

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE
ENGINEERING AND TECHNOLOGY

**EVALUATING THE ANTIOXIDANT POTENTIAL AND *IN VITRO*
BIOAVAILABILITY OF DIFFERENT PHENOLICS IN MODEL SYSTEMS**

M.Sc.THESIS

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Department of Food Engineering

Food Engineering Programme

JANUARY 2014

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

**MODEL SİSTEMLERDE FARKLI FENOLİK BİLEŞİKLERİNİN
ANTİOKSİDAN POTANSİYALİ VE BİYİYARARLILIĞININ
DEĞERLENDİRİLMESİ**

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FOREWORD

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ABBREVIATIONS

ABTS	: 2,2- azinobis 3-ethylbenzothiazoline-6-sulfonice acid
ANOVA	: Analysis of Variance
CH	: Catechin Hydrate
CUPRAC	: Cupric Reducing Antioxidant Capacity
C3G	: Cyanidin 3-O-glucoside
C3Ga	: Cyanidin 3-O-galactoside
C3R	: Cyanidin 3-O-rutinoside
DMSO	: Dimethyl Sulfoxide
DPPH	: 2,2-diphenyl-1-picrylhydrazyl
EC	: Epicatechin
ECG	: Epicatechingallate
EGCG	: Epigallocatechingallate
GAE	:Gallic Acid Equivalent
HPLC	: High Performance Liquid Chromatography
IN	: Solution Entering The Dialysis Tubing
M3G	: Malvidin 3-O-glucoside
M3Ga	: Malvidin 3-galactoside
NG	: (±)- Naringenin
PCA	: Protocatechuic Acid
PDA	: Photodiode Array Detector
PG	: Post Gastric
P3G	: Pelargonidin 3-O-glucoside
pHBA	: P-Hydroxy Benzoic Acid
P3R	: Pelargonidin 3-rutinoside
SD	: Standard Deviation
SPSS	: Statistical Package for the Social Science
TEAC	: Trolox Equivalent Antioxidant Capacity
TPC	: Total Phenolic Content
Trolox	: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
AO	: antioxidant

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EVALUATING THE ANTIOXIDANT POTENTIAL AND IN VITRO BIOAVAILABILITY OF DIFFERENT PHENOLICS IN MODEL SYSTEMS

SUMMARY

Phenolic compounds are a large group of plant constituents. To date, more than 6000 flavonoids have been identified, although a smaller number is important in respect to diet. In the 1990s, interest in these compounds truly commenced and has been growing ever since. Polyphenols have been the center of huge research interest over the past decade. They have been attributed to a wide range of beneficial properties regarding human health, including protective effects on cancer, cardiovascular diseases, inflammation, and the neurodegenerative disorders because of being potent antioxidants.

The main objective of this study is to compare the bioavailability and antioxidant (AO) capacity of different phenolic compounds in model systems. Previous studies have only investigated the bioavailability and AO activity of phenolic compounds that are found in certain food products together with the food matrix effect. In this research, analysis is carried out on single standards of phenolic compounds. In literature, there is limited research on pure phenolic compounds and their *in vitro* bioavailability. Totally 32 standards in concern are: isoflavones, phenolic acids, flavonols, flavanone, catechins and anthocyanins. All the standards are compared with each other with respect to their total phenolic contents using Folin Ciocalteu method and total AO capacities using commonly applied methods such as 2,2-azinobis (3-ethylbenzothiazoline)-6-sulfonic acid (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and copper reducing antioxidant capacity (CUPRAC) both before and after *in vitro* gastrointestinal digestion to determine their bioavailability besides the change in their phenolic profiles.

To form the models pure phenolic compounds were dissolved in water to simulate the conditions in the body. *In vitro* digestion method which consist of two stages was adapted from McDougall et al. (2005) was applied to evaluate potential bioavailability of phenolic compounds. First stage gastric digestion was performed at 37°C for 2 hours with pepsin enzyme and second stage pancreatic digestion was applied at 37°C for 2 hours with bile salts and pancreatin enzyme. Samples were collected as PG, IN and OUT fractions. PG is the fraction after gastric digestion, IN sample is representing the materials entering into serum and OUT sample is showing the material remaining in the gastrointestinal tract. All fractions were analyzed for phenolic profiles, besides their total phenolic content and AO activity.

All the data obtained were evaluated statistically by Statistical Package for the Social Sciences (SPSS) Programme version 21.0. Significant differences between the samples were analyzed by one way Analysis of Variance (ANOVA) at 0.05 significant level followed by Duncan's New Multiple Range Test as post hoc tests. The results were reported as mg equivalents/ g standard. Each analyses were repeated

in duplicate for each sample and the results were reported as mean value $\pm$ standard deviation.

As a result of this study; before digestion Cyanidin 3-O-glucoside showed the highest total phenolic content value (1901.22 ± 51.47 mg GAE/g) and glycitin the lowest (178.23 ± 2.36 mg GAE/g). Gallic acid had the highest AO capacity for all the AO activity analysis, 4022.43 ± 186.06 for DPPH, 4397.81 ± 53.59 for CUPRAC, 5646.67 ± 40.35 mg TEAC/g for ABTS, respectively. When the total antioxidant activity within the phenolic groups was evaluated by DPPH method the order of AO capacity is catechins > flavanols > phenolic Acids > anthocyanins > isoflavones > naringenin. Overall, the DPPH method is less sensitive than the other methods for hydrophilic antioxidants. In respect to flavanols for TAC results, generally quercetin showed the highest AO capacity in all TAC analysis. The highest AO capacities in the CUPRAC method were observed for epicatechin gallate, epigallocatechin gallate, quercetin, epigallocatechin, catechin, caffeic acid, epicatechin and gallic acid. According to the ABTS results, aglycones of anthocyanins had lower AO activity than corresponding glycoside forms except for Malvidin chloride.

According to total phenolic content analysis after digestion, generally postgastric (PG) fractions were higher than the IN and OUT fractions. Both IN and OUT values of phenolic acids had highest levels in comparison to the other standards. Percent recovery values for OUT fractions in isoflavones were high for CUPRAC. Besides, phenolic acids and catechins had higher PG % recovery values. On the other hand, Flavanols had high recovery% values for OUT similar to Naringenin which was Flavanone. Anthocyanins had high recovery % values for PG than recovery % values for IN and OUT in this study. In respect to ABTS analysis, within the anthocyanins, when glycoside forms and aglycone forms compared with each other, it was obtained that the values of aglycones were higher than the values of glycosides in PG fractions. Besides, anthocyanins were found to have lower bioavailability during digestion when compared to other phenolic compounds.

According to the HPLC-PDA results, glycitin had the highest values for IN and OUT fractions (96.3 ± 3.2 ; 158.1 ± 14.1 $\mu\text{g/ml}$, respectively). In respect to PG fractions; (+)-catechin hydrate had the highest value as 109.3 ± 17.7 $\mu\text{g/ml}$. Generally PG values were found to be higher than IN and OUT fractions. On the other hand, epigallocatechin had very low value as 0.3 ± 0.1 ; 0.2 ± 0.0 $\mu\text{g/ml}$, for the IN and OUT fractions, respectively. When PG results were examined value of myricetin was found to be lower than other compounds as 0.8 ± 0.2 $\mu\text{g/ml}$. Moreover, compounds such as delphinidin, cyanidin, cyanidin-3-O-glucoside, cyanidin-3-galactoside, pelargonidin and malvidin could not be determined in IN, OUT and PG fractions. Besides, for quercetin, caffeic acid, myricetin, catechin hydrate, EGC, C3R, P3G, M3Ga and M3G compounds, the peaks could only be detected in PG fraction.

Besides, there was an important relation between total phenolic content and all of total antioxidant activity methods, CUPRAC ($r=0.606$), DPPH ($r=0.590$) and ABTS ($r=0.447$), ($p<0.01$). The relation between CUPRAC and DPPH, CUPRAC and ABTS were also statistically significant ($p<0.01$). There was weak relation (0.392) between ABTS and DPPH, but according to statistical evaluation it was significant again.

Overall, the antioxidant capacity and bioavailability of anthocyanin, isoflavone, phenolic acid, flavanone and flavanols were compared both between classes and within the classes. It was seen that antioxidant activity and bioavailability differs

greatly from one polyphenol to another due to their structure, interactions and solubility according to method performed. In this regard, the study may be helpful in understanding the method responses and efficiencies against the targeted phenolic compounds so that proper method selection for the specific phenolics will be possible as well. The results clearly show wide variability in the bioavailability of different polyphenols.

MODEL SİSTEMLERDE FARKLI FENOLİK BİLEŞİKLERİNİN ANTIOKSİDAN POTANSİYALİ VE BİYUYARARLILIĞININ DEĞERLENDİRİLMESİ

ÖZET

Fenolik bileşikler bitkilerin büyük bir kısmını oluşturmaktadır. Bugüne kadar 6000'den fazla fenolik bileşik tanımlanmış fakat bunun çok küçük bir kısmı diyet açısından önemli bulunmuştur. Bu bileşiklere ilgi gerçek anlamda 1990'lı yıllarda başlamış ve giderek artmıştır. Son yıllarda polifenoller araştırmacılar ve tüketiciler arasında önem kazanmıştır. Fenolik bileşikler antioksidan özelliklerinden dolayı sağlığa yararlı etkileri, kanser,kardiyovasküler hastalıklar, iltihap ve nörodejenaratif hastalıklar üzerindeki olumlu etkileri nedeniyle dikkat çekmektedir.

Literatürde saf halde bulunan fenolik bileşiklerin in vitro biyoyararlılığı ile ilgili az sayıda çalışmaya rastlanmaktadır. In vivo metodlar karmaşık, pahalı, uzun zaman alan çalışmalardır ve çalışma boyunca etik sorunlar ile karşılaşmaktadır. In vitro metodlar ise ucuz, hızlı ve basittir. Bu çalışmada amaç; antosiyanin, izoflavon, fenolik asit ve bazı flavanoid standartlarının mevcut antioksidan aktiviteleri ve toplam fenolik madde miktarına etkilerinin değerlendirilmesinin ardından in vitro olarak sindirimleri sonrasında biyoyararlılıklarının model sistemlerde karşılaştırılmasıdır. Önceki çalışmalarda belli ürünlerde bulunan antosiyanin ve diğer fenolik bileşenlerin biyoyararlılıkları ve antioksidan aktivitelerine ilişkin incelemeler yapılmışken, bu çalışmada standart haldeki antosiyanin, izoflavon, fenolik asit ve flavanoidler üzerinde analizler yapılacaktır. Bu sayede antosiyanin, izoflavon, fenolik asit ve flavanoid fenolik madde sınıflarının antioksidan kapasiteleri ve biyoyararlılıkları hem sınıfların kendileri içlerinde hem de sınıflar arasında kıyaslama yapılarak bu konuda önemli bulgular elde edileceği düşünülmektedir. Analizlenen standartlar izoflavonlar, fenolik asitler, flavonoller, flavanonlar, kateşinler ve antosiyaninlerdir. *In vitro* sindirim öncesi ve sonrasında tüm bu standartlar Folin Ciocalteau metodu ile toplam fenolik madde içeriği ve ,2-azinobis (3-ethylbenzothiazoline)-6-sulfonik asit (ABTS), 1,1-diphenyl-2- picrylhydrazyl (DPPH) and CUPRAC metodu ile antioksidan kapasitesi açısından kıyaslanmıştır.

Saf haldeki fenolik bileşikler quercetin hariç suda çözülmüş ve bu örneklerle in vitro sindirim metodu uygulanmıştır. Bu yöntem McDougall ve diğ. (2005) gerçekleştirdiği çalışmadan uyarlanmıştır. Metod iki basamaktan oluşmaktadır. Mide sindirimini taklit etmek amacıyla pepsin/HCl sindirimi 2 saat 37°C olarak gerçekleştirilmiş ardından ince bağırsak sindirimini taklit etmek için ise pankreatin enzimi ve safra tuzları eklenerek 37°C de 2 saat gerçekleştirilmiştir. Örnekler PG, IN ve OUT kısımlarından alınmıştır. PG örnekleri mide sonrası, IN kana geçen kısmı, OUT ise mide bağırsak sindirimi sonrasında kalan maddeyi temsil etmektedir. Tüm fraksiyonlarda antosiyanin ve fenolik madde miktarları ve toplam antioksidan aktivite belirlenmiştir.

Analiz sonuçlarının tümü Sosyal Bilimler için İstatistik Paketi (SPSS) 21.0 versiyonu yazılım yardımı ile tek yönlü varyans analizi (ANOVA) uygulaması ile Duncan Yeni Çoklu Aralık Testi seçilerek 0.05 önem derecesinde değerlendirilmiştir. Sonuçlar, mg eş değerleri/g kuru madde olarak belirtilmiştir. Her bir analiz her örnek için iki kez tekrarlanmış ve sonuçlar ortalama değer $\pm$ standard sapma olarak verilmiştir.

Yapılan çalışma sonucunda; toplam fenolik içerik bakımından en yüksek değeri Syanidin-3-O-glukozit (1901.22 ± 51.47 mg GAE/g), en düşük değeri ise glisitin (178.23 ± 2.36 mg GAE/g) göstermiştir. Fenolik içerik sırasına göre sonuçlar yaklaşık olarak flavanoller > antosiyaninler > kateşinler > naringenin > fenolik asitler > isoflavonlar şeklindedir. Tüm antioksidan aktivite analizlerinde gallik asit en yüksek antioksidan kapasiteye sahip olup DPPH için 4022.43 ± 186.06 ; CUPRAC için 4397.81 ± 53.59 ve ABTS için 5646.67 ± 40.35 mg TEAC/g aktivite göstermiştir. Fenolik gruplar içerisinde toplam antioksidan aktivite DPPH metoduna göre değerlendirildiğinde kateşinler > flavonoller > fenolik asitler > antosiyaninler > isoflavonlar > naringenin şeklinde bir sıralama elde edilir. Fenolik bileşikler arasında cyanidin 3-glukozit 995.47 ± 23.09 mg TEAC/g olarak en düşük, gallik asit ise 4022.43 ± 186.06 mg TEAC/g olarak en yüksek antioksidan aktiviteyi göstermiştir. Sonuç olarak DPPH yöntemi diğer yöntemlere kıyasla hidrofilik antioksidanlar için daha düşük hassasiyet göstermiştir. Tüm antioksidan analizlerinde genellikle quercetin en yüksek aktivite göstermiştir. Epikateşin gallat, epigallokateşin gallat, quercetin, epigallokateşin, kateşin hidrat, kafeik asit, epikateşin ve gallik asit CUPRAC yönteminde yüksek antioksidan aktiviteye sahiptir. ABTS yöntemiyle elde edilen sonuçlar değerlendirildiğinde malvidin klorit dışında antosiyaninlerin aglikon yapıları glikozit yapılarına göre daha düşük antioksidan aktiviteye sahiptir.

Sindirim sonrasında yapılan toplam fenolik içerik analizlerine göre genellikle bağırsak öncesi (PG) fraksiyonları IN ve OUT fraksiyonlarından daha yüksek olduğu görülmüştür. Fenolik asitlerin IN ve OUT değerleri diğer standartlara kıyasla daha yüksek fenolik madde içeriğine sahiptir. CUPRAC yöntemine göre isoflavonlar içerisinde OUT fraksiyonların % gerikazanım değerleri daha yüksektir. Fenolik asitler arasında pHBA yüksek % gerikazanım değerine sahiptir. Ayrıca fenolik asitlerin ve kateşinlerin PG % gerikazanım değerleri daha yüksektir. Diğer taraftan flavonoller ve benzer şekilde naringenin bileşiklerinin OUT fraksiyonları daha yüksek % gerikazanım değerlerine sahiptir. Bu çalışmada antosiyaninlerin PG fraksiyonları IN ve OUT kısımlarında daha yüksek % gerikazanım değerlerine sahiptir. ABTS analizleri açısından antosiyaninler içerisinde glikozit yapıları aglikon yapıları ile kıyaslandığında PG fraksiyonların aglikon formları glikozit formlarından daha yüksek antioksidan aktiviteye sahip olduğu görülmüştür. Ayrıca fenolik bileşikler aralarında kıyaslandığında sindirim sırasında en düşük biyoyararlılığı antosiyaninler göstermiştir.

