrin (0.3-64.3 mg/g EEP), and chrysin (0.3-68.7 mg/g EEP) but low levels of non-flavonoid structures of caffeic acid (1.1 – 5.5 mg/g EEP), *p*-coumaric acid (0.9-7.1 mg/g EEP), ferulic acid (0.3-2.3 mg/g EEP) (Kumazawa et al., 2010) which are in accordance to our results.

It is also important to understand the relationship between individual phenolics and total antioxidant capacity of propolis samples. Galangin characterized by high antioxidant properties at low amounts; on the contrary pinocembrin show high concentration but poor antioxidant characteristics compared to galangin (Gregoris and Stevenato, 2010). In our study, the highest content of galangin (31.31±3.92 mg/g EEP) and *t*-cinnamic acid (3.64±1.11 mg/g EEP) was observed in sample #23 from Istanbul province of Turkey which also possess the highest antioxidant capacity measured by all spectrophotometric methods (1392.27±131.33 μM/g EEP, 1510.64±55.4 μM TE/g EEP, 4220.24±161.75 μM TE/g EEP and 557.49±20.47 μM TE/g EEP measured by DPPH, ABTS, CUPRAC and FRAP methods, respectively). Moreover, the other phenolic compounds identified were measured in high amounts including caffeic acid (5.87±4.66 mg/g EEP), chrysin (35.11±2.89 mg/g EEP), ferulic acid (2.74±3.94 mg/g EEP), *p*-coumaric acid (5.53±0.57 mg/g EEP), pinobanksin (6.6±4.92 mg/g EEP), pinocembrin (52.04±26.03 mg/g EEP), pinostrobin (25.45±2.17 mg/g EEP). It can be concluded that the amount of total phenolic compounds results in high total antioxidant capacity. Although the total antioxidant capacity of sample #52 was significantly higher than many other samples, these values are lower than the results of sample #23. This is because of the lower results of other phenolic compounds observed in sample #52 than observed in sample #23 including 1.83±0.04 mg/g for caffeic acid, 20.67±1.63 mg/g for chrysin, 1.5±2.45 mg/g for ferulic acid, 3.09±0.22 mg/g for galangin, 0.87±0.05 mg/g for *p*-coumaric acid, 13.08±0.14 for pinostrobin, 1.32±0.08 for *t*-cinnamic acid contents. These results are substantially agree with previous reports (Kumazawa et al., 2004; Gregoris and Stevenato, 2010).

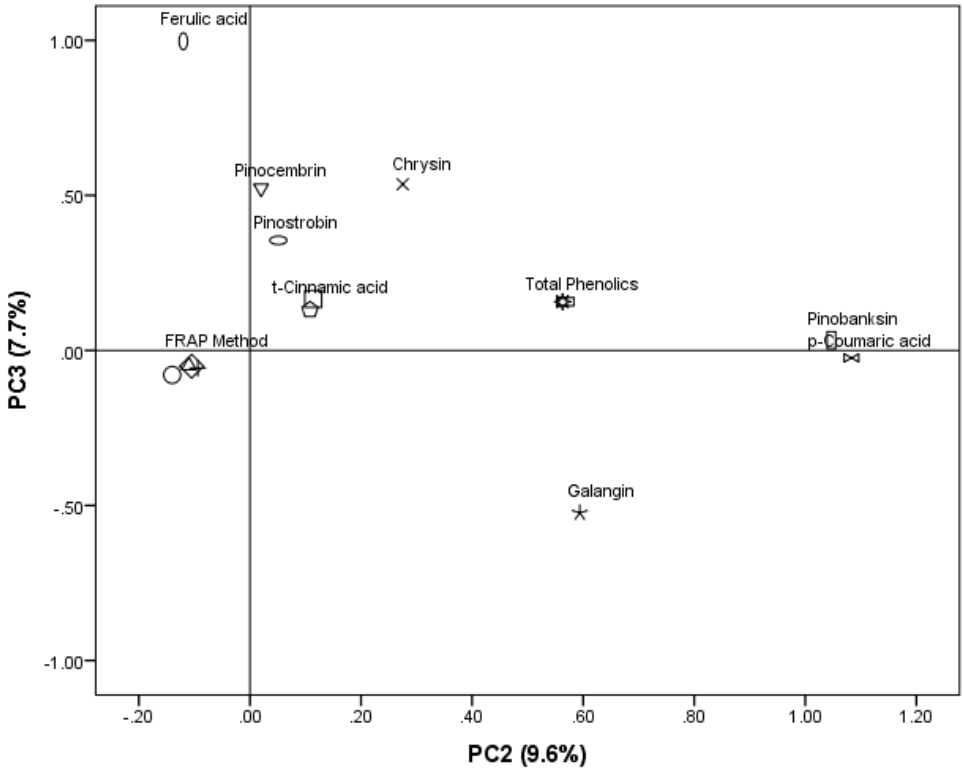
4.3 Multivariate Statistical Analysis of Total Phenolics and Flavonoids, Phenolic Profiles And Antioxidant Capacities of Propolis Samples

The amounts of total phenolics and flavonoids, phenolic profiles and antioxidant capacities (with 4 different methods) of propolis samples were analyzed using PCA to investigate the differences for the samples collected from 6 different regions of Turkey. The first three principal components (PCs) explained 82.8% of the total variance, where PC1 explained 65.5%, PC2 9.6% and PC3 7.7%, respectively (Table 4.6). The maximal amount of variance contained in the original data set is concentrated in the first principal components. The factor loading plots and regression factor score plots of the PCs are shown in Figures 4.1 and 4.2, respectively.

The first principal component (PC1) corresponding to 65.5% of total variation was strongly correlated with the values of antioxidant activities as measured with all four methods (ABTS, DPPH, CUPRAC and FRAP), caffeic acid, *t*-cinnamic acid and pinostrobin. For the second principal component (PC2, 9.6% variation), the dominant variables were pinobanksin, *p*-coumaric acid, and the amounts of total phenolics and total flavonoids (Figure 4.1). On the other hand, ferulic acid, chrysin, and pinocembrin were observed to be dominant in the third PC corresponding to 7.7% variation. Figure 4.2 shows PC1 and PC2 grouping samples studied into 6 different groups, each corresponding to geographical regions where propolis samples were collected. Accordingly, propolis samples from the Marmara region were mostly characterized by positive values of PC1, presenting contributions of caffeic acid, cinnamic acid, pinostrobin, galangin, amounts of total phenolics and total flavonoids, pinocembrin and chrysin (Figure 4.2). On the other hand, Black Sea propolis was well clustered in the negative region of PC1 but in positive region in PC2 by higher levels of total phenolics and total flavonoids, chrysin, and pinostrobin. The regions of Aegean, Central and East Anatolia, and Mediterranean were partially overlapped each other. Based on PCA plot of samples and variables, there seems to be a change in antioxidant properties and phenolic profile of propolis depending on the regions starting from North to the south and east of Turkey.



(a)



(b)

Figure 4.1: Loading plot of all variables for (a) the first and second principal components (b) the second and third principal components.

Table 4.6 : Principal component analysis; Eigenvalues, explained and cumulative variance, loadings of the variables for the first three principal components (PC).

Variance Explained	**PC1**	**PC2**	**PC3**
Eigenvalues	9.827	1.444	1.155
% of Variance	65.5	9.6	7.7
Cumulative %	65.5	75.1	82.4
Factor Loading[1]			
ABTS Method	1.091	-0.140	-0.079
CUPRAC Method	1.078	-0.110	-0.049
FRAP Method	1.075	-0.105	-0.051
DPPH Method	1.056	-0.094	-0.057
Caffeic acid	0.687	0.114	0.166
t-Cinnamic acid	0.670	0.108	0.131
Pinostrobin	0.581	0.051	0.355
p-Coumaric acid	-0.206	1.083	-0.024
Pinobanksin	-0.222	1.047	0.033
Galangin	0.478	0.594	-0.524
Total Phenolics	0.379	0.568	0.157
Total Flavonoids	0.384	0.563	0.157
Ferulic acid	-0.158	-0.120	0.997
Chrysin	0.204	0.275	0.536
Pinocembrin	0.372	0.020	0.523
KMO Probability[2]		0.866	
Bartlett Sphericity Probability[3]		< 0.0001	

[1]Rotated component matrix
[2] Kaiser-Meyer-Olkin Value measure of sampling adequacy
[3] Bartlett Sphericity test

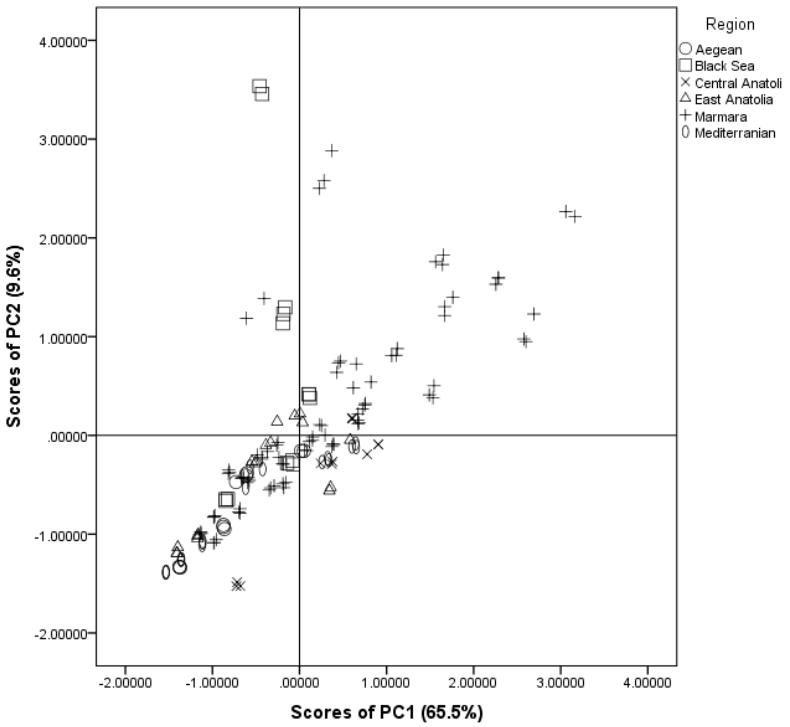


Figure 4.2 : Principal component analysis, distribution of propolis samples from six regions in score plots.

Discriminant Analysis (DA) was used for classifying the propolis based on their sample collection regions. The grouping variables were 54 propolis samples collected from different regions (Aegean, Black Sea, Central Anatolia, East Anatolia, Marmara, and Mediterranean) were subjected to UNIVARIATE analysis to determine which phenolic compounds significantly differed according to the geographical origins (Table 4.1 and Table 4.6). Accordingly, six of the nine phenolic compounds were found to be significant ($p < 0.05$) for the geographical discrimination of propolis samples. Thus, those predictors were subjected to Linear Discriminant Analysis (LDA) if they can be reliable predictors to discrimate the geographical regions of propolis.

Table 4.7 provides strong statistical evidence of significant differences between means of geographical regions for all predictors with pinocembrin, *t*-cinnamic acid, *p*-coumaric acid, and chrysin producing high value F's. However, test of equality of covariance matrices (Box M index, -2log M = 880.46, χ^2=733.28, df=105, $p < 0.0001$) was significant at the 95% confidence level and the log determinants were also not close to each other (Appendix E, Table E.4). Therefore, it was obvious that the group variances were not equal violating the assumptions of the discriminant analysis. However, the available literature indicates that violation of the homogeneity of covariances assumption is not of major importance to conducting a valid discriminant analysis (Brown and Wicker, 2000) as separate group variances can be used instead of pooled-variance. Therefore Quadratic Discriminant Analysis, which is to base the classification not on the combined covariance matrices but on the separate ones, was used by assuming unequal group variances (XLSTAT ver. 24.0).

An overall chi-square test was found to be significant (Wilk's lambda (λ) = 0.291, F = 7.054, df = 30, $p<0.0001$) showing significant differences between groups and 29.1% variance of dependent variable (geographical regions) that is not explained by the discriminant function. Table 4.7 provides information on each of the discriminate functions (equations) produced. As the maximum number of discriminant functions produced is the number of groups minus 1 and there are 6 groups (regions), five functions are displayed. According to Bartlett's test results, three statistically significant ($p < 0.001$) discriminant functions were formed. The first discriminant function (F1) accounted for 71.50% of total variance while the second and third accounted for 14.31% and 8.29%. All three functions accounted for 94.9% of the

total variance. A canonical correlation of 0.739 for the first function suggests the model explains 54.6% of the variation in the grouping variable (geographical regions) (Table 4.7).

Table 4.7 : Discriminant Analysis: Eigenvalues, Bartlett's test for eigenvalue significancy and canonical correlations.

	F1	**F2**	**F3**	**F4**	**F5**
Eigenvalue	1.205	0.248	0.147	0.070	0.016
Discrimination (%)	71.5	14.7	8.8	4.2	0.96
Cumulative %	71.5	86.1	94.9	99.0	100.0
Bartlett's statistic	184.998	66.384	33.187	12.552	2.395
p-value	0.000	0.000	0.001	0.051	0.302
Canonical correlations	0.739	0.446	0.359	0.256	0.126

Standardized discriminant coefficients were used to determine the degree to which each discriminator variable is associated with differences among the groups and the relative importance of each discriminator variable to group discrimination. Table 4.8 presents the standardized discriminant function coefficients for selected phenolic compounds. Accordingly, lower level of galangin and higher levels of *t*-cinnamic acid and *p*-coumaric acid with a large coefficient is an important predictor in the first discriminant function (F1 - 71.5% variation). Higher amounts of phenolic acids are good predictors of discriminating propolis of Black Sea and Marmara regions (Table 4.4). Similarly, low levels of flavonoid galangin discriminated Central Anatolia and Aegean regions from other regions. Although higher levels of *p*-coumaric acid, chrysin and a lower level of pinocembrin were observed in the second discriminant function, they were less successful predictors as the contribution of the function is about 14.7% (Table 4.8). In addition, factor loadings on three discriminant functions were consistent with those predictions except for the higher values of pinocembrin on the first discriminant function (Figure 4.3) showing to be a good predictor for discriminating propolis of regions of Black Sea, Marmara, and Central Anatolia from the other regions (Table 4.6).

Table 4.8 : Discriminant Analysis: Standardized canonical discriminant function coefficients.

	F1	F2	F3
Chrysin	0.305	0.963	0.262
Galangin	-1.195	-0.120	0.615
p-Coumaric Acid	0.677	1.043	-0.916
Pinobanksin	-0.015	-0.522	0.539
Pinocembrin	0.162	-0.786	-0.210
t-Cinnamic Acid	0.677	-0.372	0.608

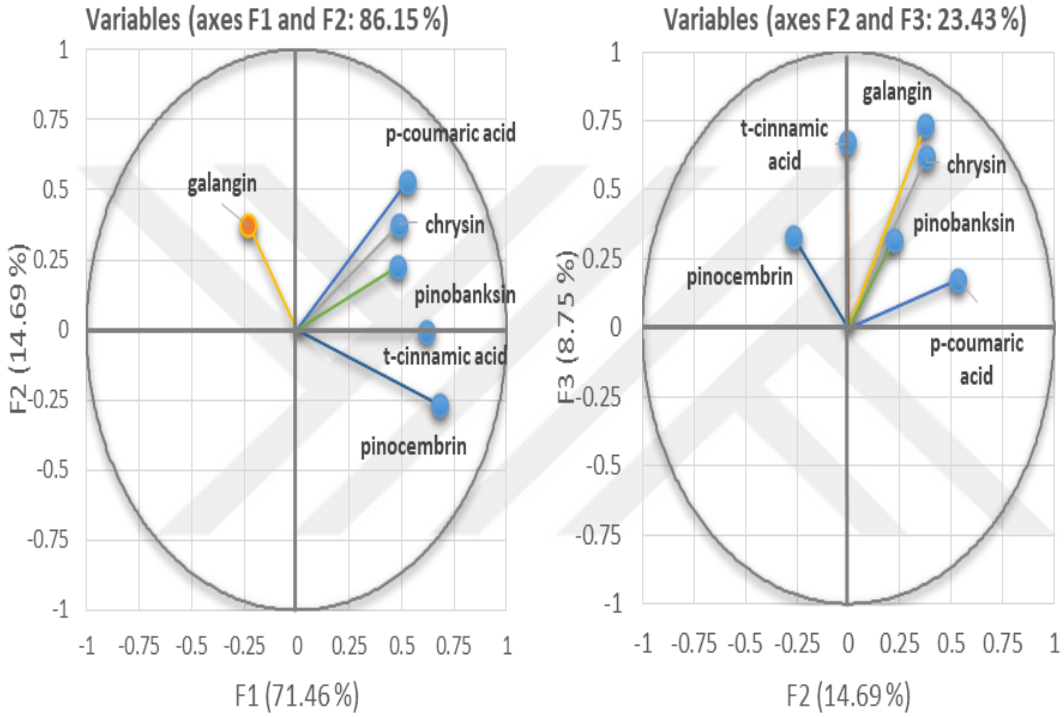


Figure 4.3 : Pearson's Coefficients (Factor Loadings) of variables on the discriminant functions: (a) Function 1 vs. Function 2 corresponding a total of 86.15% variation (b) Function 2 vs. Function 3 corresponding a total of 23.43% variation.

The scatter plot of propolis samples defined by the discriminant functions for each case is shown in Figure 4.4. Accordingly, the Black Sea, Marmara and Central Anatolia regions were differentiated from East Anatolia, Mediterranean and Aegean regions. However, it was obvious that the groups of Mediterranean and Aegean propolis are intertwined. It is noteworthy to indicate that those regions are neighboring to each other presenting a similar botanical flora (Figure 3.1). The overall correct classification rate was 80.25% using the original and 70.06 % the cross-validation method (Table 4.9), while East Anatolia and Marmara regions were correctly classified at 88.24% and 78.82%, respectively.

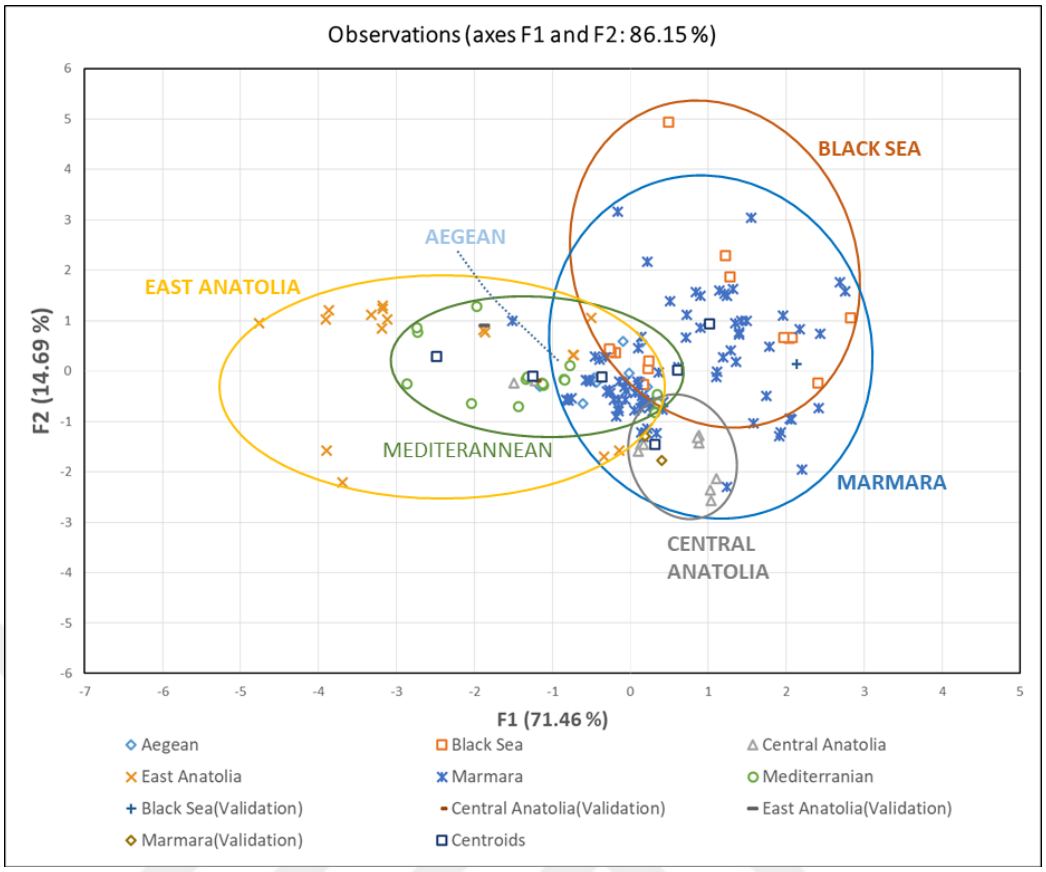


Figure 4.4 : Quadratic discriminant analysis score plots of the functions.

Table 4.9 : Cross-validation results for the classification of propolis samples based on geographical regions.

From \ To	Aegean	Black Sea	Central Anatolia	East Anatolia	Marmara	Medit.	Total	% correct
Aegean	8	2	0	0	2	0	12	66.67%
Black Sea	0	6	0	0	5	3	14	42.86%
Central Anatolia	1	0	6	0	3	1	11	54.55%
East Anatolia	0	0	0	15	0	2	17	88.24%
Marmara	6	9	0	3	67	0	85	78.82%
Mediterranean	1	0	3	6	0	8	18	44.44%
Total	16	17	9	24	77	14	157	70.06%

Furthermore, the group centroids reveal how much and in what ways the groups are differentiated on each function. The absolute magnitude of the group centroids indicates the degree to which a group is differentiated on a function, and the sign of the centroid indicates the direction of the differentiation. Figure 4.5 and Appendix E, Table E.5 shows that Function 1 discriminates the regions of Black Sea, Marmara, and Central Anatolia from other regions as they scored at the positive end on the bipolar function, and Aegean, East Anatolia, and Mediterranean regions at the

negative end of the function. Function 2 discriminated Central Anatolia from Black Sea and Marmara regions as it was at the negative end of the function and the other two regions were at the positive end of the function. The second function also discriminated East Anatolia region (at the positive end of the function) from Mediterranean and Aegean regions (at the negative end of the function). Finally, Black Sea region discriminated from Marmara region as it scored at the negative end of the function and the other was at the positive end of the function (Appendix E, Table E.5). The discrimination of Black Sea region was associated with slightly higher levels of *p*-coumaric acid and pinobanksin and lower levels of *t*-cinnamic acid (Figure 4.3 and Table 4.6).

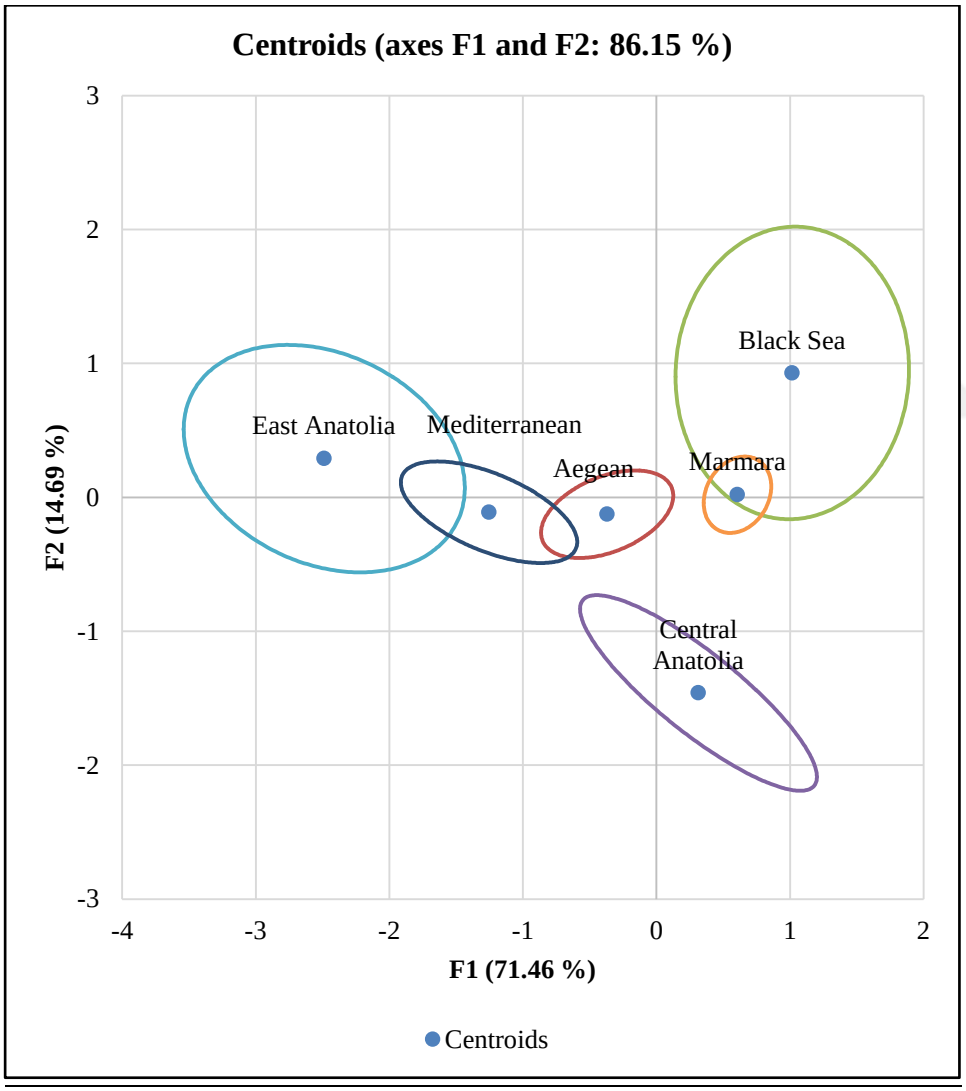


Figure 4.5 : Group average discriminant scores on two functions (F1 and F2).

4.4 Antiproliferation Effects of Propolis on Cells Lines

4.4.1 Phenolic content and profile, total antioxidant capacity of propolis concentrate

Total phenolic content, total flavonoid content, total antioxidant capacities analyzed by four different methods as ABTS, DPPH, CUPRAC and FRAP of propolis concentrate were determined as mg gallic acid equivalent (GAE)/g propolis, mg quercetin equivalent (QE)/g propolis, mg trolox equivalent (TE)/g propolis, respectively. Results are shown in Table 4.8 Total phenolic and total flavonoid contents were found to be at high levels between 314.36 mg GAE/g and 522.71 mg QE/g, respectively.

Table 4.10 : Total phenolic content, total flavonoid content, total antioxidant capacities of Turkish propolis. [1]

Total phenolic and flavonoid contents and antioxidant capacities		Turkish Propolis Sample
Total Phenolic Content (mg GAE/g)		314.36 ± 3.65
Total Flavonoid Content (mg QE/g)		522.71 ± 11.45
Total Antioxidant Capacity (mg TE/g)	ABTS	422.83 ±13.71
	DPPH	391.73 ± 12.55
	CUPRAC	1184.94 ± 63.27
	FRAP	156.59 ± 2.56

[1] Results were expressed as the mean of triplicates ± standard deviation (S.D.) and as mg GAE/g propolis for total phenolic content, mg QE/g propolis for total flavonoid content and mg TE/g propolis for total antioxidant capacities

The results of Yeşiltaş et al. (2015) also presents high antioxidant capacity values measured by four different methods. According to their results, antioxidant capacities of Turkish propolis samples varied with respect to the method applied. Antioxidant capacity values of Turkish propolis samples were 74-671 mg TE/g by ABTS, 575-1433 mg TE/g by CUPRAC, 135-454 mg TE/g by DPPH, and 140-362 mg TE/g by FRAP methods. The highest antioxidant capacity value was obtained using CUPRAC method (Yeşiltaş et al., 2015).

In our study it was observed that mixture of Turkish propolis extract is characterized with a complex nature of several compounds similar to the poplar type propolis found in other regions. According to our results, Turkish propolis represents high concentration of phenolic acids and flavonoids such as chyrisin, pinobanksin, pinostrobin, galangin and pinocembrin. The results are presented in Table 4.11.

Table 4.11 : Phenolic profile of Turkish propolis concentrate. [1]

Phenolic Compounds		mg / g propolis
Flavonoids	Pinocembrin	41.36 ± 0.41
	Pinostrobin	17.59 ± 0.78
	Chrysin	27.93 ± 6.83
	Pinobanksin	8.35 ± 0.17
	Galangin	6.66 ± 0.28
Phenolic acids	Caffeic acid	4.24 ± 0.01
	t-cinnamic acid	1.47 ± 0.03
	Ferulic acid	0.97 ±0.01
	p-coumaric acid	0.89 ± 0.01

[1] Results were expressed as the mean of triplicates ± standard deviation (S.D.)

The amounts of major phenolic compounds of propolis was consistent with our previous results (Table 4.11) : pinocembrin (41.36±0.41 mg/ g EEP), pinostrobin (17.59±0.78 mg / g EEP), chrysin (27.93±6.83 mg / g EEP), pinobanksin (8.35±0.17 mg/ g EEP), galangin (6.66±0.28 mg/ g EEP), in a descending order, respectively. The mixed and concentrated sample also contained phenolic acids including caffeic acid (4.24±0.01 mg/g EEP), *t*-cinnamic acid (1.47±0.03 mg/g EEP), ferulic acid (0.97±0.01 mg/g EEP), *p*-coumaric acid (0.89±0.01 mg/g EEP) and, respectively.

Turkish propolis extract showed high total phenolic and flavonoid content results. Sarikaya et al. (2009) also observed 313 mg GAE/g propolis in Turkish propolis sample which is very close to our total phenolic content result (Sarikaya et al., 2009). Moreira et al. (2008) analyzed the total phenolic content of Brazilian propolis from Bornos and Fundão regions and observed the values as 329 mg GAE/g in Bornos sample and 151 mg GAE/g in Fundão sample (Moreira et al., 2008). On the other hand, Moreno et al. (2000) reported total flavonoid content of propolis samples from Argentina as 13.3 to 42.6 mg QE/g of propolis (Moreno et al., 2000). Our total flavonoid result is very high compared to the Argentina propolis samples. Kumazawa et al. (2004) analyzed propolis samples collected from very different geographies (Argentina, Brazil, China, New Zealand, South Africa, Tayland Ukraine, United

States, Uzbekistan) and the results of total phenolic and flavonoid contents were observed to vary between 31.2-299 mg GAE/g and 2.5-176 mg QE/g, respectively (Kumazawa et al., 2004). Boisard et al. (2014) has reported 246.3 mg GAE/g total phenolic content for France poplar type propolis (Boisard et al., 2014). The reason for the wide range of total phenolic and total flavonoid values is that the phenolic content depends on geography, climate and plant origin (Kumazawa et al., 2004; Choi et al., 2006; Marcucci et al., 2001; Mot et al., 2011).

Turkish propolis extract also showed high antioxidant capacity values measured by four different methods. Yesiltas et al. (2015) also measured the antioxidant capacities of propolis samples supplied from Argentina and 3 samples from various regions of Turkey (Yesiltas et al., 2015). According to their results, antioxidant capacities of Turkish propolis samples varied with respect to the method applied. Our results are also in agreement with their findings as the CUPRAC method gives the highest total antioxidant capacity compared to other methods. This might be explained by differences in the solubility in different solvent systems (Arnao, 2000; Apak et al., 2007). As explained by Apak et al. (2004), CUPRAC method is advantageous over the FRAP method because the redox chemistry of copper (II)-as opposed to that of ferric ion involve faster kinetics. Besides, when compared with ABTS and DPPH methods CUPRAC reagent is much more stable and easily accessible than the chromogenic radical reagents (Apak et al., 2004).

4.4.2 Cell Culture

Antiproliferative effects of Turkish propolis extract were tested on two different breast cancer cell lines; MDA-MB-231 (ER-) and UACC-3199 (c-erbB-2 positive). Cell proliferation was measured by Tetrazolium XTT methods in propolis treated cancer cell lines after 24 h, 48 h, and 72 h treatment. The proliferation of the cell lines was also analyzed with different concentrations of propolis; the analyzed concentrations were between 0 μM and 200 μM. All time-dependent and dose-dependent results are given as the percentage value of untreated control group. These results are shown in Figures 4.1- 4.4. The most important result is that Turkish propolis has antiproliferative effect on MDA-MB-231 cell line especially at 100, 150, and 200 μM concentration after 72 h treatment (Figure 4.6).

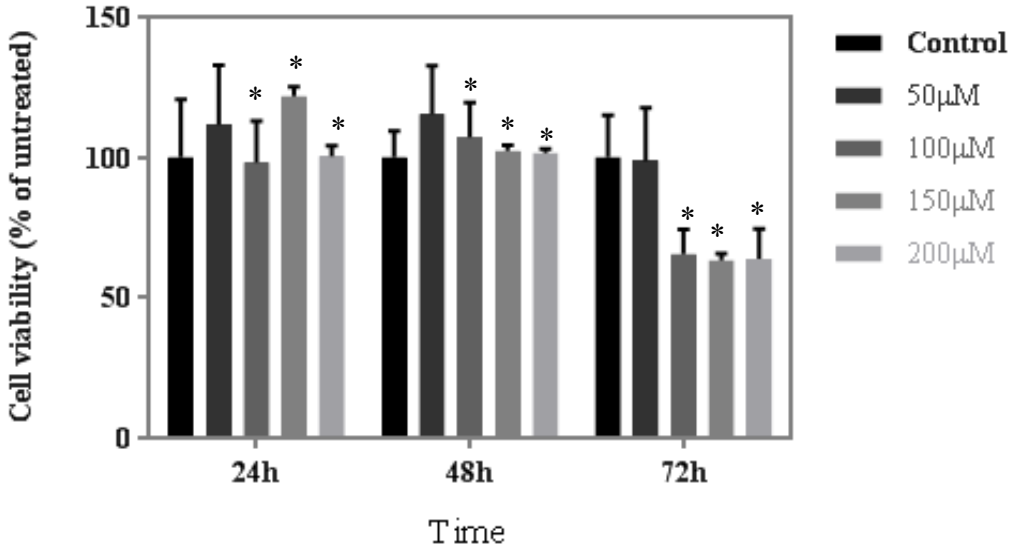


Figure 4.6 : Antiproliferative effect of propolis extract on MDA-MB-231 breast cancer cell line.

In the inhibition of proliferation in MDA-MB-231 cell line following propolis treatment was statistically significant for 72 h treatment at 100, 150, and 200 μM concentration ($p<0.001$). The inhibition of UACC cell line following 72 h treatment was less but also statistically significant ($p<0.001$) (Figure 4.7).

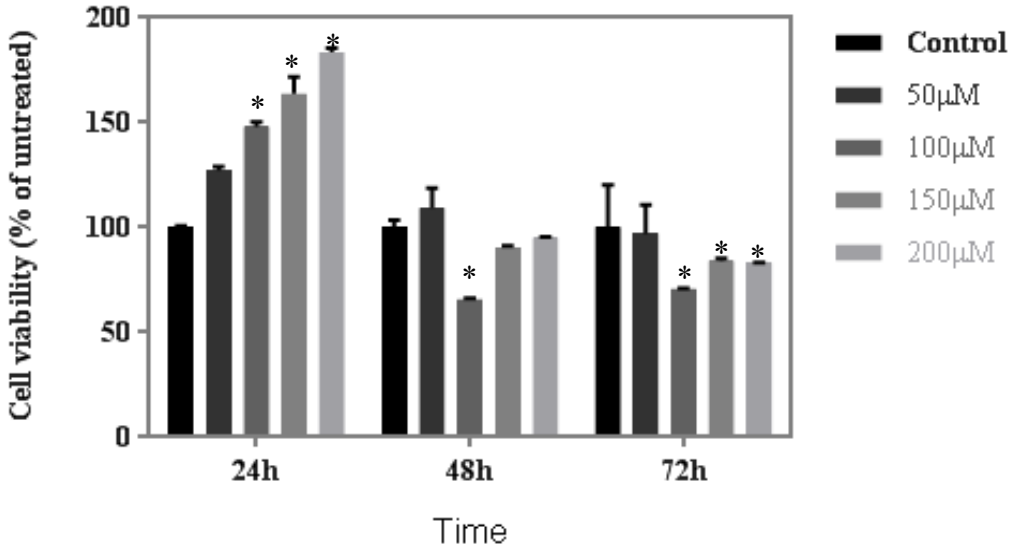


Figure 4.7 : Antiproliferative effect of propolis extract on UACC breast cancer cell line.

In the present work, the effect of propolis sample on normal fibroblast viability was also analyzed as a control group. Interestingly, the results showed that the propolis

sample has quite proliferative effect on proliferation of normal fibroblasts at 24, 48, and 72h following high-dose treatments as 200µM. (Figure 4.8).

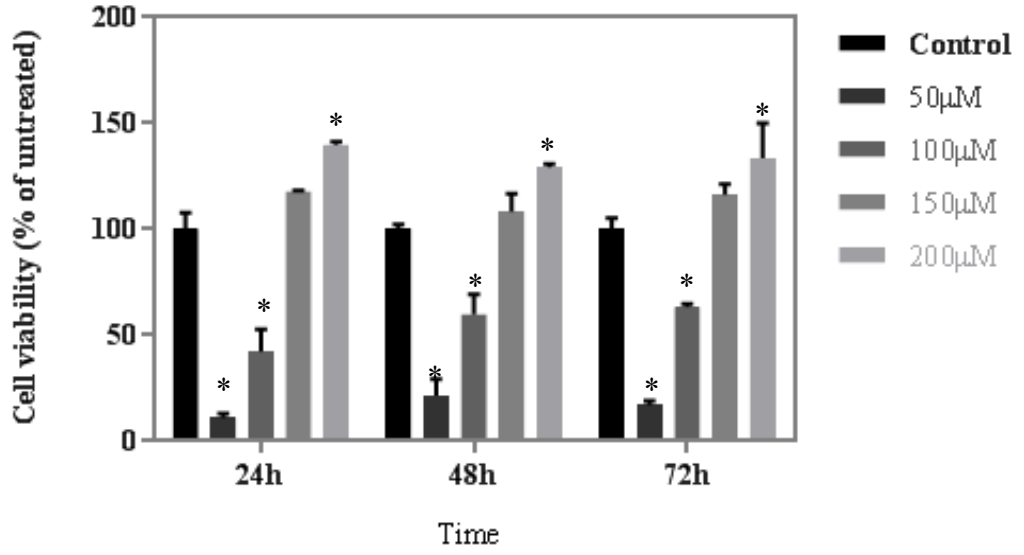


Figure 4.8 : Proliferative effect of propolis extract on normal fibroblasts.

Also, significantly higher proliferation was detected following treating strain C57B/6 mouse bone marrow derived MSCs with Turkish propolis extract. Proliferation was significant in a dose and time dependent manner (Figure 4.9).

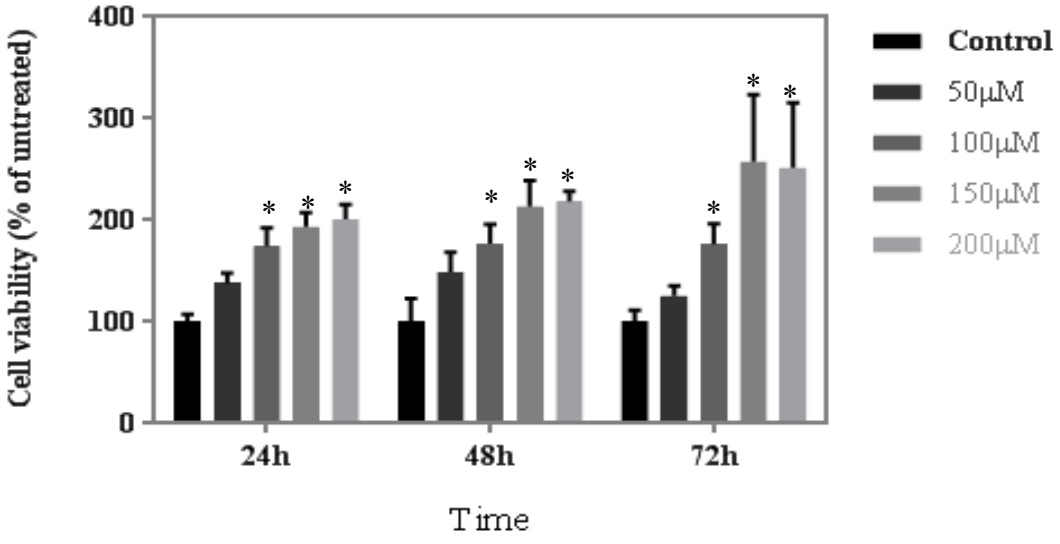


Figure 4.9 : Proliferative effect of propolis extract on C57B/6 mouse bone marrow derived MSCs.

Propolis has desirable effects with its therapeutic nature as wound healing, immunomodulation, antioxidant and antimicrobial capacity (De Castro, 2001).

Because of these valuable properties, propolis has been analyzed and evaluated in the scope of anti-cancer treatment and cell proliferation studies (Wu et al., 2011; Draganova-Filipova et al., 2008; Khacha-Ananda et al., 2013; Lotfy, 2006). As the compounds of propolis samples are variable, propolis extracts may also have different effects on different cell types *in vivo* and *in vitro* (Burdock, 1998). Relevant to this manner, it has been reported that propolis extracts with higher phenolic acids, flavonoids and CAPE causes more cytotoxic effects (Zizic et al., 2013). According to previous *in vitro* studies, methanolic extract of propolis was shown to inhibit the proliferation of U937 cells in a dose dependent manner (Motomura et al., 2008); and ethanolic extract of propolis inhibits the proliferation of lung cancer cells at around a concentration of 70 μg/ml (Frión-Herrera et al., 2015). Zizic et al. (2013) also reported that the analyzed ethanolic extract of propolis sample has inhibitory effect on HTC-116 cell line and they reached IC_{50} value at 26.33±2.97 μgmL^{-1} after 72h treatment (Zizic et al., 2013).