HPLC-PDA sonuçlarına göre glisitin için IN ve OUT fraksiyonlarında sırasıyla 96.3 ± 3.2 ; 158.1 ± 14.1 $\mu\text{g/ml}$ oranında yüksek değerler elde edilmiştir. PG fraksiyonları açısından (+)-kateşin hidrat 109.3 ± 17.7 $\mu\text{g/ml}$ oranında yüksek sonuçlar göstermiştir. Genel olarak PG değerleri IN ve OUT değerlerine göre daha yüksek elde edilmiştir. IN ve OUT fraksiyonlarında sırasıyla 0.3 ± 0.1 ; 0.2 ± 0.0 $\mu\text{g/ml}$ oranında epigallokateşin düşük sonuçlar vermiştir. PG fraksiyonlarında ise myricetin 0.8 ± 0.2 $\mu\text{g/ml}$ oranında düşük değerler elde edilmiştir. Ayrıca delfinidin, siyanidin, siyanidin-3-O-glukozit, pelargonidin ve malvidin IN, OUT ve PG fraksiyonlarında tespit edilememiştir. Bunun dışında kuersetin, kafeik asit,

myricetin, kateşin hidrat, EGC, C3R, P3G, M3Ga ve M3G bileşikleri yalnızca PG fraksiyonlarında belirlenebilmiştir.

Ayrıca toplam fenolik madde analizleri ve tüm antioksidan aktivite yöntemleri arasındaki ilişki $p<0.01$ düzeyinde önemli bulunmuş olup CUPRAC için $r=0.606$, DPPH için $r=0.590$ ve ABTS için $r=0.447$ şeklindedir. CUPRAC ve DPPH ile CUPRAC ve ABTS arasındaki ilişki istatistiksel olarak önemli bulunmuştur ($p<0.01$). bunun dışında ABTS ve DPPH arasındaki ilişkide (0.392) istatistiksel değerlendirmeye göre önemli olarak tespit edilmiştir.

Sonuç olarak, antosiyanin, isoflavon, fenolik asit, flavanon ve flavonol sınıfları hem sınıflar arasında hem de sınıflar içerisinde antioksidan kapasite ve biyoyararlılık açısından kıyaslanmıştır. Elde edilen sonuçlara göre antioksidan aktivite ve biyoyararlılık polifenoller arasında kimyasal yapı, etkileşim ve çözünürlük gibi faktörlere bağlı olarak önemli oranda değişiklik göstermiştir. Bu açıdan yapılan çalışma fenolik bileşiklerin metotlara verdiği yanıtları değerlendirebilme imkanı sağlayarak belirli fenolikler için uygun metot seçimine olanak sağlamaktadır. Biyoyararlılık sonuçları polifenoller arasında geniş ölçüde farklılık göstermiştir.

1. INTRODUCTION

Polyphenols are known as plant pigments for centuries. The first studies on the biological activity of phenolics was published by Rusznyak and Szent- Gyorgyi in 1936 (Rusznyak, 1936). Formerly polyphenols were discussed due to the impact on the color and flavor characteristics of plants. In recent years, researchers and food manufacturers became increasingly interested in polyphenols. The chief reason for this interest is the recognition of the antioxidant properties of polyphenols, their great abundance in our diet, and their probable role in the prevention of various diseases associated with oxidative stress, such as cancer and cardiovascular and neurodegenerative diseases (Middleton et al., 2000).

Flavonoids and their glycosides are the major constituents of polyphenols that are present in edible plants such as fruits, vegetables, nuts, seeds, tea, olive oil, and red wine (Monfilliette-Cotelle, 2006). Flavonoids comprise more than 5000 compounds (Harborne and Williams, 2000) and can be categorized into two groups according to their chemical structures as phenolic acids and flavanoids which are divided into 6 subclasses flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols (catechins and proanthocyanidins) (Middleton et al., 2000). Flavonoids are usually associated with a sugar moiety, though occasionally they occur in plants as aglycones. During metabolism, flavonoids can undergo modifications such as addition of hydroxyl groups, methylation, sulfatation, or glucuronidation.

Furthermore, not all polyphenols are absorbed with equal efficacy. They are extensively metabolized by intestinal and hepatic enzymes and by the intestinal microflora. Knowledge of the bioavailability and metabolism of the various polyphenols is necessary to evaluate their biological activity within target tissues.

One of the main objectives of bioavailability studies is to determine, among the hundreds of dietary polyphenols, which are better absorbed and which leads to the formation of active metabolites. Many researchers have investigated the kinetics and extent of polyphenol absorption by measuring plasma concentrations and/or urinary

excretion among adults after the ingestion of a single dose of polyphenol, provided as pure compound, plant extract, or whole food/beverage.

Besides in vivo studies, in vitro digestion methods which are used for investigating biochemical and physiochemical effects are performed as real metabolism by using enzymes. Gastric digestion performed with using pepsin and HCl and bile salts and pancreatin was used for pancreatic digestion. During digestion, pH are changing so this alteration affects the structure of phenolic compounds and anthocyanins. Anthocyanins are stable in acidic conditions such as in stomach however, in neutral pH their structure change into flavilium cation and losing their stability. This effect could be observed in pancreatic digestion. Besides that food matrices are also affecting the bioavailability.

However, existing studies in the literature are mostly based on plant extracts instead of direct phenolic substances. Generally phenolic compounds which were isolated from plants were analyzed by in vivo and in vitro methods. There are limited studies on the bioavailability of single phenolic standards. Thus, interactions are ignored in those studies. This study will be original to examine and compare lots of phenolic compounds in model systems. The total phenolic content, antioxidant activity and bioavailability of each phenolic compound after gastrointestinal digestion will be revealed.

The purposes of this study were to (1) determine the phenolic content and antioxidant activity of each phenolic compound with the commonly applied methods (Folin cicalteau method, 2,2 diphenyl -1-picrylhydrazyl (DPPH) radical scavenging method, Cupric reducing antioxidant capacity (CUPRAC) analysis method, 2,2-azinobis (3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) analysis method) and (2) investigate the stability and bioavailability of pure phenolic acids, isoflavones, catechins, flavanone, flavonols and anthocyanins after simulating gastro-intestinal conditions. Moreover, the changes in the total phenolic content of phenolic standards were investigated after and before digestion. After digestion, the samples were filtered and HPLC-PDA analysed to determine the content of total recovered phenolics.

2. LITERATURE REVIEW

2.1 Phenolic Compounds and Chemical Structures

Several thousand molecules having a polyphenol structure (ie, several hydroxyl groups on aromatic rings) have been identified in higher plants, and several hundred are found in edible plants. These molecules are secondary metabolites of plants and are generally involved in defence against ultraviolet radiation or aggression by pathogens. These compounds may be classified into two different groups as a function of the number of phenol rings that they contain and of the structural elements that bind these rings to one another. These are phenolic acids and flavonoids. The flavonoids, which share a common structure consisting of 2 aromatic rings (A and B) that are bound together by 3 carbon atoms that form an oxygenated heterocycle (ring C), may themselves be divided into 6 subclasses as a function of the type of heterocycle involved: flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols (catechins and proanthocyanidins) (Middleton et al., 2000).

2.1.1 Phenolic acids

Phenolic acids are a subclass of a larger category of metabolites commonly referred to as “phenolics”. The term phenolics encompasses approximately 8000 naturally occurring compounds, all of which possess one common structural feature, a phenol (an aromatic ring bearing at least one hydroxyl substituent). Although the basic skeleton remains the same, the numbers and positions of the hydroxyl groups on the aromatic ring create the variety (Table 2.1). Two classes of phenolic acids can be distinguished: derivatives of benzoic acid and derivatives of cinnamic acid (Figure 2.1) (Robbins, 2003).

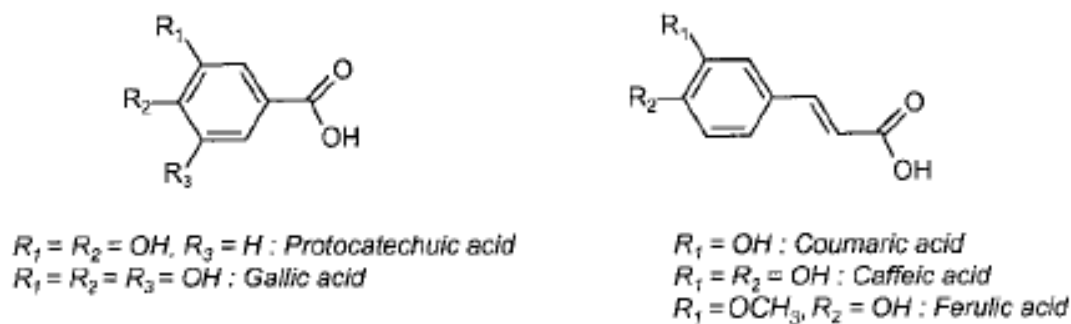


Figure 2.1: Chemical structures of phenolic acids (Middleton et al., 2000).

The hydroxybenzoic acid content of edible plants is generally very low, with the exception of certain red fruits, black radish, and onions, which can have concentrations of several tens of milligrams per kilogram fresh weight (Middleton et al., 2000). Tea is an important source of gallic acid: tea leaves may contain up to 4.5 g/kg fresh weight (Tomas-Barberan et al., 2000). Furthermore, hydroxybenzoic acids are components of complex structures (Clifford et al., 2000). Because these hydroxybenzoic acids, both free and esterified, are found in only a few plants eaten by humans, they have not been extensively studied and are not currently considered to be of great nutritional interest.

The hydroxycinnamic acids are more common than are the hydroxybenzoic acids and consist chiefly of p-coumaric, caffeic, ferulic, and sinapic acids. These acids are rarely found in the free form, except in processed food that has undergone freezing, sterilization, or fermentation. The bound forms are glycosylated derivatives or esters of quinic acid, shikimic acid, and tartaric acid. (Clifford et al., 1999). Caffeic acid, both free and esterified, is generally the most abundant phenolic acid and represents between 75% and 100% of the total hydroxycinnamic acid content of most fruit.

Hydroxycinnamic acids are found in all parts of fruit, although the highest concentrations are seen in the outer parts of ripe fruit. Concentrations generally decrease during the course of ripening, but total quantities increase as the fruit increases in size (Manach et al., 2004).

Ferulic acid is the most abundant phenolic acid found in cereal grains, which constitute its main dietary source. The ferulic acid content of wheat grain is 0.8–2 g/kg dry weight, which may represent up to 90% of total polyphenols (Sosulskiet al., 1982; Lempereur et al., 1997). Ferulic acid is found chiefly in the outer parts of the grain. The aleurone layer and the pericarp of wheat grain contain 98% of the total ferulic acid. The ferulic acid content of different wheat flours is thus directly related to levels of sieving, and bran is the main source of polyphenols (Hatcher et al., 1997). Rice and oat flours contain approximately the same quantity of phenolic acids as wheat flour (63 mg/kg), although the content in maize flour is about 3 times as high (Shahidi et al., 1995). Only 10% of ferulic acid is found in soluble free form in wheat bran (Lempereur et al., 1997).

Table 2.1: Structures of the prominent naturally occurring phenolic acids (Robbins, 2003).

$\text{Xa} = \text{---CH=CH-COOH}$ $\text{Xb} = \text{---C(=O)COOH}$ $\text{Xc} = \text{---C(=O)H}$						
R ₂	R ₃	R ₄	R ₅	X	code	common name
H	H	H	H	a	1	cinnamic acid
–OH	H	H	H	a	2	<i>o</i> -coumaric acid
H	H	–OH	H	a	3	<i>p</i> -coumaric acid
H	–OH	H	H	a	4	<i>m</i> -coumaric acid
H	–OCH ₃	–OH	H	a	5	ferulic acid
H	–OCH ₃	–OH	–OCH ₃	a	6	sinapic acid
H	–OH	–OH	H	a	7	caffeic acid
H	H	H	H	b	8	benzoic acid
–OH	H	H	H	b	9	salicylic acid
H	H	–OH	H	b	10	<i>p</i> -hydroxybenzoic acid
H	–OCH ₃	–OH	H	b	11	vanillic acid
H	–OCH ₃	–OH	–OCH ₃	b	12	syringic acid
H	–OH	–OH	H	b	13	protocatechuic acid
–OH	H	H	–OH	b	14	gentisic acid
–OH	–OH	–OH	–OH	b	15	gallic acid
H	–OCH ₃	–OCH ₃	H	b	16	veratric acid
H	–OCH ₃	–OH	–OCH ₃	c	17	syringaldehyde
H	–OCH ₃	–OH	H	c	18	vanillin

2.1.2 Isoflavones

Isoflavones are flavonoids with structural similarities to estrogens. Although they are not steroids, they have hydroxyl groups in positions 7 and 4' in a configuration analogous to that of the hydroxyls in the estradiol molecule. This confers pseudohormonal properties on them, including the ability to bind to estrogen receptors, and they are consequently classified as phytoestrogens. Isoflavones are found almost exclusively in leguminous plants (Coward et al., 1998).

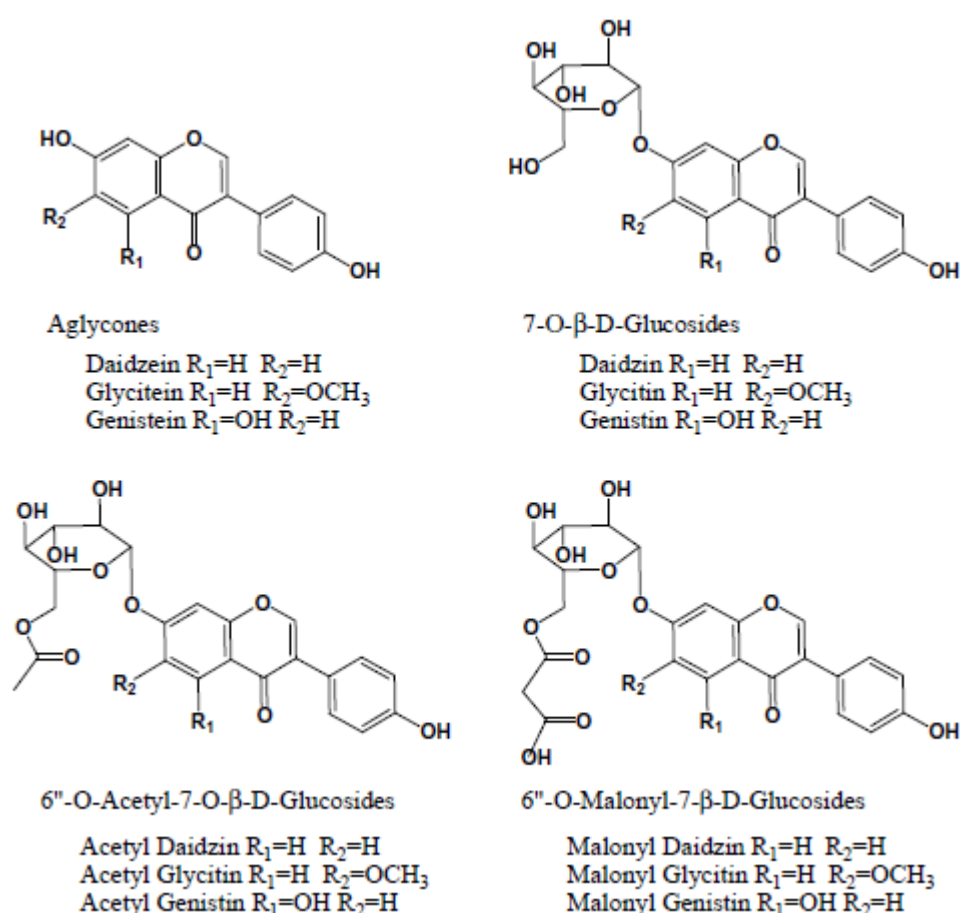


Figure 2.2: Chemical structures of isoflavones (Shao et al., 2009).