Breast cancer is the most common cancer type among women and the reason of many deaths. Breast cancer is a disease which has different subtypes and these subtypes show different prognosis. The amplification of the human epidermal growth factor receptor 2 gene (HER2 or c-erbB-2) was detected in approximately 20% of breast cancer cases. The protein of HER2 gene is a transmembrane receptor tyrosine kinase which is a large family with crucial roles in cell signaling. HER2 positivity is related with worse clinical outcome as higher rate of recurrence and mortality (Wolff et al., 2006). Estrogen receptor status is also another important finding and approximately 75% of breast cancer cases are treated according to ER (Fisher et al., 1996). Since ER plays a crucial role during tumor initiation, formation and progression, it was accepted as a promising target for cancer therapy (Ferguson et al., 1995). ER positive (ER+) cases are responsive to tamoxifen treatment, and the disease-free survival (DFS) increases significantly after treatment (Fisher et al., 1996). ER protein expression could be silenced via DNA methylation in breast cells which in return constitutes an ER negative (ER-) phenotype. ER- cells are not responsive to hormonal signaling cascade initiated by estrogen. It was shown that ER- breast cancer cell line, MDA-MB-231, can be sensitized via expressing exogenous ER (Ferguson et al., 1995). According to these data, it can be concluded that while HER2+ breast cancer cells show worse clinical progression, ER+ breast

cancers have milder phenotype. Interestingly, Xuan et al. (2014) showed that MDA-MB-231 cell line is more sensitive to propolis extracts in comparison to ER+ MCF-7 cell line (Xuan et al., 2014). Similar with the results of Xuan et al. (2014), in the present work, our results also showed antiproliferative effect on MDA-MB-231 cell line after 72 h treatment. ER- breast cancer cells are not responsive to hormone therapy because of not having ER receptor (Fisher et al., 1996) but it seems like they are more sensitive to propolis. As expected from the fact that HER positivity is related with worse clinical outcome, the proliferation inhibition of HER+ UACC cell line was less than ER- MDA-MB-231 cell line. But the interesting point is that propolis treatment caused a biphasic effect on UACC cell line. The proliferation was increased after 24 h treatment but decreased after 72 h treatment (Figure 4.2). Mutlu-Altundag et al. (2016) also observed a similar biphasic effect on human papillary thyroid cancer cell line after 24 h treatment with quercetin.

Previously, Turkish propolis extracts were analyzed as functional food ingredient candidates as reported by several groups (Barlak et al., 2011; Turan et al., 2015; Vatansever et al., 2015). Propolis extracts from different regions of Turkey were applied to PC-3 prostate cancer cell line and water-extracted Turkish propolis sample showed significant antiproliferative effect at 20 μg/mL concentration. Further proteomics studies after treating PC-3 cells with 20 μg/mL Turkish propolis extract indicated that water extracted propolis and DMSO extracted propolis samples caused different protein expression patterns (Vatansever et al., 2015). Another study investigated the cytotoxic effects of ethanol-extracted propolis samples of Trabzon region (Turkey) on PC-3, MCF-7, hepatocellular carcinoma (HepG2), colon adenocarcinoma (WiDr) and cervix adenocarcinoma (HeLa) cell lines (Turan et al., 2015). IC_{50} value of Trabzon EEP sample was 27.0±0.8 μg/mL for HepG2, 62.2±2.2 μg/mL for WiDr, 20.7±1.3 μg/mL for PC-3, 36.0±0.7 μg/mL for HeLa, and 28.0±2.0 μg/mL for MCF-7 (Turan et al., 2015). According to the results of Barlak, et al. (2011) and Turan, et al. (2015), Turkish propolis sample and propolis sample obtained from Trabzon region have similar cytotoxic effects on PC-3 cell line. Vatansever, et al. (2010) also worked with MCF-7 cell line with propolis samples from a different region of Turkey and had significant cell death at 125 and 63 μg/mL dilutions (Vatansever et al., 2010). It can be concluded that since the composition of propolis is strongly dependent on geographical region, season, etc., the samples

collected from different regions of the same country may also show different effects on the same cell line. Also, further studies should be performed not only to investigate the antiproliferative effects of functional foods including propolis, but also for analyzing their effects on cellular pathways which are involved in cellular homeostasis.

The selectivity of propolis extracts is as important as its cytotoxicity; in other words, propolis extracts should enhance or not affect the proliferation of normal cells or tissues while it has anti-cancer activity. Stem cells have a crucial role for the regenerative medicine studies and propolis extracts from different origins have been evaluated during the last decade. Previously, Ahangari et al. (2012) showed the anti-inflammatory effect of propolis extracts on dental pulp stem cells and Fung et al. (2015) also presented the proliferative effect of Malaysian propolis extract on stem cells from human exfoliated deciduous teeth (Ahangari et al., 2012; Fung et al., 2015).

Since cell culture studies are generally followed by *in vivo* studies with animal studies, the effect of Turkish propolis extract on mouse mesenchymal stem cells was also studied. Turkish propolis extract showed proliferative effect on strain C57B/6 mouse bone marrow derived MSCs. Considering the previous data (Ahangari et al., 2012; Fung et al., 2015), it can be speculated that propolis extracts from different sources or lands may have proliferative effects on MSCs originated from different tissues. Since sometimes *in vitro* benefits cannot be detected *in vivo*, our Turkish propolis extract should also be evaluated with *in vivo* studies to investigate its proliferative effect on stem cells in tissue scars or wound healing.

4.5 Analysis of Propolis Added Dry Fermented Sucuks

In this study, physicochemical (pH, moisture content, fat content, salt content, moisture/protein content, collagen content, color, lipid oxidation as thiobarbautiric acid reactive substances) and microbiological analyses (total aerobic bacteria, *E. coli*, total coliform, total mold and yeast, *Lactobacillus* spp., *Salmonella* spp. *Listeria monocytogenes*) were performed starting from the preparation of 9 different formulations of sucuk samples until the end of 200th day of storage period. Besides, sensory analysis was performed to understand whether there are statistically

significant differences between propolis added samples and no propolis added sample (R: reference sample).

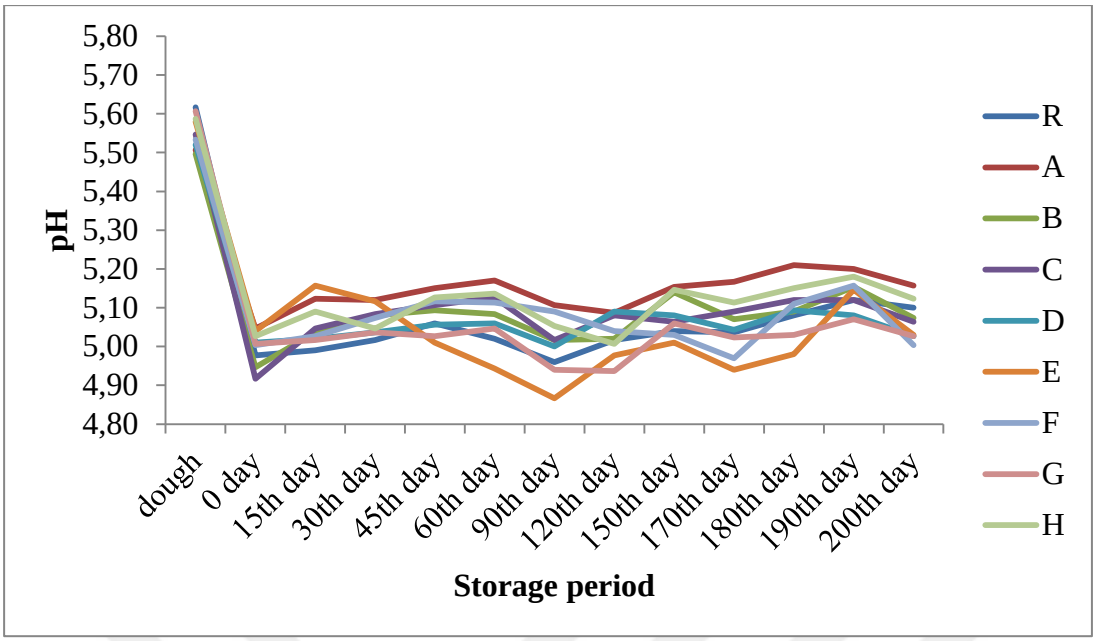
Statistical analysis was performed to investigate the differences between products on the same day of storage period and additionally between storage time intervals relevant to quality and safety parameters of heat-treated dry fermented sucuk products til the end of storage period.

4.5.1 Physicochemical analysis of heat-treated dry fermented sucuks

All chemical analyses (pH, moisture content, fat content, salt content, moisture/protein content, collagen content, color, thiobarbituric acid reactive substances) of nine heat-treated dry fermented sucuk samples were performed in triplicate and given as mean ± standard deviations. One way ANOVA analysis (SPSS ver. 24.0) was performed to understand the differences between nine treatments (products) and storage time periods. Furthermore, a General Lineer Model (GLM) of UNIVARIATE analysis was applied to selected variables where nitrite and propolis are accepted as independent two factors. Such analysis was aimed to understand the effects of those parameters and the presence of any interaction at their various levels on pH.

4.5.1.1 Change in pH values

The changes of pH values of heat-treated dry fermented sucuk samples during fermentation and storage are given in Figure 4.10. Results of triplicate analysis were given as mean ± standard deviation and statistically significant differences between each formulations of samples and storage time intervals were given in Table 4.12. pH is an important parameter in the production of fermented meat products such as sucuk. The impact of pH on meat proteins is the main determinant of water holding capacity, color, tenderness, taste and durability (Wasilewski et al., 2009).



[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

Figure 4.10 : Changes of Ph values during storage of heat-treated dry fermented sucuks. [1]

The pH values of heat-treated dry fermented sucuk formulation of dough before fermentation varied between 5.50±0.02 and 5.62±0.02. Also, there were statistically significant differences between samples at dough preparation step. The highest pH value was observed with the reference sample, while the lowest pH value with sample contains 0.50% propolis and no nitrate. However, considering the data including the changes during storage, the Univariate analysis showed that the pH differences between samples were dependent on the interactions between nitrite concentration and propolis % of samples (Figure 4.11 and Appendix E, Table E.6). In addition, Figure 4.12 presents the individual effects of propolis % and nitrite concentration on pH values showing an increase as their levels increased, except for 75 ppm level of nitrite concentration, although those effects were insignificant statistically ($p > 0.05$) (Appendix E, Table E.6). However, differences in pH of samples appeared to be related depending on interactions between levels of propolis and nitrite. It was observed that increasing propolis to 0.5% suppressed the increase in pH as expected due to the increase in nitrite concentration to 150 ppm (Figure 4.11). In cured meat products, acidity is influential mainly through its interaction

with nitrite and other factors involved in the manufacture of these products. During fermentation nitrite is depleted as nitrous acid is formed depending on decrease in the pH of the product (Sofos, 1982).

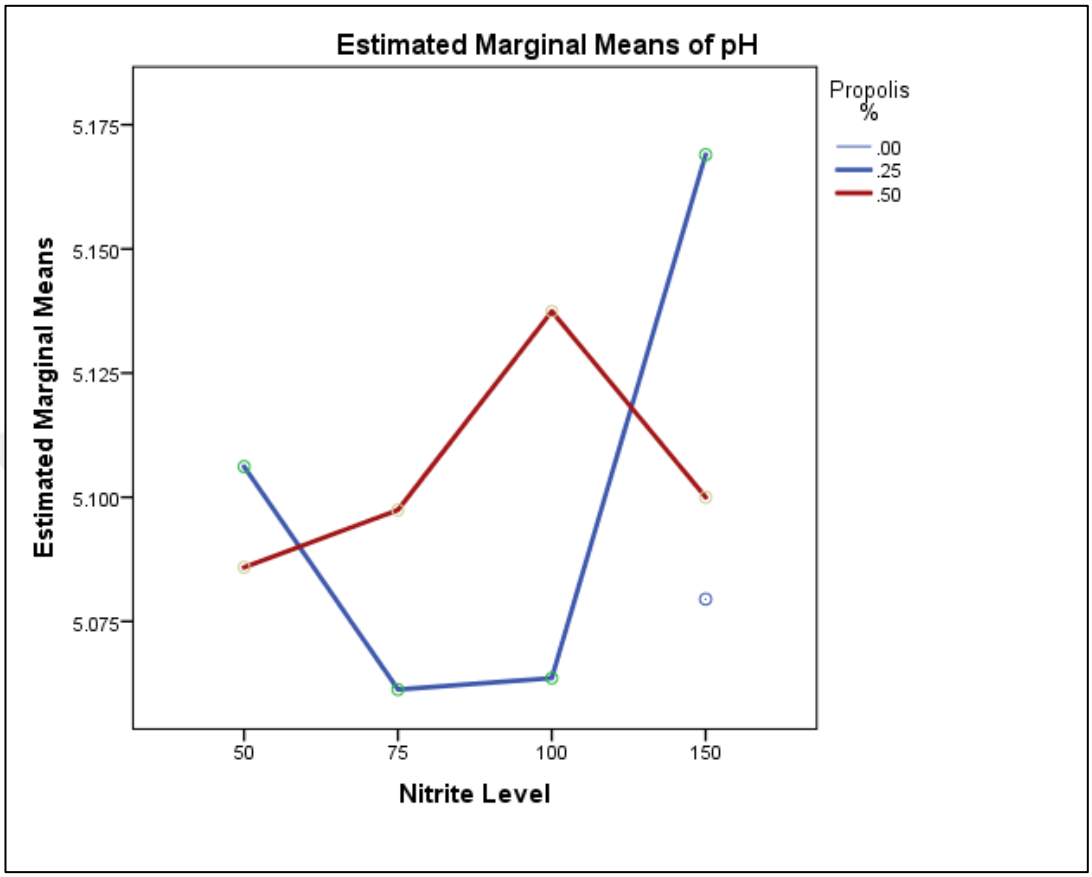
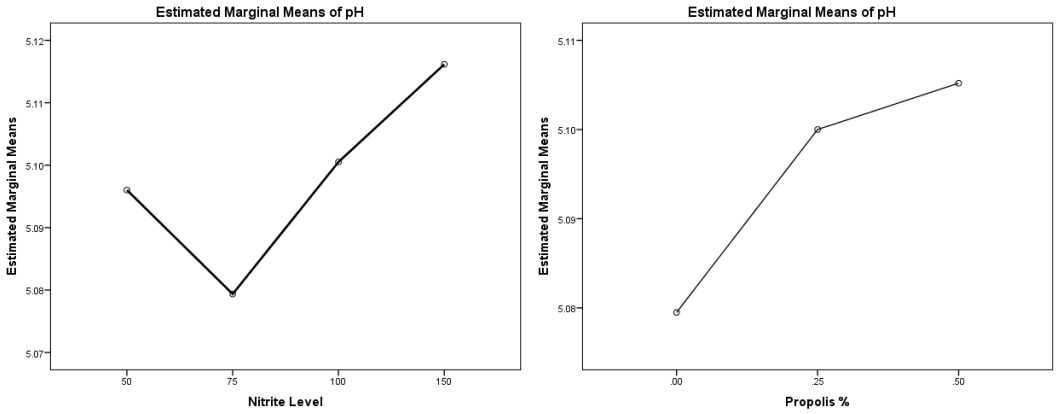


Figure 4.11 : Interaction plot of nitrite concentration and propolis % on pH values during storage of nine products ($p < 0.05$).



(a) (b)

Figure 4.12 : Change in estimated marginal means of pH depending on (a) nitrite concentration and (b) Propolis % ($p < 0.05$) in samples during storage.

With the initiation of fermentation, pH values of all heat-treated dry fermented sucuks decreased significantly ($p < 0.05$) in a range between 5.50±0.02 – 5.62±0.02 to 5.00±0.01 – 5.16±0.01 owing to the action of lactic acid bacteria at the end of the storage period. Lowering pH at the beginning of fermentation is an essential requirement, since it contributes to the inhibition of undesirable microorganisms, accelerates the development of a red colour, affects taste and reduces the water-binding capacity of proteins (Baka et al., 2011). During fermentation, a rapid decrease in pH induces conformational changes of muscle proteins, and might have caused acid-induced gelation (Zeng et al., 2013). There are statistically significant differences between products and also between sucuk doughs ($p < 0.05$). Our results are also in agreement with Bozkurt and Erkmen (2004) who studied the pH values of sucuk during fermentation and storage time.

Turkish Food Codex Communique on Meat and Meat Products (No:2012/74, 2012) states that high quality heat-treated dry fermented sucuks should have a pH value below 5.6. Our results for pH values of all heat-treated dry fermented sucuk samples have a pH value below 5.6 and all of them are acceptable according to their pH values during storage.

In general, Sebranek and Bacus (2007) describe the effect of natural additives on pH of meat products to be particularly low. However, an increase in pH with alternative curing agents in the production of meat products is often described in the literature. Increasing pH values in ham containing celery powder and concentrate, beet root were reported in conventionally cured ham during fermentation (Jackson et al., 2011; Horsch et al., 2014; Jin et al., 2014). Our findings were also in accordance with the those observations since we detected slight increases in the pH level in the samples containing propolis extracts starting from day zero to day 200.

Table 4.12 : pH Values of heat-treated dry fermented sucuk samples during 200 days storage. [1-4]

SAMPLES	R	A	B	C	D	E	F	G	H
Dough mix	5.62±0.02aA	5.51±0.01aCD	5.50±0.02aD	5.55±0.02aBC	5.52±0.01aCD	5.58±0.01aAB	5.53±0.03aCD	5.61±0.01aA	5.59±0.02aA
0 day	4.98±0.02CD	5.05±0.01hA	4.95±0.02eDE	4.92±0.02hE	5.01±0.01hiABC	5.04±0.01dAB	5.00±0.03fgBC	5.01±0.01fABC	5.03±0.02efAB
15th day	4.99±0.02ghD	5.12±0.02deAB	5.04±0.01dC	5.05±0.02fgC	5.02±0.01ghiCD	5.16±0.01bA	5.03±0.02efCD	5.02±0.02efCD	5.09±0.01dB
30th day	5.02±0.02fgD	5.12±0.01eA	5.08±0.02cABC	5.08±0.03deAB	5.04±0.01efgCD	5.12±0.02cA	5.07±0.03dBC	5.04±0.01cdeCD	5.05±0.02eBC
45th day	5.06±0.01deCD	5.15±0.02cdA	5.09±0.02cBC	5.11±0.01bcdB	5.06±0.02deCD	5.01±0.01deE	5.12±0.01cAB	5.03±0.02defDE	5.13±0.02cAB
60th day	5.02±0.01fgE	5.17±0.02cA	5.08±0.025cCD	5.13±0.01bB	5.06±0.02cdDE	4.94±0.03gF	5.11±0.03cBC	5.05±0.01bcdDE	5.14±0.02cAB
90th day	4.96±0.01hD	5.11±0.01efA	5.02±0.02d1C	5.02±0.02gC	5.00±0.01iC	4.87±0.01hE	5.09±0.01cdA	4.94±0.02gD	5.05±0.03eB
120th day	5.02±0.02fgB	5.09±0.02fA	5.02±0.01dB	5.08±0.02defA	5.09±0.01bA	4.98±0.02fC	5.04±0.01eB	4.94±0.01gD	5.01±0.02fBC
150th day	5.04±0.01efD	5.15±0.01cA	5.14±0.00bA	5.06±0.01efC	5.08±0.00bcB	5.01±0.01deE	5.03±0.00efD	5.06±0.00bcC	5.15±0.01bcA
170th day	5.04±0.01efEF	5.17±0.01cA	5.07±0.01cD	5.09±0.01cedC	5.04±0.01defE	4.94±0.01gH	4.97±0.01gG	5.02±0.01defF	5.11±0.01cdB
180th day	5.08±0.01cdD	5.21±0.01bA	5.09±0.01cD	5.12±0.01bcC	5.09±0.01bD	4.98±0.01efF	5.11±0.01cC	5.03±0.01defE	5.15±0.01bcB
190th day	5.12±0.01bD	5.2±0.01bA	5.15±0.01bC	5.12±0.01bcD	5.08±0.01bcE	5.15±0.01bC	5.16±0.01bC	5.07±0.01bE	5.18±0.01bB
200th day	5.10±0.01bcC	5.16±0.01cA	5.07±0.01cD	5.06±0.01efD	5.03±0.01fghE	5.03±0.00dE	5.00±0.01fgF	5.03±0.01defE	5.12±0.01cdB

[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

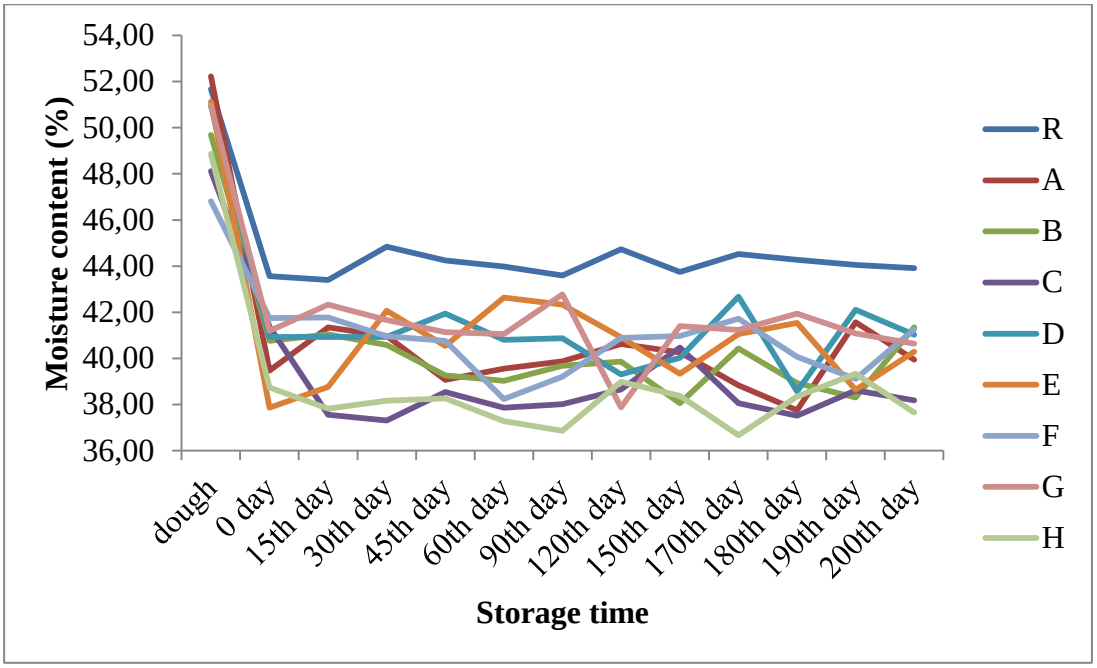
[2] Values in columns represent the mean of 3 replications and standard deviation.

[3] Values followed by the same small letters in columns are not significantly different ($p < 0.05$).

[4] Values followed by the same capital letters in rows are not significantly different ($p < 0.05$).

4.5.1.2 Moisture content

The changes of moisture contents of heat-treated dry fermented sucuk samples duirng fermentation and storage were represented in Figure 4.13. Results of triplicate analysis and statistically significant differences between each formulations of heat-treated dry fermented sucuk products and storage time intervals were shown in Table 4.13.



[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

Figure 4.13 : Changes of moisture contents during storage of heat-treated dry fermented sucuks. [1]

Moisture contents of doughs were observed to be between 46.81±0.01 – 52.23±0.02%, and there were statistically significant differences between the recipes ($p < 0.05$). After heat-treatment water content of heat-treated sucuks significantly decreased to 37.86±0.02 – 43.57±0.01% ($p < 0.05$) while the highest water content was observed in control sample that contained no propolis extract (Appendix E Table E.6). The firmness of samples is associated with the extent water removed during fermentation and ripening. Therefore, textural formation is dominated by the amount of released water in addition to acid-induced gelation of proteins (Zeng et al., 2013).

Table 4.13 : Moisture analysis results of heat-treated dry fermented sucuk samples. [1-4]

SAMPLES	R	A	B	C	D	E	F	G	H
dough	51.66±0.02aB	52.23±0.02aA	49.69±0.04aE	48.12±0.02aG	50.95±0.02aD	51.13±0.03aC	46.81±0.01aH	50.96±0.021aD	48.87±0.01aF
0 day	43.57±0.01iA	39.48±0.01hG	40.76±0.02dF	41.37±0.02bC	40.93±0.02efE	37.86±0.02lI	41.74±0.01bB	41.19±0.01gD	38.74±0.01dH
15th day	43.4±0.03jA	41.35±0.02cD	41.05±0.01cE	37.56±0.02gI	40.93±0.02efF	38.76±0.01kG	41.78±0.01bC	42.33±0.04cB	37.82±0.01hH
30th day	44.85±0.03bA	40.97±0.01dD	40.59±0.03eE	37.31±0.01gG	40.93±0.02efD	42.06±0.02dB	40.96±0.01eD	41.66±0.01eC	38.17±0.02gF
45th day	44.25±0.02eA	39.07±0.02iG	39.27±0.01iF	38.55±0.26dH	41.95±0.02dB	40.54±0.02hE	40.76±0.02dD	41.14±0.03ghC	38.27±0.01fI
60th day	43.98±0.02fgA	39.56±0.02hE	39.03±0.01jF	37.86±0.26fH	40.8±0.02gD	42.64±0.02bB	38.23±0.02hG	41.06±0.03hC	37.28±0.01jI
90th day	43.59±0.02iA	39.88±0.02gE	39.68±0.01hF	38.01±0.26efH	40.88±0.02fgD	42.33±0.02cC	39.21±0.02gG	42.78±0.03bB	36.86±0.01kI
120th day	44.73±0.03cA	40.63±0.01fC	39.86±0.03gD	38.65±0.01dG	39.31±0.02iE	40.91±0.02gB	40.88±0.01dB	37.88±0.01jH	38.99±0.02cF
150th day	43.74±0.13hA	40.26±0.09eE	38.06±0.06lI	40.47±0.06cD	40.02±0.09hF	39.34±0.09jC	40.98±0.06dG	41.41±0.05fB	38.37±0.03eH
170th day	44.52±0.05dA	38.83±0.07jG	40.44±0.07fF	38.06±0.08efH	42.68±0.09bB	41.05±0.07fE	41.72±0.12bC	41.24±0.07gD	36.68±0.08lI
180th day	44.27±0.05eA	37.75±0.16kH	38.93±0.05jE	37.51±0.05gI	38.57±0.07jF	41.54±0.05eC	40.07±0.04fD	41.94±0.07dB	38.35±0.09efG
190th day	44.05±0.03fA	41.57±0.09bC	38.3±0.12kI	38.62±0.04dH	42.12±0.09cB	38.64±0.14kG	39.12±0.10gF	41.09±0.07hD	39.34±0.09bE
200th day	43.91±0.09gA	39.95±0.07gF	41.35±0.09bB	38.18±0.01eG	41.03±0.07eC	40.29±0.12iE	41.26±0.07cB	40.64±0.09iD	37.65±0.05iH

[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

[2] Values in columns represent the mean of 3 replications and standard deviation.

[3] Values followed by the same small letters in columns are not significantly different ($p < 0.05$).

[4] Values followed by the same capital letters in rows are not significantly different ($p < 0.05$).

At the end of 200 days, the highest moisture content was observed in the control sample (R) corresponding about 15% moisture loss. On the other hand, the moisture losses of propolis samples were varied in the range of 15 – 24%, except for the sample F (12% moisture loss) (Table 4.13).

The Turkish Food Codex (TFC) Communique on Meat and Meat Products states that heat treated sucuks must have moisture content below 50% (Turkish Food Codex Communique on Meat and Meat Products (No:2012/74, 2012). All heat-treated sucuk products have a moisture content below 50% and our products are acceptable with Turkish Food Codex Communique on Meat and Meat Products.

Bilenler et al. (2017) used microencapsulated starter cultures in fermented and heat-treated sucuk formulations and analysed specific product characteristics of fermented and heat treated sucuks. They have observed a faster drying in fermented sucuk groups than heat treated ones. This might be due to the formation of a strict structure that prevents water evaporation during storage as a result of the protein denaturation at higher temperatures. They have observed moisture contents in the range of 37.11 – 38.82% and 43.02 – 46.27% after 45 days of storage for fermented and heat treated groups, respectively. Our results for heat-treated sucuk control product (44.25±0.017 % moisture content) was in accordance with their results at 45^{th} day of storage.

In addition, data obtained to the end of storage (200 days) was analysed to investigate the effects of nitrite and propolis additions on moisture contents using an GLM procedure of UNIVARIATE analysis. The results of the analysis showed that the differences in moisture values between samples were dependent on the on propolis levels and also on interactions between nitrite concentration ($p < 0.05$) (Figure 4.14, Appendix E, Table E.6). The increase in propolis percentages decreased the moisture levels in products. As propolis can only be soluble at 70% alcohol presenting hydrophobicity, its increasing levels might be facilitating the removal of moisture in products upon fermentation. On the other hand, a significant interaction observed between propolis levels and nitrite concentration (Figure 4.15) shows that the decrease in moisture contents can be influenced by the lower nitrite level (50 ppm). It is suggested that using lower amounts of sodium nitrite and addition of propolis extract may help decreasing the moisture content of heat treated sucuks.

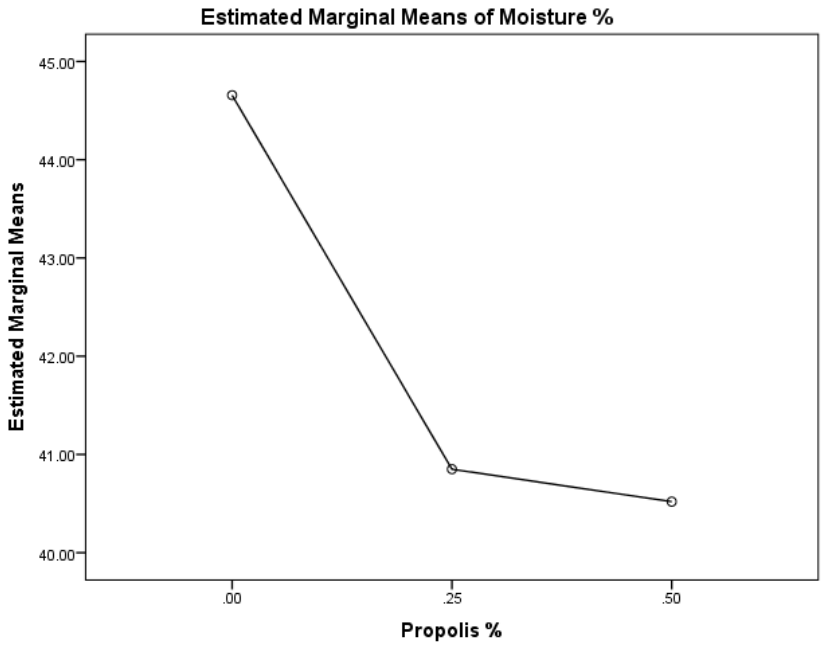


Figure 4.14 : Change in estimated marginal means of moisture contents depending on propolis % ($p < 0.05$).

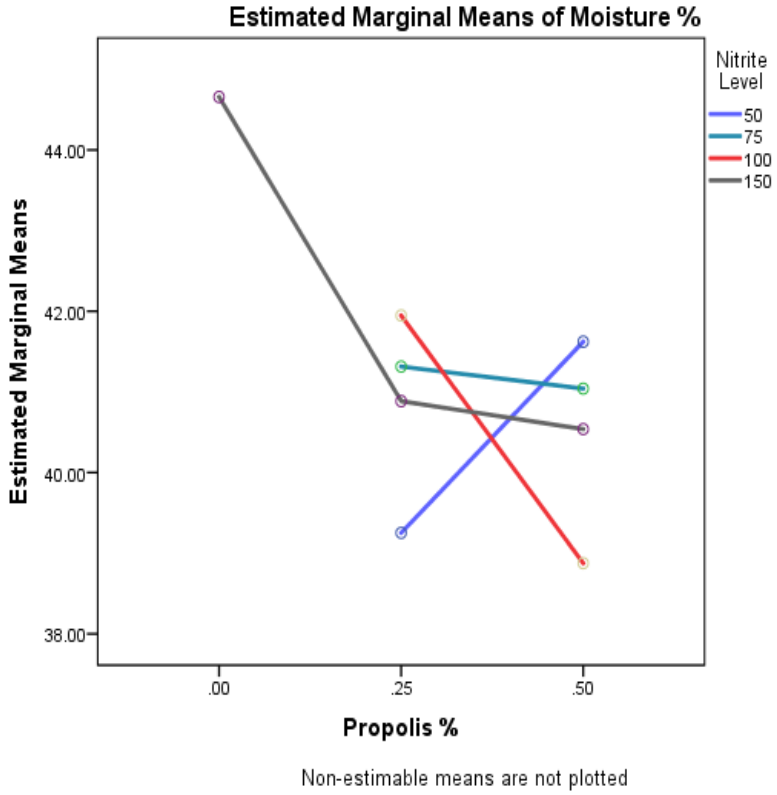
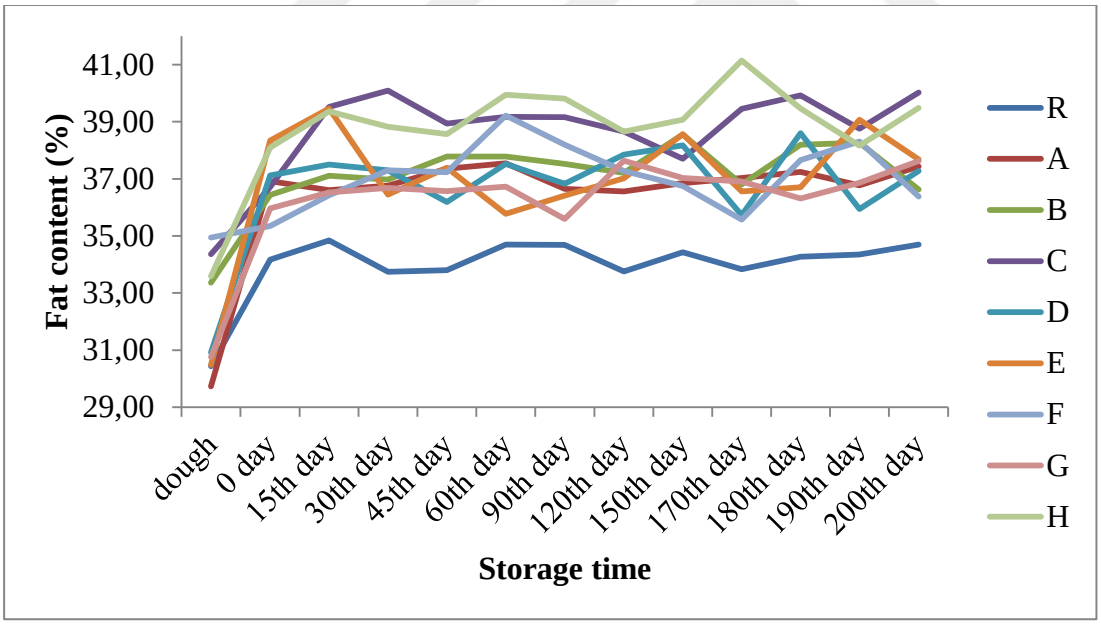


Figure 4.15 : Interaction plot of nitrite concentration and propolis % on moisture contents during storage of nine products ($p < 0.05$).

4.5.1.3 Fat content

The changes of fat contents of heat-treated dry fermented sucuk samples during fermentation and storage were given in Figure 4.16. Fat amounts based on dry matter contents and statistically significant differences between samples and storage time intervals were presented in Table 4.12. Additionally, fat amounts based on as is basis and statistically significant differences between samples and storage time intervals were presented in Appendix F, Table F.1.

Fat contents of dough samples were between 29.73±0.16% – 34.95±0.02% with significant differences according to their recipes ($p < 0.05$). Following heat-treatment their fat content increased significantly by about 7-29% in the range of 34.17±0.25 % - 38.35±0.17 %, and there were statistically significant differences between products and storage intervals ($p < 0.05$). At the end of 200 days, the increase in the fat contents of all samples was observed to be between 4-26% (Table 4.14). However, in general, the lowest fat content was observed in control sample (30.43±0.16% - 34.7±0.04%) compared to other samples during storage time.



[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

Figure 4.16 : Changes of fat contents during storage of heat-treated dry fermented sucuks. [1]

The increases in fat content of all samples are supported by the findings of Ercoşkun et al. (2010) who investigated the optimum fermentation time of heat-treated sucuks. They reported an increase in the fat content of heat-treated sucuks from 32.46±0.25% to 33.35±0.18 % after 2 days of fermentation time.

The Univariate analysis of data showed that the differences in fat values between samples were dependent on propolis %, nitrite concentration and also on interactions between those factors ($p < 0.05$) (Appendix E, Table E.6). The fat contents of samples increased as propolis addition level increased (Figure 4.18a) while increasing the nitrite level presented a decrease in fat contents of samples (Figure 4.18b). However, the fat contents were decreased with a gradual increase in nitrite concentration at 0.25% propolis level, while an increase at 0.50% propolis addition. Those results suggest that the increasing effect of propolis on fat content depends on nitrite concentration (Figure 4.17). Nitrite reacts with heme proteins (myoglobin, hemoglobin), nonheme proteins, lipids, and carbohydrates. During fermentation, it binds myoglobin to form nitrosylmyoglobin and stabilizes the porphyrin ring and prevents the release and oxidation of Fe^{2+}. Nitrite acts as a very active antioxidant during those complex set of reactions (Ford, 2012). It is apparent that there is an interaction mechanism between of propolis extract (Table 4.17) and nitrites in meat products depending on their high antioxidant activity properties. However, further studies are needed to establish that mechanism in meat products.

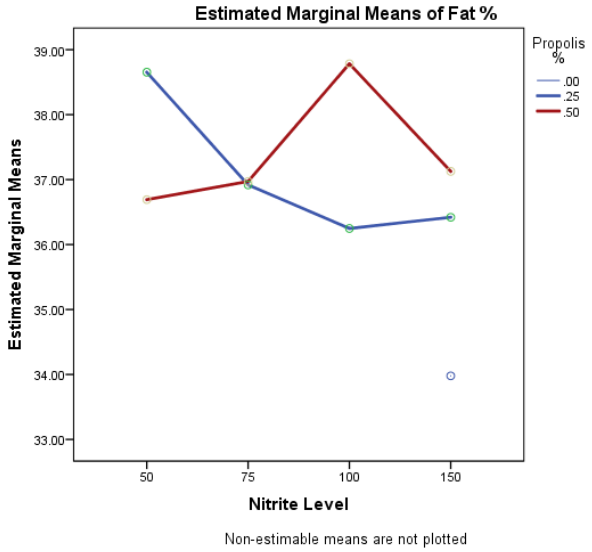
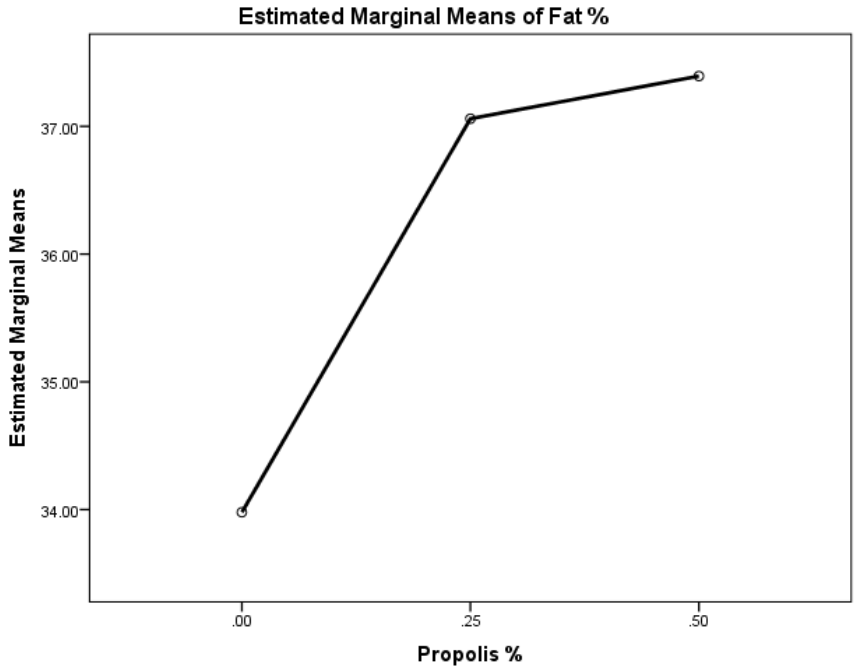
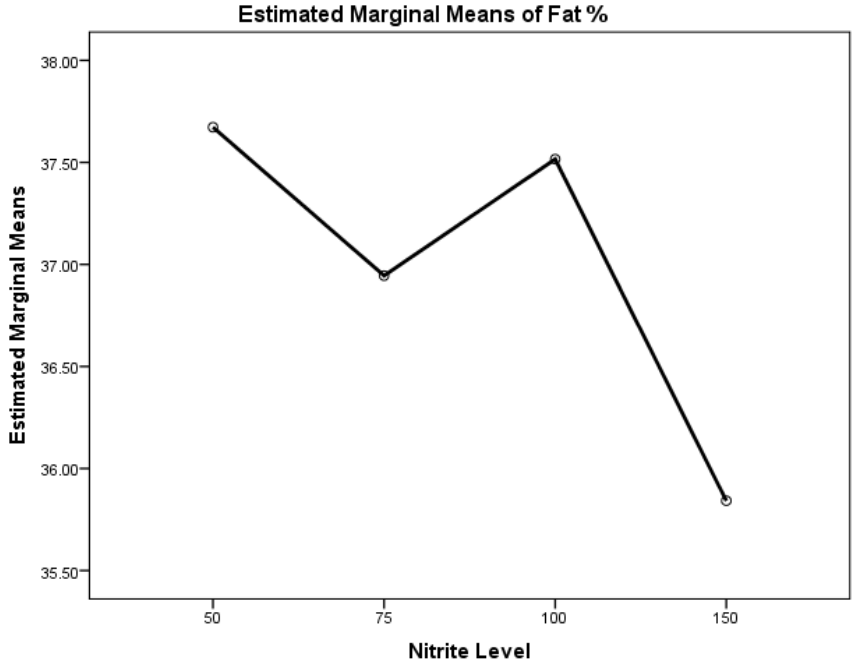


Figure 4.17 : Interaction plot of nitrite concentration and propolis % on fat contents during storage of nine products ($p < 0.05$).