Soya and its processed products are the main source of isoflavones in the human diet. They contain 3 main molecules: genistein, daidzein and glycitein, generally in a concentration ratio of 1:1:0.2. These isoflavones are found in 4 forms: aglycone, 7-O-glucoside, 6''-O-acetyl-7-O-glucoside, and 6''-O-malonyl-7-O-glucoside (Coward

et al., 1998). The 6"-Omalonylglucoside derivatives have an unpleasant, bitter, and astringent taste. They are sensitive to heat and are often hydrolyzed to glycosides during the course of industrial processing, as in the production of soya milk (Kudou et al., 1991). The fermentation carried out during the manufacturing of certain foods, such as miso and tempeh, results in the hydrolysis of glycosides to aglycones. The aglycones are highly resistant to heat. The isoflavone content of soya and its manufactured products varies greatly as a function of geographic zone, growing conditions, and processing. Soybeans contain between 580 And 3800 mg isoflavones/kg fresh wt, and soymilk contains between 30 and 175 mg/L (Reinli et al., 1996).

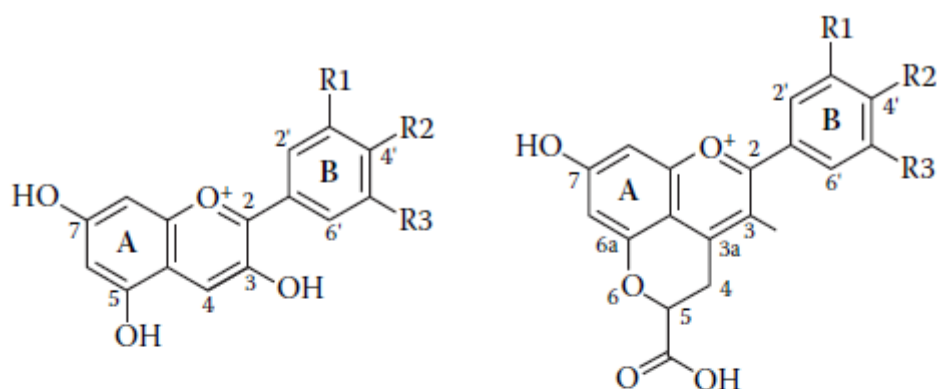
2.1.3 Anthocyanins

Anthocyanins are glycosides of anthocyanidin, aglycone possessing a fundamental skeleton of 2-phenylbenzopyrylium, known as the flavylum cation (Figure 2.3). More than 90% of all anthocyanins isolated in nature are based only on the following six anthocyanidins: pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin, which as shown in the figure, are differentiated by the substitution pattern on the B ring. Recently, the structure of a novel type found especially in wine and called pyroanthocyanin was described (Figure 2.3) (Mercadante & Bobbio, 2008).

Anthocyanins are pigments dissolved in the vacuolar sap of the epidermal tissues of flowers and fruit, to which they impart a pink, red, blue, or purple color (Mazza et al., 1993). They exist in different chemical forms, both colored and uncolored, according to pH. Although they are highly unstable in the aglycone form (anthocyanidins), while they are in plants, they are resistant to light, pH, and oxidation conditions that are likely to degrade them. Degradation is prevented by glycosylation, generally with a glucose at position 3, and esterification with various organic acids (citric and malic acids) and phenolic acids.

In addition, anthocyanins are stabilized by the formation of complexes with other flavonoids (copigmentation). In the human diet, anthocyanins are found in red wine, certain varieties of cereals, and certain leafy and root vegetables (aubergines, cabbage, beans, onions, radishes), but they are most abundant in fruit. Cyanidin is the most common anthocyanidin in foods. Food contents are generally proportional to

color intensity and reach values up to 2–4 g/kg fresh weight in blackcurrants or blackberries (Table 2.2). These values increase as the fruit ripens. Anthocyanins are found mainly in the skin, except for certain types of red fruit, in which they also occur in the flesh (cherries and strawberries). Wine contains 200– 350 mg anthocyanins/L, and these anthocyanins are transformed into various complex structures as the wine ages (Clifford et al., 2000; Es-Safi, 2002).



Anthocyanidins	Substitution pattern		
	R1	R2	R3
Pelargonidin (plg)	H	OH	H
Cyanidin (cyd)	OH	OH	H
Delphinidin (dpd)	OH	OH	OH
Peonidin (pnd)	OCH ₃	OH	H
Petunidin (ptd)	OCH ₃	OH	OH
Malvidin (mvd)	OCH ₃	OH	OCH ₃

Figure 2.3: Chemical structures of anthocyanins (Mercadante & Bobbio, 2008).

2.1.4 Other flavanoids

Flavonols are the most ubiquitous flavonoids in foods, and the main representatives are quercetin and kaempferol. They are generally present at relatively low concentrations of 15–30 mg/kg fresh weight. The richest sources are onions (up to 1.2 g/kg fresh weight), curly kale, leeks, broccoli, and blueberries (Table 2.2). Red wine and tea also contain up to 45 mg flavonols/L. These compounds are present in glycosylated forms. The associated sugar moiety is very often glucose or rhamnose,

but other sugars may also be involved (eg, galactose, arabinose, xylose, glucuronic acid). Fruit often contains between 5 and 10 different flavonol glycosides. Other flavonols in the diet include kaempferol (broccoli), myricetin (berries), and isorhamnetin (onion) (Macheix et al., 1990).

Flavonols accumulate in the outer and aerial tissues (skin and leaves) because their biosynthesis is stimulated by light. Marked differences in concentration exist between pieces of fruit on the same tree and even between different sides of a single piece of fruit, depending on exposure to sunlight (Price et al., 1995). Similarly, in leafy vegetables such as lettuce and cabbage, the glycoside concentration is 10 times as high in the green outer leaves as in the inner light-colored leaves (Herrmann et al., 1976). This phenomenon also accounts for the higher flavonol content of cherry tomatoes than of standard tomatoes, because they have different proportions of skin to whole fruit.

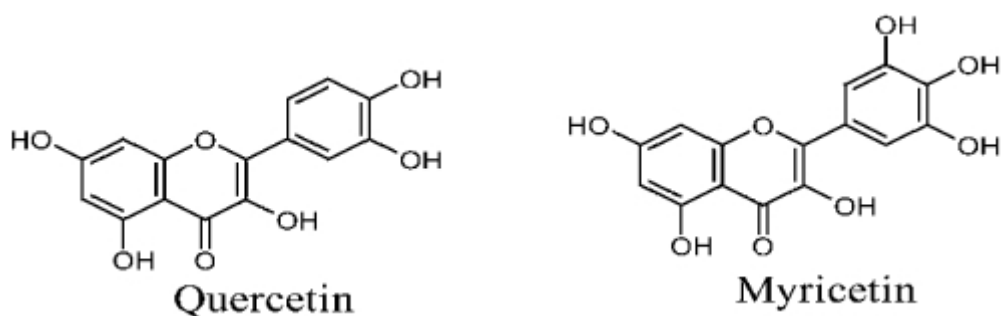


Figure 2.4: Chemical structures of flavonols (Strobel et al., 2005).

The other group which is flavanones occur almost exclusively in citrus fruits. The highest concentrations are found in the solid tissues, but concentrations of several hundred milligrams per liter are present in the juice as well (Tomas-Barberan, 2000). The main aglycones are naringenin in grapefruit, hesperetin in oranges, and eriodictyol in lemons. Flavanones are generally glycosylated by a disaccharide at position 7: either a neohesperidose, which imparts a bitter taste (such as to naringin in grapefruit), or a rutinose, which is flavorless. Low concentrations of naringenin are also found in tomatoes and tomato-based products. Fresh tomatoes, especially tomato skin, also contain naringenin chalcone, which is converted to naringenin during processing to tomato ketchup (Krause et al., 1992).

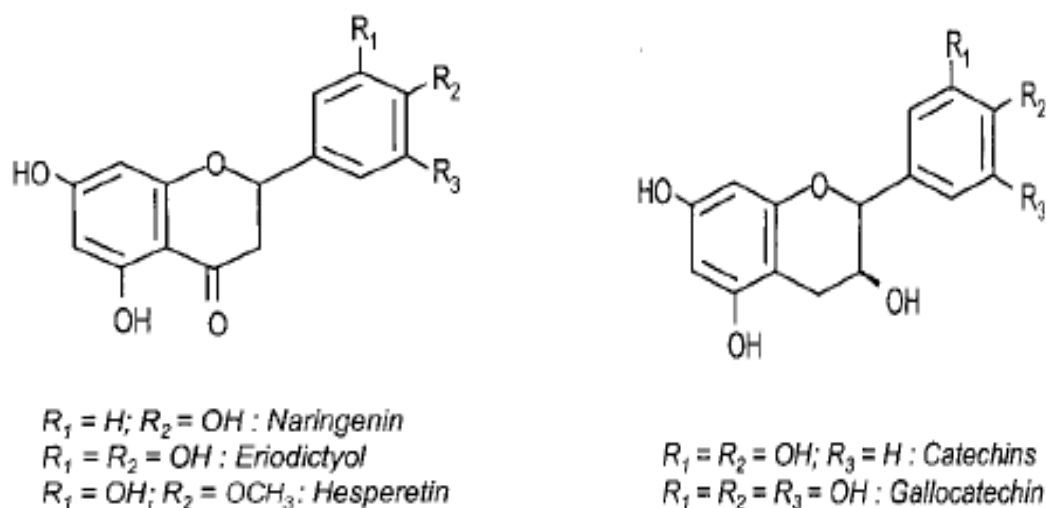


Figure 2.5: Chemical structures of flavanones and flavanols (Manach et al., 2004).

Flavanols exist in both the monomer form (catechins) and the polymer form (proanthocyanidins). Catechins are found in many types of fruit (apricots, which contain 250 mg/kg fresh weight, are the richest source; Table 2.2). They are also present in red wine (up to 300 mg/L), but green tea and chocolate are by far the richest sources. An infusion of green tea contains up to 200 mg catechins (Lakenbrink et al., 2000). Black tea contains fewer monomer flavanols, which are oxidized during “fermentation” (heating) of tea leaves to more complex condensed polyphenols known as theaflavins (dimers) and thearubigins (polymers). Catechin and epicatechin are the main flavanols in fruit, whereas gallocatechin, epigallocatechin, and epigallocatechin gallate are found in certain seeds of leguminous plants, in grapes, and more importantly in tea (Van De Putte et al., 2000). In contrast to other classes of flavonoids, flavanols are not glycosylated in foods. The tea epicatechins are remarkably stable when exposed to heat as long as the pH is acidic: only 15% of these substances are degraded after 7 h in boiling water at pH 5 (Tsang et al., 1997).

Table 2.2: Polyphenols in foods (Manach et al., 2004).

<i>Polyphenol content</i>			
Source (serving size)		By wt or vol	By serving
		<i>mg/kg fresh wt (or mg/L)</i>	<i>mg/serving</i>
Hydroxybenzoic acids	Blackberry (100 g)	80–270	8–27
Protocatechuic acid	Raspberry (100 g)	60–100	6–10
Gallic acid	Black currant (100 g)	40–130	4–13
p-Hydroxybenzoic acid	Strawberry (200 g)	20–90	4–18
Hydroxycinnamic acids	Blueberry (100 g)	2000–2200	200–220
Caffeic acid	Kiwi (100 g)	600–1000	60–100
Chlorogenic acid	Cherry (200 g)	180–1150	36–230
Coumaric acid	Plum (200 g)	140–1150	28–230
Ferulic acid	Aubergine (200 g)	600–660	120–132
Sinapic acid	Apple (200 g)	50–600	10–120
	Pear (200 g)	15–600	3–120
	Chicory (200 g)	200–500	40–100
	Artichoke (100 g)	450	45
	Potato (200 g)	100–190	20–38
	Corn flour (75 g)	310	23
	Flour: wheat, rice, oat (75 g)	70–90	5–7
	Cider (200 mL)	10–500	2–100
	Coffee (200 mL)	350–1750	70–350
Anthocyanins	Aubergine (200 g)	7500	1500
Cyanidin	Blackberry (100 g)	1000–4000	100–400
Pelargonidin	Black currant (100 g)	1300–4000	130–400
Peonidin	Blueberry (100 g)	250–5000	25–500
Delphinidin	Black grape (200 g)	300–7500	60–1500
Malvidin	Cherry (200 g)	350–4500	70–900
	Rhubarb (100 g)	2000	200
	Strawberry (200 g)	150–750	30–150
	Red wine (100 mL)	200–350	20–35
	Plum (200 g)	20–250	4–50
	Red cabbage (200 g)	250	50
Flavonols	Yellow onion (100 g)	350–1200	35–120
Quercetin	Curly kale (200 g)	300–600	60–120
Kaempferol	Leek (200 g)	30–225	6–45
Myricetin	Cherry tomato (200 g)	15–200	3–40
	Broccoli (200 g)	40–100	8–20
	Blueberry (100 g)	30–160	3–16
	Black currant (100 g)	30–70	3–7
	Apricot (200 g)	25–50	5–10
	Apple (200 g)	20–40	4–8
	Beans, green or white (200 g)	10–50	2–10
	Black grape (200 g)	15–40	3–8
	Tomato (200 g)	2–15	0.4–3.0
	Black tea infusion (200 mL)	30–45	6–9

	Green tea infusion (200 mL)	20–35	4–7
	Red wine (100 mL)	2–30	0.2–3
Flavones	Parsley (5 g)	240–1850	1.2–9.2
Apigenin	Celery (200 g)	20–140	4–28
Luteolin	Capsicum pepper (100 g)	5–10	0.5–1
Flavanones	Orange juice (200 mL)	215–685	40–140
Hesperetin	Grapefruit juice (200 mL)	100–650	20–130
Naringenin	Lemon juice (200 mL)	50–300	10–60
Eriodictyol			
Isoflavones	Soy flour (75 g)	800–1800	60–135
Daidzein	Soybeans, boiled (200 g)	200–900	40–180
Genistein	Miso (100 g)	250–900	25–90
Glycitein	Tofu (100 g)	80–700	8–70
	Tempeh (100 g)	430–530	43–53
	Soy milk (200 mL)	30–175	6–35
Monomeric flavanols	Chocolate (50 g)	460–610	23–30
Catechin	Beans (200 g)	350–550	70–110
Epicatechin	Apricot (200 g)	100–250	20–50
	Cherry (200 g)	50–220	10–44
	Grape (200 g)	30–175	6–35
	Peach (200 g)	50–140	10–28
	Blackberry (100 g)	130	13
	Apple (200 g)	20–120	4–24
	Green tea (200 mL)	100–800	20–160
	Black tea (200 mL)	60–500	12–100
	Red wine (100 mL)	80–300	8–30
	Cider (200 mL)	40	8

2.2 Antioxidant Activity

The first detailed kinetic study of antioxidant activity was conducted by Boland and ten-Have (1947) who postulated Reaction 2.1 and Reaction 2.2 for free radical terminators. Phenolic antioxidants (AH) interfere with lipid oxidation by rapid donation of a hydrogen atom to lipid radicals (Reaction 2.1 and Reaction 2.2). The latter reactions compete with chain propagation Reaction 2.5:



These reactions are exothermic in nature. The activation energy increases with increasing A–H and R–H bond dissociation energy. Therefore, the efficiency of the antioxidants (AH) increases with decreasing A–H bond strength. The resulting phenoxyl radical must not initiate a new free radical reaction or be subject to rapid oxidation by a chain reaction. In this regard, phenolic antioxidants are excellent hydrogen or electron donors; in addition, their radical intermediates are relatively stable due to resonance delocalization and lack of suitable sites for attack by molecular oxygen (Sherwin, 1978; Nawar, 1986; Belitz and Grosch, 1987).