(a)



(b)

Figure 4.18 : Change in estimated marginal means of fat contents depending on (a) propolis % and (b) nitrite concentration ($p < 0.05$).

Table 4.14 : Fat amounts of heat-treated dry fermented sucuk samples. [1-4]

SAMPLES	R	A	B	C	D	E	F	G	H
dough	62.96±0.32aC	62.24±0.34bE	66.3±0.32aA	66.22±0.16aA	63.04±0.08deC	62.38±0.21defDE	65.71±0.05aB	62.71±0.1bcCD	65.69±0.11aB
0 day	60.56±0.44eG	61±0.38efEFG	61.51±0.3gDE	62.62±0.18eAB	62.84±0.12eA	61.71±0.25fCD	60.69±0.02fFG	61.16±0.15fDEF	62.18±0.14hBC
15th day	61.55±0.11bcD	62.42±0.27bC	62.95±0.03bBC	63.3±0.13dB	63.49±0.14abB	64.44±0.73aA	62.57±0.34cdeC	63.34±0.25aB	63.31±0.13eB
30th day	61.19±0.25cdD	62.27±0.14bC	62.24±0.26cdefC	63.96±0.03cA	63.15±0.17cdB	62.92±0.14bcdeBC	63.18±0.74bcB	62.88±0.21bBC	62.8±0.13fBC
45th day	60.63±0.12eE	61.31±0.26deD	62.22±0.02cdefBC	63.36±0.17dA	62.35±0.16fBC	62.88±0.77bcdeAB	62.86±0.27bcdAB	62.14±0.01eC	62.47±0.16gBC
60th day	61.93±0.47bB	62.12±0.37bcB	61.96±0.32efB	63.05±0.43dA	63.38±0.13bcA	62.37±0.28defB	63.49±0.02bA	62.32±0.13deB	63.69±0.15dA
90th day	61.5±0.12bcdCD	60.95±0.27efD	62.21±0.02cdefBC	63.18±0.17dA	62.3±0.16fB	63.13±0.79bcdA	62.84±0.33bcdAB	62.21±0.23eBC	63.05±0.13fA
120th day	61.07±0.25cdeFG	61.58±0.14dEF	61.86±0.25efgDE	63.02±0.03dAB	62.37±0.16fCD	62.66±0.14cdeBC	63.06±0.74bcAB	60.59±0.19gG	63.36±0.13eA
150th day	61.21±0.12cdG	61.68±0.12cdF	62.25±0.04cdeE	63.33±0.02dCD	63.66±0.06aA	63.57±0.11bcAB	62.27±0.07deE	63.2±0.06aD	63.41±0.08eBC
170th day	61±0.08deG	60.55±0.06fH	61.79±0.15fgF	63.7±0.06cB	62.33±0.07fD	62.02±0.04efE	61.03±0.11fG	62.81±0.07bcC	64.97±0.07bA
180th day	61.49±0.04bcdE	59.84±0.16gD	62.55±0.07cC	63.9±0.04cA	62.84±0.11eB	62.79±0.1bcdeB	62.83±0.09bcdB	62.54±0.12cdC	64.02±0.16cA
190th day	61.4±0.06bcdF	62.94±0.1aC	62.03±0.09defE	63.14±0.03dB	62.1±0.12fE	63.68±0.08abA	62.92±0.15bcdC	62.57±0.12bcdD	62.91±0.06fC
200th day	61.87±0.03bE	62.33±0.05bD	62.45±0.12cdD	64.74±0.01bA	63.22±0.09bcdBC	63.11±0.12bcdC	61.94±0.09eE	63.4±0.06aB	63.34±0.1eB

[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

[2] Values in columns represent the mean of 3 replications and standard deviation.

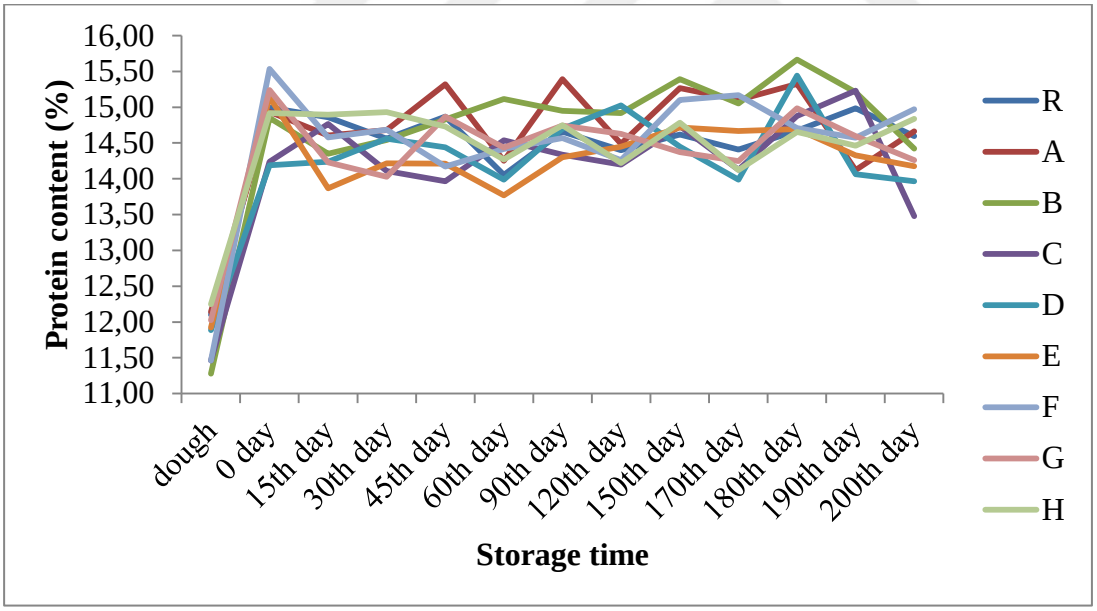
[3] Values followed by the same small letters in columns are not significantly different ($p < 0.05$).

[4] Values followed by the same capital letters in rows are not significantly different ($p < 0.05$).

4.5.1.4 Protein Content

The changes of protein contents of heat-treated dry fermented sucuk samples during fermentation and storage were given in Figure 4.19. Protein amounts based on dry matter contents and statistically significant differences between samples and storage time intervals were presented in Table 4.15. Additionally, protein amounts based on as is basis and statistically significant differences between samples and storage time intervals were presented in Appendix F, Table F.2.

Protein contents of heat-treated sucuk doughs were between 11.28±0.04%-12.25±0.01%. Following heat-treatment protein contents of heat-treated sucuks increased by about 19-34 % in the range of 14.19±0.01% – 15.53±0.02% due to the decrease in their moisture contents. According to Table 4.13 amounts of protein in dough samples varying between 21.55±0.02% – 25.42±0.07% on dry matter basis. The protein contents of samples at the end of 200 days ranged between 21.8±0.02% – 26,02±0,03% on dry matter basis (Table 4.15) showing a slight change.



[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

Figure 4.19 : Changes of fat contents during storage of heat-treated dry fermented sucuks. [1]

Table 4.15 : Protein analysis results of heat-treated dry fermented sucuk samples in dry matter basis. [1-4]

SAMPLES	R	A	B	C	D	E	F	G	H
dough	25.03±0.06fB	25.42±0.07bA	22.42±0.09hG	22.09±0.02iH	24.23±0.05fE	24.39±0.04efD	21.55±0.02kI	24.52±0.02fC	23.96±0.03cF
0 day	26.57±0.03bB	24.68±0.05eE	25.07±0.02cD	24.29±0.01bG	24.02±0.02hH	24.37±0.03fF	26.66±0.04aA	25.92±0.05aC	24.35±0.01aFG
15th day	26.25±0.02dA	24.9±0.07dC	24.34±0.03gE	23.65±0.01dH	24.11±0.04gF	22.64±0.03lI	25.04±0.02eB	24.68±0.03eD	23.96±0.02cG
30th day	26.4±0.01cA	24.87±0.04dB	24.48±0.03fE	22.5±0.01hH	24.65±0.02dC	24.54±0.03dD	24.87±0.04fB	24.04±0.03hG	24.15±0.02bF
45th day	26.67±0.04abA	25.14±0.03cC	24.42±0.03fgE	22.73±0.09gG	24.88±0.02bD	23.9±0.01iF	23.93±0.08iF	25.26±0.02cB	23.86±0.03dF
60th day	25.09±0.04fA	23.58±0.07hE	24.79±0.02dB	23.39±0.09eF	23.63±0.02iE	24±0.03hD	23.34±0.01jF	24.49±0.01fC	22.75±0.03gG
90th day	25.99±0.01eA	25.6±0.02aC	24.78±0.01dD	23.13±0.14fG	24.86±0.03bD	24.79±0.01cD	23.97±0.02iE	25.77±0.06bB	23.36±0.04fF
120th day	26.05±0.01eA	24.4±0.04fC	24.81±0.04dB	23.14±0.02fG	24.75±0.01cB	24.46±0.05eC	24.12±0.07hD	23.55±0.05iE	23.31±0.01fF
150th day	25.99±0.07eA	25.56±0.04aB	24.85±0.03dC	24.75±0.03aD	24.09±0.05ghG	24.26±0.05gF	25.58±0.03cB	24.53±0.06fE	23.99±0.02cH
170th day	25.97±0.09eA	24.69±0.03eD	25.27±0.03bB	22.81±0.04gG	24.41±0.06eE	24.88±0.03bC	26.02±0.05bA	24.24±0.03gF	22.3±0.02hH
180th day	26.32±0.16cdA	24.62±0.06eE	25.65±0.02aC	23.81±0.03cF	25.14±0.05aD	25.14±0.03aD	24.54±0.04gE	25.81±0.02bB	23.77±0.03eF
190th day	26.78±0.04aA	24.16±0.05gE	24.65±0.03eC	24.81±0.01aB	24.3±0.03fD	23.35±0.06kH	23.94±0.05iF	24.78±0.01dB	23.84±0.04dG
200th day	26.02±0.03eA	24.42±0.06fD	24.59±0.05eC	21.8±0.02jH	23.68±0.04iG	23.74±0.02jFG	25.49±0.03dB	24.02±0.02hE	23.8±0.05deF

[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

[2] Values in columns represent the mean of 3 replications and standard deviation.

[3] Values followed by the same small letters in columns are not significantly different ($p < 0.05$).

[4] Values followed by the same capital letters in rows are not significantly different ($p < 0.05$).

The Turkish Food Codex (TFC) Communique on Meat and Meat Products states that heat treated sucuks must have protein content more than 14% (Turkish Food Codex Communique on Meat and Meat Products (No:2012/74), 2012). All heat-treated sucuk products have a protein content above 14% until the end of 190th day and our products are acceptable with Turkish Food Codex Communique on Meat and Meat Products. However, only two samples, C (50 ppm sodium nitrite + 0.25% propolis) and D (50 ppm nitrite + 0.50% propolis), did not meet that requirement as their protein contents reached to 13.48±0.016% and 13.96±0.012%, respectively at 200th day of storage (Appendix F, Table F.2).

Ercoşkun et al. (2010) similarly observed an increase in the protein contents of sucuks from 15.49±0.06% to 16.45±0.09% after 2 days of fermentation. Although our results were slightly lower than their results for protein content, all the protein contents increased gradually throughout fermentation as a result of drying. They have also found that for each fermentation interval heat treatment caused significant increases in salt, ash, fat and protein contents ($p < 0.05$). They have observed higher protein contents when fermentation time increased to 5 days (Ercoşkun et al., 2010).

In some recent studies, Turkish dry-fermented sucuks are reported to have slightly higher protein content values in the range of 17.01 – 21.83% compared to our results (Özer et al., 2016; Kılıç & Özer, 2017). It is suggested that such increases are associated with protein denaturation and evaporation of moisture during heat treatment. Furthermore, univariate analysis of data revealed that nitrite levels affected protein content (Figure 4.20). As nitrite concentration increased a higher protein content of samples was observed proposing interaction of nitrites with heme proteins rendering denaturation of protein moiety (globin) or displacement of protein with nitric oxide at one of the coordination sites of heme iron (Xiong, 2012).

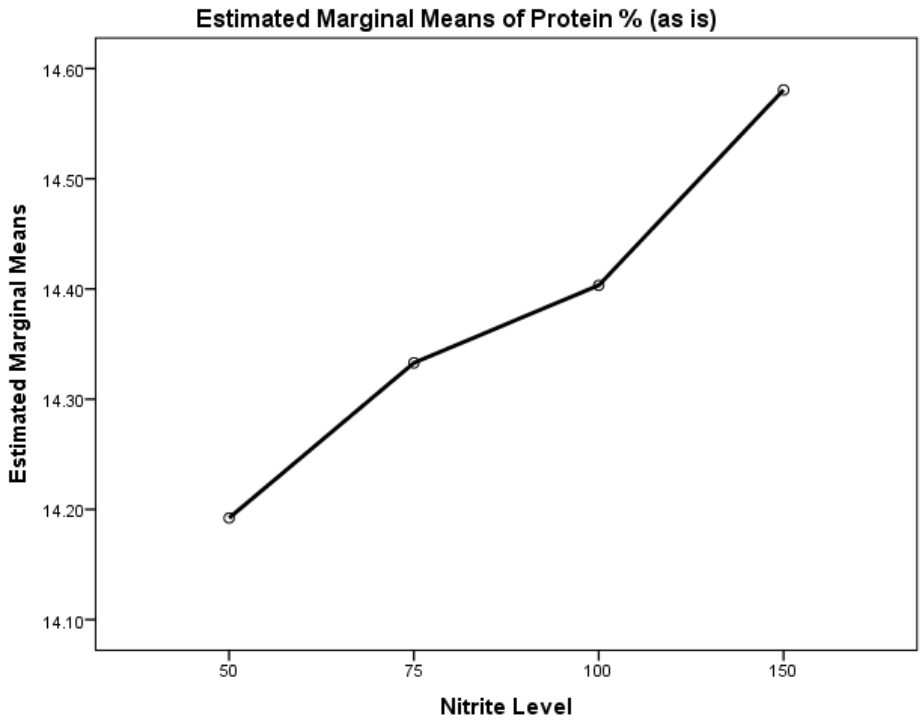


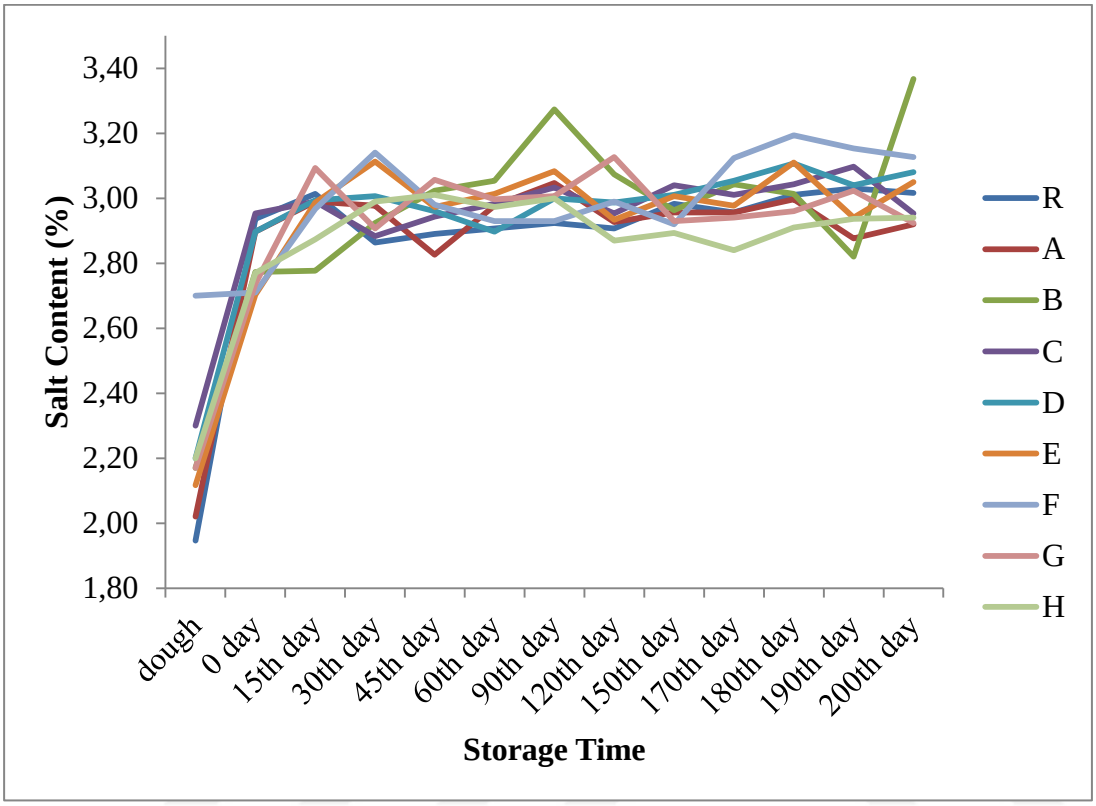
Figure 4.20 : Change in estimated marginal means of protein % depending on nitrite levels in samples ($p < 0.05$).

4.5.1.5 Salt Content

The changes of salt contents of heat-treated dry fermented sucuk samples during fermentation and storage were given in Figure 4.21. Salt amounts based on dry matter contents and statistically significant differences between samples and storage time intervals were presented in Table 4.16. Additionally, salt amounts based on as is basis and statistically significant differences between samples and storage time intervals were presented in Appendix F, Table F.3.

Salt content of heat-treated sucuks were between 1.95±0.02% – 2.3±0.02% and there were statistically significant differences in sucuk doughs. After heat-treatment salt content of heat-treated sucuks increased significantly ($p < 0.05$) due to the increase in dry matter content. After heat treatment, at the beginning of storage period, the salt content was between a range from 2.7±0.041% to 2.94±0.033% salt content. The salt content was increased during 200 day of storage as moisture content decreases. The highest salt content was reached at sample C that contains 150 ppm sodium nitrite and 0.50% propolis extract as 3.37±0.406% salt content at the end of 200 day of storage.

Ercoşkun et al. (2010) also found similar results before heat treatment (2.97 ± 0.01%) and after heat treatment (3.18 ± 0.02%) of sucuks. They have also observed that salt content increase significantly after heat treatment.



[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

Figure 4.21 : Changes of salt contents during storage of heat-treated dry fermented sucuks. [1]

Table 4.16 : Salt analysis results of heat-treated dry fermented sucuk samples in dry matter basis. [1-4]

SAMPLE	R	A	B	C	D	E	F	G	H
dough	4.03±0.04dE	4.23±0.02gD	4.31±0.03eC	4.43±0.03eB	4.49±0.03dB	4.33±0.05fC	5.08±0.05bcA	4.42±0.01iB	4.3±0.05eCD
0 day	5.2±0.06bcA	4.79±0.02eD	4.68±0.04cdeE	5.04±0.04abB	4.9±0.04cC	4.35±0.06fG	4.65±0.06fE	4.65±0.02hE	4.52±0.06dF
15th day	5.32±0.07abcA	5.1±0.06aB	4.71±0.02cdeDE	4.79±0.06cdCD	5.07±0.01bB	4.88±0.05deC	5.1±0.02bcB	5.36±0.04aA	4.62±0.04cdE
30th day	5.19±0.04cB	5.04±0.02abCD	4.92±0.04cdEF	4.6±0.04deG	5.09±0.02bC	5.37±0.07aA	5.32±0.06aA	4.98±0.02fgDE	4.84±0.06abF
45th day	5.18±0.13cA	4.64±0.02fE	4.98±0.04bcdBC	4.79±0.03cdD	5.1±0.06bAB	5±0.07cdBC	5.03±0.06cdB	5.19±0.02bcA	4.88±0.06aCD
60th day	5.19±0.06cAB	4.92±0.02cDE	5.01±0.04bcdCD	4.8±0.03cdEF	4.89±0.02cDE	5.25±0.07abA	4.74±0.06efF	5.08±0.02deBC	4.74±0.15abcF
90th day	5.18±0.04cC	5.07±0.02aD	5.43±0.04abA	4.89±0.03bcE	5.07±0.06bD	5.35±0.07aAB	4.82±0.06eEF	5.25±0.02bBC	4.75±0.06abcF
120th day	5.26±0.06abcA	4.93±0.02cDE	5.11±0.04bcB	4.81±0.04cF	4.92±0.02cE	4.96±0.07cdCDE	5.06±0.06bcdBC	5.03±0.02efBCD	4.7±0.06bcG
150th day	5.3±0.15abcA	4.95±0.06bcBC	4.78±0.04cdCD	5.11±0.17aB	5.02±0.02bB	4.96±0.07cdBC	4.95±0.06dBC	5±0.05fgB	4.69±0.03bcD
170th day	5.33±0.09abcA	4.83±0.07cdeD	5.11±0.04bcB	4.86±0.16bcCD	5.33±0.02aA	5.05±0.07cB	5.36±0.05aA	5±0.06fBC	4.48±0.02dE
180th day	5.4±0.08aA	4.81±0.09deDE	4.93±0.1cdBCD	4.87±0.15bcCDE	5.06±0.01bBC	5.32±0.09aA	5.33±0.05aA	5.1±0.06deB	4.72±0.03bcE
190th day	5.42±0.03aA	4.92±0.06cdDE	4.57±0.11deF	5.04±0.08abCD	5.25±0.11aAB	4.79±0.1eE	5.18±0.07bBC	5.13±0.06cdBC	4.84±0.05abE
200th day	5.38±0.02abAB	4.86±0.06cdeBCD	5.74±0.7aA	4.78±0.1cdCD	5.22±0.06aABCD	5.11±0.04bcBCD	5.32±0.06aABC	4.92±0.04gBCD	4.72±0.09bcD

[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

[2] Values in columns represent the mean of 3 replications and standard deviation.

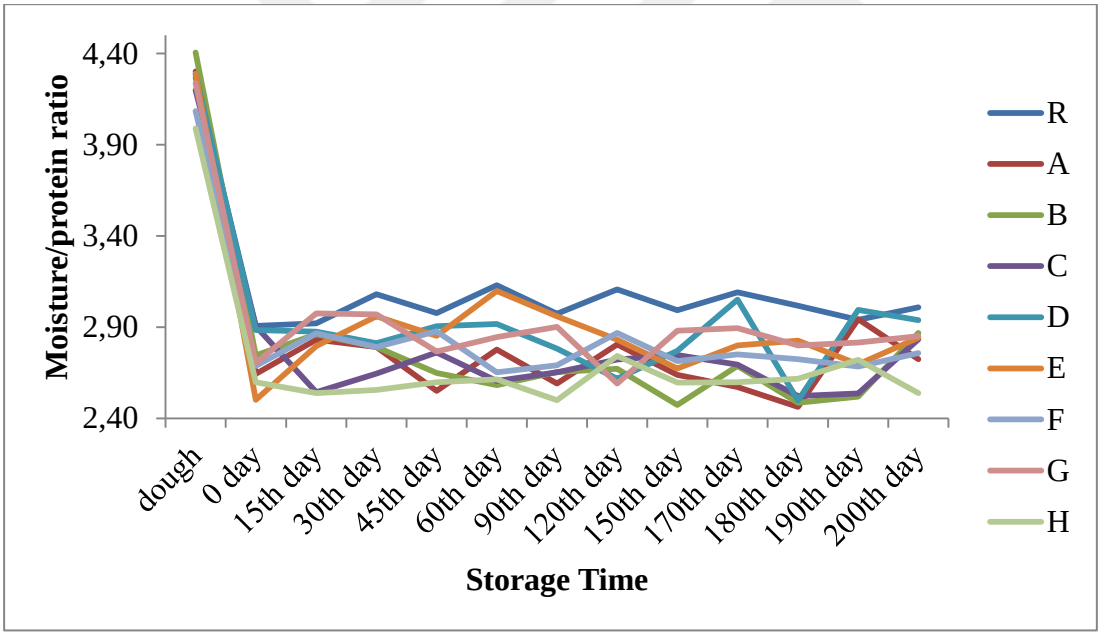
[3] Values followed by the same small letters in columns are not significantly different ($p < 0.05$).

[4] Values followed by the same capital letters in rows are not significantly different ($p < 0.05$).

4.5.1.6 Moisture/Protein ratio

The changes of moisture/protein values of heat-treated dry fermented sucuk samples during fermentation and storage were given in Figure 4.22 and Table 4.17.

Moisture/protein ratios of heat-treated sucuk doughs were between 3.99±0.01 - 4.4±0.01 and there were statistically significant differences between samples ($p < 0.05$). After heat treatment moisture/protein ratios were decreased in all products by about 34 – 38% in a range between 2.5±0.00 and 2.91±0.002 significantly ($p < 0.05$). However following day zero to the end of 200th day of storage moisture/protein ratios decreased and reached to 2.54±0.01 and 3.01±0.01 (Table 4.17). The highest moisture/protein value was observed in control sample at the end of 200th day of storage (3.01±0.01). In addition, the moisture/protein values of all samples containing propolis was lower than the control sample in the range of 2.54±0.00 – 2.87±0.01 (Table 4.17).



[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

Figure 4.22 : Changes of moisture/protein values during storage of heat-treated dry fermented sucuks. [1]

Table 4.17 : Moisture/protein ratio of heat-treated dry fermented sucuk samples [1-4]

SAMPLES	R	A	B	C	D	E	F	G	H
dough	4.27±0.01aC	4.30±0.01aB	4.40±0.01aA	4.20±0.00aE	4.29±0.01aBC	4.29±0.01aB	4.08±0.01aF	4.24±0.01aD	3.99±0.01aG
0 day	2.91±0.00hA	2.64±0.01gF	2.74±0.00dC	2.91±0.00bA	2.88±0.00fB	2.50±0.00jH	2.69±0.00fE	2.70±0.01iD	2.60±0.00eG
15th day	2.92±0.01hB	2.83±0.01cE	2.86±0.01bD	2.54±0.001iG	2.87±0.00gC	2.80±0.01gF	2.87±0.00bCD	2.97±0.00bA	2.54±0.00gG
30th day	3.08±0.01dA	2.79±0.01deE	2.79±0.00cE	2.65±0.00gF	2.81±0.00hD	2.96±0.00cC	2.79±0.00cE	2.97±0.00bB	2.56±0.00fG
45th day	2.98±0.00fA	2.55±0.00jH	2.65±0.00gF	2.76±0.02dE	2.91±0.00eB	2.85±0.00dD	2.88±0.01bC	2.77±0.01hE	2.60±0.00eG
60th day	3.13±0.00bA	2.78±0.01eE	2.58±0.00hH	2.60±0.020hG	2.92±0.00eC	3.10±0.01bB	2.65±0.00gF	2.84±0.00eD	2.61±0.00dG
90th day	2.97±0.00fA	2.59±0.00hG	2.65±0.00gF	2.65±0.01gF	2.78±0.00iD	2.96±0.00cB	2.69±0.00fE	2.90±0.00cC	2.50±0.00hH
120th day	3.11±0.01cA	2.80±0.01dD	2.67±0.00fG	2.72±0.00eF	2.62±0.00jH	2.83±0.00efC	2.87±0.01bB	2.59±0.01jI	2.74±0.00bE
150th day	2.99±0.015fA	2.64±0.01gG	2.47±0.00jI	2.75±0.00dD	2.77±0.01iC	2.67±0.01iF	2.71±0.01eE	2.88±0.01dB	2.60±0.00eH
170th day	3.09±0.01cdA	2.57±0.01iH	2.69±0.01eF	2.69±0.01fF	3.05±0.01bB	2.80±0.01gD	2.75±0.01dE	2.89±0.01cC	2.60±0.01eG
180th day	3.02±0.013eA	2.46±0.01kH	2.49±0.01jG	2.52±0.00jF	2.50±0.01kG	2.83±0.00fB	2.72±0.00eD	2.80±0.01gC	2.62±0.01dE
190th day	2.94±0.01gB	2.94±0.01bB	2.52±0.01iH	2.54±0.00ijG	2.99±0.01cA	2.70±0.01hE	2.68±0.01fF	2.81±0.01fC	2.72±0.01cD
200th day	3.01±0.01eA	2.72±0.00fG	2.87±0.01bC	2.83±0.00cE	2.94±0.01dB	2.84±0.01deDE	2.76±0.01dF	2.85±0.01eD	2.54±0.00gH

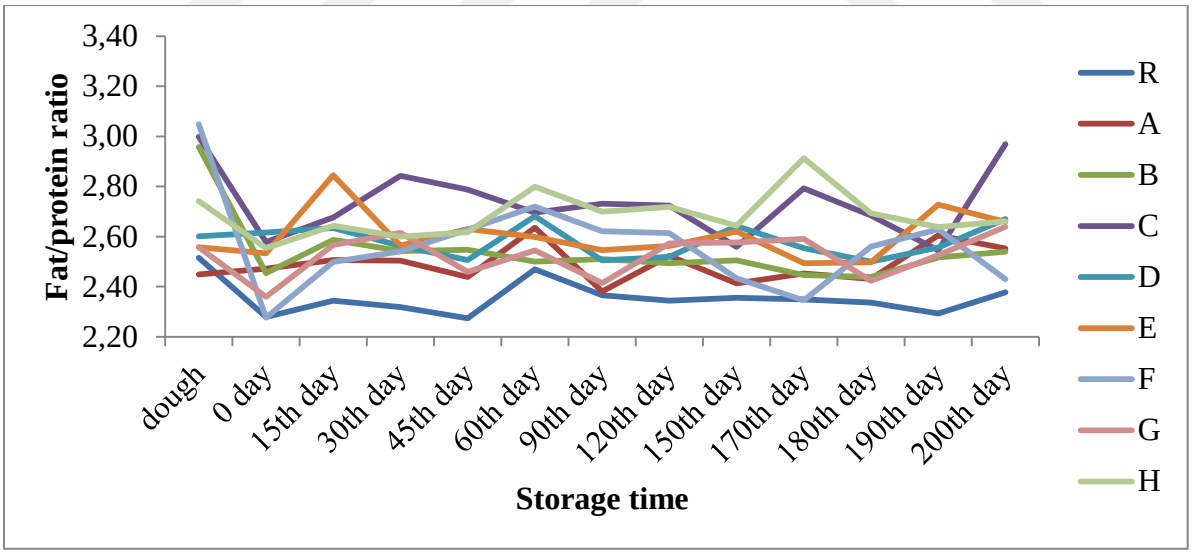
[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.
[2] Values in columns represent the mean of 3 replications and standard deviation.
[3] Values followed by the same small letters in columns are not significantly different ($p < 0.05$).
[4] Values followed by the same capital letters in rows are not significantly different ($p < 0.05$).

It has been shown that the water/protein ratio has a significant influence on the textural characteristics of sucuk (Colmenero et al., 1995). The sucuk will lose more water when cooked due to the higher water/protein ratio. This higher water/protein ratio will also cause the ionic strength to be lower, decreasing the extraction of meat proteins (Colmenero et al., 1995; Bengtsson et al., 2011). Accordingly, as all the propolis extract containing sucuk samples presented a lower water/protein ratio than the control sample, addition of propolis positively contributes to the quality of sucuk product. As expected lower moisture/protein ratio will improve the acceptance of product as they will lose less water upon cooking.

The Turkish Food Codex Communique on Meat and Meat Products states that heat treated sucuks must have a moisture/protein value below 3.6 to maintain its microbiological stability during shelf life (Turkish Food Codex Communique on Meat and Meat Products (No: 2012/74, 2012). Therefore, it is apparent that all heat-treated sucuk products with a moisture/protein values below 3.6 fulfill that requirement in the Turkish legislation.

4.5.1.7 Fat/Protein ratio

The changes of fat/protein values of heat-treated dry fermented sucuk samples during fermentation and storage were given in Figure 4.23 and Table 4.18.



[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

Figure 4.23 : Changes of fat/protein values during storage of heat-treated dry fermented sucuks. [1]

Table 4.18 : Fat/protein ratio of heat-treated dry fermented sucuk samples. [1-4]

SAMPLES	R	A	B	C	D	E	F	G	H
dough	2.52±0.02aG	2.45±0.02efH	2.96±0.00aC	3.00±0.00aB	2.60±0.01dE	2.56±0.01fF	3.05±0.01aA	2.56±0.00eF	2.74±0.01cD
0 day	2.28±0.02gF	2.47±0.02eD	2.45±0.01fD	2.58±0.01hB	2.62±0.01cA	2.53±0.01fgC	2.28±0.00iF	2.36±0.01jE	2.55±0.01jC
15th day	2.34±0.00deF	2.51±0.02dE	2.59±0.00bD	2.68±0.01gB	2.63±0.01bC	2.85±0.03aA	2.50±0.02eE	2.57±0.01deD	2.64±0.01gC
30th day	2.32±0.01fE	2.50±0.01dD	2.54±0.01cC	2.84±0.00cA	2.56±0.01eC	2.56±0.01efC	2.54±0.03dC	2.62±0.01bB	2.60±0.01iB
45th day	2.27±0.01gF	2.44±0.01fgE	2.55±0.00cC	2.79±0.01dA	2.51±0.01fgD	2.63±0.03cdB	2.63±0.02cB	2.46±0.00hE	2.62±0.01hB
60th day	2.47±0.02bH	2.63±0.02aD	2.50±0.01eG	2.70±0.01fC	2.68±0.01aC	2.60±0.01deE	2.72±0.00bB	2.54±0.00fF	2.80±0.01bA
90th day	2.37±0.00cdG	2.38±0.01hG	2.51±0.00deE	2.73±0.01eA	2.51±0.00gE	2.55±0.03fD	2.62±0.01cC	2.41±0.00iF	2.70±0.00eB
120th day	2.34±0.01deE	2.52±0.01dD	2.49±0.01eD	2.72±0.00eA	2.52±0.01fD	2.56±0.01efC	2.61±0.03cB	2.57±0.00dC	2.72±0.01dA
150th day	2.36±0.01cdeH	2.41±0.00gG	2.50±0.00deE	2.56±0.00iD	2.64±0.01bA	2.62±0.00dB	2.43±0.01fF	2.58±0.01dC	2.64±0.00gA
170th day	2.35±0.01deG	2.45±0.00efF	2.45±0.00fF	2.79±0.00dB	2.55±0.00eD	2.49±0.00iE	2.35±0.00gG	2.59±0.01cC	2.91±0.00aA
180th day	2.34±0.01efF	2.43±0.00fgDE	2.44±0.00fD	2.68±0.00fgA	2.50±0.00gC	2.50±0.00ghC	2.56±0.00dB	2.42±0.01iE	2.69±0.00eA
190th day	2.29±0.01gH	2.60±0.00bD	2.52±0.00dG	2.54±0.002jF	2.56±0.00eE	2.73±0.00bA	2.63±0.00cC	2.53±0.00gG	2.64±0.00gB
200th day	2.38±0.00cH	2.55±0.01cE	2.54±0.00cF	2.97±0.00bA	2.67±0.00aB	2.66±0.00cC	2.43±0.00fG	2.64±0.00aD	2.66±0.00fC

[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

[2] Values in columns represent the mean of 3 replications and standard deviation.

[3] Values followed by the same small letters in columns are not significantly different ($p < 0.05$).

[4] Values followed by the same capital letters in rows are not significantly different ($p < 0.05$).

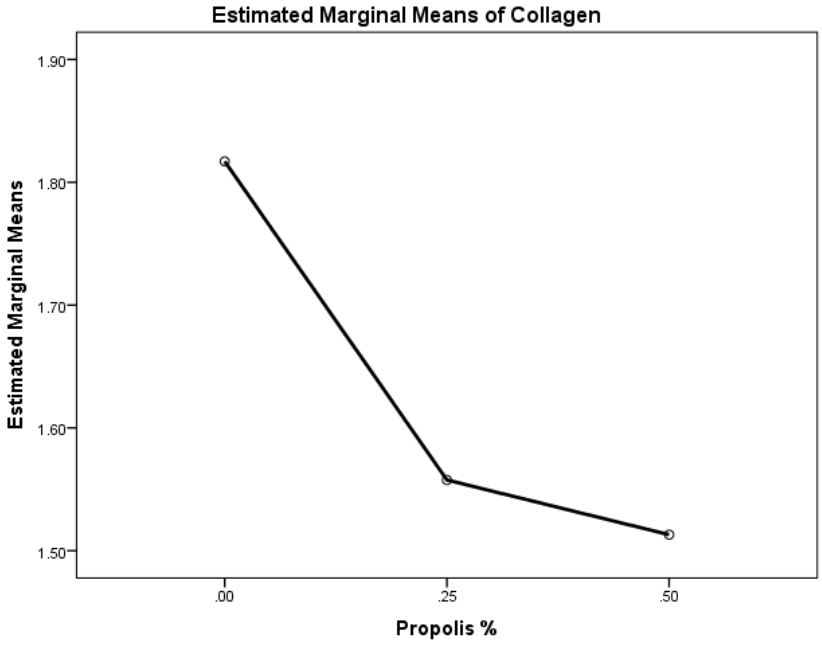
Fat/protein ratios of sucuk doughs were between 2.45±0.02 - 3.05±0.01, and there were statistically significant differences between samples ($p < 0.05$). After heat treatment fat/protein ratios presented a decreased in most of the samples by about 7-14% in a range between 2.28±0.02 and 2.62±0.01. significantly ($p < 0.05$). Upon heating the lowest value for fat/protein ratio was observed in control sample (2.28±0.02) with an about 10% decrease. In addition, the fat/protein ratios of all samples containing propolis were higher than that of control sample in the range of 2.43±0.00 - 2.97±0.00 (Table 4.16) during storage of 200 days.

4.5.1.8 Collagen Content

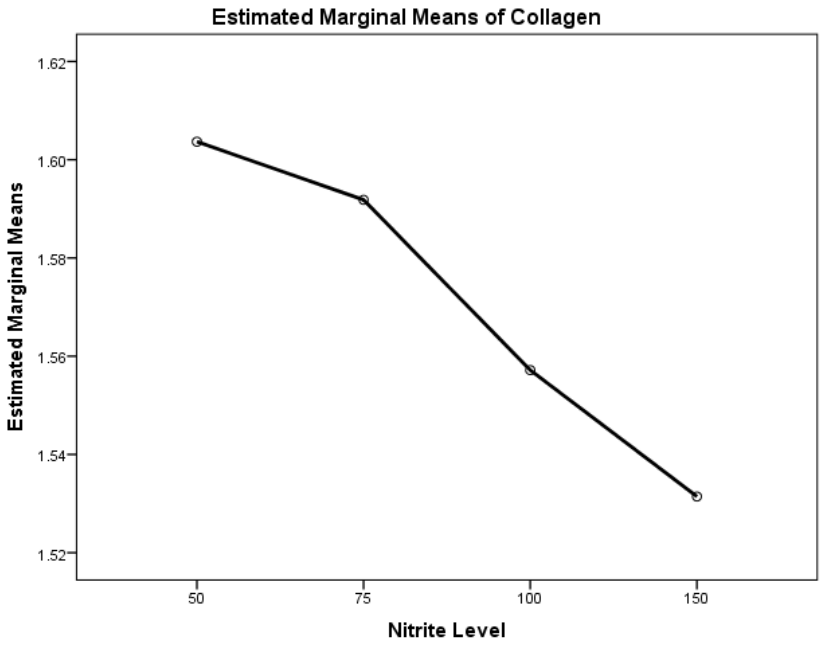
The amount of collagen in heat-treated dry fermented sucuk samples during fermentation and storage were given in Figure 4.26 and Table 4.19. Data is collected from the 30th day to 200th day of storage.

Collagen contents of sucuk samples were in the range of 1.84±0.01% and 2.21±0.02% (Table 4.19) at day 30th. There are statistically significant differences among products starting at day 30 till the end of storage period ($p < 0.05$). As storage time reaches to 200 days, a decreasing trend in the amount of collagen was observed in all sucuk samples. Those amounts decreased by about 22% – 51% and reached to a range of 0.75±0.03 and 1.71±0.05 % in all samples at the end of 200 days. However, collagen in control sample showed the highest value than other samples at day 200 (Table 4.19).

The Univariate analysis of data revealed that nitrite concentration and propolis % affected the collagen amount (Figure 4.24). The addition of propolis extract and sodium nitrite content resulted in lower amounts of collagen as their amounts increased (Appendix E, Table E.6). In addition, the interactions between levels of those two factors were effective on collagen amounts (Figure 4.25). Accordingly, the decrease in collagen amount with 0.5% propolis addition was lower when nitrite levels are above 100 ppm. This finding might be associated to a possible interaction of propolis with hydrolysis of collagen upon heating during processing of sucuks. However, further research is necessary to explain that mechanism.



(a)



(b)

Figure 4.24 : Change in estimated marginal means of collagen amount depending on (a) propolis % and (b) nitrite concentration during 200 days ($p < 0.05$).

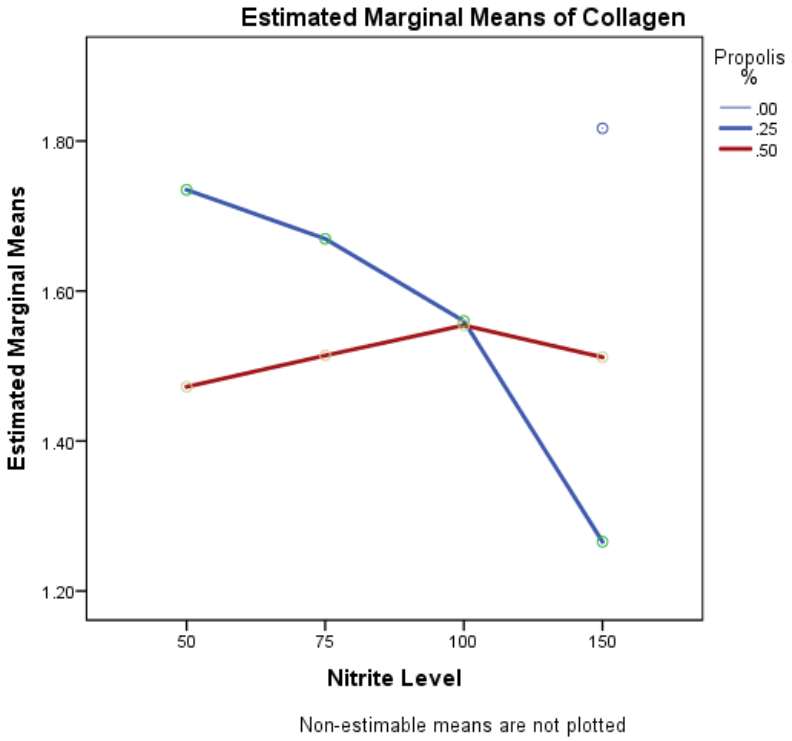
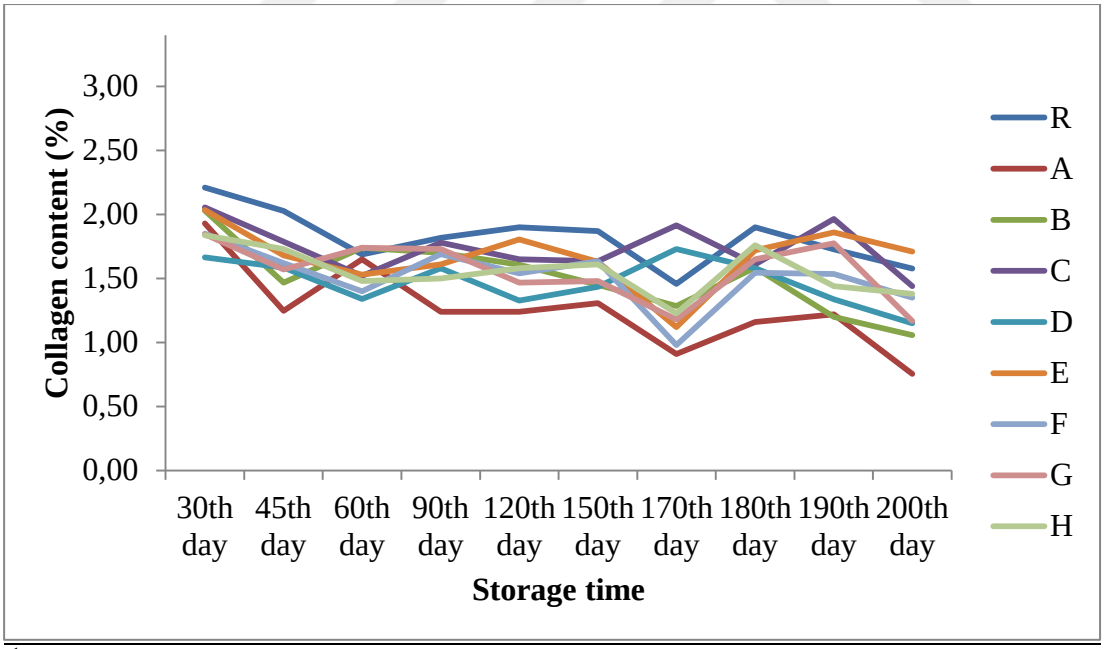


Figure 4.25 : Interaction plot of nitrite concentration and propolis % on collagen amounts during storage of nine products ($p < 0.05$).



[a1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

Figure 4.26 : Changes of collagen contents during storage of heat-treated dry fermented sucuks. [1]

Table 4.19 : Collagen amounts (%) of heat-treated dry fermented sucuk samples. [1-4]

SAMPLES	R	A	B	C	D	E	F	G	H
30th day	2.21±0.02aA	1.93±0.01aC	2.03±0.04aB	2.05±0.02aB	1.66±0.02bE	2.03±0.04aB	1.85±0.05aD	1.84±0.04aD	1.84±0.01aD
45th day	2.03±0.04bA	1.25±0.11cdF	1.47±0.09deE	1.79±0.06cB	1.58±0.05cDE	1.68±0.01cdBCD	1.62±0.04bcCD	1.57±0.07dDE	1.73±0.05bBC
60th day	1.69±0.03dAB	1.65±0.04bB	1.74±0.03bA	1.52±0.02eC	1.34±0.03eE	1.53±0.02eC	1.40±0.02dD	1.74±0.02bA	1.48±0.01deC
90th day	1.82±0.03cA	1.24±0.02cdE	1.70±0.02bcB	1.78±0.02cA	1.58±0.02cC	1.61±0.02dC	1.69±0.02bB	1.73±0.02bB	1.50±0.02dD
120th day	1.90±0.02cA	1.24±0.03cdH	1.61±0.03bcdCD	1.65±0.02dC	1.33±0.02eG	1.80±0.02bB	1.54±0.02cE	1.47±0.01eF	1.58±0.04cDE
150th day	1.87±0.09cA	1.31±0.03cC	1.45±0.18eBC	1.63±0.06dB	1.43±0.02dBC	1.63±0.06dB	1.63±0.15bcB	1.48±0.03eBC	1.61±0.01cB
170th day	1.46±0.01fC	0.91±0.01eI	1.28±0.04fD	1.91±0.01bA	1.73±0.01aB	1.12±0.01fG	0.98±0.02eH	1.18±0.02fF	1.23±0.01gE
180th day	1.90±0.02cA	1.16±0.04dE	1.58±0.02cdeCD	1.61±0.03dCD	1.58±0.02cD	1.72±0.03cB	1.54±0.04cD	1.65±0.03cC	1.76±0.02bB
190th day	1.73±0.02dD	1.22±0.01cdH	1.20±0.01fH	1.96±0.01bA	1.34±0.03eG	1.86±0.03bB	1.53±0.02cE	1.77±0.02bC	1.44±0.03eF
200th day	1.58±0.01eB	0.75±0.03fG	1.06±0.02gF	1.44±0.01fC	1.15±0.03fE	1.71±0.05cA	1.35±0.01dD	1.17±0.02fE	1.38±0.03fD

[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

[2] Values in columns represent the mean of 3 replications and standard deviation.

[3] Values followed by the same small letters in columns are not significantly different ($p < 0.05$).

[4] Values followed by the same capital letters in rows are not significantly different ($p < 0.05$).

The Turkish Food Codex (TFC) Communique on Meat and Meat Products states that heat treated sucuks must have a collagen content below 25% of total protein content (Turkish Food Codex Communique on Meat and Meat Products (No: 2012/74), 2012). All heat-treated sucuk products have a collagen content in limits and our products are acceptable with Turkish Food Codex legislation on Meat and Meat Products (Table 4.19).

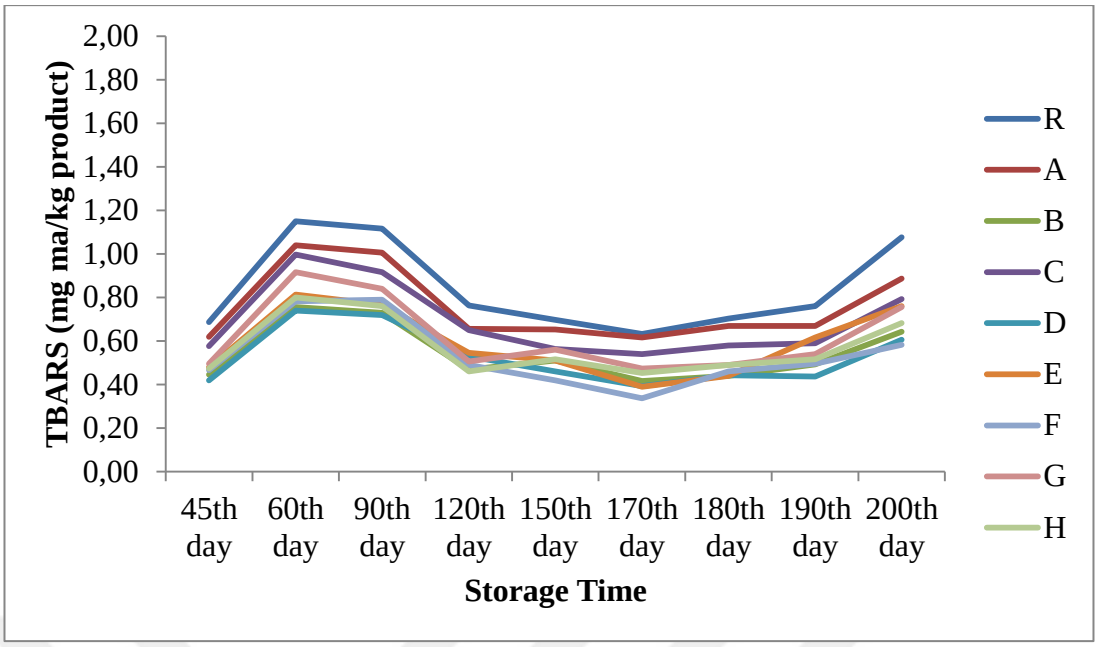
4.5.1.9 Lipid oxidation measured as Thiobarbautiric Acid Reactive Substances (TBARS)

The changes of TBARS contents of heat-treated dry fermented sucuk samples during 45 – 200 days of storage period were given in Figure 4.27. Results of triplicate analysis and statistically significant differences between each formulations of heat-treated dry fermented sucuk products and storage time intervals were shown in Table 4.20.

Lipid oxidation in sucuk samples were expressed as the TBARS values in kg of product. At the 45th day of storage, sucuk products have TBARS values in the range 0.42±0.02 mg/kg product and 0.69±0.01 mg/kg product. Those values increased significantly until 60th day of storage ($p < 0.05$) which was followed by a gradual decrease til 120th days of storage and then again a slight increase towards the end of storage.

There were statistically significant differences between products and during storage time intervals ($p < 0.05$). The highest TBARS values were observed at control sample reaching to a maximum TBARS value of 1.08±0.012 mg/kg product at 200th day of storage.

Those results show that TBARS values were affected ($p < 0.05$) by the addition of propolis extracts to the formulations. Sucuks containing propolis extracts had significantly lower ($p < 0.05$) TBARS value compared to that of control sample. Besides, increasing the addition level of propolis extract from 0.25% to 0.50% changed the TBARS values significantly ($p > 0.05$). It was observed that propolis extracts appeared to be more effective than sodium nitrate most probabaly due its high antioxidant capacity. It is well known that phenolic/flavonoids compounds have antioxidative properties (Papotti et al., 2012) and the presence of these compounds may retard ($p < 0.05$) the lipid oxidation during the ripening periods of sucuks.



[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

Figure 4.27 : Changes of TBARS contents during storage of heat-treated dry fermented sucuks. [1]

In addition, all data including storage time was analysed to investigate the effects of nitrite and propolis additions on lipid oxidation using GLM procedure of Univariate analysis. The results of the analysis showed that the differences in TBARS values were affected by propolis levels, nitrite concentration, and also by interactions between propolis and nitrite levels ($p < 0.05$) (Appendix E, Table E.6). According to Figure 4.28 lipid oxidation decrease as propolis % increases, while nitrite levels at 150 ppm showed an increasing effect on TBARS values (Figure 4.28). On the other hand the interaction between propolis % and nitrite levels resulted in an increase in lipid oxidation only at 0.25% propolis addition at 150 ppm nitrite concentration (Figure 4.29). When propolis percentage increased to 0.50, lipid oxidation was lowered again.

Table 4.20 : Thiobarbituric acid reactive substances (TBARS) analysis results of heat-treated dry fermented sucuk samples. [1-4]

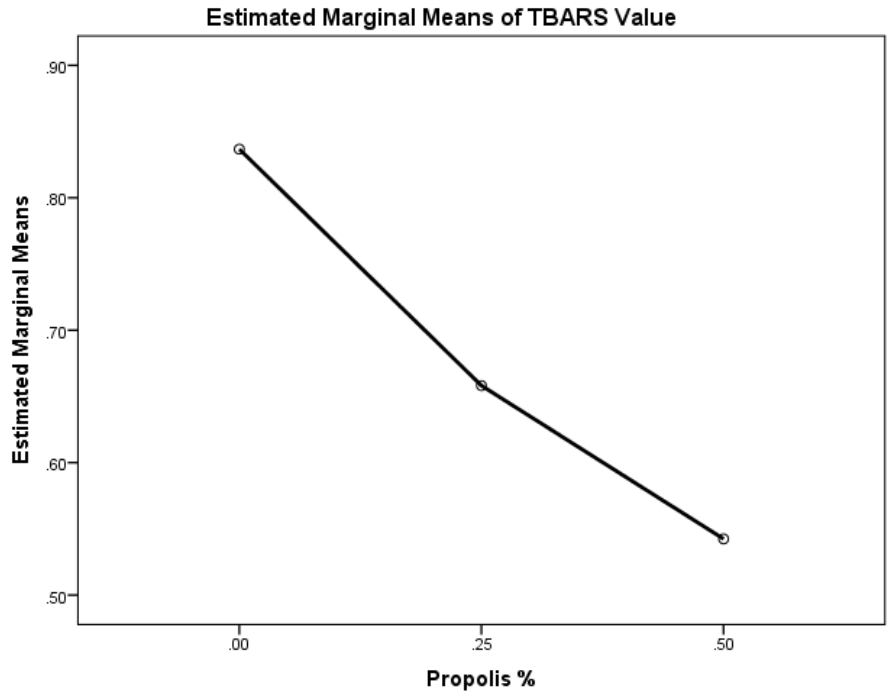
SAMPLES	R	A	B	C	D	E	F	G	H
45th day	0.69±0.01eA	0.62±0.02eB	0.45±0.02efE	0.58±0.01eC	0.42±0.02fF	0.48±0.01fDE	0.47±0.01dDE	0.50±0.02efD	0.47±0.02efDE
60th day	1.15±0.01aA	1.04±0.02aB	0.76±0.02aFG	1.00±0.01aC	0.74±0.02aH	0.81±0.03aE	0.78±0.02aE	0.92±0.01aD	0.80±0.02aEF
90th day	1.12±0.01bA	1.01±0.01bB	0.73±0.02bFG	0.92±0.02bC	0.72±0.01aH	0.77±0.01bE	0.79±0.01aE	0.84±0.02bD	0.76±0.02bEF
120th day	0.76±0.03dA	0.66±0.02dB	0.47±0.01efF	0.65±0.02dB	0.53±0.01cCD	0.55±0.02dC	0.49±0.01cEF	0.51±0.01eDE	0.46±0.02fF
150th day	0.70±0.02eA	0.65±0.01dB	0.51±0.00dD	0.56±0.01efC	0.46±0.00dE	0.51±0.01eD	0.42±0.00eF	0.56±0.00dC	0.52±0.01dD
170th day	0.63±0.01fA	0.62±0.01eB	0.42±0.01efF	0.54±0.01fC	0.39±0.01gEF	0.39±0.01hF	0.34±0.01fE	0.47±0.01fD	0.45±0.01fD
180th day	0.70±0.01eA	0.67±0.01dB	0.44±0.01fF	0.58±0.01eC	0.44±0.01deEF	0.44±0.01gF	0.46±0.01dE	0.49±0.01efD	0.49±0.01deD
190th day	0.76±0.01dA	0.67±0.01dB	0.49±0.01deG	0.59±0.01eD	0.44±0.01efH	0.62±0.01cC	0.50±0.01cG	0.54±0.01dE	0.52±0.01dF
200th day	1.08±0.01cA	0.89±0.01cB	0.64±0.01cF	0.79±0.01cC	0.61±0.01bG	0.76±0.00bD	0.58±0.01bH	0.76±0.01cD	0.68±0.01cE

[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

[2] Values in columns represent the mean of 3 replications and standard deviation.

[3] Values followed by the same small letters in columns are not significantly different ($p < 0.05$).

[4] Values followed by the same capital letters in rows are not significantly different ($p < 0.05$)



(a)

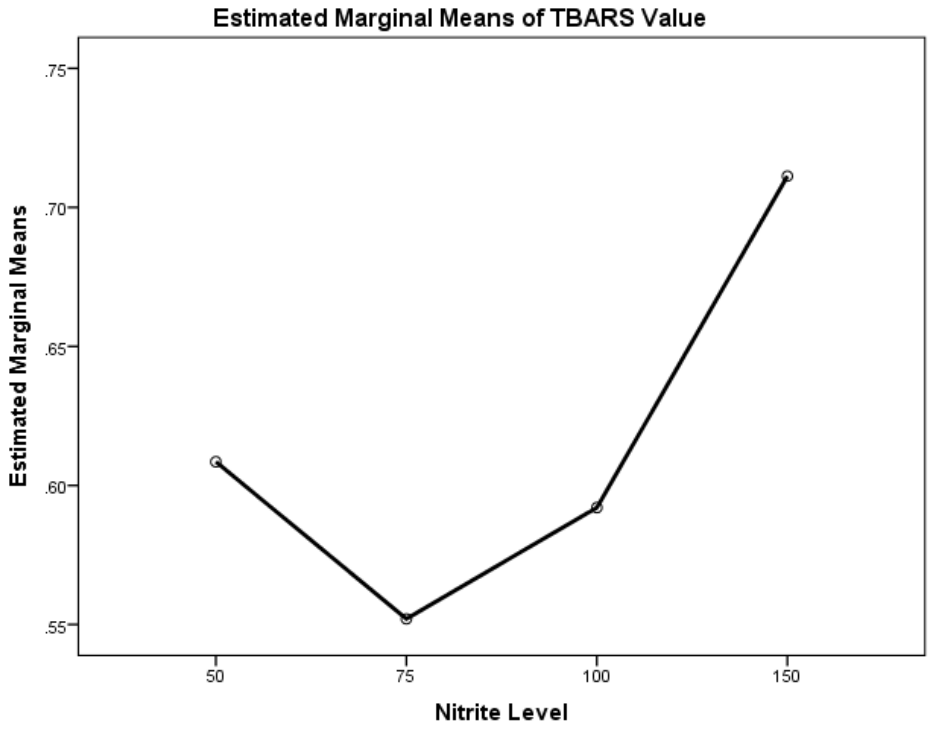


Figure 4.28 : Change in estimated marginal means of TBARS values depending on (a) propolis % and (b) nitrite concentration during 200 days ($p < 0.05$).

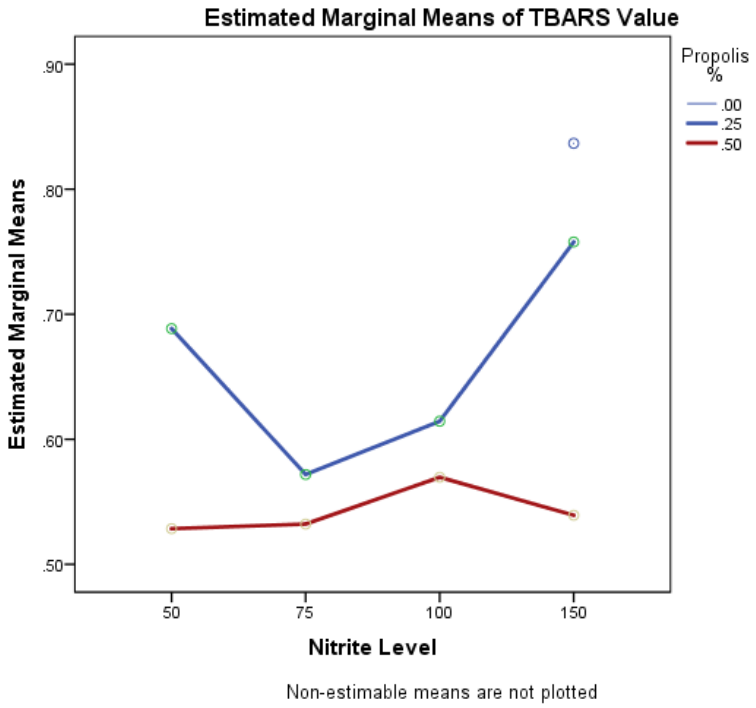


Figure 4.29 : Interaction plot of nitrite concentration and propolis % on TBARS values during storage ($p < 0.05$).

In the study of Karabacak & Bozkurt (2008) the addition of *Urtica dioica* and *Hibiscus sabdariffa* in sucuk formulations also decreased the TBARS values significantly. The authors proposed that addition of antioxidants might have reduced lipid deterioration (TBA) through the inhibition of formation of malonaldehyde (Karabacak & Bozkurt, 2008). Gök et al. (2011) also observed that ripening time and antioxidant treatment have a significant effect on TBARS value. They have found that control sample that contains no additional antioxidant have higher TBARS values during ripening and storage (Gök et al., 2011). Moreover, Bozkurt & Bayram (2006) similarly reported lower TBARS content of Turkish sucuk added with green tea extract and *Thymbra spicata* oil.

Wu et al. (1991) reported that if the TBARS value is higher than 1 mg/ kg generally off-odors are formed and it is considered as the beginning of organoleptic perception of lipid oxidation. TBARS values of sucuk were found to be lower than this value until the end of 200 days (Table 4.20). On the 200th day only control sample slightly exceeded 1 mg/kg reaching 1.08±0.012 mg/kg TBARS.

4.5.1.10 Color Analysis

Color changes of heat-treated dry fermented sucuk samples at 0^{th}, 90^{th}, and 200^{th} days of storage were tested. The statistically significant differences between each formulations of heat-treated dry fermented sucuk products and storage time intervals were given in Table 4.21.

Addition of propolis and sodium nitrite influence the color parameters (L*, a* and b*), and significant differences observed between samples ($p < 0.05$) at all storage time intervals. Following heat treatment, lightness (L*) values of the sucuk samples ranged between 45.80±0.50 – 50.20±0.10. Those values slightly increased to a range of 50.33±0.21 – 57.46±0.12 at day 90 and then to 53.14±0.11 – 64.90±0.09 at day 200 (Table 4.19). Yalınkılıç et al. (2012) also observed that an increase in L* value in relation to the amount of added orange fiber. In addition those researchers reported that L* value increased as fat level increased (Yalınkılıç et al. 2012). Two-way variance analysis used to explain the effect of propolis% and nitrite concentration on L values presented a significant interaction between those factors ($p < 0.05$) (Appendix E, Table E.6). According to Figure 4.30 the addition of propolis decreased lightness values of sucuk samples depending on nitrite concentration.

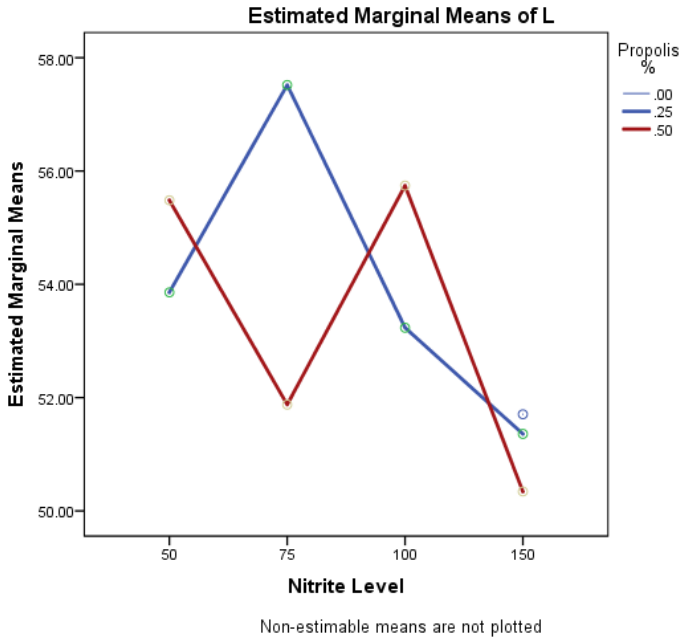


Figure 4.30 : Interaction plot of nitrite concentration and propolis % on L values during storage ($p < 0.05$).

Statistically significant differences between samples ($p < 0.05$) observed at each time of analysis show that addition of sodium nitrite and propolis influence the redness value (a* values) of heat-treated dry fermented sucuk samples. Following heat treatment at day zero, redness values (a*) of the sucuk samples ranged between 23.10±0.18 – 25.60±0.22. Those values slightly decreased to a range of 17.02±0.09 – 23.45±0.11 at day 90 and then to 10.70±0.03 – 21.40±0.04 at day 200 (Table 4.21). However, the control sample exhibited the highest redness values during 200 days of storage consistently (Table 4.21). In other words, addition of propolis to samples slightly reduced the redness value of sucuk samples. Univariate analysis of data during storage was carried out to examine the effect of propolis % and nitrite concentration on redness value of samples. The results of test revealed that the nitrite level and the interactions between propolis % and nitrite levels significantly affected the redness values (Appendix E, Table E.6). Figure 4.31 shows that as the level of nitrite concentration increases the redness value increases. However, this effect is subject to the interactions with nitrite concentration levels. According to Figure 4.32 the increase in redness value may become higher at 0.5% propolis level with an increase in nitrite concentration.

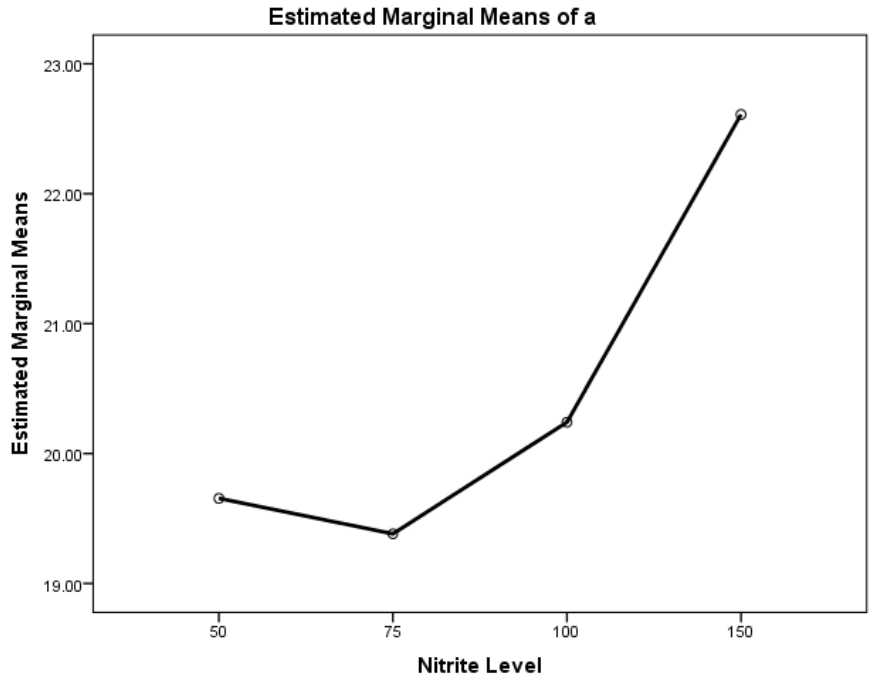


Figure 4.31 : Change in estimated marginal means of a* values depending on nitrite concentration during 200 days ($p < 0.05$).

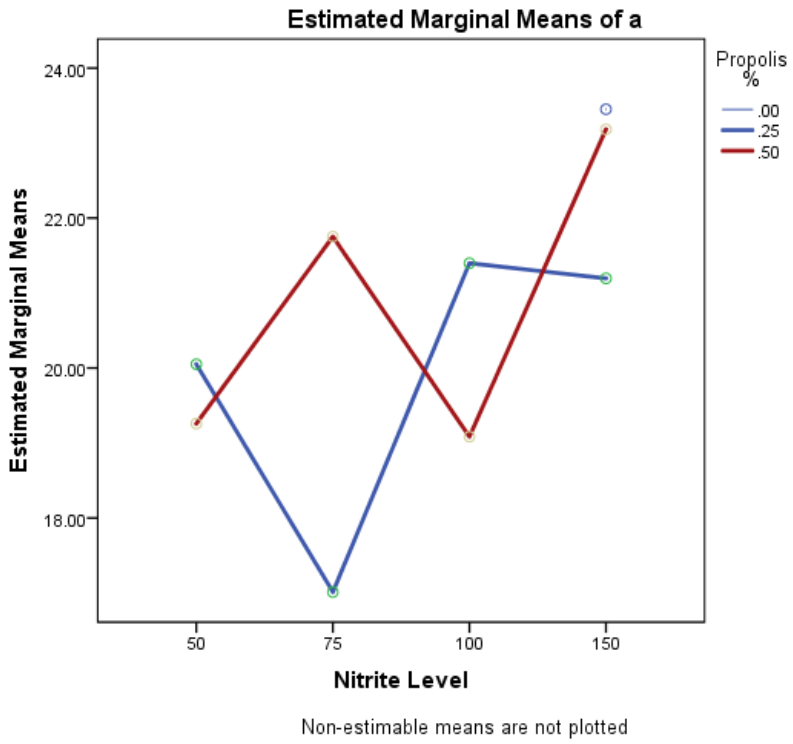
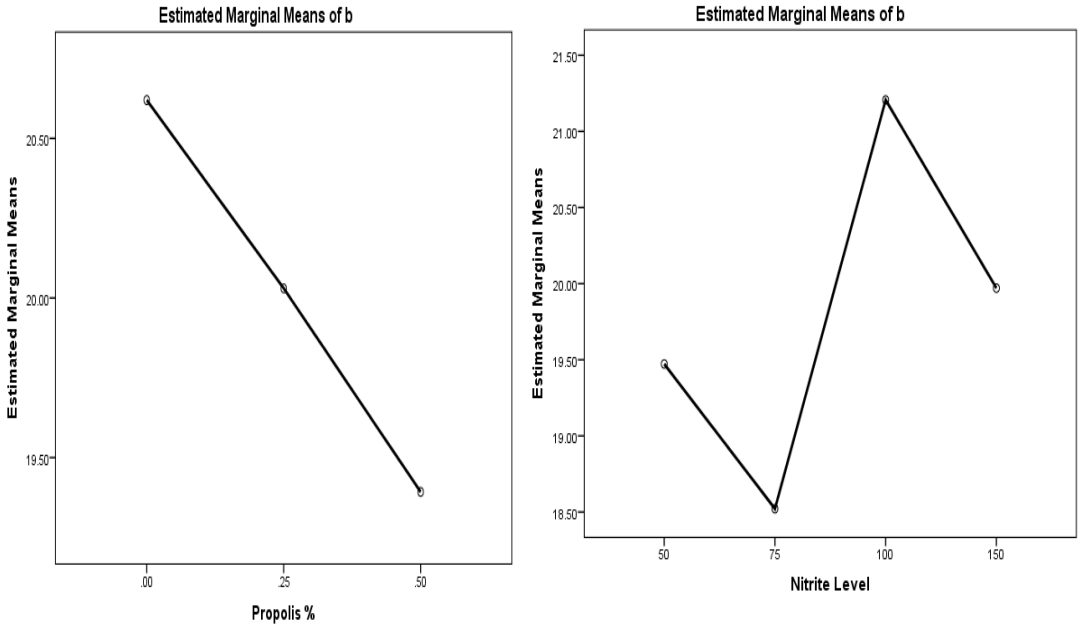


Figure 4.32 : Interaction plot of nitrite concentration and propolis % on a* values during storage ($p < 0.05$).

Ekici et al. (2015) observed that a* values of samples added with nitrite was found to be slightly higher compared to samples having no nitrite. Similar to our findings, they reported that increase of black carrot concentrate amount in the samples containing nitrite decreased the a* values of the samples significantly ($p < 0.05$) (Ekici et al., 2015). Furthermore, our results were similar to the data of Kayaardı and Gök (2003) who have observed that redness (a*) values of sucuk increased during the first 5 days of ripening and then decreased during further ripening. Similarly, the a* value of Spanish type dry-cured sucuk increased during the fermentation period, and then decreased during the ripening period (Perez-Alvarez et al., 1999).

Kayaadi and Gok (2003) proposed that the reason of increase in a* values during first days of ripening is related to combination of nitrogenous compounds present in meat with myoglobin to reach the desired red-colored pigment. According to the authors, after 5 days of ripening period, a* values decrease in sucuk due to the denaturation of red-colored pigment. Perez-Alvares et al. (1999) also stated that the possible reason for decreasing a* values might be partial or total denaturation of nitrosomyoglobin.

Yellowness values (b*) were observed between 19.0±0.1 and 21.4±0.1 at the beginning of storage period. Following day zero, yelowness values (b*) of the sucuk samples ranged between 19.04±0.07 – 21.40±0.11. Those values slightly decreased to a range of 17.20±0.10 – 23.30±0.00 at day 90 and then to 14.52±0.20 – 26.25±0.10 at day 200 (Table 4.21). The decrease in yellowness (b* value) may be related to browning reactions as melanoidins are brown-colored. It indicates that the sucuk samples were turned into blueish rather than yellow. In addition, univariate ANOVA was carried out to examine any possible effect of propolis and nitrite concentration on yellowness values. The test results showed significant differences in yellowness values depending on propolis percentage, nitrite ppm values and possible interactions between those factors (Appendix E, Table E.6). Figure 4.33 presents that yellowness values decreases as the level of propolis % increases. However, due to the interactions between two factors, the yellowness values increased as nitrite concentration increased only lower level of propolis (Figure 4.34).



(a) (b)

Figure 4.33 : Change in estimated marginal means of yellowness values depending on (a) propolis % and (b) nitrite concentration during 200 days ($p < 0.05$).

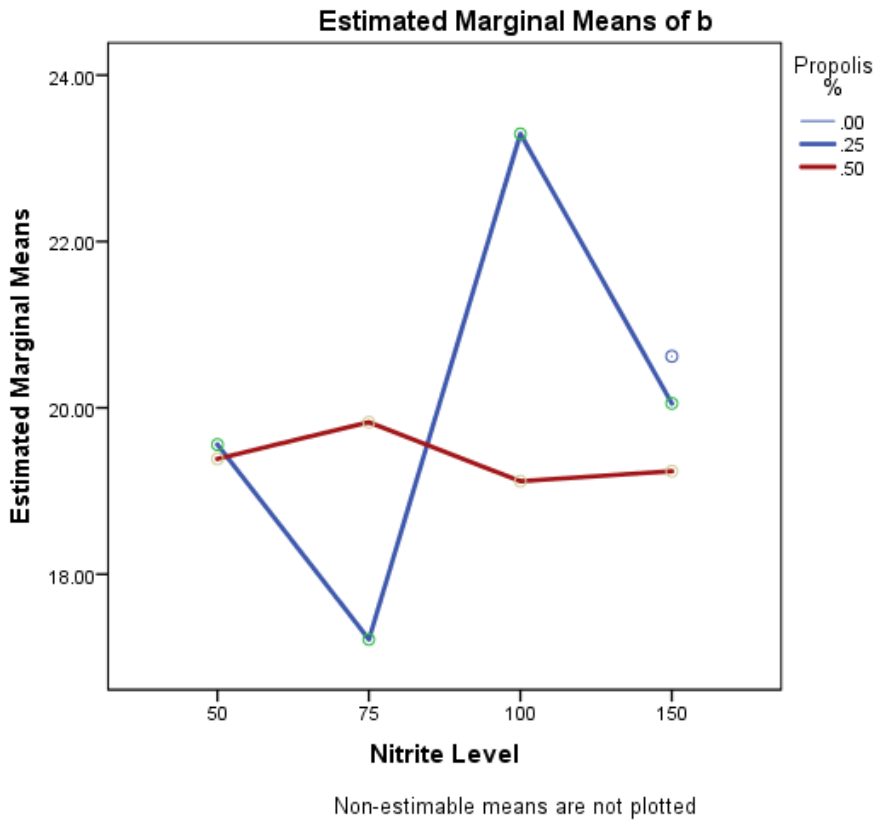


Figure 4.34 : Interaction plot of nitrite concentration and propolis % on b* values during storage ($p < 0.05$).

Similar results for decrease in b* values during storage has been reported by Kayaardı and Gök (2003) and Perez-Alvarez et al. (1999). Yalınkılıç et al. (2012) also observed that b* value also increased as to the amount of added orange fiber into sucuk. Our results are also supported by the results of Karabacak and Bozkurt (2008) who investigated the effect of *Urtica dioica*, *Hibiscus sabdariffa*, butylatedhydroxytoluene (BHT) and nitrite/nitrate in sucuk (Turkish dry-fermented sucuk) during the ripening periods. They reported increase in the Hunter a* value (and a decrease in b* values during the ripening periods ($p < 0.05$).The decrease of yellowness in sucuk samples was explained as a decrease in oxymyoglobin that contributes to yellowness as a result of oxygen consumption of microorganisms by Bozkurt & Bayram (2006).

Table 4.21 : Color analysis results of heat-treated dry fermented sucuk samples. [1-4]

	L*			a*			b*		
	0 day	90th day	200th day	0 day	90th day	200th day	0 day	90th day	200th day
R	47.80±0.20dC	51.75±0.21eB	55.57±0.04fA	25.60±0.22aA	23.45±0.11aB	21.40±0.04aC	21.40±0.11aA	20.60±0.10bB	19.90±0.10cC
A	49.10±0.20bC	51.37±0.20fB	53.58±0.15hA	23.50±0.16deA	21.17±0.19eB	19.00±0.03dC	19.90±0.11dB	20.10±0.10cA	20.20±0.10bA
B	47.60±0.30dC	50.33±0.21gB	53.14±0.11iA	25.00±0.05bA	23.18±0.06bB	21.40±0.05aC	20.57±0.10bA	19.20±0.08gB	17.93±0.10eC
C	45.80±0.50fC	53.91±0.21cB	61.90±0.11dA	24.40±0.20cA	20.04±0.09fB	15.70±0.10eC	21.56±0.08aA	19.60±0.00eB	17.55±0.10fC
D	46.60±0.20eC	55.53±0.09bB	64.30±0.23bA	23.60±0.14deA	19.28±0.05gB	14.90±0.03fC	19.04±0.07eC	19.40±0.00fB	19.73±0.20cA
E	50.20±0.10aC	57.46±0.12aB	64.90±0.09aA	23.30±0.08eA	17.02±0.09hB	10.70±0.03hC	19.91±0.21dA	17.20±0.10hB	14.52±0.20gC
F	49.20±0.10bC	51.84±0.16eB	54.60±0.11gA	23.70±0.33deA	21.77±0.11cB	19.90±0.07bC	20.57±0.12bA	19.80±0.10dB	19.10±0.10dC
G	48.70±0.10cC	53.23±0.12dB	57.80±0.12eA	23.10±0.18eA	21.38±0.08dB	19.70±0.05cC	20.34±0.11bcC	23.30±0.00aB	26.25±0.10aA
H	49.20±0.20bC	55.74±0.17bB	62.30±0.10cA	23.90±0.75dA	19.19±0.16gB	14.20±0.06gC	20.26±0.27cA	19.10±0.00gB	17.98±0.10eC

[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

[2] Values in columns represent the mean of 3 replications and standard deviation.

[3] Values followed by the same small letters in columns are not significantly different ($p < 0.05$).