Flavonoids usually occur in living cells as glycosides and may break down to their respective aglycone and sugar by enzymes or acid-heat treatments. Pratt and Watts

(1964) and Pratt (1965, 1972) have considered flavonoids as primary antioxidants. Many of the flavonoid and related phenolic acids have shown marked antioxidant characteristics (Table 2.3) (Mehta and Seshadri, 1959).

The antioxidant behavior of phenolic compounds show variations based on solvent type and polarity, reaction mechanism, solubility parameters as well as an essential structural property (i.e. electron-transfer capability) (Özyürek et al., 2011).

Flavonoids and cinnamic acids are known as primary antioxidants and act as free radical acceptors and chain breakers. Flavonols are known to chelate metal ions at the 3-hydroxy-4-keto group and/or 5-hydroxy-4-keto group (when the A-ring is hydroxylated at the fifth position) (Pratt and Hudson, 1990). All flavonoids with 3',4'-dihydroxy configuration possess antioxidant activity (Dziedzic and Hudson, 1983b).

It has been established that the position and degree of hydroxylation are of primary importance in determining antioxidant activity of flavonoids. The o-dihydroxylation of the B-ring contributes to the antioxidant activity. The p-quinol structure of the B-ring has been shown to impart an even greater activity than o-quinol; however, para and meta hydroxylation of the B-ring do not occur naturally (Pratt and Hudson, 1990). All flavonoids with 3',4'-dihydroxy configuration possess antioxidant activity (Dziedzic and Hudson, 1983b).

Robinetin and myricetin have an additional hydroxyl group at their 5' position, thus leading to enhanced antioxidant activities over those of their corresponding flavones that do not possess the 5' hydroxy group, namely, fisetin and quercetin. Two flavanones (naringenin and hesperitin) have only one hydroxyl group on the B-ring and possess little antioxidant activity. Hydroxylation of the B-ring is the major consideration for antioxidant activity (Pratt and Hudson, 1990). Other important features include a carbonyl group at position 4 and a free hydroxy group at position 3 and/or 5 (Dziedzic and Hudson, 1983b).

Uri (1961) has investigated the importance of other sites of hydroxylation. It has been shown that the o-dihydroxyl grouping on one ring and p-dihydroxyl grouping on the other (e.g., 3,5,8,3',4'- and 3,7,8,2',5'-pentahydroxy flavones) produce very potent antioxidants, while 5,7 hydroxylation of the A-ring apparently has little influence on the antioxidant activity of the compounds (Pratt and Hudson, 1990). Thus, quercetin and fisetin have almost the same activity, while myricetin possesses an activity similar to that of robinetin (Table 2.3). The 3-glycosylation of flavonoids with monosaccharides/disaccharides reduces their activity compared with that of the corresponding aglycones (e.g., rutin is less active than quercetin).

In the isoflavone, it is clear that both hydroxyl groups in 4' and 5 positions are needed for significant antioxidant activity as genistein. Even 6,7,4'-trihydroxyisoflavone is marginally active when compared to analogous flavone apigenin, which is inactive as an antioxidant. Genistein is particularly active. The resonance-stabilized quinoid structures show that for isoflavone the carbonyl group at position four remains intact and can interact with the 5-hydroxy group, if present; however, in flavone, the carbonyl group at position four loses its functionality. This may explain the superior antioxidant activity of genistein compared with that of apigenin (Dziedzic and Hudson, 1983a).

Table 2.3 Antioxidant activity of some flavonoid-related compounds.

<i>Compound</i>	<i>Time to Reach Peroxide Value of 50 (h)^a</i>	<i>Induction Period by Rancimat (h)^b</i>
Control		
Stripped corn oil	105	-
Lard	-	1.4
Aglycones		
Quercetin (3,5,7,3'-4'-pentahydroxy)	475	7.1
Fisetin (3,7,3',4'-tetrahydroxy)	450	8.5
Myricetin (3,5,7,3',4',5'-hexahydroxy)	552	-
Robinetin (3,7,3',4',5'-pentahydroxy)	750	-
Eriodictyol (3,7,3',4'-tetrahydroxy)	-	-
Naringenin (5,7,4'-tetrahydroxy)	198	-
Hesperitin (5,7,3'-trihydroxy-4 methoxy)	125	-
Glycosides		
Quercetrin (3-rhamnoside)	475	1.9
Rutin (3-rhamnoglucoside)	195	-
Isoflavones		
Daidzein (7,4'-dihydroxy)	1.4	-
Genistein (5,7,4'-trihydroxy)	2.6	-
Phenolic acids		
Protocatechuic acid (3,4-dihydroxybenzoic acid)	-	4.8
Gallic acid (3,4,5-trihydroxybenzoic acid)	-	28.6
Coumaric acid (p-hydroxycinnamic acid)	120	0.8
Ferulic acid (4-hydroxy-3-methoxy-cinnamic acid)	145	2.0
Caffeic acid (3,4-dihydroxycinnamic acid)	495	23.3
Dihydrocaffeic acid (3,4-dihydroxyphenylpropionic acid)	-	31.4
Chlorogenic acid (caffeoyl quinic ester)	505	-
a (5×10^{-4} M in stripped corn oil).		
b (2.3×10^{-4} M in lard).		

The antioxidant activity of phenolic acids and their esters depends on the number of hydroxyl groups in the molecule; this will be strengthened by steric hindrance (Dziedzic and Hudson, 1983b). Hydroxylated cinnamic acids have been found to be more effective than their benzoic acid counterparts.

2.3 Bioavailability

2.3.1 Definitions and methods

Bioavailability can be defined as absorption and transportation of nutrient to body tissues where they are converted to physical activity (Benito and Miller, 1998). According to FDA report; bioavailability is described as absorption of active component of food or drug to be available for physical activity (FDA, 2003).

Bioavailability can be determined in three different methods as in vitro digestion Gastrointestinal (GIT) model, CaCo-2 in vitro model and in vivo models. In vivo models are giving accurate results but they are time consuming and expensive methods and it is hard to perform these models because of ethical restrictions. However, in vitro models are demonstrating reliable results and leading researcher to investigate about bioavailability. The differences between assessment of bioavailability by in vitro and in vivo studies are based on mechanisms, because in vivo assays are defined as opened systems whereas in vitro methods as closed systems. In vivo studies can be affected by reducing properties of metabolites which are formed after absorption and excretion (Bermudez-Soto, et al. 2004).

In vitro digestion gastrointestinal model has two parts; digestion which is performed by using commercial digestive enzymes such as pepsin, pancreatin and absorption which is commonly based on CaCo-2 cells (Colon Adeno Carcinoma Cell) (Parada and Aguilera, 2007). In vitro digestion can be also performed by followings:

- Preparation of food
- Simulating stomach digestion (with pepsin)
- Mimicking intestinal digestion (using pancreatin and bile salts)

CaCo-2 cells which have small intestine villus cell properties are isolated from human colon cells. This model system is used for intestinal studies to determine organization and function of intestine cells. In the study which is relevant to drug bioavailability it was reported that CaCo-2 cell permeability and extent of absorption shows excellent correlation with in vivo studies (Mandagere et al. 2002).

Current literature showed that the factors affecting the bioavailability of flavonoids are related with the flavonoid class, its chemical structure, and the glycoside group attached to its structure. In addition, dosage, vehicle of administration, diet, sex differences, individual genetic properties, microbial population of the colon, and other compounds present in the food material were also found to affect absorption and bioavailability. Factors affecting antioxidant bioavailability is shown in Table 2.4 (Porrini and Riso, 2008).

Table 2.4 : Factors affecting bioavailability of antioxidants in humans.

➤ Related to the antioxidant Chemical structure Species/form Molecular linkage Concentration in foods Amount introduced Interaction with other compounds	➤ Related to the food preparation Matrix characteristics Technological processing Presence of positive effectors of absorption: fat, protein, lecithin Presence of negative effectors of absorption: fiber, chelating agents Duration of storage Hormonal status Intestinal transit time Microflora
➤ Related to the host Nutritional and antioxidant status Physiological condition Secretion of HCl	➤ External Exposure to different environment Food availability

CaCo-2 cells which have small intestine villus cell properties are isolated from human colon cells. This model system is used for intestinal studies to determine organization and function of intestine cells. In the study which is relevant to drug bioavailability it was reported that CaCo-2 cell permeability and extent of absorption shows excellent correlation with in vivo studies (Mandagere et al. 2002).

2.3.2 Bioavailability of phenolic compounds

Phenolic compounds include two major groups: derivatives of cinnamic acid and benzoic acid depending on their structure. Benzene binds to carboxylic group is referred as benzoic acids and if the propionic acid binds it is called as cinnamic acids (Lafay and Gil-Izquierdo, 2008).

Phenolic acids are available as aglycons or glycosides. The aglycones can be absorbed from the small intestine. However, most polyphenols are present in food in the form of esters, glycosides, or polymers that cannot be absorbed in their native form.

Bouayed et al (2012) assessed bioaccessible and dialysable apple polyphenols available for potential uptake by intestinal epithelial cells, an in vitro gastrointestinal (GI) digestion method was developed and main polyphenols investigated by UPLC. Polyphenolic profiles in the gastric medium were similar to those natively occurring in apples; however, bioaccessible polyphenols were at lower concentrations than those in the apples. The polyphenolic profile was altered during intestinal digestion, with a considerable decrease of total polyphenols. Flavan-3-ols were completely unstable in the intestinal medium, owing to their pH sensitivity. In addition, 41–77% of bioaccessible chlorogenic acid, the major abundant hydroxycinnamic acid in apples, was degraded during intestinal digestion, with partial isomerisation to cryptochlorogenic acid and neochlorogenic acid.

Gumienna et al. (2011) studied the qualitative and quantitative changes of wine polyphenols during in vitro digestion process conducted in a gastrointestinal tract model. Following the stages of in vitro digestion—stomach, small and large intestine—qualitative and quantitative changes particularly in phenolic acids were

monitored. Decomposition of resveratrol and chlorogenic acid, secretion of caffeic acid and formation of other derivatives characterized with high antioxidant activity were determined. As a second focus of this work the evaluation of interactions between human fecal microflora (*Enterobacteriaceae*, *Lactobacillus*, *Enterococcus* and *Bifidobacterium*) and polyphenolic compounds and their derivatives secreted during the digestion were performed.

In another study, the *in vitro* gastrointestinal stability of (poly)phenolic compounds in Concord grape juice was compared with recoveries in ileal fluid after the ingestion of the juice by ileostomists. Recoveries in ileal fluid indicated that 67% of hydroxycinnamate tartarate esters, and smaller percentages of the intake of other (poly)phenolic compounds, pass from the small intestine to the colon. Peak plasma concentrations (C_{max}) ranged from 1.0 nmol/L for petunidin-3-O-glucoside to 355 nmol/L for dihydrocoumaric acid. Urinary excretion, as an indicator of bioavailability, varied from 0.26% for total anthocyanins to 24% for metabolites of hydroxycinnamate tartarate esters. The C_{max} times of the anthocyanins indicated that their low level absorption occurred in the small intestine in contrast to hydroxycinnamate metabolites which were absorbed in both the small and the large intestine where the colonic microflora appeared responsible for hydrogenation of the hydroxycinnamate side chain (Stalmach et al., 2012).

Perez-Vicente et al. (2002) determined the *in vitro* availability of anthocyanins, vitamin C, and total phenols from the pomegranate by *in vitro* digestion. Results had shown that pomegranate phenolic compounds were available during the digestion in a quite high amount (29%). Nevertheless, due to pH, anthocyanins were largely transformed into non-red forms and/or degraded (97%), and similar results were obtained for vitamin C (>95% degradation).

Chiang et al. (2013) investigated the potential effects of the digestion process on antioxidant properties and individual phenolic compounds of two European gooseberries: Tixia and Invicta. Gooseberries were digested via an *in vitro* digestive model with active or heat-inactivated enzymes. Results revealed that digestion enhances the availability of antioxidants in both gooseberries, where the digested fruits showed higher total phenolic content and antioxidant capacity. Eight phenolic antioxidants were identified and quantified in Tixia and Invicta gooseberries. Among

the identified phenolic compounds, only quercetin hydrate was significantly improved by digestion.

In another study, stability and composition of phenolic compounds are determined in chokeberry juice by *in vitro* gastric and pancreatic digestion in the absence of light and under N₂. Recovery for total soluble phenolics were analyzed by HPLC-DAD/HPLC-MS-MS. There was no effect on flavan-3-ols, flavonols and caffeic acid derivatives after gastric digestion. There was considerable decrease for anthocyanin, flavonols, flavan-3-ols and neochlorogenic acid, 43%, 26%, 19% and 28%, respectively, whereas chlorogenic acid increased 24%. Losses of anthocyanins and phenolic compounds are based on alkaline conditions in intestine and it is not related with digestive enzymes (Bermúdez-Soto et al. 2007).

In another study, an *in vitro* model simulating gastrointestinal (GI) digestion, including dialysability, was adapted to assess free soluble polyphenols from apples (four varieties). Results indicated that polyphenol release was mainly achieved during the gastric phase (ca. 65% of phenolics and flavonoids), with a slight further release (<10%) during intestinal digestion. Anthocyanins present after the gastric phase (1.04–1.14 mg/100 g) were not detectable following intestinal digestion. Dialysis experiments employing a semipermeable cellulose membrane showed that free soluble dialysable polyphenols and flavonoids were 55% and 44% of native concentrations, respectively, being approximately 20% and 30% lower than that of the GI digesta. Similar results were found for the antioxidant capacity of dialysable antioxidants, being 57% and 46% lower compared to total antioxidants in fresh apples (FRAP and ABTS test, respectively) (Bouayed et al., 2011).

3. MATERIALS AND METHODS

3.1 Materials

Isoflavones pure standards (daidzein, daidzin, genistin, glycitin) from Extrasynthese BP62-69726 GENAY cedex and genistein from Labor Dr. Ehrenstorfer-Schafers, were taken. Phenolic acids standards (gallic acid, p-hydroxybenzoic acid, cafeic acid, p-coumaric acid, syringic acid, sinapic acid, protocatechuic acid) and the other flavanoids (+)- catechin hydrate, (-)-epicatechin, epigallocatechin gallate, (-)-epigallocatechin, quercetin, myricetin, ($\pm$)- naringenin were obtained from Sigma-Aldrich Chemie GmbH company. The other standard epicatechingallate were from HWI Analytik GmbH. Ferulic acid, vanilic acid and anthocyanins such as delphinidin, cyanidin, pelargonidin, malvidin, cyanidin 3-O-glucoside=kuromanin, cyanidin 3-O-galactoside=idein, cyanidin 3-O-rutinoside=keracyanin, pelargonidin 3-O-glucoside=callistephin, malvidin 3-galactoside, malvidin 3-O-glucoside=oenin were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Totally, 32 standards were obtained and they were stored at -20 °C or 2-8 °C. Magnetic stirrer-IKA RH basic 2 and Vorteks minishakers-IKA were used for the mixing purposes.

3.2 Chemicals

Methanol ($\geq 99.9\%$), formic acid ($\geq 98\%$), sodium carbonate (Na_2CO_3), hydrochloric acid (37%), ammonium acetate (NH_4Ac), Copper (II) chloride (CuCl_2), potassium persulfate ($\text{K}_2\text{S}_2\text{O}_8$), dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4) and trifluoroacetic acid (99%) were obtained from Merck KGaA (Darmstadt, Germany).