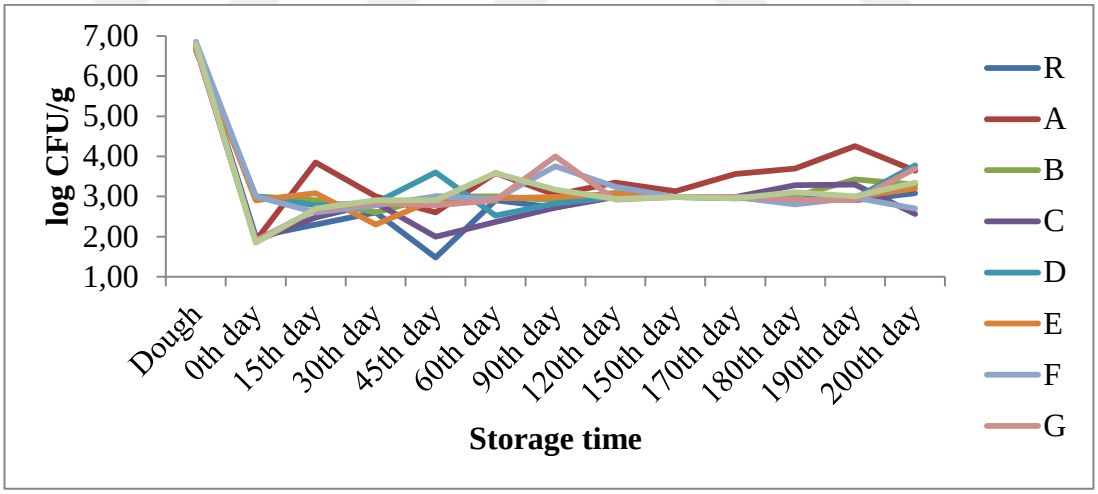
[4] Values followed by the same capital letters in rows are not significantly different ($p < 0.05$).

4.5.2 Microbiological analysis of heat-treated dry fermented sucuks

All microbiological analyses (total aerobic bacteria, *E. coli*, total coliform, total mold and yeast, *Lactobacillus* spp., *Salmonella* spp. *L. monocytogenes*) of nine heat-treated dry fermented sucuk samples were performed in triplicate and given as mean ± standard deviations. Statistical analysis were performed to investigate the differences between products and storage time intervals. Univarite analysis were performed to understand any possible effect of nitrite concentration and propolis % in the results of total aerobic bacteria, *E. coli*, total coliform, total mold and yeast, *Lactobacillus* spp., *Salmonella* spp. *L. monocytogenes*. The model was based on two way ANOVA where interaction of factors were also considered.

4.5.2.1 Total aerobic bacteria

The changes of total aerobic bacteria counts of heat-treated dry fermented sucuk samples during storage period were given in Figure 4.35. Results of triplicate analysis and statistically significant differences between each formulations of heat-treated dry fermented sucuk products and storage time intervals were presented in Table 4.22.



[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

Figure 4.35 : Changes of total aerobic bacteria counts (log cfu/g) during storage of heat-treated dry fermented sucuks. [1]

Total aerobic bacteria counts of all sucuk dough formulations didn't differ significantly ($p > 0.05$) varying between 6.7±0.0 log cfu/g to 6.9±0.0 log cfu/g.

However, heat-treatment decreased total aerobic bacteria counts significantly ($p < 0.05$) ranging between 1.9±0.1 log cfu/g to 3.0±0.0 log cfu/g. Ercoşkun et al. (2010) also observed the total aerobic bacteria counts of 2 days fermented heat-treated sucuk decreased from 8.17±0.59 log cfu/g to 2.99±0.06 log cfu/g after fermentation.

During 200 days of storage total aerobic counts increased gradually. At the end of 200 days of storage time total aerobic to bacteria counts of heat-treated sucuk samples reached to 2.6±0.1 log cfu/g 3.8±0.2 log cfu/g. The total aerobic to bacteria counts of control sample was observed to be 3.1±0.3 differing slightly from propolis added sucuk samples ($p < 0.05$).

In addition, all data including storage time was analysed to investigate the effects of nitrite and propolis additions on total aerobic bacteria counts using a GLM procedure of Univatriate analysis. The results of the analysis showed that the differences in total aerobic bacteria counts between samples were independent from propolis % levels and nitrite concentration and also on interactions between propolis% and nitrite concentration ($p > 0.05$) (Appendix E, Table E.7). Figure 4.36 presents the interaction plot of nitrite concentration and propolis % on total aerobic bacteria counts during storage of nine products. According to Figure 4.36 the increase in propolis concentration decreased total aerobic bacteria count of heat treated sucuk samples depending on nitrite concentration, although those effects were insignificant statistically ($p > 0.05$). It is suggested that using lower amounts of sodium nitrite (100 ppm rather than 150 ppm) and addition of propolis extract (0.5%) may help decreasing the total aerobic bacteria counts of heat treated sucuks.

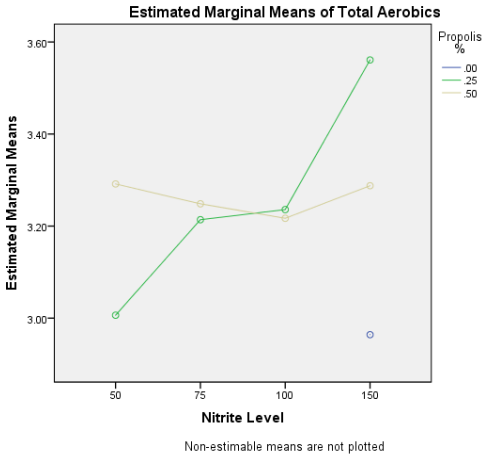


Figure 4.36 : Interaction plot of nitrite concentration and propolis % on total mold counts during storage of nine products ($p < 0.05$).

Table 4.22 : Total aerobic bacteria analysis results of heat-treated dry fermented sucuk samples (log CFU/g) during production and storage. [1-4]

SAMPLE	R	A	B	C	D	E	F	G	H
Dough	6.80±0.00aA	6.73±0.04aA	6.63±0.02aA	6.79±0.01aA	6.70±0.02aA	6.74±0.01aA	6.85±0.01aA	6.72±0.01aA	6.81±0.58aA
0 day	2.00±0.00fC	1.90±0.06iD	3.00±0.00cdA	1.90±0.06gD	3.00±0.00dA	2.90±0.00cdB	3.00±0.00cdA	1.90±0.06fD	1.85±0.06eD
15th day	2.30±0.24eF	3.85±0.06cA	2.90±0.06dBC	2.48±0.15efEF	2.78±0.07gCD	3.08±0.04bcB	2.60±0.11dDE	2.67±0.16eDE	2.70±0.09dCD
30th day	2.60±0.11dC	3.00±0.04gA	2.60±0.11eC	2.85±0.06cB	2.85±0.06efgB	2.30±0.00eD	2.78±0.07dB	2.85±0.06deB	2.90±0.06cdAB
45th day	1.48±0.16gF	2.60±0.11hD	3.00±0.00cdB	2.00±0.00gE	3.60±0.11cA	2.94±0.08bcdB	3.00±0.00cdB	2.78±0.07deC	2.90±0.06cdBC
60th day	2.90±0.06bcB	3.59±0.03eA	3.00±0.00cdB	2.37±0.10fD	2.52±0.07hC	2.95±0.05bcdB	2.94±0.06cdB	2.90±0.23deB	3.59±0.01bA
90th day	2.73±0.25cdD	3.04±0.07gBC	2.90±0.16dBCD	2.73±0.30dD	2.82±0.04fgCD	2.99±0.07bcdBC	3.75±0.13bA	4.00±0.00bA	3.17±0.13cB
120th day	3.04±0.07bBC	3.35±0.13fA	3.09±0.09cBC	2.99±0.03cBC	2.99±0.03deBC	3.07±0.13bcBC	3.24±0.26cdAB	2.92±0.17deC	2.94±0.13cdC
150th day	2.99±0.03bB	3.13±0.09gA	2.99±0.03cdB	2.99±0.03cB	2.99±0.03deB	2.99±0.03bcdB	2.99±0.03cB	2.99±0.03dB	2.99±0.03cdB
170th day	2.95±0.05bB	3.56±0.07eA	2.97±0.03cdB	2.99±0.03cB	2.99±0.03deB	2.99±0.03bcdB	2.99±0.03cdB	2.99±0.03dB	2.95±0.03cdB
180th day	2.99±0.03bBC	3.70±0.07cdA	3.00±0.04cdBC	3.28±0.04bB	2.88±0.15defgBC	2.82±0.38dC	2.80±0.58dC	2.92±0.12deBC	3.10±0.20cdBC
190th day	2.90±0.23bcA	4.26±0.23bC	3.43±0.17bB	3.30±0.00bB	2.95±0.09defC	2.99±0.03bcdC	2.97±0.06cdC	2.90±0.11deC	3.00±0.00cdC
200th day	3.08±0.26bC	3.64±0.05deAB	3.30±0.00bBC	2.56±0.13deD	3.78±0.15bA	3.20±0.23bC	2.70±0.36dD	3.70±0.31cAB	3.35±0.38bcBC

[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

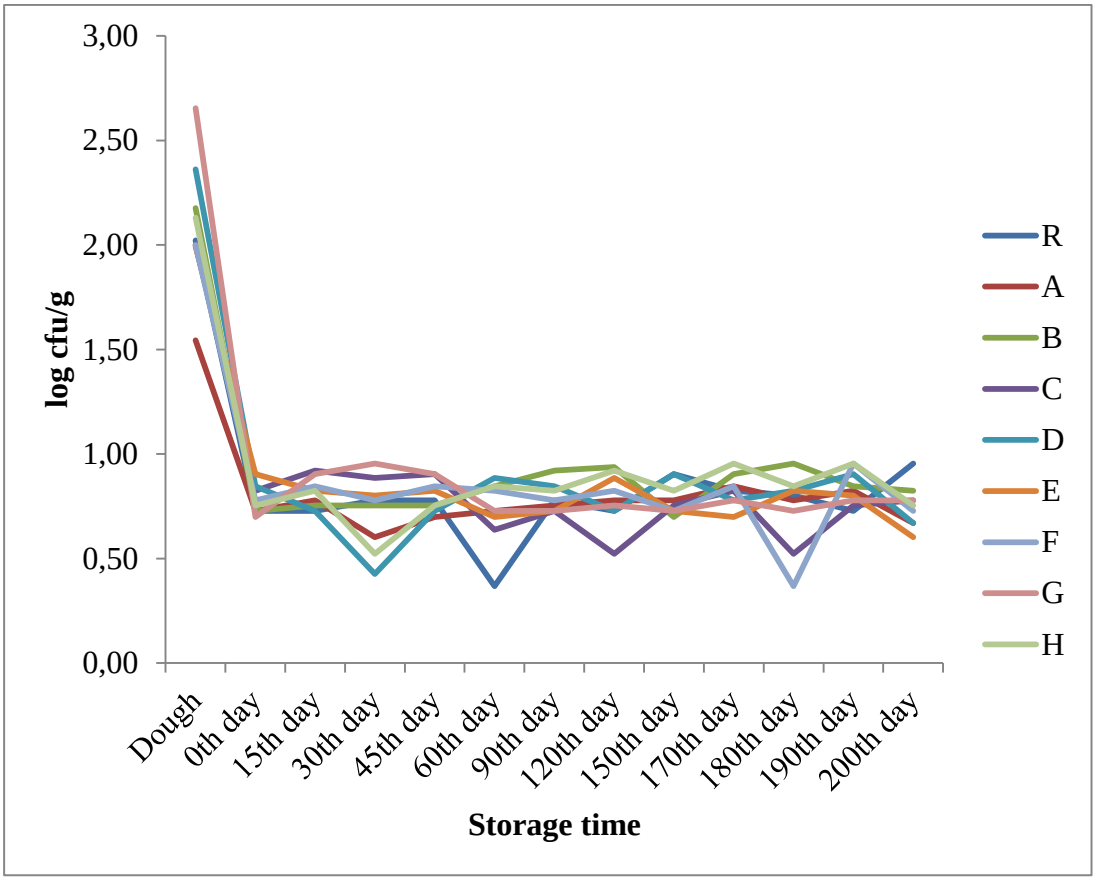
[2] Values in columns represent the mean of 3 replications and standard deviation.

[3] Values followed by the same small letters in columns are not significantly different ($p < 0.05$).

[4] Values followed by the same capital letters in rows are not significantly different ($p < 0.05$).

4.5.2.2 *Eschericia coli*

The changes of *E. coli* counts of heat-treated dry fermented sucuk samples during fermentation and storage were given in Figure 4.37. Results of triplicate analysis and statistically significant differences between each formulations of heat-treated dry fermented sucuk products and storage time intervals were shown in Table 4.23.



[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

Figure 4.37 : Changes of *E. coli* counts (log cfu/g) during storage of heat-treated dry fermented sucuks. [1]

Table 4.23 : *Eschericia coli* analysis results of heat-treated dry fermented sucuk samples (log CFU/g) during production and storage. [1-4]

SAMPLE	R	A	B	C	D	E	F	G	H
Dough	2.02±0.00aE	1.54±0.00aF	2.18±0.00aC	2.00±0.00aE	2.36±0.02aB	1.99±0.03aE	2.00±0.02aE	2.65±0.00aA	2.13±0.03aD
0 day	0.73±0.51bcA	0.73±0.20bcA	0.73±0.51bA	0.82±0.13bA	0.85±0.10bA	0.90±0.10bA	0.78±0.24bcA	0.70±0.33bA	0.75±0.05bA
15th day	0.73±0.51bcA	0.78±0.18bcA	0.75±0.05bA	0.92±0.06bA	0.73±0.51bcA	0.82±0.13bA	0.85±0.10bA	0.90±0.10bA	0.82±0.13bA
30th day	0.78±0.23bcAB	0.60±0.49cAB	0.75±0.05bAB	0.88±0.09bA	0.43±0.45cB	0.80±0.15bAB	0.78±0.00bAB	0.95±0.00bA	0.52±0.39cAB
45th day	0.78±0.24bcA	0.70±0.33bcA	0.75±0.05bA	0.90±0.06bA	0.73±0.51bcA	0.82±0.13bA	0.85±0.10bA	0.90±0.10bA	0.75±0.24bA
60th day	0.37±0.30cB	0.73±0.05bcAB	0.85±0.06bA	0.64±0.45bAB	0.88±0.15bA	0.70±0.17bAB	0.82±0.38bcAB	0.73±0.05bAB	0.85±0.06bA
90th day	0.78±0.18bcA	0.75±0.05bcA	0.92±0.06bA	0.73±0.51bA	0.85±0.10bA	0.73±0.51bA	0.78±0.24bcA	0.73±0.51bA	0.82±0.13bA
120th day	0.73±0.33bcAB	0.78±0.00bcAB	0.94±0.03bA	0.52±0.39bB	0.73±0.33bcAB	0.88±0.09bA	0.82±0.18bAB	0.75±0.05bAB	0.92±0.06bA
150th day	0.90±0.10bA	0.78±0.24bcA	0.70±0.33bA	0.75±0.05bA	0.90±0.06bA	0.73±0.51bA	0.73±0.20bcA	0.73±0.51bA	0.82±0.13bA
170th day	0.82±0.13bcAB	0.85±0.10bAB	0.90±0.10bAB	0.82±0.13bAB	0.78±0.15bcAB	0.70±0.49bB	0.85±0.13bAB	0.78±0.00AB	0.95±0.00bA
180th day	0.80±0.15bcAB	0.78±0.00cbcAB	0.95±0.00bA	0.52±0.39bBC	0.82±0.38bcAB	0.82±0.26bAB	0.37±0.30cC	0.73±0.05bAB	0.85±0.06bAB
190th day	0.73±0.51bcA	0.82±0.13bcA	0.85±0.10bA	0.75±0.53bA	0.90±0.16bA	0.80±0.04bA	0.95±0.00bA	0.78±0.00bA	0.95±0.00bA
200th day	0.95±0.00bA	0.67±0.16bcA	0.82±0.38bA	0.78±0.24bA	0.67±0.30bcA	0.60±0.24bA	0.73±0.51bcA	0.78±0.18bA	0.75±0.05bA

[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

[2] Values in columns represent the mean of 3 replications and standard deviation.

[3] Values followed by the same small letters in columns are not significantly different ($p < 0.05$).

[4] Values followed by the same capital letters in rows are not significantly different ($p < 0.05$).

The initial values of *E.coli* in sucuk samples were in a range from 1.54±0 log cfu/g to 2.65±0 before heat-treatment. Following heat treatment *E.coli* counts were decreased significantly ($p < 0.05$) into a range of 0.73±0.51 log cfu/g to 0.9±0.10 log cfu/g. Our results were also compatible with Ercoşkun et al. (2010) who reported <1 log cfu/g *E.coli* count after heat treatment of sucuks. At the end of the 200 days of storage *E.coli* counts were stable and there were no statistically significant differences between sucuk samples at the end of 200 days of storage.

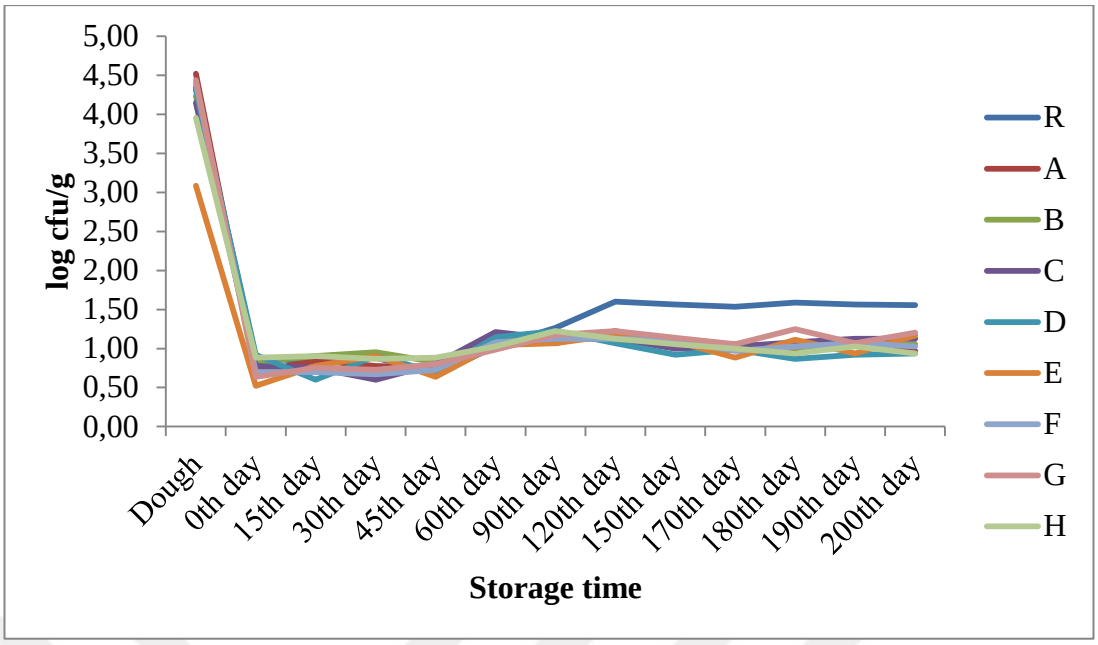
In addition, all data including storage time was analysed to investigate the effects of nitrite and propolis additions on *E.coli* counts using a GLM procedure of Univariate analysis. The results of the analysis showed that the differences in *E.coli* counts between samples were independent from propolis % levels and nitrite concentration and also on interactions between propolis % and nitrite concentration ($p > 0.05$) (Appendix E, Table E.7).

4.5.2.3 Total coliform bacteria

The changes of total coliform bacteria counts of heat-treated dry fermented sucuk samples during fermentation and storage were given in Figure 4.38. Results of triplicate analysis and statistically significant differences between each formulations of heat-treated dry fermented sucuk products and storage time intervals were shown in Table 4.24.

Total coliform bacteria counts were in a range between 3.09±0.09 log cfu/g and 4.52±0.01 log cfu/g before heat treatment (Table 4.24). After heat-treatment total coliform bacteria counts decreased significantly and reached to 0.52±0.52 – 0.92±0.03 log cfu/g. The total coliform count was not different between sucuk samples during 200 days of storage time and there were no statistically significant differences ($p > 0.05$) between products (Table 4.24).

In addition, all data including storage time was analysed to investigate the effects of nitrite and propolis additions on total coliform bacteria counts using a GLM procedure of univariate analysis. The results of the analysis showed that the differences in total coliform bacteria counts between samples were independent from propolis % levels and nitrite concentration and also on interactions between propolis % and nitrite concentration ($p > 0.05$) (Appendix E, Table E.7).



[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

Figure 4.38 : Changes of total coliform bacteria counts (log cfu/g) during storage of heat-treated dry fermented sucuks. [1]

In addition, Figure 4.39 presents the individual effects of propolis % and nitrite concentration on total coliform bacteria counts showing a decrease with propolis addition, but showing an increase on total coliform bacteria counts as their nitrite concentration increase from 75 ppm to 150 ppm, although those effects were insignificant statistically ($p > 0.05$).

According to Figure 4.40 propolis addition (0.25%) decreased total coliform bacteria count of heat treated sucuk samples depending on nitrite concentration, although those effects were insignificant statistically ($p > 0.05$). It is suggested that using lower amounts of sodium nitrite (75 ppm) and addition of propolis extract (0.25%) may help decreasing the total coliform bacteria counts of heat treated sucuks.

Table 4.24 : Total coliform bacteria analysis results of heat-treated dry fermented sucuk samples (log CFU/g) during production and storage. [1-4]

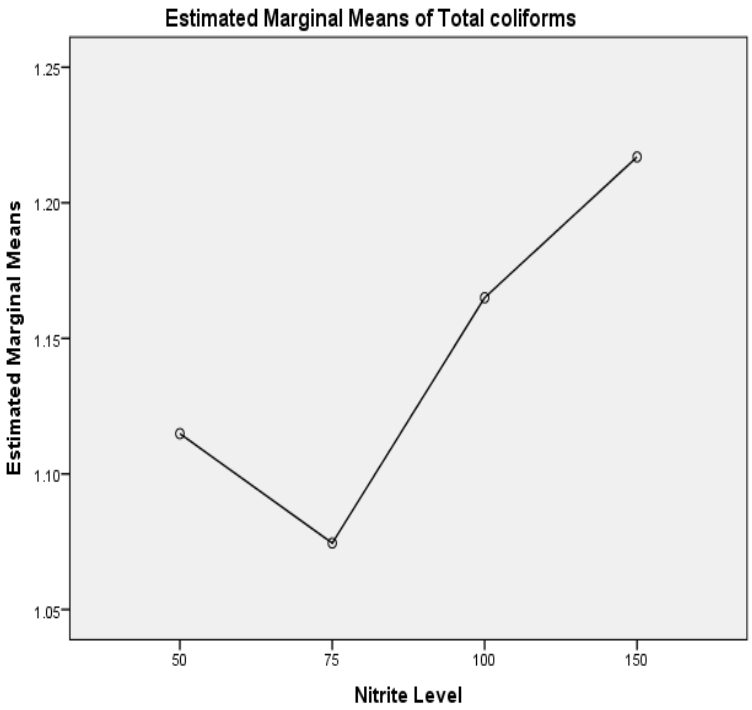
SAMPLE	R	A	B	C	D	E	F	G	H
Dough	4.34±0.02aC	4.52±0.01aA	4.23±0.05aD	4.15±0.06aE	4.31±0.01aCD	3.09±0.09aG	4.40±0.04aBC	4.44±0.05aB	3.95±0.05aF
0 day	0.88±0.15bA	0.70±0.17bAB	0.82±0.38bAB	0.78±0.00bAB	0.92±0.03bA	0.52±0.52cB	0.70±0.19bAB	0.64±0.45cAB	0.88±0.15bA
15th day	0.82±0.13bA	0.85±0.10bA	0.90±0.10bA	0.75±0.24bA	0.60±0.24bA	0.78±0.24bcA	0.70±0.33bA	0.75±0.05bcA	0.90±0.06bA
30th day	0.88±0.15bAB	0.78±0.00bAB	0.95±0.00bA	0.60±0.43bB	0.90±0.10bAB	0.90±0.06bcAB	0.67±0.48bAB	0.73±0.05bcAB	0.87±0.09bAB
45th day	0.67±0.16bA	0.75±0.34bA	0.82±0.13bA	0.80±0.25bA	0.70±0.33bA	0.64±0.15bcA	0.73±0.33bA	0.80±0.04bcA	0.88±0.09bA
60th day	1.03±0.30bA	1.20±0.30bA	1.08±0.38bA	1.21±0.41bA	1.15±0.77bA	1.04±0.78bcA	1.08±0.38bA	0.99±0.20bcA	1.03±0.70bA
90th day	1.26±0.31bA	1.07±0.10bA	1.18±0.23bA	1.12±0.17bA	1.22±0.28bA	1.07±0.10bA	1.12±0.17bA	1.18±0.23bA	1.22±0.28bA
120th day	1.60±0.58bA	1.22±0.28bA	1.12±0.17bA	1.12±0.17bA	1.07±0.10bA	1.18±0.23bA	1.12±0.17bA	1.22±0.28bA	1.12±0.17bA
150th day	1.56±0.76bA	1.05±0.30bA	1.04±0.29bA	1.00±0.20bA	0.92±0.63bA	1.09±0.36bA	1.10±0.20bA	1.14±0.43bcA	1.05±0.27bA
170th day	1.54±1.08bA	1.01±0.33bA	1.05±0.27bA	1.03±0.70bA	0.99±0.24bA	0.88±0.36bcA	0.97±0.50bA	1.05±0.30bcA	1.00±0.41bA
180th day	1.59±0.64bA	0.97±0.29bA	1.01±0.33bA	1.08±0.24bA	0.87±0.60bA	1.11±0.35bA	1.03±0.30bA	1.25±0.34bA	0.94±0.65bA
190th day	1.56±0.76bA	1.05±0.30bA	1.04±0.29bA	1.12±0.32bA	0.92±0.63bA	0.94±0.26bcA	1.07±0.27bA	1.07±0.27bcA	1.03±0.41bA
200th day	1.56±0.82bA	0.97±0.50bA	1.05±0.30bA	1.12±0.51bA	0.94±0.46bA	1.17±0.40bA	1.03±0.30bA	1.20±0.30bA	0.94±0.65bA

[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

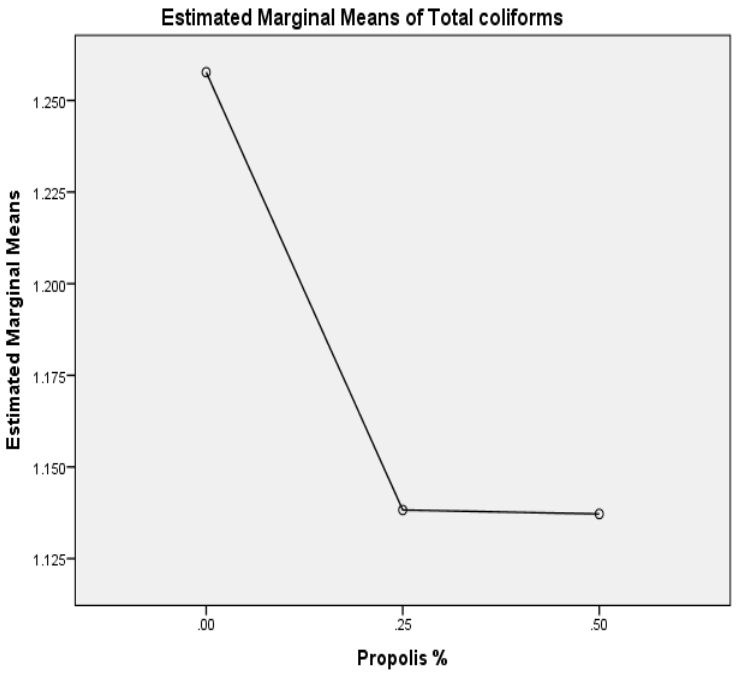
[2] Values in columns represent the mean of 3 replications and standard deviation.

[3] Values followed by the same small letters in columns are not significantly different ($p < 0.05$).

[4] Values followed by the same capital letters in rows are not significantly different ($p < 0.05$).



(a)



(b)

Figure 4.39 : Change in estimated marginal means of total coliform bacteria count depending on (a) nitrite concentration and (b) Propolis % ($p < 0.05$) in samples during storage.

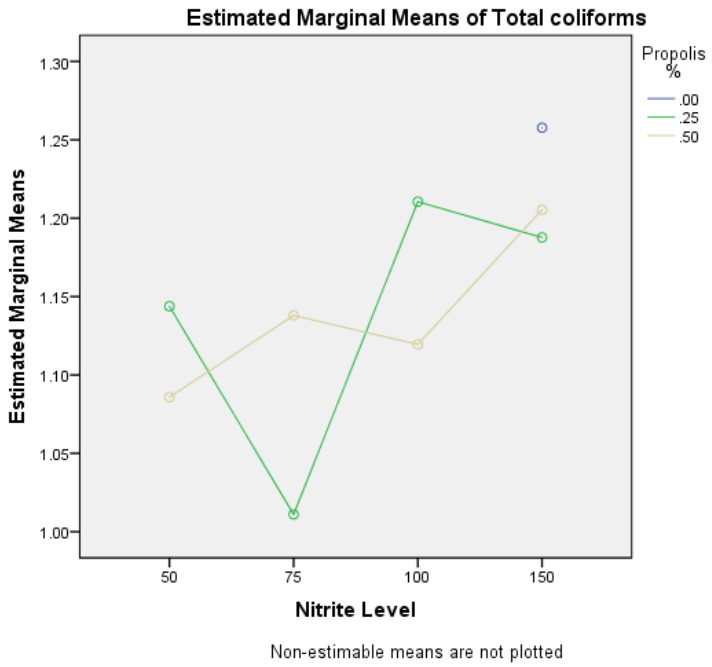


Figure 4.40 : Interaction plot of nitrite concentration and propolis % on total coliform bacteria counts during storage of nine products ($p < 0.05$).

4.5.2.4 Total yeast count

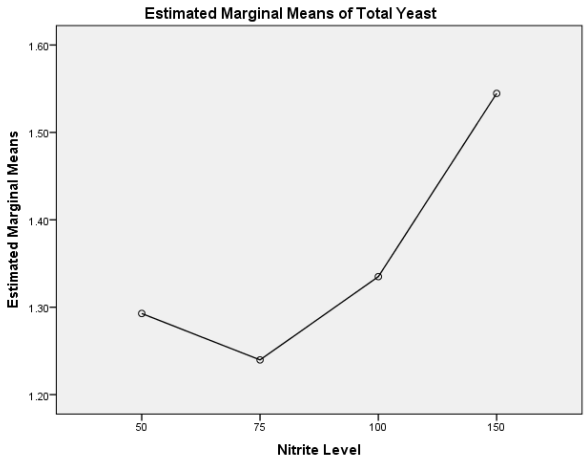
The changes of total yeast counts of heat-treated dry fermented sucuk samples during fermentation and storage were given in Figure 4.43. Results of triplicate analysis and statistically significant differences between each formulations of heat-treated dry fermented sucuk products and storage period intervals were presented in Table 4.25.

Before heat treatment, sucuk dough samples contain a total yeast amount in a range from 1.05±0.022 log cfu/g to 1.9±0.016 log cfu/g. After heat treatment total yeast amounts of samples were declined significantly in a range from 0.30±0.241 log cfu/g to 0.94±0.03 log cfu/g. There were no statistically significant differences between samples from 180th day to 200th day. Turkish Food Codex Microbiological Criteria Regulation states that total yeast count should not exceed log 2 /cfu/g. All samples fit to the rules of the Regulation on Turkish Food Codex Microbiological Criteria (No: 5996, 2011).

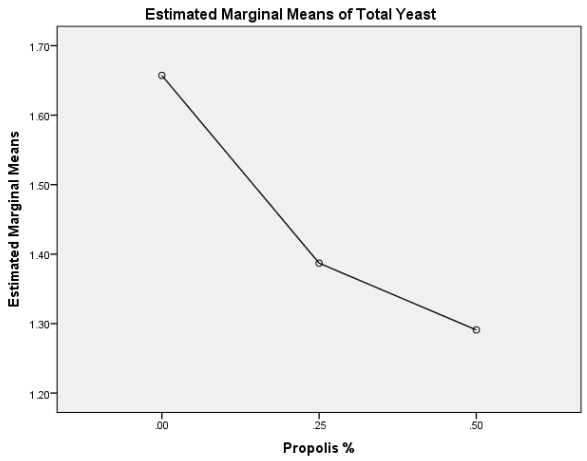
In addition, data was analysed to investigate the effects of nitrite and propolis additions on total yeast counts using a GLM procedure of univariate analysis. The results of the analysis showed that the differences in total yeast counts between samples were independent from propolis % levels and nitrite concentration and also on interactions between propolis% and nitrite concentration ($p > 0.05$) (Appendix E,

Table E.7). Figure 4.41 presents the individual effects of propolis % and nitrite concentration on total yeast counts showing a decrease as their propolis % levels increased, but showing an increase on total yeast counts as their nitrite concentration increase from 75 ppm to 150 ppm, although those effects were insignificant statistically ($p > 0.05$).

On the other hand, Figure 4.42 presents the interaction plot of nitrite concentration and propolis % on total yeast counts during storage of nine products. According to Figure 4.42 the increase of propolis % level decreased total yeast count of heat treated sucuk samples depending on nitrite concentration, although those effects were insignificant statistically ($p > 0.05$). It is suggested that using lower amounts of sodium nitrite and addition of propolis extract may help decreasing the total yeast counts of heat treated sucuks.



(a)



(b)

Figure 4.41 : Change in estimated marginal means of total yeast count depending on (a) nitrite concentration and (b) Propolis % ($p < 0.05$) in samples during storage.

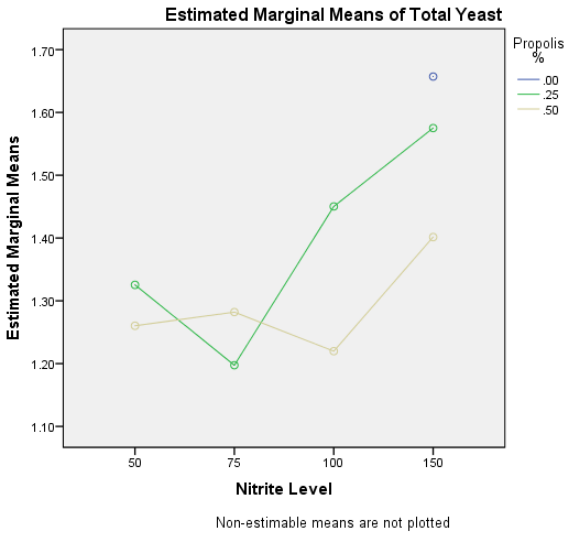
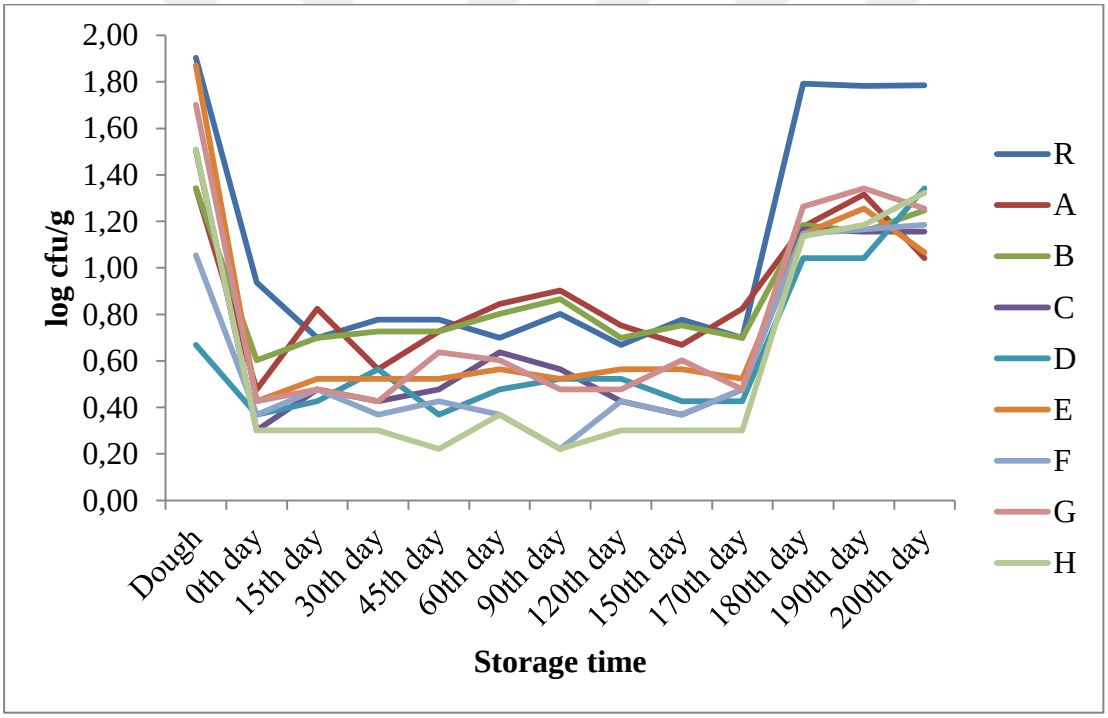


Figure 4.42 : Interaction plot of nitrite concentration and propolis % on total yeast counts during storage of nine products ($p < 0.05$).



[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

Figure 4.43 : Changes of total yeast counts (log cfu/g) during storage of heat-treated dry fermented sucuks. [1]

Table 4.25 : Total yeast analysis results of heat-treated dry fermented sucuk samples (log CFU/g) during production and storage. [1-4]

SAMPLE	R	A	B	C	D	E	F	G	H
Dough	1.90±0.02aA	1.34±0.02aD	1.34±0.02aD	1.51±0.01aC	0.67±0.16bcF	1.87±0.01aA	1.05±0.02aE	1.70±0.03aB	1.51±0.008aC
0th day	0.94±0.03bcAB	0.48±0.15cA	0.60±0.11cAB	0.30±0.24dCD	0.37±0.10cCD	0.43±0.17dBC	0.37±0.10bBC	0.43±0.10cBC	0.3±0.241cdC
15th day	0.70±0.09cAB	0.82±0.04bcA	0.70±0.09bcAB	0.48±0.15cdCD	0.43±0.10cCD	0.52±0.07cdBC	0.48±0.00bBC	0.48±0.00cBC	0.3±0.241cdD
30th day	0.78±0.23cA	0.56±0.07cAB	0.73±0.10bcA	0.43±0.45dB	0.56±0.07bcAB	0.52±0.17cdAB	0.37±0.10bAB	0.43±0.10cAB	0.3±0.241cdB
45th day	0.78±0.07bcA	0.73±0.05bcAB	0.73±0.05bcAB	0.48±0.15cdCD	0.37±0.10cDE	0.52±0.20cdBCD	0.43±0.10bCD	0.64±0.06cABC	0.22±0.275dE
60th day	0.70±0.09cAB	0.85±0.06bcA	0.80±0.04bcA	0.64±0.13bcdAB	0.48±0.15cBC	0.56±0.07cdBC	0.37±0.10bC	0.60±0.24cBC	0.37±0.102cdC
90th day	0.80±0.16bcAB	0.90±0.00abcA	0.87±0.03bcA	0.56±0.13bcdBC	0.52±0.17cBC	0.52±0.07cdBC	0.22±0.17bD	0.48±0.23cCD	0.22±0.275cdD
120th day	0.67±0.16cAB	0.75±0.21bcA	0.70±0.09bcA	0.43±0.32dBC	0.52±0.07cABC	0.56±0.07cdABC	0.43±0.10bABC	0.48±0.00cABC	0.3±0.241cdC
150th day	0.78±0.07bcA	0.67±0.06bcAB	0.75±0.05bcA	0.37±0.30dCD	0.43±0.10cBCD	0.56±0.21cdABCD	0.37±0.10bBCD	0.60±0.11cABC	0.3±0.241cdD
170th day	0.70±0.09cAB	0.82±0.04bcA	0.70±0.09bcAB	0.48±0.15cdCD	0.43±0.10cCD	0.52±0.07cdBC	0.48±0.00bBC	0.48±0.00cBC	0.3±0.241cdD
180th day	1.79±0.68abA	1.18±0.35abA	1.19±0.30abA	1.17±0.41abA	1.04±0.40abA	1.15±0.58bcA	1.15±0.58aA	1.26±0.41bA	1.14±0.751bcA
190th day	1.78±0.95abcA	1.32±0.68abA	1.16±0.48abcA	1.16±0.48abcA	1.04±0.40abA	1.26±0.46bA	1.17±0.40aA	1.34±0.40bA	1.19±0.302abA
200th day	1.79±0.85abcA	1.04±0.40abA	1.25±0.53abA	1.16±0.48abcA	1.34±0.40aA	1.07±0.28bA	1.19±0.30aA	1.26±0.46bA	1.32±0.577abA

[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

[2] Values in columns represent the mean of 3 replications and standard deviation.

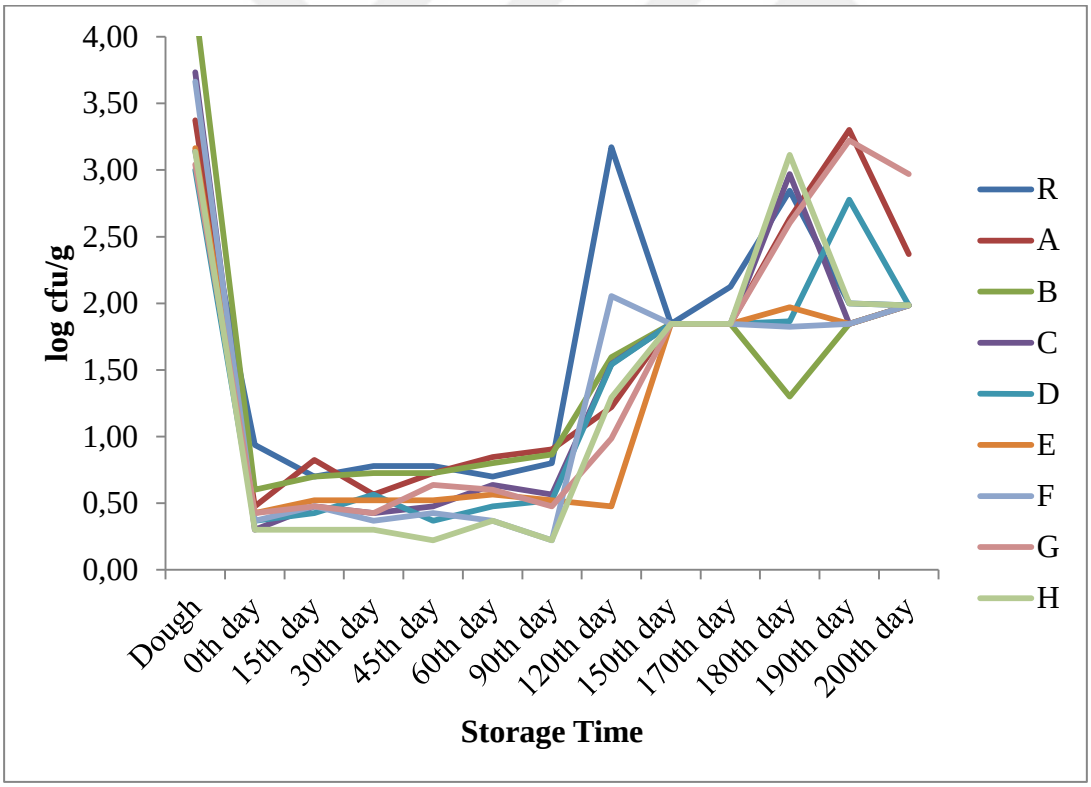
[3] Values followed by the same small letters in columns are not significantly different ($p < 0.05$).

[4] Values followed by the same capital letters in rows are not significantly different ($p < 0.05$).

4.5.2.5 Total mold count

The changes of total mold counts of heat-treated dry fermented sucuk samples during storage period were given in Figure 4.44. Results of triplicate analysis and statistically significant differences between each formulations of heat-treated dry fermented sucuk products and storage time intervals were given in Table 4.26.

Before heat treatment, sucuk dough samples contain a total mold amounts in a range from 3.04±0.08 log cfu/g to 4.23±0.01 log cfu/g. After heat treatment total mould amounts of samples were declined significantly in a range from 0.3±0.241 log cfu/g to 0.94±0.03 log cfu/g. There were no statistically significant differences between samples from 180th day to 200th day. The Regulation on Turkish Food Codex Microbiological Criteria (No: 5996, 2011) states that total yeast count should not exceed log 3 /cfu/g. All samples were acceptable according to the Regulation on Turkish Food Codex Microbiological Criteria (No: 5996, 2011).



[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

Figure 4.44 : Changes of total mould counts (log cfu/g) during storage of heat-treated dry fermented sucuks. [1]

Table 4.26 : Total mould analysis results of heat-treated dry fermented sucuk samples (log CFU/g) during production and storage. [1-4]

SAMPLE	R	A	B	C	D	E	F	G	H
Dough	3.14±0.00abD	3.37±0.03aC	4.23±0.01aA	3.73±0.01aB	3.00±0.09aE	3.17±0.03aD	3.66±0.03aB	3.04±0.08abE	3.14±0.10aD
0th day	0.94±0.03eA	0.48±0.15eBC	0.60±0.11eB	0.30±0.24eC	0.37±0.10cBC	0.43±0.17cBC	0.37±0.10cBC	0.43±0.10dBC	0.30±0.24cdC
15th day	0.70±0.09eAB	0.82±0.04deA	0.70±0.09deAB	0.48±0.15eCD	0.43±0.10cCD	0.52±0.07cBC	0.48±0.00cBC	0.48±0.00dBC	0.30±0.24cdD
30th day	0.78±0.23eA	0.56±0.07eAB	0.73±0.10deA	0.43±0.45eB	0.56±0.07cAB	0.52±0.17cAB	0.37±0.10cAB	0.43±0.10dAB	0.30±0.24cdB
45th day	0.78±0.07eA	0.73±0.05eAB	0.73±0.05deAB	0.48±0.15eCD	0.37±0.10cDE	0.52±0.20cBCD	0.43±0.10cCD	0.64±0.06dABC	0.22±0.28dE
60th day	0.70±0.09eAB	0.85±0.06deA	0.80±0.04deA	0.64±0.13eAB	0.48±0.15cBC	0.56±0.07cBC	0.37±0.10cC	0.60±0.24dBC	0.37±0.10cdC
90th day	0.80±0.16eAB	0.90±0.00deA	0.87±0.03deA	0.56±0.13eBC	0.52±0.17cBC	0.52±0.07cBC	0.22±0.17cD	0.48±0.23dCD	0.22±0.28dD
120th day	3.17±0.03aA	1.22±0.17dBC	1.59±0.60cdBC	1.54±0.93deC	1.54±0.98cC	0.48±0.15bC	2.05±0.08bB	0.99±0.55dC	1.29±0.78cC
150th day	1.85±0.58dA	1.85±0.58cA	1.85±0.58bcA	1.85±0.58cdA	1.85±0.58bA	1.85±0.58bA	1.85±0.58bA	1.85±0.58cA	1.85±0.58bA
170th day	2.12±0.04cA	1.85±0.58cA	1.85±0.58bcA	1.85±0.58cdA	1.85±0.58bA	1.85±0.58bA	1.85±0.58bA	1.85±0.58cA	1.85±0.58bA
180th day	2.85±0.12bAB	2.64±0.06bB	1.30±0.00cdD	2.97±0.06bA	1.87±0.40bC	1.97±0.06bC	1.82±0.20bC	2.60±0.00bB	3.11±0.06aA
190th day	2.00±0.00cB	3.30±0.00aA	1.85±0.58bcB	1.85±0.58cdB	2.78±0.00bA	1.85±0.58bB	1.85±0.58bB	3.22±0.03aA	2.00±0.00bB
200th day	1.99±0.03cC	2.37±0.28bB	1.99±0.03bC	1.99±0.03cC	1.99±0.03bC	1.99±0.03bC	1.99±0.03bC	2.97±0.06abA	1.99±0.03bC

[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

[2] Values in columns represent the mean of 3 replications and standard deviation.

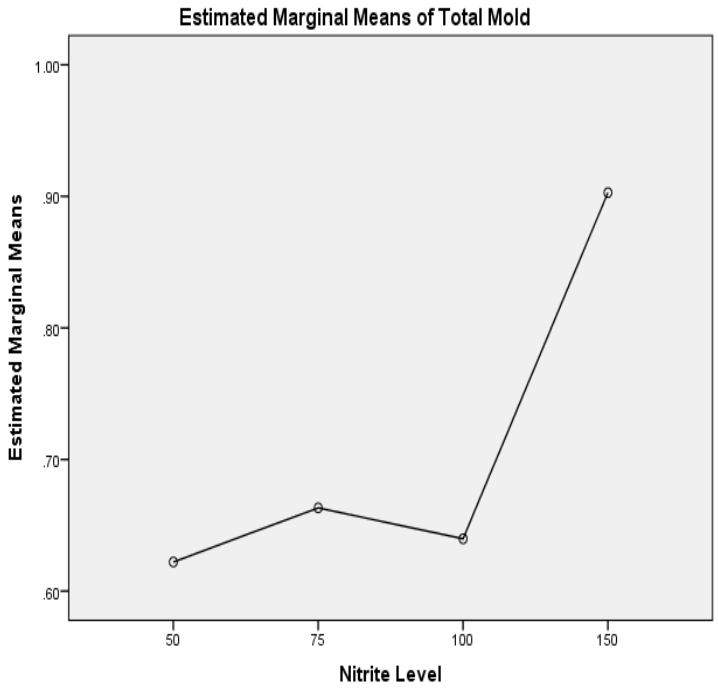
[3] Values followed by the same small letters in columns are not significantly different ($p < 0.05$).

[4] Values followed by the same capital letters in rows are not significantly different ($p < 0.05$).

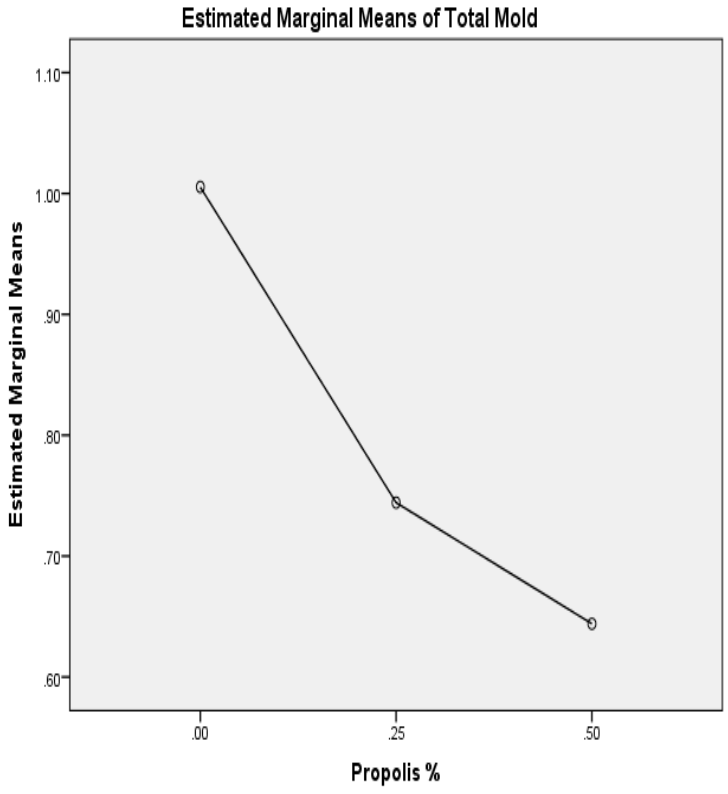
Erkmen and Bozkurt (2004) examined the quality characteristics of commercially available sucuks that had been produced at the factory (19 samples) and butchers (31 samples). Mould and yeast counts of factory and butcher's sucuks varied from 3.15 to 5.53 log CFU/g and from 3.48 to 5.80 log CFU/g, respectively. In this respect, mould and yeast counts of our samples were found considerably lower than those reported. Unsanitary production conditions, poor quality of raw materials, low temperature and humidity increase the number of mold and yeast (Bilenler et al., 2017).

In addition, all data including storage time was analysed to investigate the effects of nitrite and propolis additions on total mold counts using an GLM procedure of univariate analysis. The results of the analysis showed that the differences in total mold contents between samples were dependent on propolis % levels and nitrite concentration ($p < 0.05$), but independent from interactions between propolis % level and nitrite concentration ($p > 0.05$) (Appendix E, Table E.7). In addition, Figure 4.45 presents the individual effects of propolis % and nitrite concentration on total mold counts showing a decrease as their propolis % levels increased, but showing an increase on total mold counts as their nitrite concentration increase to 150 ppm. On the other hand, Figure 4.46 presents the interaction plot of nitrite concentration and propolis % on total mold counts during storage of nine products. Accordingly, the addition of propolis decreased total mold count of heat treated sucuk samples depending on nitrite concentration, although those effects were insignificant statistically ($p > 0.05$). It is suggested that using lower amounts of sodium nitrite and addition of propolis extract may help decreasing the total mold counts of heat treated sucuks.

Additionally regression analysis with curve estimation (linear, logarithmic, quadratic and cubic) were performed to understand the effect on propolis on total mold count during storage time. The results of this analysis presented in Appendix E, Table E.8 and Table E.9 proved that there is a linear curve estimation and propolis addition to formulations of heat treated sucuks statistically decrease the total mold counts during storage. Stepwise linear regression analysis was also performed (Appendix E, E.10 and E.11) and same results proved out that propolis addition decrease the total mould counts of heat treated sucuk formulations ($p < 0.05$).



(a)



(b)

Figure 4.45 : Change in estimated marginal means of total yeast count depending on (a) nitrite concentration and (b) Propolis % ($p < 0.05$) in samples during storage.

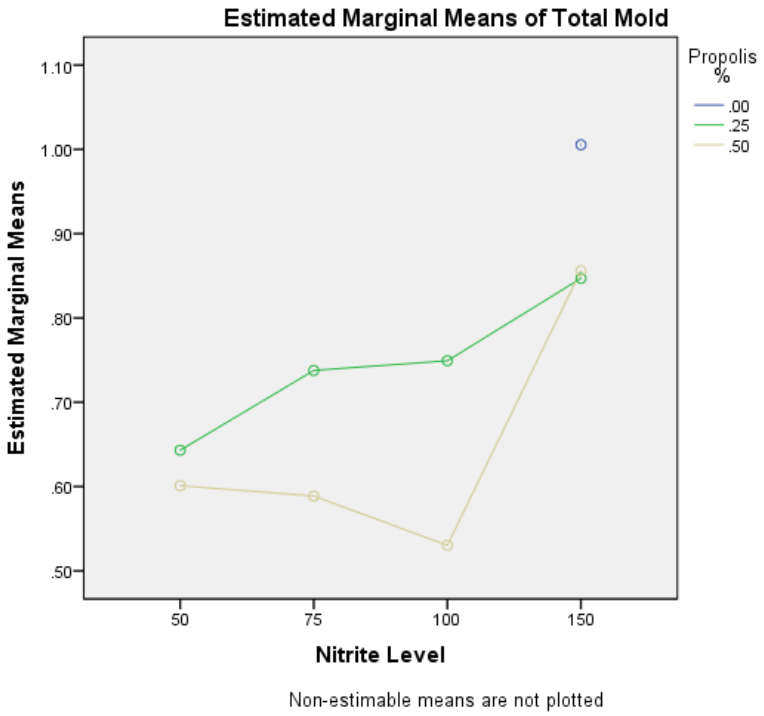


Figure 4.46 : Interaction plot of nitrite concentration and propolis % on total mold counts during storage of nine products ($p < 0.05$).

4.5.2.6 *Lactobacillus* spp. test

The changes of *Lactobacillus* spp. counts of heat-treated dry fermented sucuk samples during fermentation and storage were given in Figure 4.47 and in Table 4.27.

Before heat-treatment, *Lactobacillus* spp. counts of sucuk doughs were between 3.74±0.02 log cfu/g and 4.00±0.22 log cfu/g. After heat-treatment of fermented sucuks *Lactobacillus* spp. counts were decreased to a range of 1.18±.00 log cfu/g and 1.67±0.40 log cfu/g. Control sample has the highest value for *Lactobacillus* spp. after heat-treatment. There were no statistically significant differences in relation to storage time period. Similarly also no statistically significant differences between samples were observed at 190th and 200th days ($p > 0.05$). Heat-treated dry fermented sucuk samples have a stable *Lactobacillus* spp. count during storage.

In addition, all data including storage time was analysed to investigate the effects of nitrite and propolis additions on *Lactobacillus* spp. counts using a GLM procedure of univariate analysis. The results of the analysis showed that the differences in *Lactobacillus* spp. counts between samples were independent from propolis % levels and nitrite concentration and also on interactions between propolis% and nitrite concentration ($p > 0.05$) (Appendix E, Table E.7).

Table 4.27 : *Lactobacillus* spp. analysis results of heat-treated dry fermented sucuk samples (log CFU/g) during production and storage. [1-4]

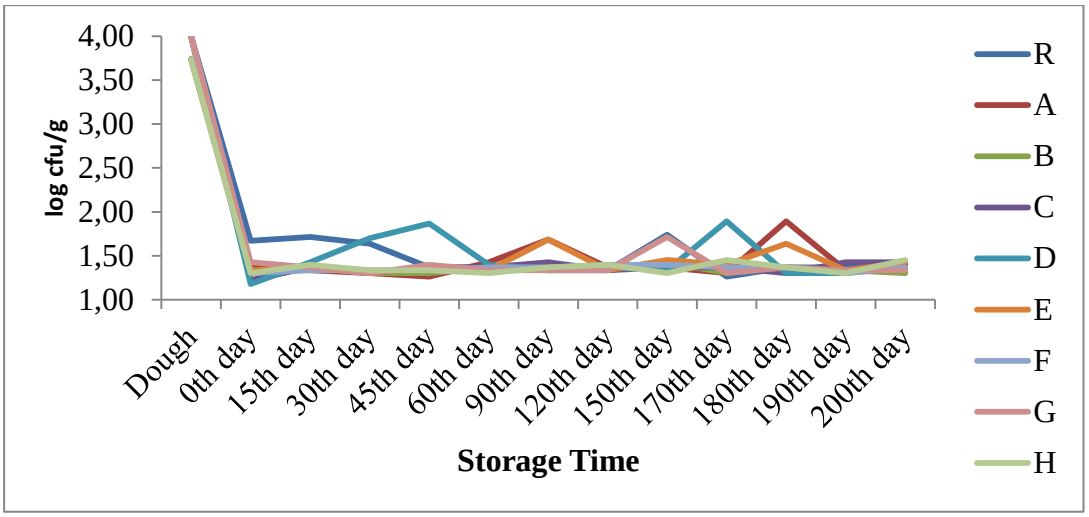
SAMPLE	R	A	B	C	D	E	F	G	H
Dough	4.00±0.02aA	3.74±0.02aB	3.74±0.02aB	4.00±0.01aA	4.00±0.02aA	4.00±0.03aA	4.00±0.01aA	4.00±0.04aA	3.74±0.01aB
0th day	1.67±0.40bA	1.37±0.10cAB	1.30±0.11bAB	1.22±0.07bB	1.18±0.00dB	1.34±0.13bAB	1.30±0.00bAB	1.43±0.10bAB	1.30±0.00bAB
15th day	1.71±0.36bA	1.34±0.15cA	1.37±0.06bA	1.37±0.19bA	1.43±0.20cdA	1.37±0.16bA	1.34±0.06bA	1.37±0.16bA	1.40±0.09bA
30th day	1.64±0.48bA	1.30±0.11cA	1.30±0.00bA	1.30±0.11bA	1.70±0.41bcdA	1.30±0.17bA	1.34±0.06bA	1.30±0.00bA	1.34±0.15bA
45th day	1.37±0.10bB	1.26±0.07cB	1.30±0.11bB	1.37±0.16bB	1.87±0.40bcA	1.37±0.10bB	1.34±0.06bB	1.40±0.19bB	1.34±0.21bB
60th day	1.37±0.19bA	1.43±0.20cA	1.37±0.16bA	1.37±0.10bA	1.40±0.19dA	1.34±0.21bA	1.37±0.16bA	1.34±0.06bA	1.30±0.00bA
90th day	1.34±0.15bA	1.68±0.38bcA	1.37±0.10bA	1.43±0.12bA	1.34±0.15dA	1.68±0.38bA	1.37±0.10bA	1.34±0.15bA	1.37±0.06bA
120th day	1.34±0.21bA	1.37±0.16cA	1.34±0.06bA	1.34±0.15bA	1.37±0.10dA	1.34±0.06bA	1.40±0.19bA	1.34±0.21bA	1.40±0.09bA
150th day	1.74±0.29bA	1.37±0.16cAB	1.43±0.12bAB	1.37±0.10bAB	1.34±0.15dAB	1.45±0.13bAB	1.40±0.09bAB	1.71±0.36bAB	1.30±0.17bB
170th day	1.26±0.07bB	1.30±0.00cB	1.30±0.11bB	1.37±0.16bB	1.89±0.26bA	1.40±0.14bB	1.37±0.10bB	1.30±0.11bB	1.45±0.13bB
180th day	1.37±0.16bB	1.89±0.26bA	1.37±0.10bB	1.30±0.00bB	1.30±0.11dB	1.64±0.48bB	1.37±0.16bB	1.37±0.19bB	1.37±0.16bB
190th day	1.37±0.10bA	1.34±0.21cA	1.34±0.21bA	1.43±0.12bA	1.30±0.17dA	1.34±0.06bA	1.30±0.00bA	1.34±0.15bA	1.30±0.00bA
200th day	1.34±0.15bA	1.40±0.09cA	1.30±0.17bA	1.43±0.12bA	1.37±0.16dA	1.43±0.12bA	1.37±0.10bA	1.34±0.15bA	1.45±0.13bA

[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

[2] Values in columns represent the mean of 3 replications and standard deviation.

[3] Values followed by the same small letters in columns are not significantly different ($p < 0.05$).

[4] Values followed by the same capital letters in rows are not significantly different ($p < 0.05$).



[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

Figure 4.47 : Changes of *Lactobacillus* spp. counts (log cfu/g) during storage of heat-treated dry fermented sucuks. [1]

In a recent study of Bilenler et al. (2017), they have found the lactic acid bacteria count after heat-treatment as 0.69±0.15 log cfu/g in control sample, 2.19±0.11 log cfu/g in starter culture added sample, and 5.72±0.33 log cfu/g encapsulated starter culture added sample. In heat treated sucuk group, the samples inoculated with encapsulated cultures showed higher lactic acid bacteria counts than those of inoculated with free cultures ($p < 0.05$). Compared to initial counts (7 log cfu/g sucuk), reduction rates of lactic acid bacteria in heat treated samples at day 0 were 1.28 log unit for encapsulated sample. The heat treatment is expected to destroy microorganisms. Ensoy et al. (2010) reported that the number of these bacteria in fermented sucuks was significantly reduced by thermal processes. The highest lactic acid bacteria counts in heat treated group were determined in the sample inoculated with encapsulated starter culture during storage periods ($p < 0.05$). Based on their results, they proposed that the encapsulation of starter cultures using alginate-starch could have successfully protected them from the harmful effect of the heat treatment.

4.5.2.7 *Salmonella* spp. test

Salmonella spp. analysis were performed at dough at 0 day and at the end of 200th day. There were no risk of *Salmonella* spp. as analysis results were shown in Table 4.28.

Table 4.28 : Salmonella spp. analysis results of heat-treated dry fermented sucuk samples during production and storage. [1, 2]

	Dough			0 day			200th day		
SAMPLE	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
R	negative	negative	negative	negative	negative	negative	negative	negative	negative
A	negative	negative	negative	negative	negative	negative	negative	negative	negative
B	negative	negative	negative	negative	negative	negative	negative	negative	negative
C	negative	negative	negative	negative	negative	negative	negative	negative	negative
D	negative	negative	negative	negative	negative	negative	negative	negative	negative
E	negative	negative	negative	negative	negative	negative	negative	negative	negative
F	negative	negative	negative	negative	negative	negative	negative	negative	negative
G	negative	negative	negative	negative	negative	negative	negative	negative	negative
H	negative	negative	negative	negative	negative	negative	negative	negative	negative

[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.
[2] Three analysis were performed at each day of analysis.

4.5.2.8 *Listeria monocytogenes* test

L. monocytogenes analysis were performed at dough at 0 day and at the end of 200th day. Before heat treatment of samples C, E and F had positive results for *L. monocytogenes.* However after heat treatment at 0th and 200th days of storage there was no risk for this pathogen bacteria. Table 4.29 shows the results of *L. monocytogenes* for all samples at dough and 0th and 200th days.

Table 4.29 : *Listeria monocytogenes* analysis results of heat-treated dry fermented sucuk samples. [1]

	Dough			0 day			200th day		
SAMPLE	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
R	negative	negative	negative	negative	negative	negative	negative	negative	negative
A	negative	negative	negative	negative	negative	negative	negative	negative	negative
B	negative	negative	negative	negative	negative	negative	negative	negative	negative
C	**positive**	**positive**	**positive**	negative	negative	negative	negative	negative	negative
D	negative	negative	negative	negative	negative	negative	negative	negative	negative
E	**positive**	**positive**	**positive**	negative	negative	negative	negative	negative	negative
F	**positive**	**positive**	**positive**	negative	negative	negative	negative	negative	negative
G	negative	negative	negative	negative	negative	negative	negative	negative	negative
H	negative	negative	negative	negative	negative	negative	negative	negative	negative

[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

4.5.3 Sensory Analysis

Sensory analysis of the nine different formulations of heat-treated dry fermented sucuk samples was carried out in a sensory analysis laboratory at 25 ˚C. Duo-trio test procedure was applied to determine whether product differences result from a change of propolis extract content in their formulations and an overall difference exist between samples. Constant reference mode is applied in which the same sample, which contains 150 ppm nitrite but no propolis extract, is always the reference. The heat-treated dry fermented sucuk samples in sensory panel are including: (R) reference sample with 150 ppm nitrite; (A) 150 ppm nitrite and 0.25% propolis extract; (B) 150 ppm nitrite and 0.50% propolis extract (C) 50 ppm nitrite and 0.25% propolis extract; (D) 50 ppm nitrite and 0.50% propolis extract; (E) 75 ppm nitrite and 0.25% propolis extract; (F) 75 ppm nitrite and 0.50% propolis extract; (G) 100 ppm nitrite and 0.25% propolis extract; (H) 100 ppm nitrite and 0.50% propolis extract. The procedure of this method is to present to each subject an identified reference sample, followed by giving two coded samples with three digit numbers, one of which matches the reference sample. Subjects were asked to indicate which coded sample matched the reference. The number of correct replies were counted and interpreted according to reference tables. According to reference table, minimum correct answers at 5% significance level should be 17.

Table 4.30 : Sensory analysis test results. [1]

Sucuk Samples [1]	Correct Results	Minimum correct answers ($p < 0.05$)
A	14	17
B	18	17
C	21	17
D	18	17
E	15	17
F	13	17
G	19	17
H	17	17

[1]A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

The samples having correct answers above 17 are statistically significant different from the reference sample. The results of sensory panels showed that samples A, E, and F have totally 14, 15, and 13 correct answers, respectively that the panelists understand the difference from the reference sample correctly. However, according

to Appendix C, Table C.1 the minimum correct answers for statistically significance level ($p \leq 0.05$) is shown as 17. All correct answers were shown in Table 4.30.

Therefore, it can be concluded that using half of the amount of nitrite than reference sample and addition of propolis as a powerful antimicrobial agent to dry-fermented sucuk formulations result in no statistically significant sensory difference.

5. CONCLUSIONS

Propolis is an apicultural product that honey bees collect plant resins to their hives and mix varying amounts of wax which they produce themselves with the resins during deposition. Propolis serves for many purposes in the hive including reducing crack and hive entrances, promoting stable temperature and humidity in the hive, preventing fungal decay of the hive walls and reducing hive microbial loads. It has been well known from ancient times for its health beneficial characteristics including antioxidant, antimicrobial, anticancer, anti-inflammatory properties. In this study, *in vitro* total phenolic contents, total flavonoid contents, total antioxidant capacities and individual phenolic contents of 54 samples of propolis collected from different geographical regions and beekeepers of Turkey were investigated.

Phenolic composition and biological activities of propolis is strongly related with the flora surrounding the hive, climate, seasonal changes. Turkey is situated in a geographical area where climate conditions are temperate, there are significant changes between seven geographical regions. Coastal regions have milder climate whereas Anatolian regions have a dry climate with hot summers, cold winters and great temperature difference between day and night. Istanbul and Marmara region has a milder climate wheras the climate in the Black Sea region is wet, warm and humid. In East Anatolia there is a long hard winter, but in Aegean region the climate is mild. According to our results there were statistically significant differences ($p < 0.05$) between total phenolic and total flavonoid contents of propolis samples collected from all regions of Turkey and it was suggested that these differences were resulted from the climate and flora around the hive. Interestingly, the highest total phenolic content, total flavonoid content and total antioxidant capacities were obtained from Marmara region, from Istanbul and Kocaeli where the temperature is mild. To our knowledge, this is the first study that evaluate the total phenolic and flavonoid contents and total antioxidant capacities of many propolis samples collected from all geographical regions of Turkey. Our results show that propolis from Turkey displays an extraordinary higher total phenolic and flavonoid contents

and total antioxidant capacities than those of other natural products (honey, berries, pomegranate etc.) in addition to their high individual flavonoid contents compared with the propolis collected from other parts of the world given in literature. The results of correlation analysis were high indicating that there is a strong correlation between the total phenolic content, total flavonoid content and antioxidant capacity values according to ABTS, DPPH, CUPRAC and FRAP methods for all propolis samples. It was suggested that the antioxidant capacity of propolis could be contributed to the phenolic compounds especially flavonoids.

All propolis samples have high total antioxidant capacities measured with four different spectrophotometrical methods. It is expected that a single method cannot determine all the antioxidant compounds available in the food matrix correctly. All the methods have their own advantages and disadvantages. Even the results of the methods sharing the same principle like ABTS and DPPH can show important differences in their response to antioxidants. It is highly recommended to apply several test procedures to evaluate antioxidant capacity. Therefore, in this study four different types of antioxidant capacity analysis methods ABTS, DPPH, CUPRAC and FRAP were performed. As a conclusion to all the antioxidant activity values, it was observed that CUPRAC method yielded consistent data and highest antioxidant activity values with respect to the results of ABTS, DPPH and FRAP methods. Moreover, the antioxidant capacities of propolis samples were found to be insignificant between years (2013 and 2014) and 6 regions (Aegean, Black Sea, Central Anatolia, East Anatolia, Marmara and Mediterranean regions).

Moreover, nine individual phenolic compounds including caffeic acid, ferulic acid, *p*-coumaric acid, *t*-cinnamic acid, galangin, chrysin, pinobanksin, pinostrobin and pinocembrin of 54 propolis samples collected from various geographical areas and beekeepers of Turkey in 2013 and 2014 have been identified and quantified. The main flavonoids including galangin, chrysin, pinobanksin, pinostrobin and pinocembrin were found to be higher in propolis collected from Istanbul and Kocaeli provinces located in Marmara region of Turkey in strong correlation with their high total antioxidant capacities. Additional *in vivo* studies are needed to determine the metabolism of these phenolic compounds in the human digestive system and their bioavailability to understand their potential health benefits clearly. There were statistically insignificant differences ($p > 0.05$) between individual phenolic contents

of propolis samples collected in 2013 compared to individual phenolic contents of propolis samples collected in 2014. However, there were statistically significant differences ($p < 0.05$) between individual phenolic contents of propolis samples including chrysin, galangin, *p*-coumaric acid, pinobanksin, pinocembrin, *t*-cinnamic acid and insignificant differences between individual phenolic contents including caffeic acid, ferulic acid and pinostrobin when different regions are considered as a factor.

As a conclusion of PCA analysis, propolis samples from the Marmara region were mostly characterized by contributions of caffeic acid, cinnamic acid, pinostrobin, galangin, amounts of total phenolics and total flavonoids, pinocembrin and chrysin. On the other hand, Black Sea propolis was well characterized by higher levels of total phenolics and total flavonoids, chrysin, and pinostrobin. The regions of Aegean, Central and East Anatolia, and Mediterranean were partially overlapped each other. Antioxidant properties and phenolic profile of propolis were changed depending on the regions starting from North to the south and east of Turkey.

As a conclusion of Linear Discriminant Analysis, there were significant differences between means of geographical regions for all predictors with pinocembrin, *t*-cinnamic acid, *p*-coumaric acid, and chrysin. According to the Quadratic Discriminant Analysis, higher amounts of phenolic acids are good predictors of discriminating propolis of Black Sea and Marmara regions. Additionally, low levels of flavonoid galangin discriminated Central Anatolia and Aegean regions from other regions. Furthermore, higher values of pinocembrin was found to be a good predictor for discriminating propolis of regions of Black Sea, Marmara, and Central Anatolia from the other regions. The Black Sea, Marmara and Central Anatolia regions were differentiated from East Anatolia, Mediterranean and Aegean regions. However, it was obvious that the groups of Mediterranean and Aegean propolis are intertwined possibly according to similar botanical flora. To our knowledge, this is the first report that evaluate these findings that can be used in standardization of Turkish propolis.

According to cytoxicity analysis results, it has been observed that MDA-MB-231 cell line is the most sensitive cell line to propolis. Further studies including isolation of major bioactive compounds with cytogenetic and molecular tests will better elucidate the antioxidant and anti-cancer activities observed here. Isolated compounds should

also be investigated regarding its biological properties against different tumor and non-tumor cell lines, using MTT-based assays. Besides, Turkish propolis sample showed proliferative effect on human fibroblast cells and mouse MSCs, but the molecular mechanism is still unclear. There is a very limited data in the literature on the anti-cancer activities of Turkish propolis, and to our knowledge it is the first report on the effects of propolis on MDA-MB-231 breast cancer cell line and UACC-3199 human infiltrating ductal carcinoma of breast, fibroblast and mouse mesenchymal stem cell line C57B/6. Further studies should focus on proliferation analysis of several different types of normal human cells and MSCs from different sources as human peripheral blood derived MSCs. The polyphenol-rich Turkish propolis can be an attractive source of nutraceuticals and medical ingredients with its great antioxidant capacity and it will be much more valuable in the future than today. However, further research is necessary to clarify precise targets of propolis in breast cancer cells.

Nine different formulations of heat-treated sucuks produced with propolis extract concentrations of 0.25% and 0.50% and lower sodium nitrite amounts (50 ppm, 75 ppm and 100 ppm) compared with heat-treated reference sucuk that contain no propolis but 150 ppm of sodium nitrite regarding their physicochemical, microbiological and sensory properties. Besides, sample A and sample B contain propolis extract of 0.25% and 0.50% and 150 ppm of sodium nitrite to compare with the reference sample. According to the pH analysis results, differences in pH of samples appeared to be related depending on interactions between levels of propolis and nitrite. It was observed that increasing propolis to 0.5% suppressed the increase in pH as expected due to the increase in nitrite concentration to 150 ppm. The increase in propolis percentages decreased the moisture levels in products. On the other hand, a significant interaction observed between propolis levels and nitrite concentration that the decrease in moisture contents can be influenced by the lower nitrite levels. Moreover, the fat contents of samples increased as propolis addition level increased while increasing the nitrite level presented a decrease in fat contents of samples. However, according to the significant interaction of propolis % and nitrite concentration on fat contents, the fat contents were decreased with a gradual increase in nitrite concentration at 0.25% propolis level, while an increase at 0.50% propolis addition.

The addition of propolis extract and sodium nitrite content resulted in lower amounts of collagen as their amounts increased. In addition, the interactions between levels of those two factors were effective on collagen amounts. Accordingly, the decrease in collagen amount with 0.5% propolis addition was lower when nitrite levels are above 100 ppm. The salt content was increased during 200 day of storage as moisture content decreases. The highest salt content was reached at sample C that contains 150 ppm sodium nitrite and 0.50% propolis extract. Furthermore, the moisture/protein value for all samples including propolis extract was significantly ($p < 0.05$) lower than the control sample. Water/protein ratio has a significant influence on the textural characteristics of sucuk. The sucuk will lose more water when cooked due to the higher water/protein ratio. This higher water/protein ratio will also cause the ionic strength to be lower, decreasing the extraction of meat proteins. Accordingly, the propolis extracts related with a lower water/protein ratio will increase the acceptance of a product as they will loose less water according to their lower water/protein ratio compared to the control sample. TBARS values were affected by propolis levels, nitrite concentration, and also by interactions between propolis and nitrite levels. According to the TBARS analysis results, it can be concluded that propolis extract addition to heat-treated sucuk formulations significantly reduce the TBARS values as it is a strong antioxidant that retards lipid oxidation. On the other hand, the interaction between propolis % and nitrite levels resulted in an increase in lipid oxidation only at 0.25% propolis addition at 150 ppm nitrite concentration and when propolis increased to 0.50%, lipid oxidation was lowered again. Addition of propolis and changing sodium nitrite content influence the color parameters (L*, a* and b*) as statistically significant differences between samples ($p < 0.05$) were observed. The addition of propolis decreased lightness values of sucuk samples depending on nitrite concentration. The nitrite level and the interactions between propolis % and nitrite levels significantly affected the redness values. Nitrite concentration increases the redness value. The increase in redness value may become higher at 0.5% propolis level with an increase in nitrite concentration. Besides, propolis percentage, nitrite concentration and possible interactions between those factors affected yellowness values. Yellowness values decreases as the level of propolis % increases. However, due to the interactions between two factors, the yellowness values increased as nitrite concentration increased only at lower level of propolis.

Propolis addition was found to be significant ($p < 0.05$) in physicochemical properties including moisture, fat, collagen contents, TBARS, color (b* value) and microbiological properties of only total mold count ($p < 0.05$). Nitrite concentration was found to be significant ($p < 0.05$) in physicochemical characteristics including fat, protein, collagen contents, TBARS values, color (a* and b* values) and microbiological characteristics of only total mold counts. The differences of physicochemical characteristics including pH, moisture, fat, collagen contents, TBARS values and color characteristics were found to be dependent on interactions between propolis % and nitrite concentration ($p < 0.05$).

According to the microbiological analysis including total aerobic bacteria, *E. coli*, total coliform, total mold and yeast, *Lactobacillus* spp., *Salmonella* spp. *L. monocytogenes* all samples are considered as acceptable according to the food safety rules. Therefore, it can be stated that propolis is a strong antibacterial agent and can be effective at very small amounts when used in heat-treated dry fermented sucuk formulations to ensure the food safety throughout 200 days of storage time. Propolis addition was found to be effective especially on decreasing total mold counts. However, the inhibitory effects of propolis extract on several microorganisms needs to be confirmed in challenge studies.

Furthermore, according to the sensory analysis results applied to samples according to duo-trio test procedure to determine whether product differences result from a change of propolis extract content in their formulations and an overall difference exist between samples, it can be concluded that using half of the amount of sodium nitrite than reference sample and addition of propolis as a powerful antimicrobial agent to heat treated sucuk formulations result in statistically insignificant sensory difference. Sensory analysis results showed that consumer acceptance for products produced with propolis extract and lower concentrations of sodium nitrite was given and that these products may be marketable. It would be more healthier alternative to use propolis as an antimicrobial agent to decrease the nitrite amount in production of heat-treated dry fermented sucuk. To our knowledge, this is the first report that evaluate the effect of propolis extract as a natural antimicrobial agent to be used in heat-treated sucuk formulations lowering the amount of sodium nitrite. In regards of all physicochemical, microbiological and sensory analysis propolis is considered as

an acceptable natural product that has many health benefits and can be used in nitrite-reduced heat treated sucuk products as a powerful antimicrobial agent.

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APPENDICES

APPENDIX A : Propolis Samples

APPENDIX B : Calibration Curves

APPENDIX C : Duo-trio Test Table

APPENDIX D : Antioxidant Properties of Propolis Samples

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APPENDIX F : Fat, Protein and Salt Contents of Heat Treated Sucuk Samples (As Is Basis)

APPENDIX A : Propolis Samples

Table A.1 : Region, beekeper and collection year of propolis samples.

Sample No	Year	Region	Beekeper
1	2013	Elazığ	Mustafa Ayaz
2	2013	Edirne	Türkmen Çapan
3	2013	İstanbul	Recep Büyükçolpan
4	2013	İstanbul	Abdülkadir Avcı
5	2013	Erzincan	M. A. Erçelik
6	2013	Kocaeli	Ali Ok
7	2013	Edirne	Zeki Kumaz
8	2013	İstanbul	Mustafa Yeniköylü
9	2013	Bingöl	Halil Baran
10	2013	Sivas	Alişan Şahin
11	2014	İstanbul	Hüseyin Kabul
12	2014	Kocaeli	Mehmet Ok
13	2013	Burdur	Bilal Er
14	2014	Erzincan	Mehmet Abdulkadir
15	2014	Elazığ	Mustafa Ayaz
16	2014	İzmir	Halil İbrahim Köydedurmaz
17	2014	Ordu	Atilla Öztürk
18	2014	İzmir	Nihat Demir
19	2013	Muğla	Ebu Aydın
20	2013	Düzce	Kudret Aynacı
21	2014	Ordu	Cengiz Güngör
22	2014	Mersin	Ali Türkoğlu
23	2013	İstanbul	Halil İbrahim Çadar
24	2013	Antalya	Davut Erdoğan
25	2013	İstanbul	Abdullah Çakmak
26	2013	İstanbul	Hüseyin Kaya
27	2013	İstanbul	Fahrettin Akbaba
28	2013	İstanbul	Cemil Karakayalı
29	2013	Kocaeli	Saim Ender
30	2013	Kocaeli	Mehmet Ok
31	2013	İstanbul	Tuncer Çalışkan
32	2013	İstanbul	Ali Işık
33	2013	İstanbul	Cemal Gül
34	2014	Bartın	Erkan Özkara
35	2014	İstanbul	Adnan Kıyıcıoğlu
36	2013	Kocaeli	Saim Ender
37	2014	Elazığ	Nurettin Düzgün
38	2014	Kocaeli	Saim Ender
39	2014	Kocaeli	Kurtuluş Çardak

Table A.1 (continued) : Region, collection year and beekeper of propolis samples.