Gallic acid ($\geq 98\%$), Folin-Ciocalteu phenol reagent, neocuproine (Nc), ethanol ($\geq 99.8\%$), DPPH, pepsin enzyme, pancreatin enzyme, bile salts, acetonitrile (99.8%) sodium bicarbonate (NaHCO_3) and sodium chloride were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Trolox (6-hydroxy-2,5,7,8-

tetramethylchroman-2-carboxylic acid were obtained from fluka Chemie (Buchs, Switzerland). ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid diammonium salt) were obtained from Applichem GmbH (Darmstadt, Germany).

3.3 Methods

Total phenolic content and antioxidant activity analysis were performed using spectrophotometric methods for each standard. Phenolic profiles for each standard were determined with HPLC/PDA. The bioavailability of all standard polyphenols was determined after *in vitro* gastrointestinal digestion. After this, total phenolic content by Folin-Ciocalteu method and antioxidant activity analysis (DPPH, ABTS and CUPRAC) were carried out in PG, OUT and IN fractions. In addition to those, HPLC phenolic profile was examined in order to identify phenolic transformations and changes in the amount of phenolic compounds.

3.3.1 Preparation of standards

The anthocyanins and other phenolic compounds which are commonly found in foods, were determined from the literature. 10 mg of standard, except for the anthocyanins and epigallocatechin (1 mg) and glycitin and pelargonidin (5 mg), was weighed to test tubes and 5 ml of 75% methanol: water solution with 0.1% formic acid was added to each tube. But for anthocyanins and epigallocatechin of 0.5 ml and glycitin and pelargonidin 2.5 ml solvent was put into the tubes. Then samples were mixed till they completely dissolved by vortex mixer. Prepared samples were stored at -20°C until performing the analysis.

3.3.2 Spectrophotometric analyses

3.3.2.1 Total phenolic content

The Folin-Ciocalteu method is commonly used to quantify total phenolic content. It does not only determine phenolic compounds but also measures several non-phenolic compounds such as some nitrogen compounds, vitamin C and Cu(I) which can react with this reagent.

Folin-Ciocalteu method was performed according to Velioglu et al. (1998). 750 µl of Folin-Ciocalteu agent (1:10 diluted with distilled water) was added to 100 µl of standard solution and was waited for 5 minutes. Then 750 µl sodium carbonate (Na_2CO_3) (60 g/L) was put into the mixture and mixed by vortex minishakers (IKA, Germany). After incubating 90 minutes at room temperature by protecting from light, absorbance values of samples were measured at 725 nm by UV-visible spectrophotometer (Shimadzu UV-1700 UV-Vis). The calibration curve was prepared with gallic acid standard solution. The results were expressed in gallic acid equivalents (GAE) per 1 g standard and reported as mean value $\pm$ SD.

3.3.2.2 Antioxidant activity methods

2,2 diphenyl -1-picrylhydrazyl (DPPH) radical scavenging method

The DPPH method determines the hydrogen bonding capacity- of -extract which react with DPPH radical and bleach the DPPH solution (Martos et al., 2010).

Antioxidants react with the stable DPPH radical and the dark purple color of DPPH radical changes into yellow color. The higher antioxidant capacity means the higher bleaching reaction. DPPH is rapid and simple method which was adapted from Martos et al. (2011). 0.1 mM of DPPH solution was prepared by using 100% methanol. 100 µl of extract was mixed with 2 ml of DPPH solution. The absorbance was- measured at 517 nm after 30 minutes in the dark. Standard curve was prepared with Trolox in 75% methanol-water containing 0.1% formic acid and the results were expressed as Trolox equivalents (TEAC) per 1 g standard and reported as mean value $\pm$ SD.

Cupric reducing antioxidant capacity (CUPRAC) analysis method

The CUPRAC method was developed by Apak et al. (2005) planned to measure total antioxidant including dietary polyphenols, vitamin C and E by using copper (II)-neocuproine [Cu(II)-Nc] reagent. This method is based on measurement of copper (II) ion reducing ability of polyphenols. CUPRAC method comprises a mixture of the antioxidant solution, copper (II) chloride solution, neocuproine alcoholic solution, and an ammonium acetate aqueous buffer solution at pH 7 (Apak et al., 2004).

The principle of -CUPRAC method is based on reducing of Cu(II) to Cu(I). Cu (I) reacts with neocuproine (2,9- dimetil-1,10- fenantrolin) and gives absorbance at 450 nm (Prior et al., 2005).

For CUPRAC assays, 1 ml each of CuCl₂ solution (1.0×10^{-2} M), neocuproine alcoholic solution (7.5×10^{-3} M), NH₄Ac buffer solution at pH 7.0 and finally 1 ml water added to 100 µl sample (Apak et al., 2006). After 30 min., the absorbance at 450 nm was measured against a reagent blank. Standard curve was prepared with Trolox in 75% methanol-water containing 1% formic acid and the results were expressed as Trolox equivalents (TEAC) per 1 g of DW and reported as mean value $\pm$ SD.

2,2-azinobis (3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) analysis method

ABTS method has the similar principle with that of DPPH. ABTS reacts with hydroxyl, peroxy, alkoxy and inorganic radicals and forms stable ABTS radical cation. Antioxidant or reactive oxygen species catch free radicals and prevents ABTS cation radical formation. The loss of colour is measured spectrophotometrically at 734 nm (Vattem and Shetty, 2007).

Analysis of antioxidant activity was performed as described by Miller and Rice-Evans (1997). ABTS reagent and potassium persulfate solutions (K₂S₂O₈) were prepared by dissolving 200 mg of ABTS in 200 ml water and 38 mg of K₂S₂O₅ in 2 ml. These solutions were mixed and left for one night to form the radical. 0.05 M buffer solution at pH 8.0 was prepared by mixing 0.05 M potassium dihydrogen phosphate (KH₂PO₄) and 0.05 M dipotassium hydrogen phosphate (K₂HPO₄). ABTS reagent mixture was prepared by mixing ABTS and KP_i solution and measuring absorbance at 0.9 ± 0.2 . 100 µl of extract was taken and 1 ml of prepared ABTS reagent mixture was added and mixed for 15 seconds. After waiting 45 seconds, absorbance was read at 734 nm against water blank. Standard curve was prepared with Trolox in 75% met-water involving 0.1% formic acid and the results were expressed as Trolox equivalents (TEAC) per 1 g standard and reported as mean value $\pm$ SD.

3.3.3 *In vitro* gastrointestinal digestion method for bioavailability

Information was necessary about absorption, metabolism, distribution in tissue, organs and excretion because of evaluating potential bioavailability of any phytochemical. Studies on humans and animals are not preferred owing to the restrictions such as complexity, expensiveness and ethical reasons (Guven et al., 2010). Besides, *in vitro* digestion method is rapid and easy for evaluating phytochemical stability in proceed food (McDougall et al., 2005a). In 1981, Miller performed the first *in vitro* digestion method. They compared the iron content of IN samples with serum iron bioavailability *in vivo* study. Then this method was adapted and used to determine the potential bioavailability of anthocyanins and other phenolic compounds in fruits in some studies such as orange juice by Gil-Izquierdo et al. (2001), pomegranate juice by Perez-Vicente et al. (2002), raspberry and red wine by McDougall (2005a, 2005b), frozen cherries by Fazzari et al. (2008).

Bioavailability procedure was adapted from McDougall et al., (2005a) with some modifications. This method comprises of two steps; gastric fraction and small intestine fraction. To mimic gastric and small intestine conditions, pepsin-HCl was applied and waited for 2 h at 37 oC in water bath and bile salts-pancreatin for 2 h at 37 oC, respectively. 1 mg pure compound was weighed in a glass beaker. All the phenolic compounds were dissolved in 2.5 ml water except quercetin which was dissolved in DMSO. Then stomach solution-, containing 2 g/l NaCl and 315 units/ml pepsin, was prepared to simulate gastric fluid. 20 ml of this solution was added to beaker. The pH of sample solution was adjusted to 2.0 with concentrated 5 N HCl and the solution was incubated at 37 oC in a shaking bath for 2 h. After gastric digestion, 2 ml sample was taken from PG to eppendorf tubes and stored at -20 oC, after centrifugation at 18000 rpm, 4 oC. The remainder was placed in a beaker, 4.5 ml of 4 mg/ml pancreatin and 25 mg/ml bile salts mixture was added. A piece of cellulose dialysis tube which was washed with distilled water due to opening was prepared (molecular mass cutoff, 12 kDa) and the bottom of the tubing was linked tightly. After 20 ml NaHCO₃ added to dialysis tube for neutralizing the sample's titratable acidity and the top of the tubing was linked tightly again. Dialysis tube was added, and the beaker was sealed with parafilm and placed again in the heated water bath at 37 oC for 2 h. After digestion, the solution outside the tubing, which was the

sample did not enter the serum, was taken in the eppendorf tubes as OUT sample. The solution in the dialysis tubing was taken as the IN sample which was represented the material that entered the serum. After IN and OUT samples were centrifuged at 18000 rpm at 4 °C, the centrifugates were stored at -20 °C for analysis. In vitro digestion procedure was applied to all phenolic compounds for each sample in duplicate. Total phenolic analysis by Folin-Ciocalteu method and the total antioxidant activity analysis by DPPH, CUPRAC, ABTS methods were carried out in PG, IN and OUT fractions for each sample in triplicate.

3.3.4 HPLC analysis of major phenolic compounds and anthocyanins

HPLC analysis were carried out by using the method adapted from Capanoglu et al. (2008b). Standard calibration curves were prepared by using daidzein, daidzin, genistein, genistin, glycitin, gallic, pHBA(-P-hydroxy benzoic acid), caffeic acid, vanilic acid, coumaric acid, ferulic acid, syringic acid, sinapic acid, protocatechuic acid, quercetin, myricetin, (±)- naringenin, (+)- catechin hydrate, (-) epicatechin, epicatechin gallate, (-)- epigallocatechin gallate, (-) epigallocatechin, delphinidin chloride, cyanidin chloride, cyanidin 3-O-glucoside, cyanidin 3-O-galactoside, cyanidin 3-O-rutinoside, pelargonidin chloride, pelargonidin 3-O-glucoside, malvidin chloride, malvidin 3-galactoside, malvidin 3-O-glucoside. 100 µl trifluoroacetic acid (TFA) was added on bioavailability samples. These samples and stock solutions were filtered through a 0.45-µm membrane filter and 1 ml of the filtered sample was placed into vials and analyzed in a Waters W600 HPLC system with PDA (Waters 996) detector, for each sample. Luna C18 column (Phenomenex) was used as the stationary phase.

The mobile phase was including solvent A, Milli-Q water with 0.1% (v/v) TFA and solvent B, acetonitrile with 0.1% (v/v) TFA, acetonitrile with 0.1% (v/v) TFA. A Linear gradient was used as follows: at 0 min, 95% solvent A and 5% solvent B; at 45 min, 65% solvent A and 35% solvent B; at 47 min, 25% solvent A and 75% solvent B; and at 54 min returns to initial conditions. The flow rate was 1 ml/min. Detections were done at 280, 312, 360, and 512 nm wavelengths. Identification was based on the retention times and characteristic UV spectra and quantification was done by external standard curves. All analyses were performed in except for epigallocatechin and anthocyanins.

3.3.5 Statistical Analyses

The results were analyzed statistically by using one way analysis of variance (ANOVA), and the differences between the samples were evaluated by Duncan's New Multiple Range Test at 0.05 significant level using Statistical Package for the Social Sciences (SPSS) 21.0 version. The differences between all samples and fractions initial, PG, IN, OUT were evaluated statistically. Duncan's New Multiple Range Test was applied to mean values to observe the difference between contents, antioxidant activity or amount of phenolics and anthocyanins ($p < 0.05$). All the comparisons and statistical evaluation by SPSS are shown in APPENDIX A. To observe the relations between methods, basic regression analysis was performed and significant relations were determined statistically ($p < 0.05$).

4. RESULTS and DISCUSSION

In this study; firstly the initial total phenolic content and antioxidant activity of 32 different phenolic compounds was determined. Afterwards, *in vitro* digestion method was applied on the same phenolic compounds belonging to anthocyanins, isoflavones, phenolic acids and flavanoid groups and reanalyzed for their total phenolic and antioxidant activity. Also phenolic profiles and quantity was determined by HPLC/PDA to calculate recovery values after digestion to compare their bioavailability. Lastly regressions between the methods were calculated and evaluated statistically.

4.1 Phenolic Contents and Antioxidant Activity for Phenolic Compounds

4.1.1 Total phenolic content

Results for total phenolic content analysis were expressed as mg gallic acid equivalents (GAE)/ g standard. The standard calibration curve of gallic acid is shown in Figure 4.1 which was prepared between 0.005-0.1 mg/ml and the equation obtained from the curve was used to calculate the equivalent gallic acid concentrations of the samples measured by UV-VIS spectrophotometer. The results are shown in Table 4.1.

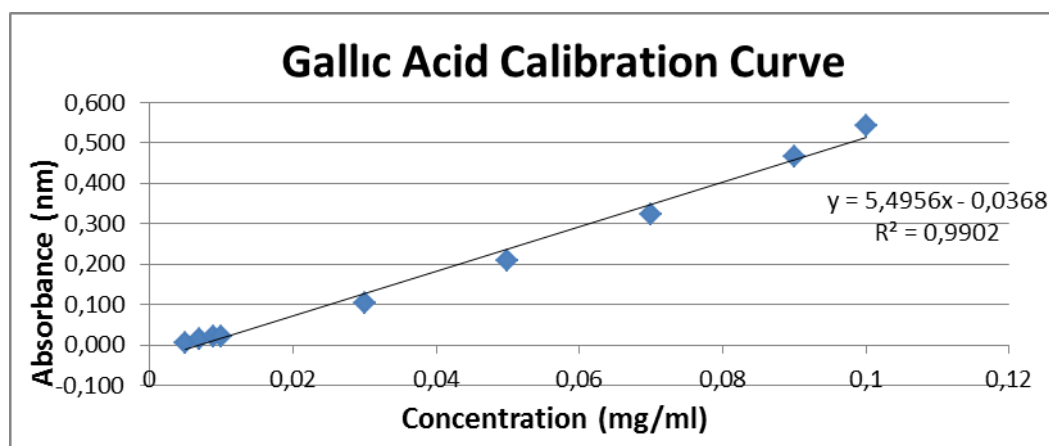


Figure 4.1: Calibration curve of gallic acid standard.

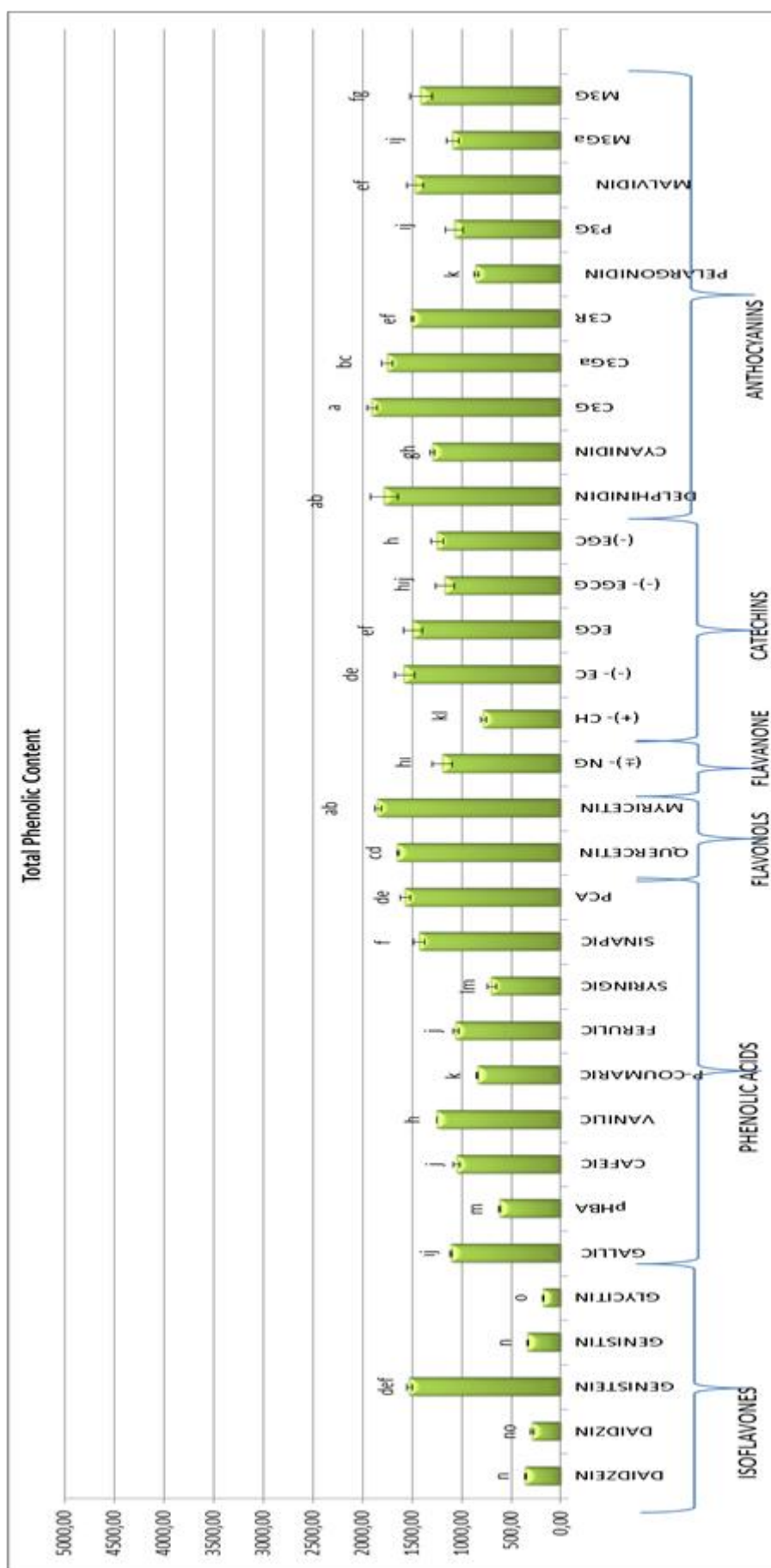


Figure 4.2: Total phenolic content of each phenolic compound.

Statistical evaluation of all samples were given in Figure 4.2. Cyanidin 3-O-glucoside showed the highest total phenolic content value (1901.2 ± 51.5 mg GAE/g standard) whereas glycitin the lowest (178.2 ± 2.4 mg GAE/g standard).

The results approximately show that generally the order of phenolic content is flavonols > anthocyanins > catechins > naringenin > phenolic acids > isoflavones according to groups. Similar to the results of this study, Singleton et al. (1999) reported that flavonols such as quercetin, but not their 3-glycosides, gave more color than predicted, probably from participation of the enolic C ring. This idea is reinforced by the behavior of flavone and flavanones. Flavonoids such as catechin closely approximated the sum of the color expected from the phloroglucinol A ring plus the reaction possibilities of their B ring .

Within the isoflavones, genistein showed the highest phenolic content, whereas glycitin represented the lowest. Moreover, significant difference was not determined statistically between the Genistin, Daidzein and Daidzin ($p < 0.05$).

In respect to phenolic acids, the highest phenolic content was observed for protocatechuic acid (PCA), on the other hand the lowest TPC (Total Phenolic Content) was obtained for p-hydroxybenzoic acid (pBHA). Similarly, syringic acid had also lower content and found to have no significant difference with pBHA statistically ($p < 0.05$). In terms of TPC, gallic, ferulic and caffeic acids showed same level of responses.

When anthocyanins are evaluated, the aglycone forms of cyanidin and pelargonidin was found to have lower TPC values than their respective glucoside forms, whereas malvidin is significantly higher than M3Ga but statistically not different than M3G which are glucoside forms of malvidin. When evaluated statistically, pelargonidin is found to be lowest from all other anthocyanins and it is followed by P3G and M3Ga ($p < 0.05$).

Table 4.1 : Total phenolic content and total antioxidant analysis of phenolic compounds.

	STANDARD	FOLIN	ABTS	CUPRAC	DPPH
ISOFLAVONES	DAIDZEIN	356.9±7.8	1110.3±69.1	326.9±1.1	47.8±0.5
	DAIDZIN	289.3±14.3	985.1±2.0	217.4±4.8	45.4±5.0
	GENISTEIN	1521.5±27.5	2829.0±34.3	573.9±13.9	11.0±1.2
	GENISTIN	333.9±8.9	838.5±27.7	255.1±65.7	43.2±0.8
	GLYCITIN	178.2±2.4	911.3±25.7	127.2±41.3	43.0±2.2
PHENOLIC ACIDS	GALLIC ACID	1104.1±11.3	5646.7±40.4	4397.8±53.6	4022.4±186.1
	pHBA	617.1±5.8	275.4±6.2	37.6±2.6	10.9±0.9
	CAFEIC ACID	1047.4±32.2	1308.3±76.7	3418.3±345.6	1455.2±107.6
	VANILIC ACID	1249.7±1.7	1625.7±128.1	1698.2±59.0	18.8±0.9
	P-COUMARIC ACID	839.7±5.4	2035.2±132.1	1011.4±84.4	11.5±0.8
	FERULIC ACID	1052.9±28.7	2013.8±116.0	1712.0±13.4	932.4±13.0
	SYRINGIC ACID	699.6±44.6	785.2±12.9	1475.5±38.9	572.1±20.4
	SINAPIC ACID	1429.0±55.8	1545.8±55.5	1629.8±122.2	2263.1±28.5
	PCA	1570.6±50.6	1304.7±19.2	3165.2±12.9	2367.8±173.8
FLAVO NOLS	QUERCETIN	1643.3±10.7	2645.7±77.7	3392.5±294.7	1517.9±148.0
	MYRICETIN	1842.7±32.2	2970.3±155.4	3345.2±294.7	2165.2±34.0
FLAVAN ONE	(±)- NG	1192.5±100.8	1802.6±75.7	323.07±174.16	10.2±0.2
CATECHINS	(+)- CH	777.8±29.2	2445.4±149.3	3306.51±85.74	909.1±83.1
	(-)- EC	1576.1±102.9	2078.8±296.6	2369.50±150.05	991.3±59.2
	ECG	1491.2±96.5	2735.0±353.1	2876.50±16.08	2716.4±172.5
	(-)- EGCG	1166.7±90.1	2586.7±123.1	2472.57±94.32	3472.2±236.3
	(-)- EGC	1242.5±61.1	2906.8±35.3	2182.80±22.77	2163.2±12.2
ANTHOCYANINS	DELPHINIDIN	1784.5±139.4	2456.0±77.7	2836.64±96.46	1794.5±31.2
	CYANIDIN	1294.7±21.4	1062.2±25.2	1309.57±198.28	838.9±16.3
	C3G	1901.2±51.5	1894.6±40.4	2794.96±37.51	995.5±23.1
	C3Ga	1751.1±53.6	1694.9±6.1	2434.98±42.87	1397.9±51.6
	C3R	1497.9±8.6	1525.9±1.0	2086.36±32.15	1193.3±9.5
	PELARGONIDIN	852.1±19.7	176.6±57.0	1061.45±32.15	365.2±4.9
	P3G	1071.8±87.9	970.9±53.5	1014.01±80.38	981.1±35.3
	MALVIDIN	1469.1±87.9	1371.1±46.4	1449.77±64.31	822.6±28.5
	M3Ga	1091.5±64.3	876.8±9.1	1055.69±32.15	970.5±4.1
	M3G	1408.4±109.4	1016.6±39.3	1241.36±5.36	1036.8±8.2

(pHBA:P-Hydroxybenzoic acid, PCA:Protocatechuic acid, (±)- NG:Naringenin, (+)- CH:Catechin hydrate, (-)- EC:Epicatechin, ECG:Epicatechin gallate, (-)-EGCG:Epigallocatechin gallate, (-)-EGC:Epigallocatechin, C3G:Cyanidin-3-O-glucoside, C3Ga:Cyanidin-3-O-galactoside, C3R:Cyanidin-3-O-rutinoside, P3G:Pelargonidin 3-O-glucoside, M3Ga:Malvidin-3-O-galactoside, M3G:Malvidin-3-O-glucoside).

4.1.2 Antioxidant Activity

4.1.2.1 2,2 diphenyl -1-picrylhydrazyl (DPPH) radical scavenging method

The antioxidant activity (AA) was determined by DPPH method for initial pure phenolic standards. Standard calibration curve was prepared for DPPH by using Trolox and it is given in Figure 4.3. The results were expressed as mg TEAC/g standard. The standard calibration curve was prepared between 0.01-0.25 mg/ml concentrations and the equation obtained from the curve was used to calculate Trolox equivalent values of sample measured by UV-VIS spectrophotometer. The results are shown in Table 4.1.

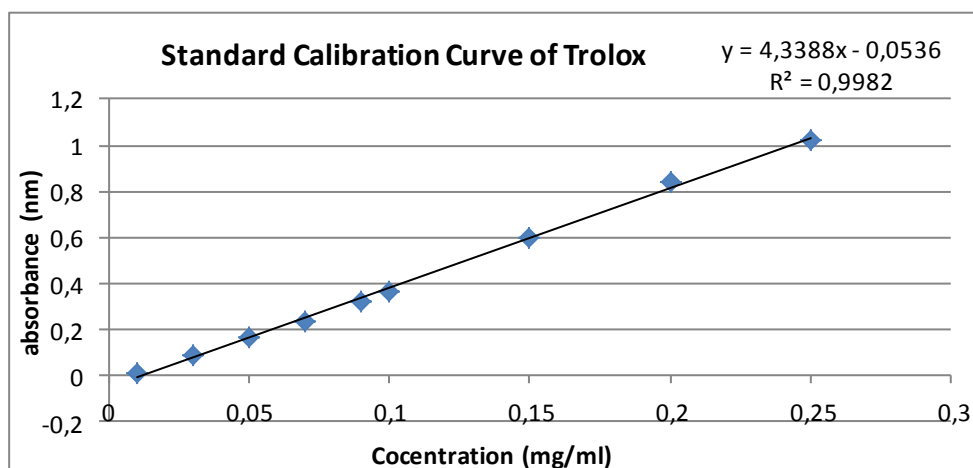


Figure 4.3 : Calibration curve of Trolox standard for DPPH method.

Total antioxidant activity results by DPPH for all standards are shown in Figure 4.4. Gallic acid showed the highest and ($\pm$)- naringenin had the lowest as 4022.4 ± 186.1 and 10.2 ± 0.2 mg TEAC/g standard respectively, as presented in Table 4.1.

According to this results, within the phenolic groups the general order of antioxidant capacity by DPPH method is catechins > flavanols > phenolic acids > anthocyanins > isoflavones > naringenin.

When examining the results, it was shown that responses of isoflavones to DPPH method is quite low than the other phenolic standards. However, the difference between isoflavones is not statistically significant ($p < 0.05$).

P-hydroxy benzoic acid showed the lowest antioxidant capacity in the group of phenolic acids. Also, vanilic acid and p-voumaric acid are approximately at the same level with pHBA. These values were significantly higher than the other acids. After gallic acid, sinapic acid and PCA had higher levels of responses in DPPH method when compared to other phenolic acids.

When responses of catechins in DPPH assay is evaluated, it was obtained that antioxidant activity of EGCG had higher response than other catechins while (+)-CH and (-)-EC had lower antioxidant capacity.

Within the anthocyanins, the order of antioxidant activity is delphinidin > C3Ga > C3R > M3G > C3G > P3G > M3Ga > Cyanidin > Malvidin > Pelargonidin. Accordingly, glucoside forms of anthocyanins are usually higher than the corresponding aglycone forms. However, there is no significant difference between C3G, P3G, M3Ga, cyanidin and malvidin chloride statistically ($p < 0.05$). Delphinidin had the significantly highest response whereas pelaorginidin the lowest response with respect to other anthocyanins ($p < 0.05$).

Nessa et al. investigated (2004) the free radical scavenging activity of pure flavonoids isolated from the leaves using DPPH radical and found out that flavonols showed higher antioxidant activities than the corresponding flavones (quercetin>luteolin) and flavanone derivatives showed lower activity than flavones and flavonols in their assay procedures. Flavanoids with free hydroxyl groups acted as free radical-scavengers, and multiple hydroxyl groups, especially on the B ring, enhance their antioxidant activity (Jovanovic, Steenken, Tosic, Marjanovie, & Simic, 1994). In that study, quercetin showed high antioxidant activity. It seems that the presence of 3',4'-hydroxyls group was essential for high antioxidant activity and the presence of a methoxyl group at the C-4' position greatly reduced its activity as observed in that experiment. Similarly, in our study quercetin had considerable value among the other phenolics in respect to antioxidant activity.

Compounds having higher numbers of phenolic hydroxyl groups exhibited stronger free radical-scavenging activities, which is in keeping with the notion that phenolic

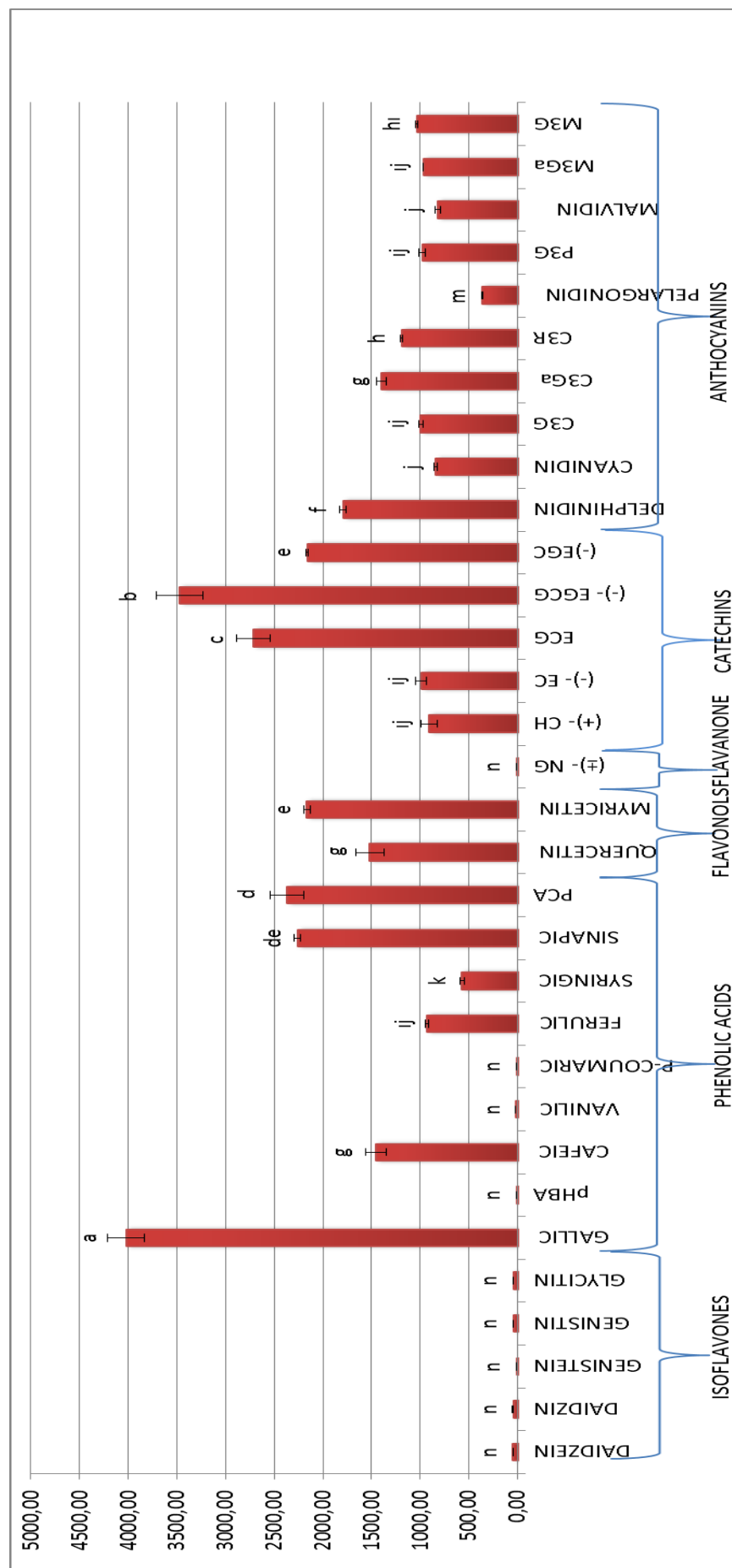


Figure 4.4 : Total antioxidant activity by DPPH method for each phenolic compound.

hydroxyl groups are able to donate hydrogens and that phenoxyl radicals, once formed, are stabilized by delocalization of electrons (Kovatcheva et al., 2001).