Sample No	Year	Region	Beekeper
40	2014	Hatay	Nurettin Düzgün
41	2014	Kocaeli	Fuat Torun
42	2014	Bursa	Halis Avcı
43	2014	Balıkesir	Hasan Sarıdere
44	2014	Mersin	Hayati Karayer
45	2014	Aydın	Fikret Şeker
46	2014	Kırşehir	Ali Bala
47	2013	Edirne	Tuncay Balcı
48	2013	Kastamonu	Adem Teke
49	2014	Sivas	Veli Reçber
50	2013	Tekirdağ	Zafer Irmakoğlu
51	2013	Balıkesir	Abdürrahim Yılancıoğlu
52	2013	Konya	Mustafa Uğur
53	2013	Hatay	Nurettin Düzgün
54	2013	İstanbul	Turgut Bektaş

APPENDIX B : Calibration Curves

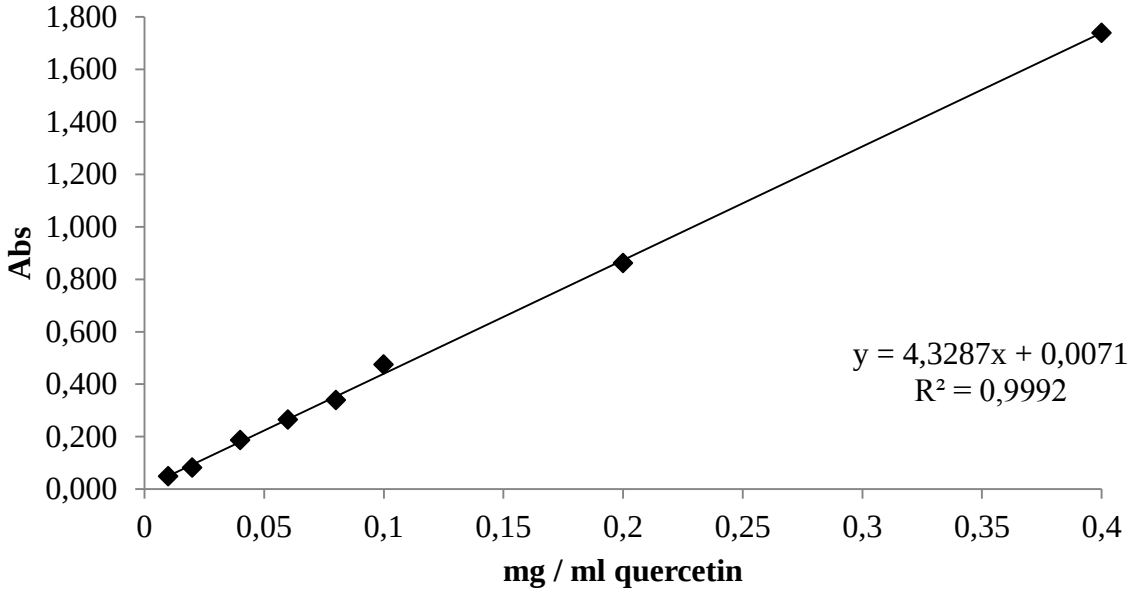

Figure B.1 : Calibration curve for total phenolics.

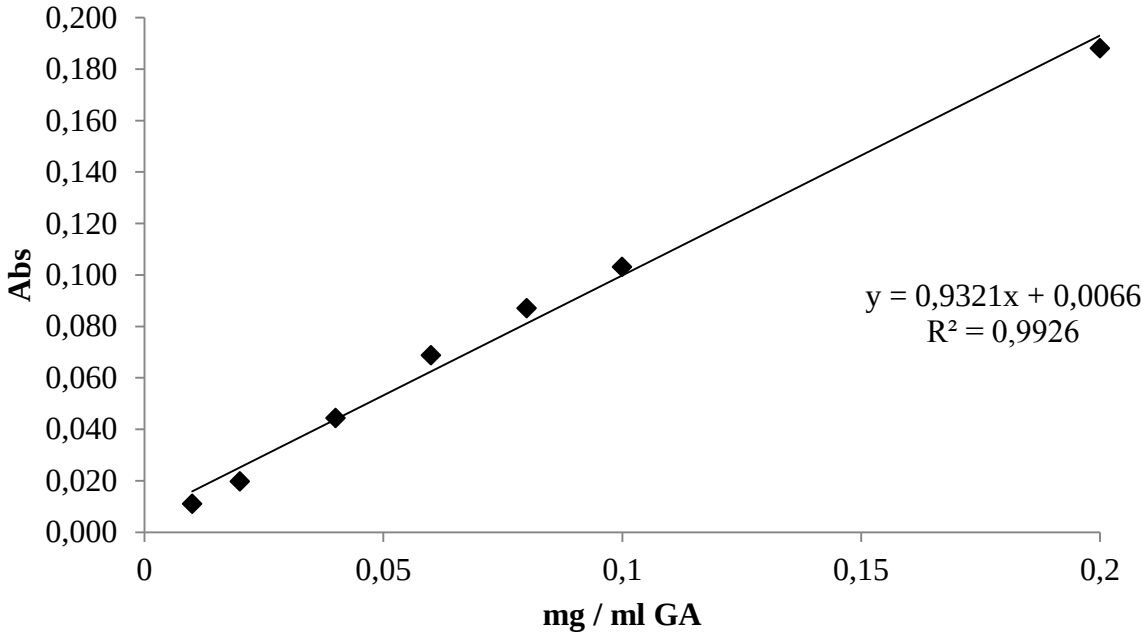

Figure B.2 : Calibration curve for total flavonoids.

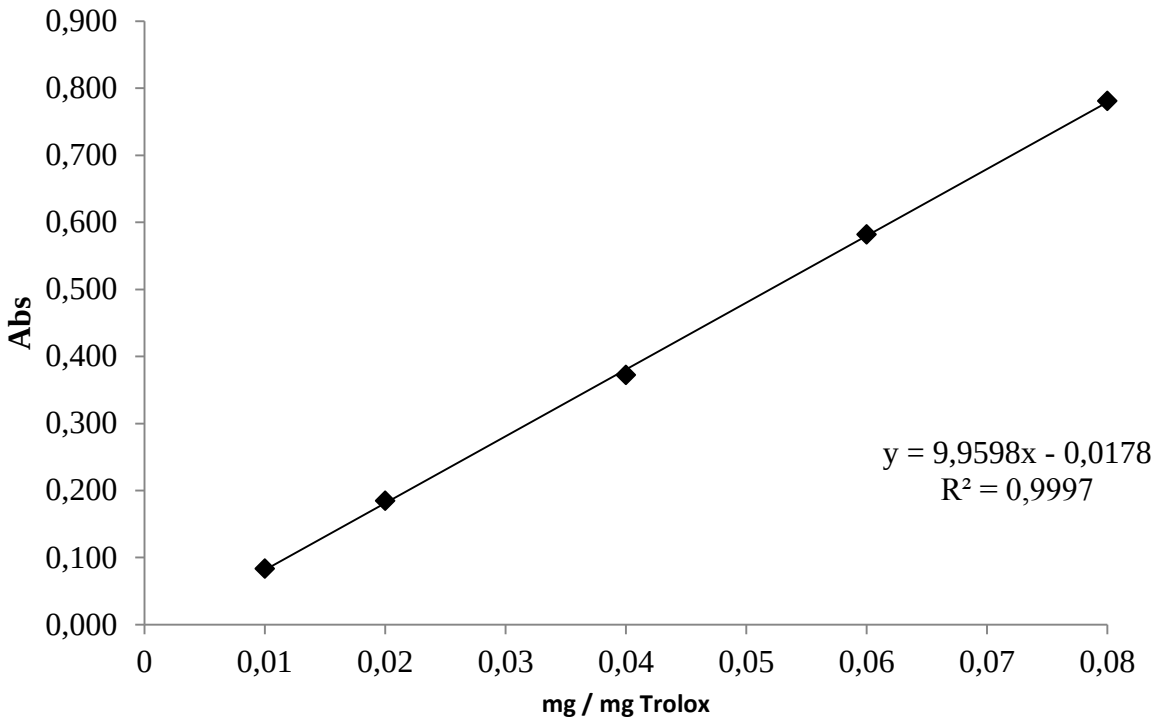

Figure B.3 : Calibration curve for ABTS method.

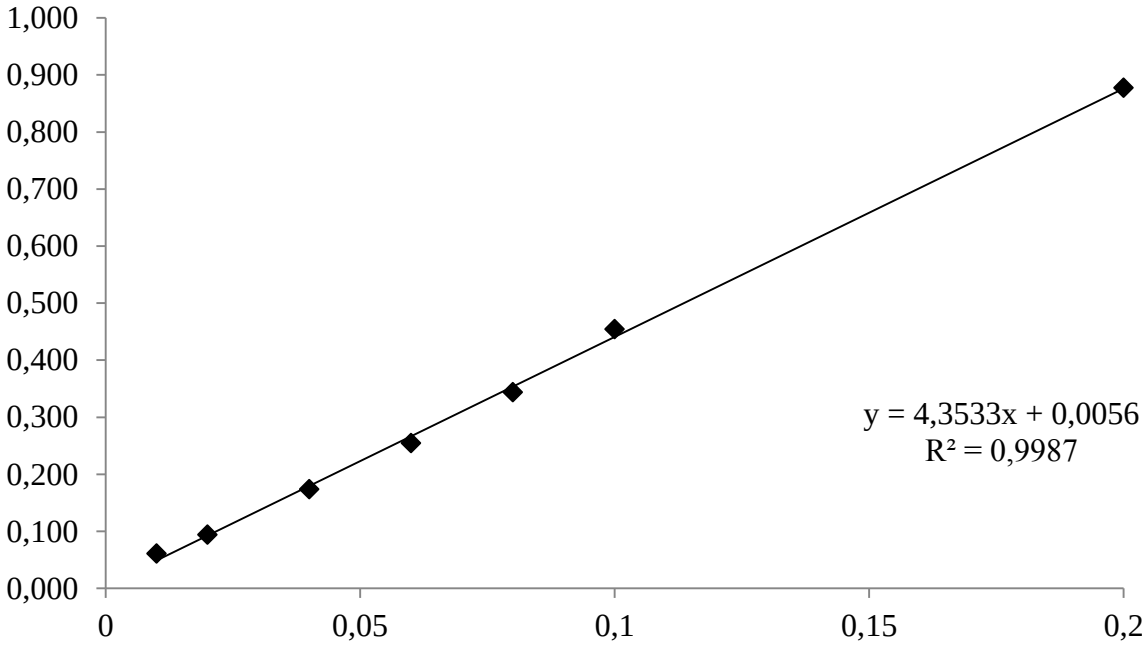

Figure B.4: Calibration curve for DPPH method.

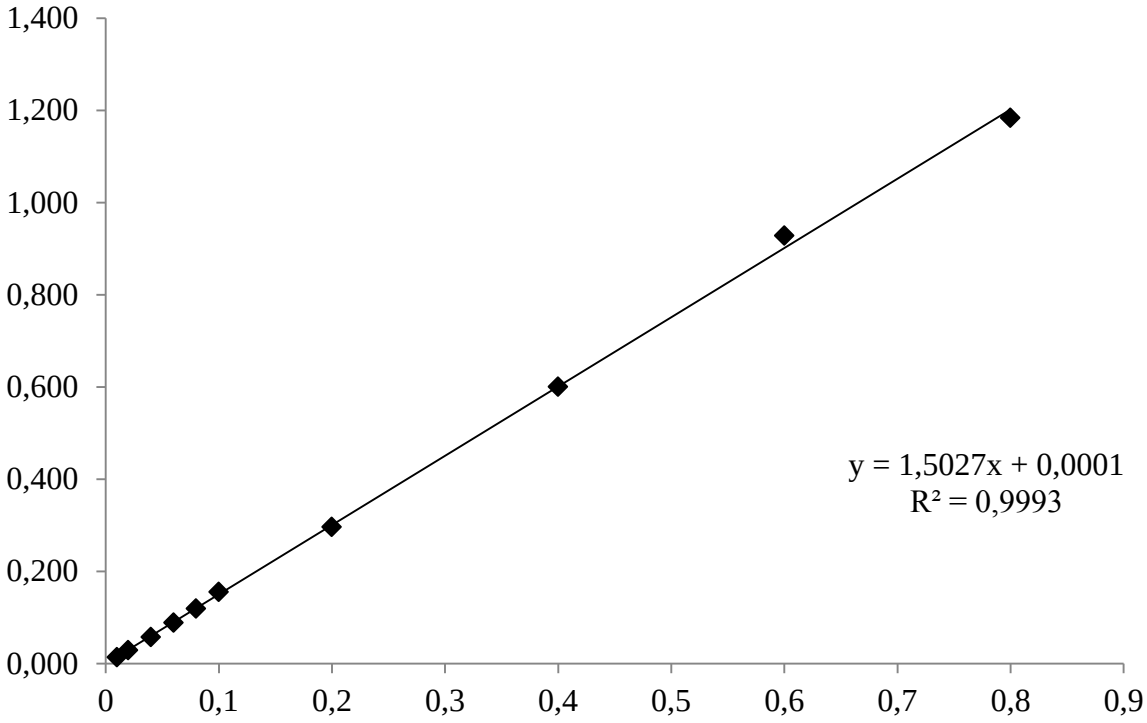

Figure B.5 : Calibration curve for CUPRAC method.

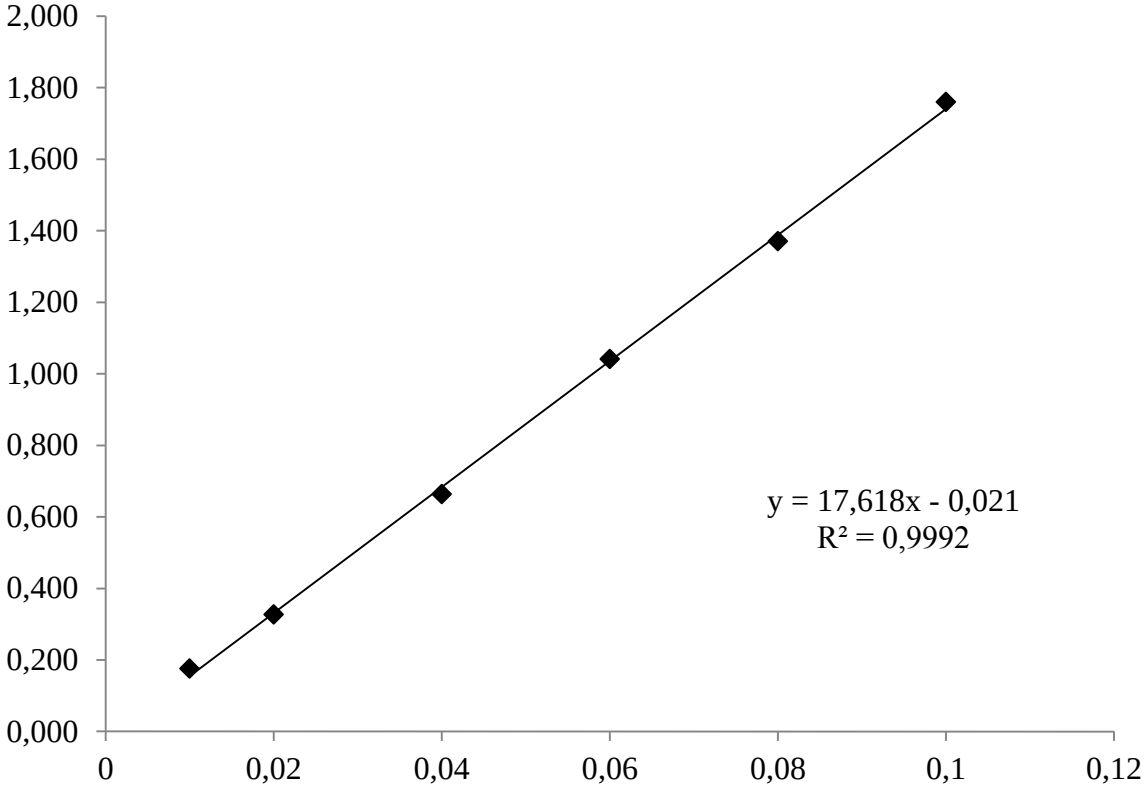

Figure B.6 : Calibration curve for FRAP method.

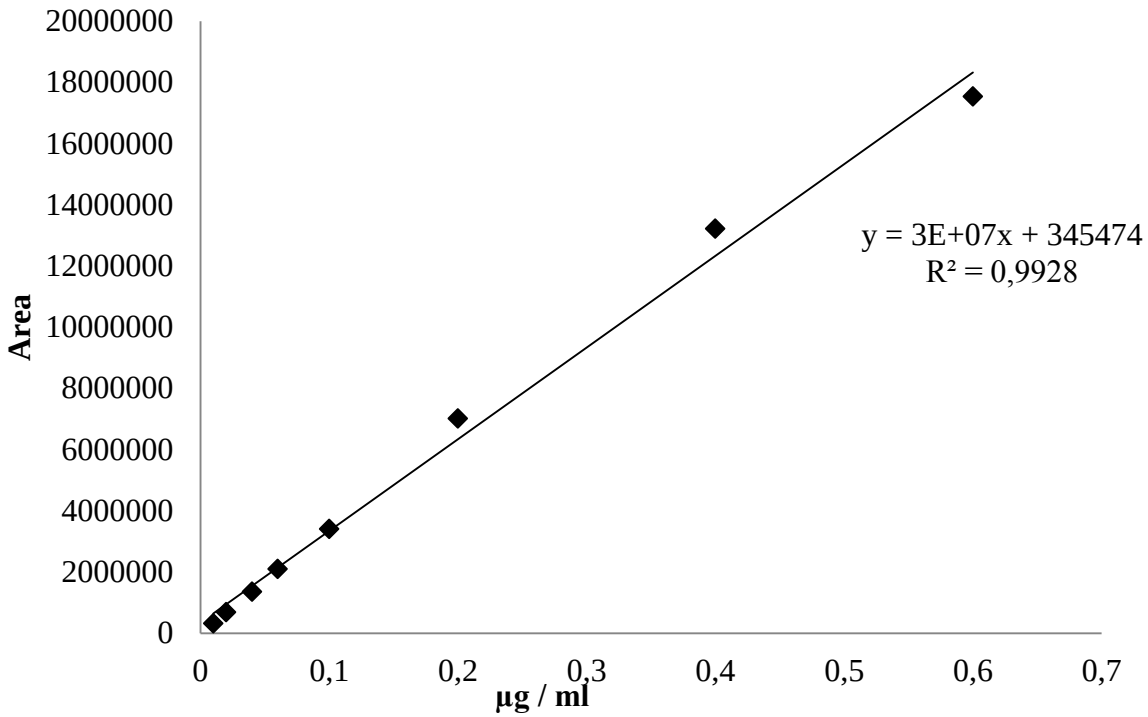

Figure B.7: Calibration curve for chyrisin.

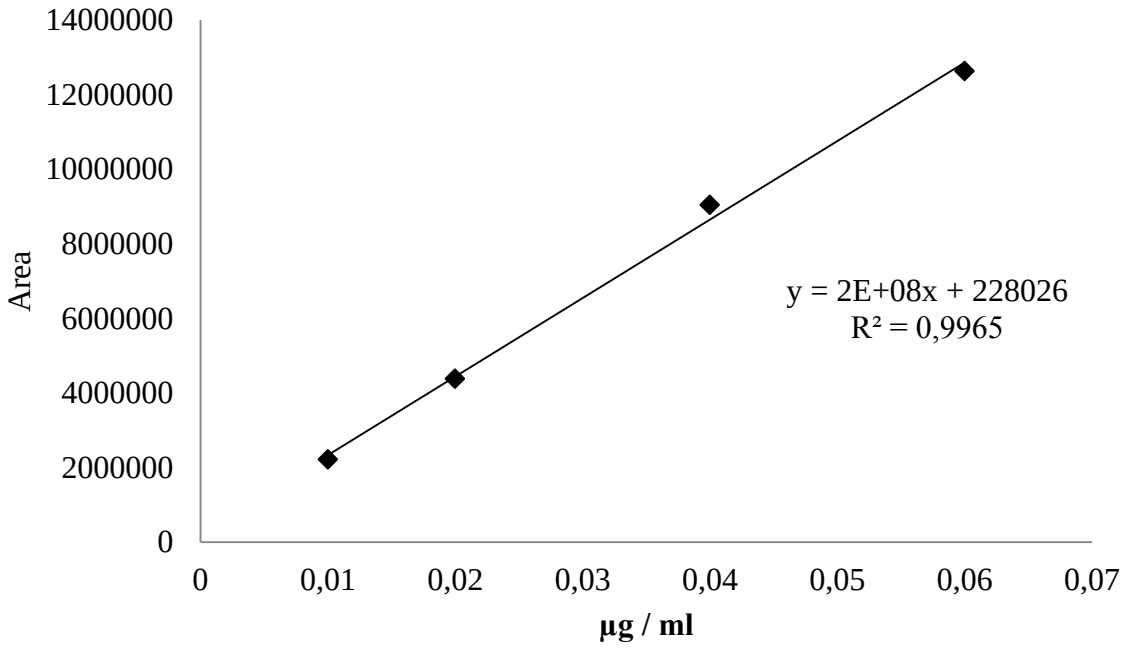

Figure B.8 : Calibration curve for galangin.

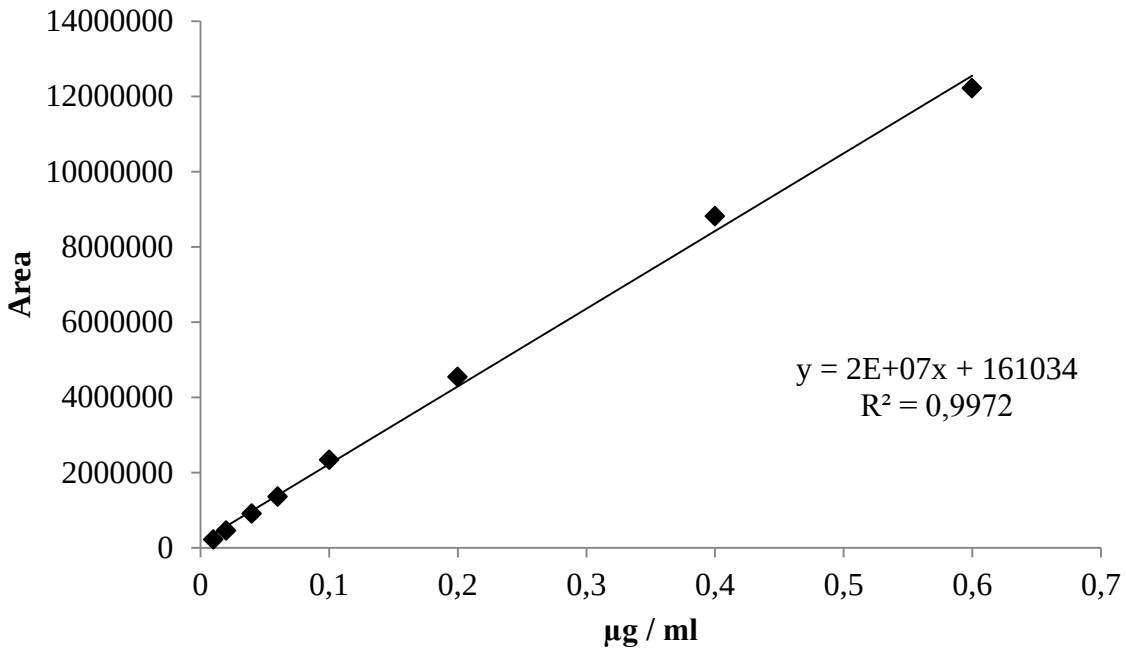

Figure B.9 : Calibration curve for pinostrobin.

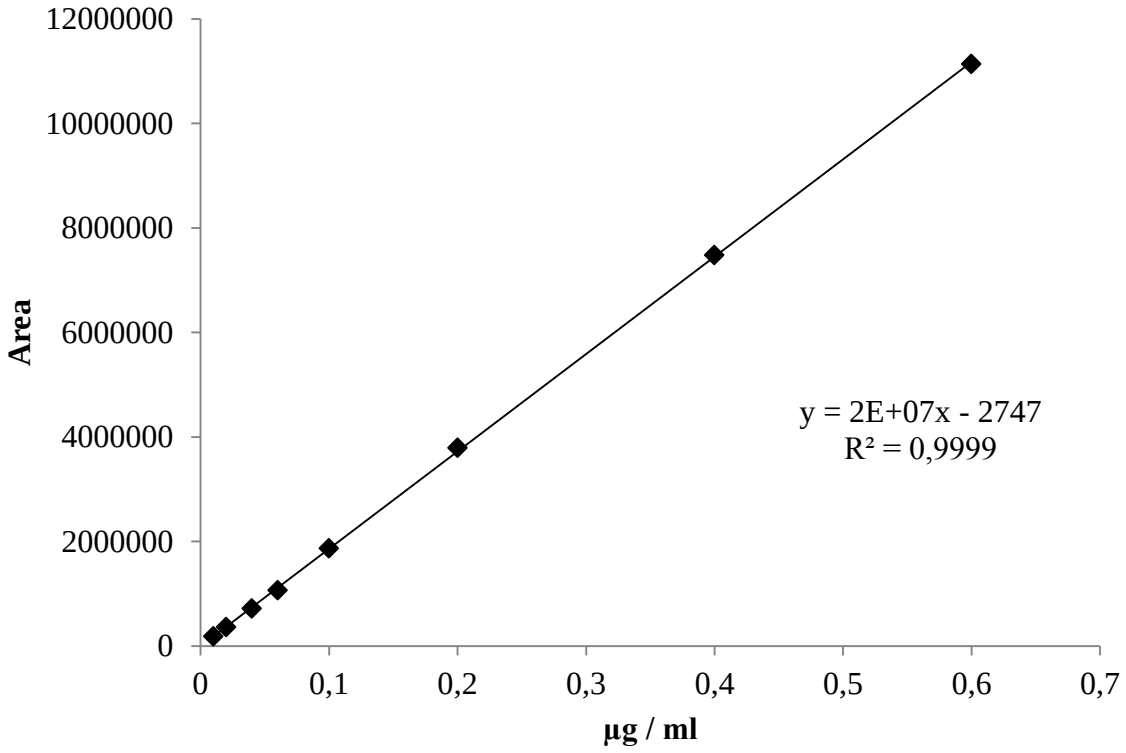

Figure B.10 : Calibration curve for pinobanksin.

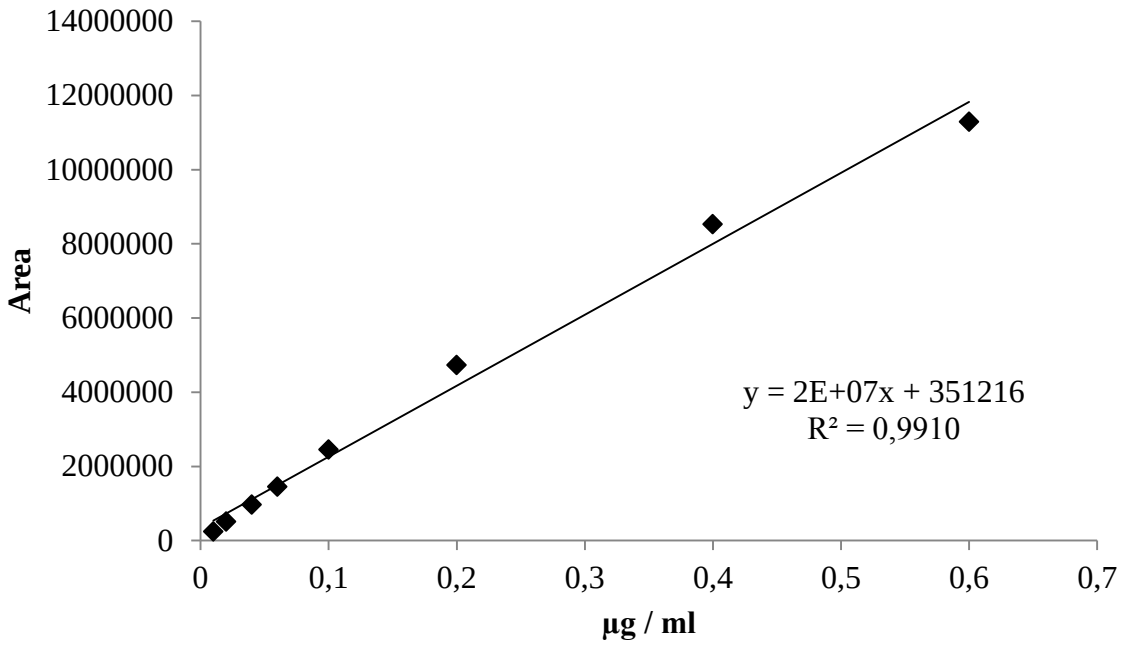

Figure B.11 : Calibration curve for pinocembrin.

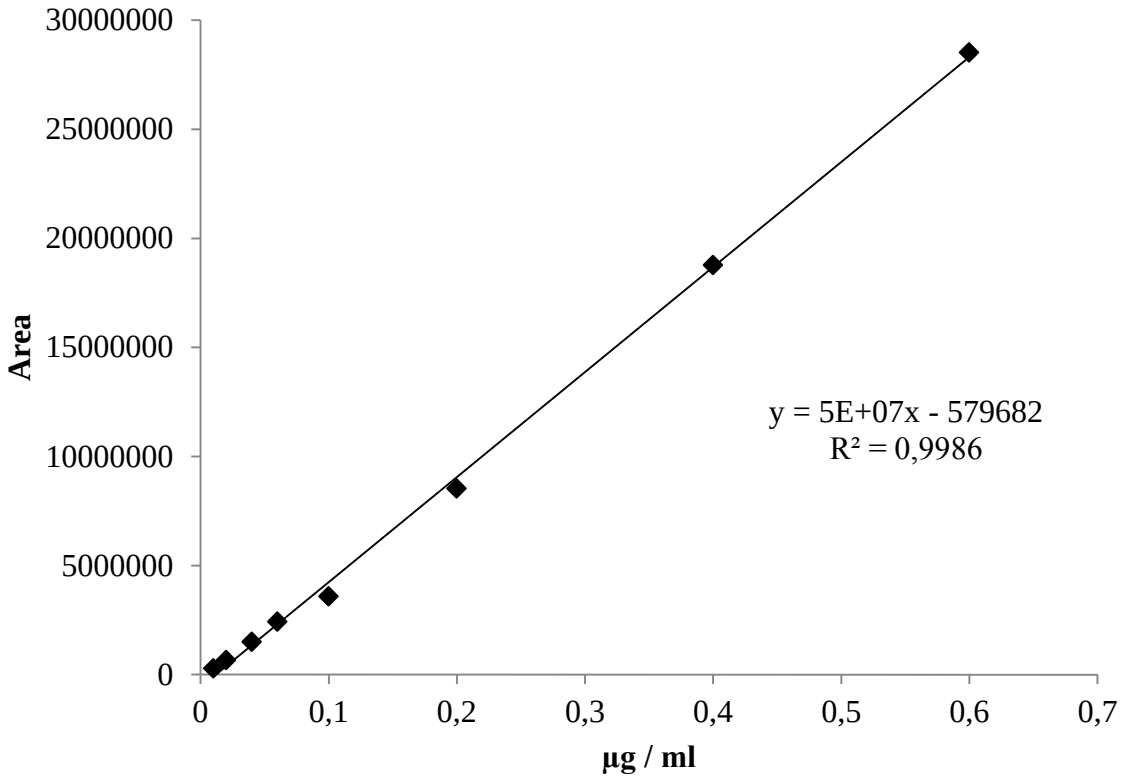

Figure B.12 : Calibration curve for caffeic acid.

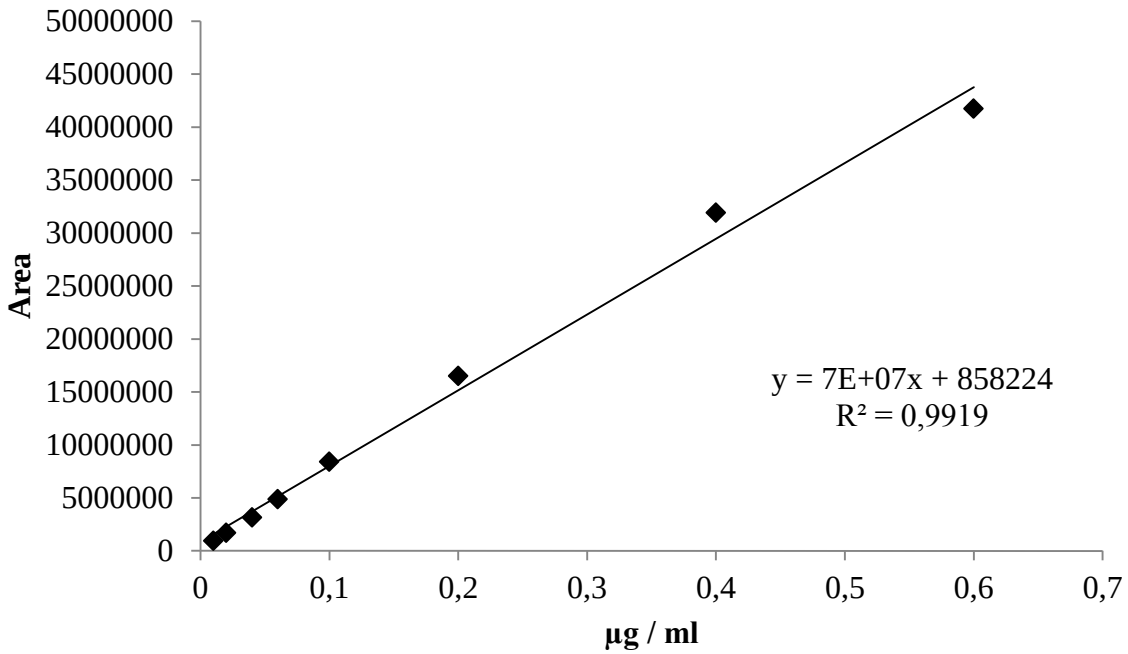

Figure B.13 : Calibration curve for *p*-coumaric acid.

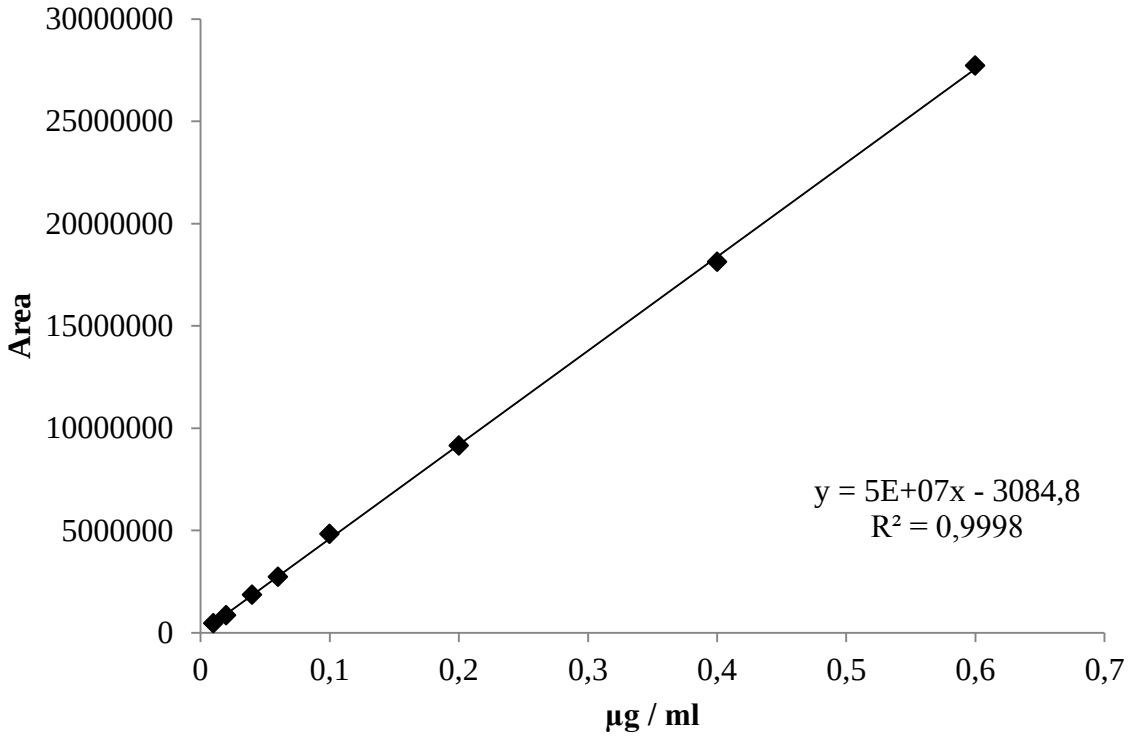

Figure B.14 : Calibration curve for ferulic acid.

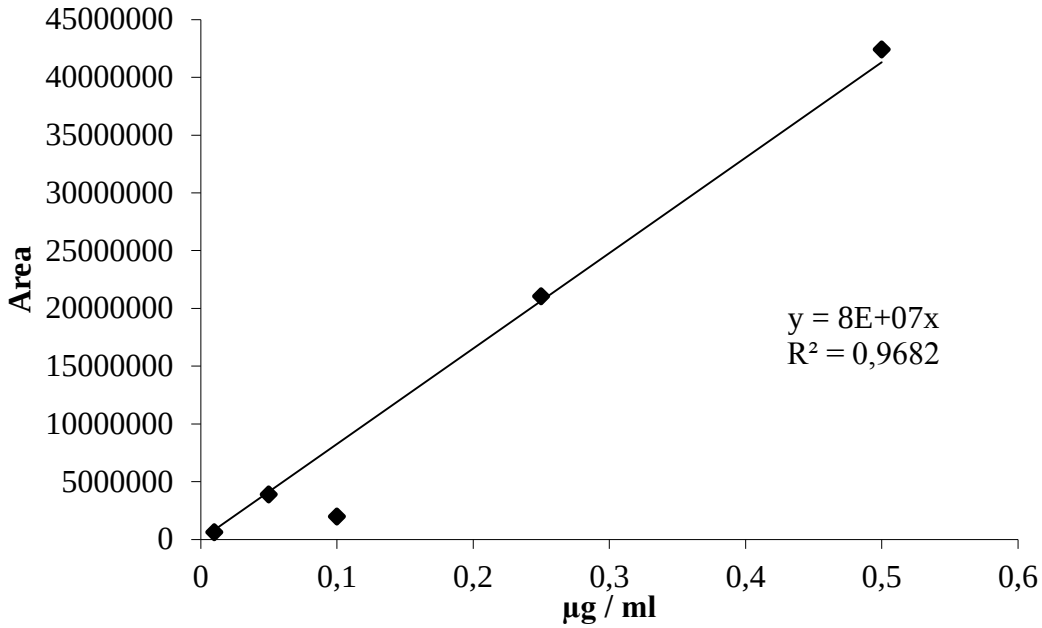

Figure B.15 : Calibration curve for *t*-cinnamic acid.

APPENDIX C : Duo-Trio Test Table

Table C.1 : Determination of statistically significant differences in duo-trio test (TS ISO-10399, 2002).

Number of answers	Minimum correct answers for statistical significance level		
	5% ($\infty \leq 0.05$)	**1% ($\infty \leq 0.01$)**	**0.1% ($\infty \leq 0.001$)**
7	7	7	-
8	7	8	-
9	8	9	-
10	9	10	10
11	9	10	11
12	10	11	12
13	10	12	13
14	11	12	13
15	12	13	14
16	12	14	15
17	13	14	16
18	13	15	16
19	14	15	17
20	15	16	18
21	15	17	18
22	16	17	19
23	16	18	20
24	17	19	20
25	18	19	21
30	20	22	24
35	23	25	27
40	26	28	31
45	29	31	34
50	32	34	37
60	37	40	43
70	43	46	49
80	48	51	55
90	54	57	61
100	59	63	66

APPENDIX D : Total Phenolic And Flavonoid Contents, Antioxidant Capacities And Individual Phenolic Contents Of Propolis Samples

Table D.1 : Total phenolic total flavonoid and total antioxidant capacity values of propolis samples collected in 2013. [1]

Sample no	Total Phenolic Content (mg GAE/g EEP)[1]	Total Flavonoid Content (mg QE/g EEP)[1]	Total Antioxidant Capacity (μmole TE/g EEP)			
			DPPH	ABTS	CUPRAC	FRAP
1	46.97±0.65	78.12±3.33	336.7±9.10	299.98±12.92	1028.82±44.85	135.95±5.83
2	50.30±1.40	83.78±1.80	277.56±4.30	293.92±5.83	831.92±17.12	109.96±2.20
3	125.39±8.25	208.44±2.89	864.04±51.24	944.57±11.58	2613.15±34.61	345.33±4.36
4	113.82±1.78	189.25±3.78	1104.19±74.01	1184.82±23.02	3336.93±65.33	440.96±8.50
5	46.64±1.22	77.4±3.51	342.11±6.71	369.56±16.51	1040.49±47.14	137.49±6.13
6	131.94±5.94	219.53±2.33	605.02±38.28	633.90±6.03	1825.85±15.56	241.39±2.08
7	68.41±5.67	113.55±8.17	129.81±18.50	320.56±16.61	858.35±57.61	113.64±6.12
8	98.65±2.93	164.1±4.76	689.66±108.32	738.65±18.53	2082.87±60.16	275.28±7.09
9	19.25±0.52	31.92±0.21	143.10±1.53	152.09±0.61	432.81±1.93	57.21±0.24
10	85.74±2.39	142.4±0.36	702.41±4.05	777.43±1.34	2144.91±2.56	283.60±0.22
13	12.89±0.55	22.09±0.98	73.35±15.23	76.63±2.82	223.50±8.64	29.54±1.03
19	10.00±0.63	16.66±0.93	83.75±24.99	91.11±5.56	256.59±16.63	33.90±2.03
20	149.03±6.52	247.9±5.29	295.56±4.12	316.25±6.58	890.41±18.76	117.63±2.45
23	145.03±8.01	241.22±9.48	1392.27±131.33	1510.64±55.40	4220.24±161.75	557.49±20.47
24	34.53±1.20	57.25±14.34	289.69±49.71	310.50±76.40	745.81±28.31	116.11±28.34
25	101.42±6.98	168.51±41.71	289.28±49.30	310.45±74.89	781.40±96.80	114.95±27.78
26	111.15±6.31	184.72±4.60	689.15±95.49	742.22±16.93	2090.25±51.10	275.91±60.00
27	50.17±0.48	83.3±3.37	245.79±9.32	265.66±10.00	748.32±28.80	98.87±3.73
28	157.25±12.2	261.61±13.60	1323.44±176.8	1417.93±68.95	3992.77±204.24	527.76±25.93

[1] Data represent average amounts ± standard deviation for each EEP collected in 2013.

Table D.1 : (continued) : Total phenolic, total flavonoid and total antioxidant capacity values of propolis samples collected in 2013. [1]

Sample no	Total Phenolic Content (mg GAE/g EEP)	Total Flavonoid Content (mg QE/g EEP)	Total Antioxidant Capacity (μmole TE/g EEP)			
			DPPH	ABTS	CUPRAC	FRAP
29	130.94±6.29	217.74±5.79	1010.88±96.19	1065.36±27.05	3032.65±79.43	400.64±9.85
30	101.98±6.49	169.58±0.62	597.88±19.49	661.09±2.32	1828.81±7.68	241.39±0.79
31	59.48±1.79	98.77±0.95	438.80±43.38	460.96±4.30	1332.50±11.72	176.00±1.79
32	83.27±3.72	138.35±1.97	578.00±46.02	636.58±7.36	1762.34±24.67	232.57±2.65
33	48.36±0.51	80.50±27.39	287.04±68.36	307.78±101.82	1040.49±32.37	114.04±37.76
36	149.14±6.05	248.02±3.24	1018.53±43.17	560.76±6.6	3112.41±37.69	411.22±5.00
47	57.34±2.72	95.34±0.64	456.65±34.39	490.83±3.37	1382.72±13.54	182.81±1.57
48	60.35±1.41	100.35±1.46	464.80±52.42	500.63±6.13	1410.78±17.91	185.96±2.10
50	67.90±5.10	112.96±2.87	363.34±89.30	366.92±8.02	1110.93±22.16	146.78±2.94
51	76.37±4.17	127.02±1.64	625.42±45.38	690.96±7.84	1915.95±20.94	252.73±2.89
52	87.94±4.10	146.22±4.14	895.14±97.57	978.00±24.18	2707.68±71.09	357.68±8.76
53	78.27±4.67	130.12±0.41	733.00±13.34	798.38±2.78	2214.33±7.68	292.42±0.79
54	26.07±0.96	43.37±0.98	151.77±7.81	164.08±3.72	457.62±10.89	60.45±1.37

[1] Data represent average amounts ± standard deviation for each EEP collected in 2013.

Table D.2 : Total phenolic, total flavonoid and total antioxidant capacity values of propolis samples collected in 2014. [1]

Sample no	Total Phenolic Content (mg GAE/g EEP)	Total Flavonoid Content (mg QE/g EEP)	Total Antioxidant Capacity (μmole TE/g EEP)			
			DPPH	ABTS	CUPRAC	FRAP
11	97.59±3.36	162.31±0.21	707.00±5.30	752.02±1.34	2122.75±2.56	280.32±0.22
12	30.05±0.41	50.04±0.27	177.37±1.34	190.91±0.76	537.39±2.34	71.04±0.29
14	13.59±1.17	22.57±0.62	70.65±13.93	77.12±2.40	212.87±7.07	28.12±0.89
15	67.64±1.46	112.48±1.15	545.36±47.20	626.33±4.63	1206.94±9.22	159.37±1.15
16	73.52±2.23	122.26±1.69	509.16±47.99	553.67±6.18	1526.00±20.94	201.83±2.36
17	37.17±1.42	61.84±3.08	224.73±13.62	243.15±12.26	681.26±35.34	90.04±4.52
18	29.60±1.99	49.27±1.98	210.97±11.65	226.08±9.25	636.80±26.67	84.12±3.45
21	78.98±5.09	131.32±1.64	525.99±50.92	538.07±6.13	1593.95±20.31	210.65±1.94
22	60.12±3.20	99.97±3.53	609.61±132.83	674.91±20.25	1855.39±62.04	245.17±7.51
34	114.89±4.04	191.28±3.94	91.71±7.72	97.31±1.87	274.17±5.55	36.25±0.70
35	47.96±0.82	79.72±5.81	339.91±15.29	363.45±26.75	1038.86±77.58	137.29±10.08
37	64.94±2.64	108.07±1.29	691.70±41.64	760.49±7.84	2105.02±21.86	278.56±2.65
38	154.89±4.34	257.55±8.41	1152.12±118.01	1222.7±38.69	3475.78±113.96	459.22±14.29
39	89.92±3.76	149.44±1.61	620.32±40.65	694.97±6.73	1877.55±17.73	247.94±2.10
40	23.53±2.28	39.02±1.07	132.75±7.55	143.84±3.04	398.24±8.72	52.60±1.12
41	39.70±4.38	65.90±1.43	259.76±5.05	278.32±6.16	787.16±17.53	104.03±2.27
42	28.93±2.57	48.08±2.86	210.46±15.22	226.30±12.24	633.84±35.23	83.79±4.50
43	15.04±0.85	25.13±0.45	111.64±6.20	120.09±1.85	333.69±5.9	44.12±0.71
44	7.12±0.22	11.84±0.16	26.15±0.92	28.39±0.36	79.29±1.11	10.48±0.13
45	35.53±0.41	59.16±2.91	235.90±12.93	250.73±12.55	709.32±36.3	93.73±4.68
46	6.18±0.36	10.24±0.33	427.24±17.76	460.27±14.9	1304.91±42.45	172.4±5.58
49	62.09±2.86	103.30±6.40	520.89±26.95	715.47±40.14	2013.44±125.44	265.96±14.62

[1] Data represent average amounts ± standard deviation for each EEP collected in 2014.

Table D.3 : Phenolic profiles of propolis samples collected in 2013. [1]

Sample No	caffeic acid	chrysin	ferulic acid	galangin	*p*-coumaric acid	Pinobanksin	Pinocembrin	Pinostrobin	*t*-cinnamic acid
1	1.13±0.01	23.15±0.77	0.08±0.01	15.09±0.24	0.37±0.00	5.02±0.25	11.65±0.73	4.76±0.25	0.21±0.01
2	1.59±0.02	18.10±1.31	0.39±0.01	2.61±0.22	0.78±0.01	6.64±0.19	33.73±3.87	7.40±0.78	0.55±0.00
3	4.64±0.01	26.42±2.43	3.45±0.03	9.14±0.58	2.78±0.03	10.91±0.38	51.21±2.63	19.45±0.82	1.79±0.07
4	3.27±0.02	20.20±1.73	0.14±0.00	5.73±2.48	1.92±0.01	11.71±0.14	56.34±9.85	11.8±2.70	2.30±0.01
5	1.25±0.03	21.67±0.95	0.06±0.01	20.39±4.55	0.73±0.02	5.54±0.08	10.80±0.47	8.31±0.08	0.76±0.01
6	3.06±0.03	25±8.54	0.68±0.01	11.85±9.57	5.81±0.06	29.47±0.38	42.71±14.3	14.57±0.45	1.02±0.02
7	3.03±0.01	22.25±0.68	0.80±0.01	4.77±0.62	0.54±0.01	6.21±0.02	38.14±0.71	11.12±0.09	1.06±0.01
8	4.24±0.01	27.93±6.83	0.97±0.01	6.66±0.28	0.89±0.01	8.35±0.17	41.36±0.41	17.59±0.78	1.47±0.03
9	0.43±0.01	12.03±0.28	0.17±0.01	5.38±0.10	0.18±0.01	1.52±0.36	4.16±0.09	1.71±0.27	0.08±0.02
10	3.38±0.02	23.87±0.30	0.36±0.00	5.31±0.01	1.25±0.01	10.86±0.18	56.94±0.65	12.43±0.08	1.40±0.01
13	0.39±0.00	5.77±0.06	0.08±0.00	1.03±0.05	0.07±0.00	1.04±0.01	12.47±0.19	1.90±0.14	0.11±0.00
19	0.31±0.00	2.49±0.01	0.03±0.00	0.52±0.01	0.13±0.00	0.68±0.01	8.02±0.01	1.68±0.24	0.11±0.00
20	1.42±0.05	28.16±6.00	0.58±0.01	12.62±0.43	6.62±0.07	24.41±20.97	42.59±13.68	8.74±5.79	0.28±0.19
23	5.87±4.66	35.11±2.89	2.74±3.94	31.31±3.92	5.53±0.57	6.6±4.92	52.04±26.03	25.45±2.17	3.64±1.11
24	1.69±0.02	15.85±4.64	0.87±1.35	12.43±1.70	0.93±0.01	3.94±0.05	24.16±11.96	8.52±0.11	0.95±0.01
25	1.49±1.18	32.21±0.69	1.40±1.19	7.00±0.21	3.10±0.12	18.34±0.59	45.20±9.01	10.4±1.30	0.62±0.09
26	3.35±0.11	46.79±9.70	5.19±0.14	10.06±0.54	1.94±0.06	7.82±0.53	54.33±4.02	23.37±12.11	1.46±0.07
27	2.39±0.01	21.18±3.43	0.54±0.01	3.55±0.30	0.65±0.01	5.05±0.18	34.17±1.26	10.48±0.84	0.79±0.01
28	7.32±0.04	41.17±9.90	1.34±0.02	12.54±0.69	1.24±0.02	13.27±0.78	60.3±0.62	37.85±0.53	2.38±0.08

[1] Data represent average amounts ± standard deviation for each EEP collected in 2013.

Table D.3 (continued) : Phenolic profiles of propolis samples collected in 2013. [1]

Sample No	Caffeic acid	Chrysin	Ferulic acid	Galangin	*p*-coumaric acid	Pinobanksin	Pinocembrin	Pinostrobin	*t*-cinnamic acid
29	6.42±0.03	32.85±6.22	1.45±0.02	12.23±0.10	3.63±0.02	11.88±0.35	56.55±9.67	30.18±1.01	2.09±0.04
30	3.94±0.11	30.13±5.73	1.23±0.03	7.93±0.76	1.87±0.05	13.30±0.25	42.30±0.18	17.41±0.42	1.26±0.02
31	2.59±0.11	17.95±0.68	0.53±0.02	3.93±0.25	0.55±0.03	4.95±0.32	35.83±0.87	9.95±0.39	0.84±0.03
32	3.11±0.01	33.16±0.51	5.02±0.22	4.57±0.26	1.92±0.02	5.23±0.15	44.87±0.33	17.76±7.46	1.06±0.01
33	2.36±0.01	16.50±0.97	0.22±0.02	5.02±0.27	1.99±0.02	5.43±0.08	46.27±6.66	9.58±0.23	2.38±0.01
36	7.61±0.08	29.41±1.81	2.29±0.98	12.36±1.39	4.22±0.02	14.85±0.17	53.23±3.30	34.92±5.81	2.55±0.07
47	4.08±0.04	27.84±3.13	1.08±0.02	6.84±0.50	0.63±0.00	8.54±0.18	48.17±6.00	13.18±0.63	1.43±0.01
48	2.27±0.04	25.16±1.89	0.81±0.01	4.78±0.39	0.97±0.01	6.21±0.73	44.25±2.31	11.05±0.54	0.81±0.04
50	3.39±0.03	21.41±0.25	0.78±0.03	5.55±0.01	0.45±0.02	6.19±0.18	41.44±1.21	10.50±0.05	1.04±0.02
51	2.40±0.04	23.62±1.56	0.48±0.02	6.90±0.18	0.93±0.01	7.32±0.22	57.20±3.72	10.36±0.28	1.22±0.03
52	1.83±0.04	20.67±1.63	1.50±2.45	3.09±0.22	0.87±0.05	10.66±0.17	65.94±3.07	13.08±0.14	1.32±0.08
53	3.56±0.06	27.86±0.92	0.98±1.13	5.08±0.33	1.08±0.05	7.49±0.23	62.87±1.76	18.33±0.28	0.63±0.01
54	0.58±0.03	13.16±0.04	0.8±0.01	0.72±0.14	0.56±0.03	2.00±0.08	23.38±0.24	3.35±0.05	0.16±0.06

[1] Data represent average amounts ± standard deviation for each EEP collected in 2014.

Table D.4 : Phenolic profiles of propolis samples collected in 2014. [1]

Sample No	Caffeic acid	Chrysin	Ferulic acid	Galangin	*p*-coumaric acid	Pinobanksin	Pinocembrin	Pinostrobin	*t*-cinnamic acid
11	4.91±0.05	33.86±1.69	1.24±0.01	6.06±0.34	1.60±0.01	7.61±0.06	42.62±1.43	15.98±0.17	1.74±0.00
12	1.10±0.01	13.71±0.27	0.33±0.02	2.36±0.23	0.50±0.01	3.62±0.03	26.70±0.20	4.42±0.28	0.35±0.01
14	0.29±0.01	9.87±3.95	1.42±0.06	0.78±0.01	0.50±0.01	0.98±0.01	11.34±0.03	2.00±0.30	0.18±0.00
15	1.85±0.45	24.89±4.05	0.49±0.15	19.79±0.27	0.44±0.11	8.71±0.16	26.10±19.77	8.56±0.23	0.28±0.01
16	2.42±0.03	27.22±3.26	0.60±0.01	5.22±0.68	0.78±0.01	6.47±0.17	25.81±20.29	11.53±0.19	0.8±0.02
17	1.55±0.03	20.27±0.05	1.27±0.01	2.84±0.10	0.69±0.01	4.48±0.09	30.20±0.80	5.39±0.08	0.39±0.01
18	1.70±0.02	13.94±2.09	0.44±0.01	2.10±0.12	0.20±0.00	3.03±0.07	25.31±1.13	4.51±0.20	0.49±0.01
21	3.11±0.02	29.70±0.76	2.28±0.10	4.25±0.07	2.59±0.02	9.94±0.07	52.17±1.77	12.15±0.39	1.51±0.03
22	2.86±0.12	19.37±2.26	0.03±0.02	14.4±0.56	0.62±0.03	4.68±0.27	20.49±7.10	15.58±0.58	0.43±0.02
34	6.54±0.03	29.95±6.63	1.54±0.01	8.38±0.48	2.97±0.00	10.36±1.04	50.94±1.20	22.29±0.30	2.69±0.07
35	2.11±0.03	19.46±1.53	0.31±0.02	4.57±0.24	0.57±0.01	4.23±0.52	40.02±2.78	12.28±0.31	1.17±0.01
37	2.17±0.02	17.24±1.02	0.05±0.00	8.88±9.93	0.52±0.02	6.13±0.10	58.07±5.11	11.69±0.72	0.39±0.03
38	8.84±0.07	30.87±1.95	1.96±0.01	12.87±1.43	2.79±0.01	16.97±0.27	54.24±1.06	30.34±0.88	3.27±0.15
39	1.40±0.01	32.00±0.31	2.32±0.01	5.97±0.08	0.69±0.01	10.89±0.09	55.98±1.16	26.41±27.71	0.8±0.00
40	0.79±0.01	10.92±1.43	0.07±0.01	1.20±0.03	0.22±0.01	1.31±0.18	21.62±0.09	7.27±0.09	0.09±0.01
41	2.07±0.01	13.73±0.29	0.82±0.02	2.86±0.21	0.55±0.01	3.01±0.20	29.35±0.23	6.50±0.63	0.68±0.02
42	0.83±0.00	8.81±0.26	0.26±0.00	1.33±0.09	0.14±0.00	2.08±0.07	22.36±0.25	2.98±0.13	0.27±0.01
43	2.41±0.02	23.31±0.14	0.48±0.02	6.83±0.24	0.92±0.01	7.17±0.12	56.33±1.40	9.59±1.21	1.22±0.01
44	0.11±0.00	1.62±0.37	0.02±0.00	0.37±0.06	0.04±0.00	0.39±0.00	3.23±0.40	1.23±0.12	0.02±0.00
45	2.01±0.01	26.44±0.78	1.53±1.78	4.69±0.26	0.74±0.03	6.56±0.15	47.83±2.94	10.07±0.36	0.78±0.01
46	0.19±0.00	3.24±0.11	0.02±0.00	1.03±0.75	0.05±0.00	0.6±0.04	7.20±0.13	1.83±1.91	0.07±0.00
49	3.18±0.05	22.13±0.5	0.13±0.01	4.78±0.03	0.66±0.03	8.32±0.06	57.49±0.58	11.64±0.13	0.86±0.01

[1] Data represent average amounts ± standard deviation for each EEP collected in 2014.

APPENDIX E : Statistical Analyses Tables

Table E.1 : Statistical analysis results for total phenolic, total flavonoid and total antioxidant capacity values of propolis samples collected in 2013 & 2014.

		Sum of Squares	df	Mean Square	F	Sig.	R^2
Total Phenolics	Model	291009.592	7	41572.799	29.554	.000	0.815
	Year	4649.893	1	4649.893	3.306	.075	
	Region	18848.545	5	3769.709	2.680	.033	
	Error	66113.757	47	1406.676			
	Total	357123.349	54				
Total Flavonoids	Model	804700.588	7	114957.227	29.552	.000	0.815
	Year	12854.082	1	12854.082	3.304	.075	
	Region	52104.926	5	10420.985	2.679	.033	
	Error	182831.498	47	3890.032			
	Total	987532.086	54				
ABTS Method	Model	15401833.920	7	2200261.988	19.734	.000	0.746
	Year	102761.354	1	102761.354	.922	.342	
	Region	892049.339	5	178409.868	1.600	.179	
	Error	5240314.266	47	111496.048			
	Total	20642148.180	54				
DPPH Method	Model	13503191.650	7	1929027.379	18.995	.000	0.739
	Year	127091.426	1	127091.426	1.251	.269	
	Region	726590.086	5	145318.017	1.431	.231	
	Error	4773029.819	47	101553.826			
	Total	18276221.470	54				
CUPRAC Method	Model	126487941.700	7	18069705.960	20.252	.000	0.751
	Year	1245302.933	1	1245302.933	1.396	.243	
	Region	7858247.556	5	1571649.511	1.761	.139	
	Error	41934524.970	47	892223.936			
	Total	168422466.700	54				
FRAP Method	Model	2207171.852	7	315310.265	20.213	.000	0.751
	Year	22175.077	1	22175.077	1.422	.239	
	Region	133920.706	5	26784.141	1.717	.149	
	Error	733157.908	47	15599.104			
	Total	2940329.760	54				

Table E.2 : Statistical analysis results for individual phenolic compounds of propolis samples collected in 2013 & 2014.

	Sum of Squares	df	Mean Square	F	Sig.	R^2
Caffeic acid	463.285	7	66.184	17.165	.000	.719
	.457	1	.457	.118	.732	
	43.710	5	8.742	2.267	.063	
	181.221	47	3.856			
	644.507	54				
Chrysin	27963.803	7	3994.829	54.280	.000	.890
	96.489	1	96.489	1.311	.258	
	975.884	5	195.177	2.652	.034	
	3459.044	47	73.597			
	31422.846	54				
Ferulic acid	61.834	7	8.833	7.637	.000	0.532
	.270	1	.270	.234	.631	
	8.444	5	1.689	1.460	.221	
	54.363	47	1.157			
	116.197	54				
Galangin	2967.365	7	423.909	13.599	.000	.669
	58.197	1	58.197	1.867	.178	
	231.820	5	46.364	1.487	.212	
	1465.116	47	31.173			
	4432.481	54				
p-coumaric acid	135.741	7	19.392	11.056	.000	.622
	6.721	1	6.721	3.832	.056	
	24.731	5	4.946	2.820	.026	
	82.437	47	1.754			
	218.178	54				
Pinobanksin	3891.497	7	555.928	15.498	.000	.698
	113.601	1	113.601	3.167	.082	
	407.078	5	81.416	2.270	.063	
	1685.978	47	35.872			
	5577.475	54				
Pinocembrin	85110.400[a]	7	12158.629	54.088	.000	.890
	58.079	1	58.079	.258	.614	
	4634.813	5	926.963	4.124	.003	
	10565.245	47	224.792			
	95675.646	54				
Pinostrobin	9134.680	7	1304.954	19.990	.000	0.749
	13.006	1	13.006	.199	.657	
	667.964	5	133.593	2.046	.089	
	3068.103	47	65.279			
	12202.783	54				
t-cinnamic acid	69.503	7	9.929	17.357	.000	0.721
	.233	1	.233	.408	.526	
	9.400	5	1.880	3.286	.013	
	26.887	47	.572			
	96.390	54				

Table E.3 : Discriminant Analysis -Tests of Equality of Group Means.

	Wilks' Lambda	F	df1	df2	Sig.
Chrysin	0.788	8.140	5	151	< 0.0001
Galangin	0.865	4.699	5	151	0.001
p-Coumaric acid	0.771	8.971	5	151	< 0.0001
Pinobanksin	0.821	6.567	5	151	< 0.0001
Pinocembrin	0.704	12.713	5	151	< 0.0001
t-Cinnamic acid	0.728	11.293	5	151	< 0.0001

Table E.4 : Discriminant Analysis - Sum of weights, prior probabilities and logarithms of determinants for each class.

Class	Sum of weights	Prior probabilities	Log (Determinant)
Aegean	12.000	0.167	-9.025
Black Sea	14.000	0.167	10.425
Central Anatolia	11.000	0.167	-6.807
East Anatolia	17.000	0.167	4.372
Marmara	85.000	0.167	13.323
Mediterranian	18.000	0.167	0.700

Table E.5 : Functions at the centroids.

Regions	F1	F2	F3
Aegean	-0.369	-0.126	-0.552
Black Sea	1.017	0.929	-0.570
Central Anatolia	0.315	-1.460	-0.180
East Anatolia	-2.487	0.289	0.438
Marmara	0.607	0.019	0.229
Mediterranean	-1.254	-0.111	-0.576

Table E.6 : Statistical analysis results for physicochemical analysis of heat treated dry fermented sucuk sample.

	Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Square
pH	Corrected Model	.376[a]	8	.047	2.248	.024	.050
	Intercept	7681.290	1	7681.290	367092.599	.000	.999
	Nitrite	.125	3	.042	1.994	.115	.017
	Propolis	.081	2	.040	1.930	.147	.011
	Nitrite * Propolis	.230	3	.077	3.672	.013	.031
	Error	7.156	342	.021			
	Total	9137.145	351				
	Corrected Total	7.532	350				
Moisture %	Model	594534.910[a]	9	66059.434	8147.978	.000	.995
	Propolis * Nitrite	288.867	3	96.289	11.877	.000	.094
	Propolis	413.130	2	206.565	25.478	.000	.130
	Nitrite	29.607	3	9.869	1.217	.303	.011
	Error	2772.752	342	8.107			
	Total	597307.662	351				
Fat	Model	477659.371[a]	9	53073.263	19180.161	.000	.998
	Propolis * Nitrite	201.653	3	67.218	24.292	.000	.176
	Propolis	211.715	2	105.857	38.256	.000	.183
	Nitrite	44.219	3	14.740	5.327	.001	.045
	Error	946.345	342	2.767			
	Total	478605.717	351				
Protein	Model	72792.413[a]	9	8088.046	11456.097	.000	.997
	Propolis * Nitrite	.806	3	.269	.381	.767	.003
	Propolis	2.150	2	1.075	1.523	.220	.009
	Nitrite	8.540	3	2.847	4.032	.008	.034
	Error	241.453	342	.706			
	Total	73033.867	351				
Collagen	Model	668.882[a]	9	74.320	1345.389	.000	.979
	Propolis	3.788	2	1.894	34.288	.000	.208
	Nitrite	1.791	3	.597	10.807	.000	.110
	Nitrite * Propolis	2.188	3	.729	13.202	.000	.132
	Error	14.418	261	.055			
	Total	683.300	270				

a. R Squared = .050 (Adjusted R Squared = .028)

Table E.6 (continued) : Statistical analysis results for physicochemical analysis of heat treated dry fermented sucuk samples.

	Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
TBARS	Model	98.030[a]	9	10.892	441.684	.000	98.030[a]
	Nitrite * Propolis	.315	3	.105	4.260	.006	.315
	Propolis	1.361	2	.680	27.586	.000	1.361
	Nitrite	.259	3	.086	3.496	.016	.259
	Error	5.771	234	.025			5.771
	Total	103.800	243				103.800
Color -L	Model	231886.746[a]	9	25765.194	1052.690	.000	.992
	Nitrite * Propolis	180.954	3	60.318	2.464	.069	.093
	Propolis	11.556	2	5.778	.236	.790	.007
	Nitrite	192.155	3	64.052	2.617	.057	.098
	Error	1762.241	72	24.476			
	Total	233648.987	81				
Color-a*	Model	35048.063[a]	9	3894.229	385.685	.000	.980
	Nitrite * Propolis	131.020	3	43.673	4.325	.007	.153
	Propolis	24.311	2	12.155	1.204	.306	.032
	Nitrite	86.613	3	28.871	2.859	.043	.106
	Error	726.978	72	10.097			
	Total	35775.041	81				
Color-b*	Model	31980.590[a]	9	3553.399	1745.438	.000	.995
	Nitrite * Propolis	105.010	3	35.003	17.194	.000	.417
	Propolis	13.019	2	6.509	3.197	.047	.082
	Nitrite	66.801	3	22.267	10.938	.000	.313
	Error	146.579	72	2.036			
	Total	32127.169	81				

a. R Squared = .050 (Adjusted R Squared = .028)

Table E.7 : Statistical analysis results for physicochemical analysis of heat treated dry fermented sucuk samples.

	Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Total Aerobic Bacteria	Model	3660.126[a]	9	406.681	334.751	.000	.898
	Nitrite	3.220	3	1.073	.883	.450	.008
	Propolis	5.511	2	2.756	2.268	.105	.013
	Nitrite * Propolis	3.069	3	1.023	.842	.472	.007
	Error	415.487	342	1.215			
	Total	4075.613	351				
E.coli	Model	248.476[a]	9	27.608	136.674	.000	.782
	Nitrite	.325	3	.108	.536	.658	.005
	Propolis	.197	2	.099	.488	.614	.003
	Nitrite * Propolis	.190	3	.063	.313	.816	.003
	Error	69.085	342	.202			
	Total	317.561	351				
Total Coliform	Model	466.776[a]	9	51.864	57.378	.000	.602
	Nitrite	.680	3	.227	.251	.861	.002
	Propolis	.097	2	.049	.054	.948	.000
	Nitrite * Propolis	.547	3	.182	.202	.895	.002
	Error	309.132	342	.904			
	Total	775.907	351				
Total Yeast	Model	670.995[a]	9	74.555	71.060	.000	.652
	Nitrite	2.675	3	.892	.850	.467	.007
	Propolis	1.462	2	.731	.697	.499	.004
	Nitrite * Propolis	1.125	3	.375	.357	.784	.003
	Error	358.823	342	1.049			
	Total	1029.818	351				
Total Mold	Model	193.797[a]	9	21.533	121.364	.000	.762
	Nitrite	2.643	3	.881	4.966	.002	.042
	Propolis	1.398	2	.699	3.941	.020	.023
	Nitrite * Propolis	.621	3	.207	1.167	.322	.010
	Error	60.679	342	.177			
	Total	254.476	351				
Lactobacillus spp.	Model	860.557[a]	9	95.617	190.550	.000	.834
	Nitrite	.082	3	.027	.054	.983	.000
	Propolis	.110	2	.055	.110	.896	.001
	Nitrite * Propolis	.195	3	.065	.130	.942	.001
	Error	171.614	342	.502			
	Total	1032.172	351				

[a]R Squared = ,898 (Adjusted R Squared = ,895)

Table E.8 : ANOVA table of regression analysis with curve estimation of total mold counts of heat treated sucuk samples.

Model		Sum of Squares	df	Mean Square	F	Sig.
Linear	Regression	4.551	1	4.551	25.001	.000
	Residual	63.533	349	.182		
	Total	68.085	350			
Logarithmic	Regression	3.952	1	3.952	21.508	.000
	Residual	64.132	349	.184		
	Total	68.085	350			
Quadratic	Regression	5.206	2	2.603	14.405	.000
	Residual	62.879	348	.181		
	Total	68.085	350			
Qubic	Regression	5.259	2	2.629	14.564	.000
	Residual	62.826	348	.181		
	Total	68.085	350			

Table E.9 : Coefficients table of regression analysis with curve estimation of total mold counts of heat treated sucuk samples.

Model		Unstandardized Coefficients		Standardized Coefficients		
		B	Std. Error	Beta	t	Sig.
Linear	Nitrite Level	.003	.001	.259	5.000	.000
	(Constant)	.437	.063		6.992	.000
Logarithmic	ln(Nitrite Level)	.255	.055	.241	4.638	.000
	(Constant)	-.425	.250		-1.700	.090
Quadratic	Nitrite Level	-.005	.004	-.479	-1.225	.221
	Nitrite Level ** 2	4.019E-5	.000	.744	1.903	.058
	(Constant)	.805	.203		3.964	.000
Qubic	Nitrite Level ** 2	-2.297E-5	.000	-.425	-.817	.415
	Nitrite Level ** 3	2.227E-7	.000	.698	1.340	.181
	(Constant)	.666	.088		7.588	.000

Table E.10 : Coefficients table of regression analysis with curve estimation of total mold counts of heat treated sucuk samples.

Model		Sum of Squares	df	Mean Square	F	Sig.
Propolis %	Regression	3.692	1	3.692	20.012	.000
	Residual	64.392	349	.185		
	Total	68.085	350			
Nitrite Level	Regression	4.551	1	4.551	25.001	.000
	Residual	63.533	349	.182		
	Total	68.085	350			

Table E.11 : ANOVA table of stepwise linear regression analysis with curve estimation of total mold counts of heat treated sucuk samples.

	Unstandardized Coefficients		Standardized Coefficients			95,0% Confidence Interval for B		Correlations	
Model	B	Std. Error	Beta	t	Sig.	Lower Bound	Upper Bound	Zero-order	Partial
Propolis	.934	.051		18.215	.000	.833	1.035		
%	-.615	.138	-.233	-4.473	.000	-.886	-.345	-.233	-.233
Nitrite	.437	.063		6.992	.000	.314	.560		
Level	.003	.001	.259	5.000	.000	.002	.004	.259	.259

APPENDIX F : Fat Content of Heat Treated Sucuks – As Is Basis

Table F.1 : Fat amounts of heat-treated dry fermented sucuk samples (as is basis). [1]

SAMPLES	R	A	B	C	D	E	F	G	H
dough	30.43±0.16eF	29.73±0.16fG	33.36±0.14iD	34.36±0.071iB	30.92±0.05kE	30.48±0.11hF	34.95±0.02fA	30.76±0.06jE	33.59±0.06hC
0 day	34.17±0.25cG	36.92±0.24cdBC	36.44±0.19hD	36.72±0.12hCD	37.12±0.08fB	38.35±0.17bA	35.35±0.01eF	35.96±0.09hE	38.09±0.09gA
15th day	34.84±0.08aE	36.61±0.17deD	37.11±0.01eC	39.52±0.09cA	37.5±0.09dB	39.46±0.45aA	36.43±0.19dD	36.53±0.12fD	39.37±0.08cA
30th day	33.75±0.12dF	36.76±0.08cdeDE	36.98±0.17efCD	40.09±0.01aA	37.3±0.09eC	36.45±0.09fE	37.3±0.45cC	36.69±0.12efDE	38.83±0.08eB
45th day	33.8±0.08dF	37.36±0.17aC	37.78±0.01cB	38.93±0.09eA	36.19±0.09hE	37.39±0.45cdC	37.24±0.17cC	36.57±0.01efD	38.56±0.09fA
60th day	34.69±0.25abF	37.55±0.24aC	37.78±0.19cC	39.18±0.12dB	37.52±0.08dC	35.78±0.17gE	39.21±0.01aB	36.73±0.09deD	39.94±0.09bA
90th day	34.69±0.08abH	36.65±0.17deEF	37.52±0.01dD	39.16±0.09dB	36.83±0.09gE	36.41±0.45fF	38.2±0.19bC	35.6±0.12iG	39.81±0.08bA
120th day	33.76±0.12dF	36.56±0.08eE	37.21±0.17eD	38.66±0.01fA	37.85±0.09cB	37.02±0.09deD	37.28±0.45cCD	37.64±0.12aBC	38.66±0.08fA
150th day	34.43±0.03bcH	36.85±0.03cdeF	38.56±0.05aB	37.7±0.04gD	38.18±0.04bC	38.56±0.02bB	36.75±0.01dG	37.03±0.01bE	39.08±0.05dA
170th day	33.84±0.02dI	37.04±0.02bcC	36.81±0.05fgE	39.45±0.03cB	35.73±0.02jG	36.56±0.02efF	35.57±0.01eH	36.91±0.01bcD	41.14±0.03aA
180th day	34.27±0.01cI	37.25±0.02abF	38.2±0.02bD	39.93±0.02bA	38.61±0.02aC	36.71±0.02efG	37.66±0.03cE	36.31±0.03gH	39.47±0.04cB
190th day	34.35±0.02cH	36.78±0.01cdeF	38.27±0.02bC	38.76±0.02fB	35.94±0.02iG	39.08±0.05aA	38.31±0.03bC	36.86±0.03cdE	38.16±0.02gD
200th day	34.7±0.04abI	37.43±0.02aE	36.63±0.01ghG	40.03±0.01abA	37.28±0.02eF	37.68±0.01cC	36.38±0.02dH	37.63±0.02aD	39.49±0.03cB

[1] R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

Values in columns represent the mean of 3 replications and standard deviation.

Values followed by the same small letters in columns are not significantly different ($p < 0.05$).

Values followed by the same

Table F.2 : Protein analysis results of heat-treated dry fermented sucuk samples (as is basis). 1

SAMPLE	R	A	B	C	D	E	F	G	H
dough	12.10±0.02hB	12.14±0.04kB	11.28±0.04kF	11.46±0.01lE	11.89±0.02jD	11.92±0.02jD	11.46±0.01aE	12.03±0.01jC	12.25±0.01kA
0 day	14.99±0.01aD	14.94±0.03eE	14.85±0.02gF	14.24±0.01gG	14.19±0.01gH	15.14±0.02aC	15.53±0.02aA	15.24±0.03aB	14.92±0.01abE
15th day	14.86±0.02bA	14.61±0.04gC	14.35±0.02jD	14.77±0.01cB	14.24±0.02fE	13.87±0.02hF	14.58±0.02aC	14.23±0.02hE	14.9±0.02bA
30th day	14.56±0.02eC	14.68±0.02fB	14.54±0.01hC	14.11±0.01iE	14.56±0.02dC	14.22±0.01fD	14.68±0.02aB	14.03±0.02iF	14.93±0.01aA
45th day	14.87±0.02bB	15.32±0.02bA	14.83±0.02gB	13.97±0.01jF	14.44±0.01eD	14.21±0.01fE	14.17±0.05aE	14.87±0.02cB	14.73±0.02eC
60th day	14.05±0.02gE	14.25±0.04iD	15.11±0.01dA	14.54±0.03eB	13.99±0.02iF	13.77±0.02iG	14.42±0.01aC	14.44±0.01fC	14.27±0.01hD
90th day	14.66±0.01cdE	15.39±0.02aA	14.95±0.01fB	14.34±0.03fG	14.70±0.02cD	14.3±0.01eG	14.57±0.01aF	14.74±0.03dC	14.75±0.02eC
120th day	14.40±0.02fE	14.49±0.02hD	14.92±0.02fB	14.2±0.01hG	15.02±0.01bA	14.45±0.02dD	14.26±0.05aF	14.63±0.03eC	14.22±0.01iFG
150th day	14.62±0.04cdeF	15.27±0.01cB	15.39±0.01bA	14.73±0.01dDE	14.45±0.03eG	14.72±0.01bE	15.10±0.03aC	14.37±0.04gH	14.78±0.01dD
170th day	14.41±0.04fE	15.10±0.01dB	15.05±0.01eC	14.13±0.01iG	13.99±0.03iH	14.67±0.01cD	15.17±0.02aA	14.25±0.03hF	14.12±0.01jG
180th day	14.67±0.08cF	15.33±0.01bC	15.66±0.02aA	14.88±0.01bE	15.44±0.02aB	14.70±0.01bcF	14.71±0.02aF	14.98±0.03bD	14.65±0.01fF
190th day	14.98±0.03aB	14.12±0.01jF	15.21±0.01cA	15.23±0.01aA	14.06±0.01hG	14.33±0.01eE	14.57±0.01aC	14.60±0.01eC	14.46±0.01gD
200th day	14.60±0.01deD	14.66±0.03fC	14.42±0.01iE	13.48±0.02kI	13.96±0.01iH	14.18±0.02gG	14.97±0.01aA	14.26±0.01hF	14.84±0.02cB

R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

Values in columns represent the mean of 3 replications and standard deviation.

Values followed by the same small letters in columns are not significantly different ($p < 0.05$).

Values followed by the same capital letters in rows are not significantly different ($p < 0.05$)

Table F.3 : Salt analysis results of heat-treated dry fermented sucuk samples (as is basis). [1]

SAMPLES	R	A	B	C	D	E	F	G	H
dough	1.95±0.02eF	2.02±0.01hE	2.17±0.02fC	2.30±0.02dB	2.2±0.02gC	2.12±0.03fD	2.7±0.02cA	2.17±0.01iC	2.2±0.02gC
0 day	2.94±0.03abcdA	2.9±0.01efA	2.77±0.03eB	2.95±0.03bcA	2.9±0.03fA	2.7±0.04eC	2.71±0.04cBC	2.74±0.01hBC	2.77±0.04fB
15th day	3.01±0.04abB	2.99±0.04abcB	2.78±0.01deD	2.99±0.04abcB	2.99±0.01cdeB	2.99±0.03cdB	2.97±0.01bB	3.09±0.03abA	2.87±0.03deC
30th day	2.86±0.03dD	2.98±0.01bcdBC	2.92±0.03cdeCD	2.88±0.03cD	3.01±0.01cdeB	3.11±0.04aA	3.14±0.04aA	2.91±0.01gD	2.99±0.04abB
45th day	2.89±0.08cdCD	2.83±0.01gD	3.02±0.03bcdeAB	2.94±0.03bcBC	2.96±0.04eBC	2.97±0.04cdABC	2.98±0.04bAB	3.06±0.01bcA	3.01±0.04aAB
60th day	2.91±0.03cdCD	2.98±0.01bcdABCD	3.05±0.03bcdA	2.98±0.03abcABCD	2.90±0.01fD	3.01±0.04bcdAB	2.93±0.04bBCD	3.00±0.01deABC	2.97±0.09abcABCD
90th day	2.92±0.03bcdD	3.05±0.01aBC	3.27±0.03abA	3.03±0.03abBC	3.00±0.04cdeC	3.08±0.04abB	2.93±0.04bD	3.01±0.01deC	3.00±0.04abC
120th day	2.91±0.03cdCD	2.93±0.01cdefBCD	3.07±0.03bcA	2.95±0.03bcBC	2.99±0.01deB	2.93±0.04dBCD	2.99±0.04bB	3.13±0.01aA	2.87±0.04deD
150th day	2.98±0.08abcdAB	2.96±0.04bcdeAB	2.96±0.03cdeAB	3.04±0.10abA	3.01±0.02cdeAB	3.01±0.05bcdAB	2.92±0.04bAB	2.93±0.03fgAB	2.89±0.02cdeB
170th day	2.96±0.05abcdBC	2.96±0.04bcdeBC	3.04±0.03bcdeABC	3.01±0.10abcBC	3.05±0.02abcAB	2.98±0.05cdBC	3.12±0.03aA	2.94±0.03fgC	2.84±0.01efD
180th day	3.01±0.04abABC	3.00±0.05AabBC	3.01±0.06bcdeABC	3.04±0.09abBC	3.11±0.01aAB	3.11±0.06aAB	3.19±0.03aA	2.96±0.03efCD	2.91±0.01bcdeD
190th day	3.03±0.02aBC	2.88±0.04fgDE	2.82±0.07cdeE	3.10±0.05aAB	3.04±0.07bcdBC	2.94±0.06dCD	3.15±0.04aA	3.02±0.04cdBC	2.94±0.03abcdCD
200th day	3.02±0.01abB	2.92±0.04defB	3.37±0.41aA	2.95±0.06bcB	3.08±0.03abAB	3.05±0.02abcAB	3.13±0.03aAB	2.92±0.03fgB	2.94±0.06abcdB

R: control. 150 ppm sodium nitrite. no propolis added; A: 150 ppm sodium nitrite and 0.25% (w/w) propolis added; B: 150 ppm sodium nitrite and 0.50% (w/w) propolis added; C: 50 ppm sodium nitrite and 0.25% (w/w) propolis added; D: 50 ppm sodium nitrite and 0.50% (w/w) propolis added; E: 75 ppm sodium nitrite and 0.25% (w/w) propolis added; F: 75 ppm sodium nitrite and 0.50% (w/w) propolis added; G: 100 ppm sodium nitrite and 0.25% (w/w) propolis added; H: 100 ppm sodium nitrite and 0.50% (w/w) propolis added.

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Values followed by the same capital letters in rows are not significantly different ($p < 0.0$)

CURRICULUM VITAE

Name-Surname: Tuğba Özdal

Date & Place of Birth: 13.02.1986, İstanbul

Address: Okan University, Department of Food Engineering, Tuzla, Istanbul

e-mail: tugba.ozdal@okan.edu.tr

BSc: Istanbul Technical University, Department of Food Engineering

M.Sc: Istanbul Technical University, Food Engineering Programme

Work Experience and Awards:

- 2010 April – 2017 May: Research Assistant, Department of Food Engineering, Okan University
- 2017 May – present : Lecturer, Department of Food Engineering, Okan University

Publications:

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