The study of Lee et al. (2005) compared the antioxidant potency of genistein and daidzein with their corresponding glycosides isolated from soybean seeds. The free radical-scavenging activity of all soybean isoflavones and their glycosides was also compared at a concentration of 12 $\mu\text{mol/l}$ (1324 mg/g). Genistin had greater activity than other isoflavones and glycosides. In general, likewise our study, all tested soybean isoflavones and their glycosides were less effective in scavenging DPPH free radical. The results are in agreement with those of Mitchell et al. (1998), who demonstrated that genistein and daidzein did not strongly scavenge DPPH or galvinoxyl free radicals. Similarly, in our study both genistein and daidzein had very low antioxidant activity by DPPH radical. The study of Lee et al. (2005) suggests that anti-free radical activity associated with consumption of soybean isoflavones and glycosides is not comparable to that achieved by drinking green tea or intake of α -tocopherol on the same molar basis.

Gil et al, (2000) calculated the contribution of the different groups of phenolics to the total antioxidant activity of pomegranate juice, the antioxidant capacity values of 1 mM solutions of gallic acid, cyanidin 3-glucoside, ellagic acid, and punicalagin were calculated and quoted as TEAC. Cyanidin 3-glucoside showed less antioxidant activity in the same range (having four free phenolic hydroxyls). Gallic acid showed a relatively high antioxidant activity (2.5 TEAC) although it had only three free phenolic hydroxyls per molecule. When contribution of different phenolics groups to the juice antioxidant activity was calculated, anthocyanins accounted for only 1.4 mM of Trolox. The results in are in agreement with our study since cyanidin 3-glucoside had low antioxidant activity whereas gallic acid had very high value as 995.5 ± 23.1 and 4022.4 ± 186.1 mg TEAC/g standard, respectively. Besides, anthocyanins had lower antioxidant activity than some other phenolic acids, catechins and flavonols. Overall, DPPH method is less sensitive than the other methods especially for hydrophilic antioxidants.

In another study, the DPPH radical scavenging activity order of the tested antioxidants was found to have order of: rosmarinic acid>>caffeic acid phenethyl

ester > caffeic acid > chlorogenic acid > α -tocopherol ferulic acid > ferulic acid
phenethyl ester > BHT (Chen and Ho,1997).

4.1.2.2 Cupric reducing antioxidant capacity (CUPRAC) method

Trolox standard calibration curve was prepared for CUPRAC method as shown in Figure 4.5 and results were expressed in mg Trolox equivalents (TEAC)/g standard for each sample. The standard calibration curve was prepared between 0.004-0.4 mg/ml and the equation was used to calculate the Trolox equivalent value measured by spectrophotometer. The results are shown in Table 4.1.

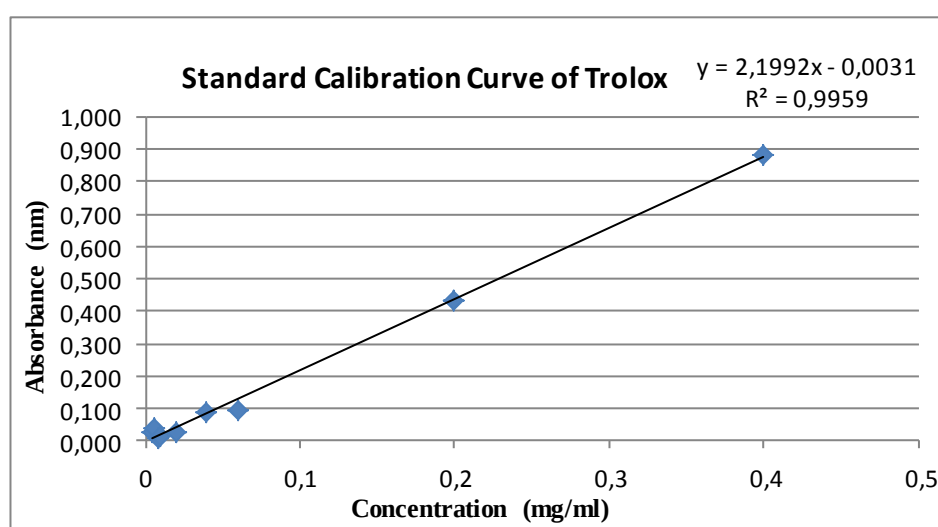


Figure 4.5 : Calibration curve of Trolox standard for CUPRAC method.

Antioxidant activity was evaluated for all phenolic compounds as shown in Figure 4.6. Gallic acid showed highest value as 4397.8 ± 53.6 and pHBA showed the lowest as 37.6 ± 2.6 mg TEAC/g standard.

According to these results, within the phenolic groups the general order of antioxidant capacity by CUPRAC assay is flavanols > catechins > phenolic acids > anthocyanins > isoflavones > naringenin.

Isoflavones had lower antioxidant capacity in comparison with the other phenolic standards similar to the DPPH method. In respect to antioxidant activity, genistein had the highest level between other isoflavones ($p < 0.05$), no significant difference was found between the rest of the isoflavones ($p < 0.05$).

For phenolic acids; after gallic acid, caffeic acid and PCA possessed high antioxidant activity with respect to other phenolic acids. Vanilic, ferulic, syringic and sinapic acids showed statistically similar values when they were compared with each other ($p < 0.05$). According to Apak et al. (2007), as for hydroxycinnamic acids which are almost the most abundant phenolic components in the citrus family and in some other fruits, the TEAC coefficients with respect to the CUPRAC method are caffeic acid: 2.9, ferulic acid: 1.2, and p-coumaric acid: 0.6. Similarly, in our study results were in order of caffeic acid > ferulic acid > p-coumaric acid as $3418,3 \pm 345,6$; $1712,0 \pm 13,4$; $1011,4 \pm 84,4$ mg TEAC/g standard, respectively. On the other hand, in the study carried out by Apak et al. (2007), gallic acid had one more –OH group than chlorogenic acid, and therefore showed higher capacity. The results of study is in agreement with this research.

Similarly, Mansouri et al. (2005) found that benzoate derivatives (e.g. gallic acid) are stronger scavengers than their hydroxycinnamic acid analogues (e.g. caffeic acid). Because structural requirements of these compounds would normally dictate that the two–OH bearing caffeic and chlorogenic acids should exhibit higher antioxidant activity than the one–OH bearing p-coumaric acid (Apak et al., 2007). The antioxidant potency of flavonoids of similar conjugation level is roughly proportional to the total number of –OH groups and is positively affected by the presence of an o-dihydroxy moiety in the B-ring (Robards et al., 1999).

In respect to flavanols when TAC results are examined, generally quercetin had showed highest antioxidant capacity in all TAC analysis. Similarly, Apak et al. (2007) reported that quercetin represented highest antioxidant capacity in all TAC tests. Because quercetin meets three criteria. Firstly, it has 3',4'-dihydroxy catechol structure in B-ring, imparting a greater stability to the formed aryloxy radicals as a result of flavonoid oxidation, possibly through H-bonding and electron delocalization (Van Acker et al., 1996). Secondly, 2,3-double bond promoting conjugation between rings, enhancing electron-transfer and radical scavenging actions through electron-delocalization (Pietta, 2000). And lastly presence of both 3- and 5-OH groups in the A ring, enabling formation of stable quinonic structures upon flavonoid oxidation (Firuza et al., 2005).

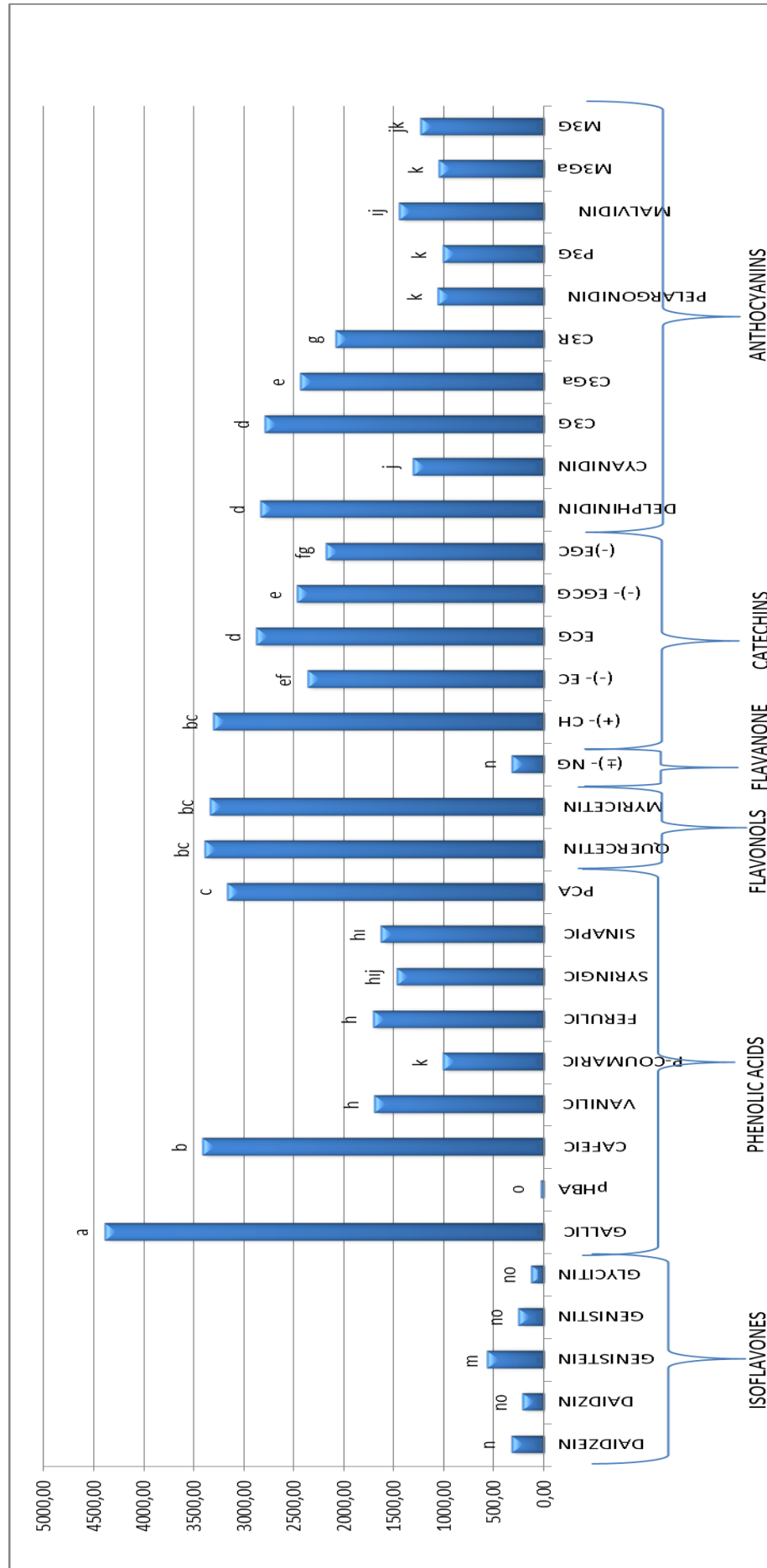


Figure 4.6 : Total antioxidantactivity by CUPRAC for phenolic compounds.

When epicatechin and epigallocatechin were compared with gallate forms, it was observed that gallate had higher total antioxidant capacity than EC and EGC. On the other hand, CH had highest antioxidant activity value in comparison with other catechins and was significantly different from others ($p < 0.05$). Although the order of antioxidant capacity is EGCG > EC and EC > EGC, difference is insignificant ($p < 0.05$). The catechin group, also known as “tea antioxidants”, gave a capacity order in accord with the number and position of their –OH groups, together with the overall extent of conjugation in the molecule (Apak et al, 2007).

For anthocyanins, it was obtained that the general order of antioxidant activity was delphinidin > C3G > C3Ga > C3R > Malvidin > Cyanidin > M3G > Pelargonidin > MGa > P3G. Accordingly, cyanidin glucosides had higher antioxidant activity than cyanidin aglycone, whereas malvidin glucoside forms had lower antioxidant activity than its aglycone form. But there is no significant difference between the malvidin aglycone and M3G ($p < 0.05$). Similarly, the difference is insignificant between M3G and M3Ga. Besides, pelargonidin glucoside had lower antioxidant activity in comparison with its aglycone form pelargonidin chloride. And in respect to statistical analysis, the difference is not significant ($p < 0.05$). It was seen that anthocyanins gave higher responses by CUPRAC method with respect to other AA methods.

Highest antioxidant capacities in CUPRAC method were observed for epicatechin gallate, epigallocatechin gallate, quercetin, epigallocatechin, catechin, caffeic acid, epicatechin, gallic acid, in accordance with theoretical expectations, because the number and position of the hydroxyl groups as well as the degree of conjugation of the whole molecule are important for easy electron transfer.

4.1.2.3 2,2-azinobis (3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) antioxidant analysis method

Standard calibration curve for ABTS was prepared by using Trolox as shown in Figure 4.7 and results were expressed as mg TEAC/g standard for each sample. The standard calibration curve was obtained between 0.001-0.01 mg/ml and the equation was used to determine the Trolox equivalent values of the samples measured by spectrophotometer.

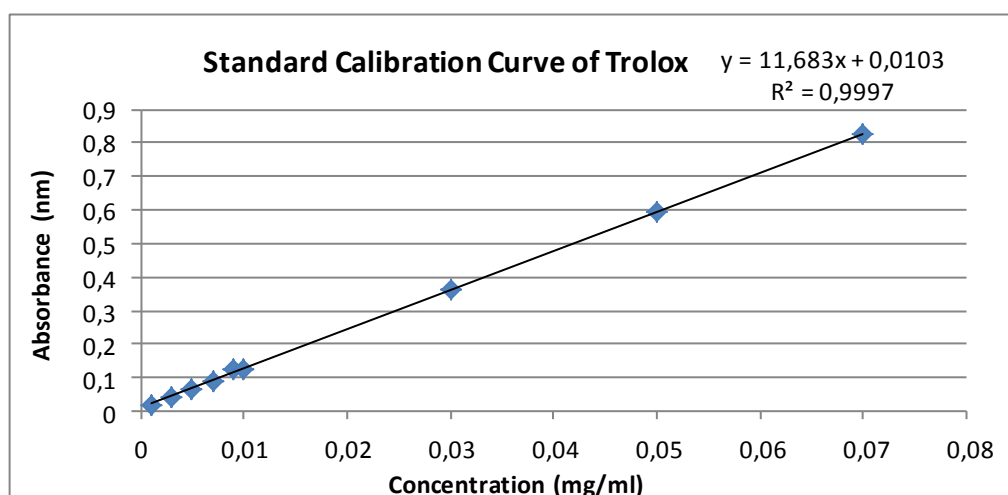


Figure 4.7: Calibration curve of Trolox standard for ABTS method.

Results for all samples obtained by ABTS method are presented in Table 4.1. Gallic acid showed the highest value as 5646.7 ± 40.4 and pelargonidin had the lowest value as 176.6 ± 57.0 mg TEAC/g standard as observed similarly in other total antioxidant activity methods.

As presented in Figure 4.8, total antioxidant activity by ABTS of phenolic standards is in the order of flavonols > catechins > phenolic acids > isoflavones > anthocyanins.

Within the isoflavones, genistein had the highest level of antioxidant activity. It is obvious that when compared with the values obtained with other AA methods isoflavones gave higher responses by the ABTS method. When aglycones forms of isoflavones were compared with their glycoside forms, genistein was higher than genistin and daidzein than daidzin. But the difference between daidzein and daidzin were not significant statistically ($p < 0.05$). The glycosylation of flavonoids reduces their activity when compared to the corresponding aglycones (Shahidi, 1992). Because of the fact that the maximum effectiveness for radical scavenging apparently requires the 3-OH group attached to the 2,3-double bond and adjacent to the 4-carbonyl in the C ring (Rice-Evans et al., 1996).

pHBA had the lowest antioxidant capacity in comparison to other phenolic acids which is followed by syringic acid. This reduction might be depending on the number of hydroxyl groups in the molecule (Dziedzic & Hudson, 1983). For

example; gallic acid has an antioxidant capacity of 3.0 mM, corresponding to three hydroxyl groups available in its structure. While substituting the 3-and 5-hydroxyl with methoxy groups in syringic acid it demonstrates a diminishing in antioxidant activity. On the other hand, antioxidant capacity might be changing according to the class of phenolic acids. So hydroxycinnamic acids tend to be more effective in AO-capacity than the corresponding hydroxybenzoic acids, possibly due to the aryloxy radical stabilizing effect of the $-\text{CH}=\text{CH}-\text{COOH}$ linked to the phenyl ring (Cai, 2006; Rice-Evans et al., 1996). Thus, pHBA and syringic acid might be showing low AO-capacity in tests because of belonging to class of hydroxybenzoic acids.

Rice-Evans et al., (1996) examined the antioxidant activity of hydroxycinnamic acids, coumaric, caffeic and ferulic acids produced from the shikimate pathway from phenylalanine or tyrosine. The antioxidant activity of caffeic acid is almost the same as that of protocatechuic acid. Likewise, in our study caffeic acid and protocatechuic acid had nearly same values as 1308.3 ± 76.7 ; 1304.7 ± 19.2 mg TEAC/g standard, respectively.

In this study, for flavonols the order of AA was as follows: myricetin > quercetin > naringenin. Similarly, Shahidi (1992) reported that flavanones with only one hydroxyl group in the B ring have been suggested to possess little antioxidant activity in lipid systems using stripped corn oil; myricetin > quercetin > naringenin.

In addition, Saija et al., (1995) has studied a report that the order of efficacy of quercetin, rutin, hesperetin and naringenin as antioxidants in three different types of preparation: a linoleic acid peroxidation system compared with a rat cerebral membrane autooxidation system and with dipalmitoyl phosphatidyl choline vehicles. The authors ascribe the conflicting sequences of antioxidant potential in the different systems to their differential abilities to penetrate and interact with the lipid bilayers.

In respect to catechins, the order of antioxidant activity was: EGC > ECG > EGCG > CH>EC. Salah et al. (1995) claimed that the antioxidant capacities of tea polyphenolic constituents in relation to their concentrations in tea are used to calculate their predicted contributions to the antioxidant potential of green tea. The contribution of catechin components to the composition of the green tea extract was as the catechin-gallate components was 26.7% dry weight of composed of epigallocatechin gallate, 11.16%; epicatechin gallate, 2.25%; epigallocatechin,

10.32%; epicatechin, 2.45%; catechin, 0.53%. Accordingly, polyphenolic constituents of green tea in relation to their relative compositions, the order of contribution to the antioxidant effectiveness in green tea is epigallocatechin~epigallocatechingallate > > epicatechin gallate = epicatechin > catechin. The reason for different results obtained by research of Salah et al. and our study might be that equal amounts of each compound were used in our study in contrast to research carried out by Salah et al.

When anthocyanins are evaluated by ABTS method, aglycones of anthocyanins were found to have lower AA than their glycoside forms except for malvidin chloride. Malvidin > M3G > M3Ga is the order of antioxidant activity for ABTS method. But the difference is insignificant between M3G and M3Ga ($p < 0.05$). Glycosylation of anthocyanins in position 3, diminishes the antioxidant activity. Malvidin glycosylation in the 3 position (oenin) reduces the value only slightly presumably because this structural feature does not make a significant contribution without the dihydroxy structure in the B ring (Rice-Evans et al., 1996). On the other hand, the antioxidant activity of C3G and C3Ga was 1894,6; 1694,9 mg TEAC/g standard, respectively. There was no significant difference between these two standards (< 0.05). Likewise, Rice-Evans et al. (1996) investigated antioxidant activity of anthocyanins in berries and grape skins and obtained that cyanidin-3-rutinoside (keracyanin) and the 3-O-galactoside (ideain) have TEAC values around 3 mM.

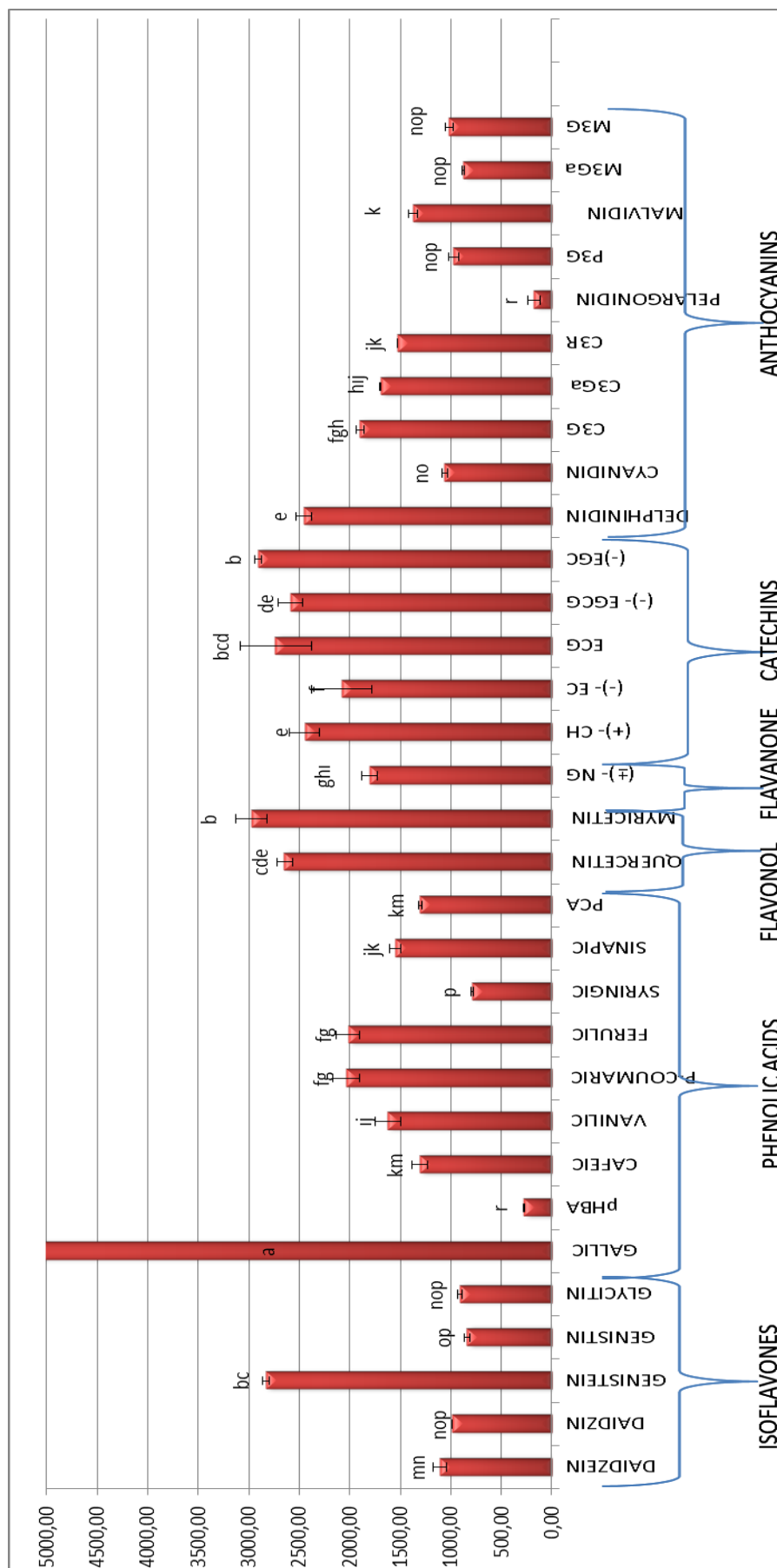


Figure 4.8: Total antioxidant activity values obtained by ABTS method for phenolic compounds.

4.2 Changes In Phenolic Contents and Antioxidant Activities After *In Vitro* GI Digestion

4.2.1 Total phenolic contents after GI digestion

Results for total phenolic content analysis were expressed as mg gallic acid equivalents (GAE)/g standard for each GI digestion samples obtained from three fractions (PG, IN and OUT). The standard calibration curve of gallic acid shown in Figure 4.9 was prepared between 0.005-0.1 mg/ml and the equation obtained from the curve was used to calculate the gallic acid equivalent values of the samples measured by UV-VIS spectrophotometer. The results are shown in Table 4.2.

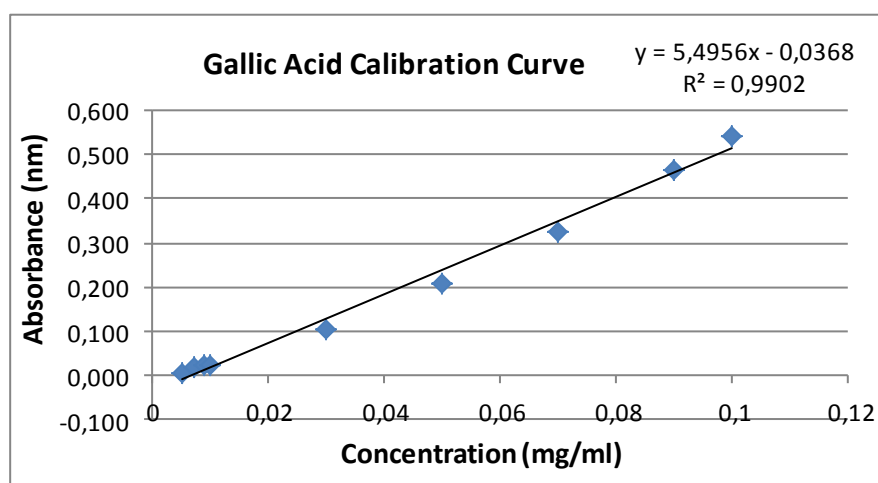


Figure 4.9: Calibration curve of gallic acid standard.

Statistical evaluation of all samples were shown on Figure 4.10. In PG fraction, (+)-catechin showed the highest total phenolic content value (5906.1 ± 692.0 mg GAE/g standard) and myricetin the lowest (-108.0 ± 1.0 mg GAE/g standard).

Generally, for phenolic acids total phenolic contents of PG fractions were higher than the values for IN and OUT fractions. Generally IN and OUT values of phenolic acids had higher levels of total phenolics in comparison to other standards.

Within the isoflavones, OUT fractions were found to be higher than IN and PG values. While the order of total phenolic content for other phenolics such as phenolic acids, catechins and anthocyanins is as $PG > OUT > IN$ fractions.

Results for total phenolic content after GI digestion and their statistical evaluation at INITIAL, IN, OUT and PG fractions for each sample are presented in Table 4.2.

Percent distribution (recovery %) of total phenolic contents obtained by Folin-Ciocalteu method according to the GI digestion fractions (IN, OUT and PG), when the initial values are assumed to be 100%, are presented in Table 4.3.

When results are evaluated statistically according to Table 4.2 it was obtained that some phenolic compounds generally had similar PG and INITIAL values except for anthocyanins. Similarly, in study of Perez-Vicente et al. (2002) which carried out evaluation of the *in vitro* availability of anthocyanins, total phenols, and vitamin C of pomegranate juice it was reported that no differences in total phenolics content were evident when the concentrations before and after pepsin digestion (292 mg of GAE/L) were compared.

For isoflavones, OUT fractions were generally higher than IN and PG fractions. Although it was obtained that IN was greater than PG, there was no significant difference between the IN and PG fractions, when evaluated statistically ($p < 0.05$). In respect to phenolic acids IN % values were low.

Besides OUT % values were high for Flavonols. In regard to Catechins, IN % values were low. PG % values were higher than IN and OUT % values except for ECG. OUT % values of ECG were higher than PG % values. However, there was no difference between the OUT and PG % values ($p < 0.05$).

When results were evaluated for anthocyanins, it was shown that PG % values were high in comparison to IN and OUT % values. But for malvidin OUT % value was high. However, the difference between the OUT and PG fractions was insignificant for malvidin ($p < 0.05$). Perez-Vicente et al. (2002) evaluated the *in vitro* availability of anthocyanins, total phenols, and vitamin C of pomegranate juice. According to total phenolic content analysis, 29% of these compounds were detected in IN fraction when the concentration after the pancreatin-bile salt digestion was analyzed. In our research, it was detected at an average value of 24.8% in IN fraction. Besides, in study of Perez-Vicente et al., it was obtained that $PG > OUT > IN$ and the results of the study is in agreement with the results of this research.

McDougall et al. (2005) assessed the recovery of raspberry anthocyanins using an *in vitro* digestion procedure that mimics the physiochemical and biochemical conditions encountered in the gastrointestinal tract. According to this study, the recovery of

phenolics from the bioavailability procedure was generally higher than the recovery of anthocyanins. Similarly, anthocyanins had low bioavailability in our research. But in study which was carried out by McDougall et al., on average, the recovery of phenolics in the IN and OUT samples approached 100% of the amount in the extract. The recovery of anthocyanins was lower, and only ~70% could be recovered in the IN and OUT samples. On the other hand, in our study for some phenolics and anthocyanins ~23.63% and ~42.02% could be recovered in IN and OUT samples, respectively. In this study, pure phenolic standards are used which does not bind to any compound, whereas McDougall used raspberry extracts which contained carbohydrate, protein, iron and such other compounds. Therefore, it can be said that for this low results in comparison to the study of McDougall, if the phenols and anthocyanins are binding to the food matrices during gastric and pancreatic digestion, this might protect the anthocyanins but still allow these particularly labile compounds to diffuse into the IN sample.

Gumienna et al. (2011) aimed to study the stability and biotransformations of the major polyphenolic compounds in grape and chokeberry wine and their antioxidant activity as well as the interactions between human fecal microflora and polyphenolic compounds and their derivatives secreted during *in vitro* digestion process in a human gastrointestinal track model simulating the conditions (pH, temperature and enzymes) in the human stomach, small and large intestine. Accordingly, the highest content of phenolic compounds was recorded for all kinds of wine after digestion carried out in the stomach similar to our study. The small intestinal digest and the large intestinal digest were characterized with significant reductions in the concentrations of the total phenolic compounds, finally averaging 78.4 mg/L with white grape wine, 504.1 mg/L red grape wine, and 508.1mg/L chokeberry wine. This is expected since small intestines are rich in the activity of glycosidases responsible for hydrolysis of glycoside bonds and formation of aglycone forms.

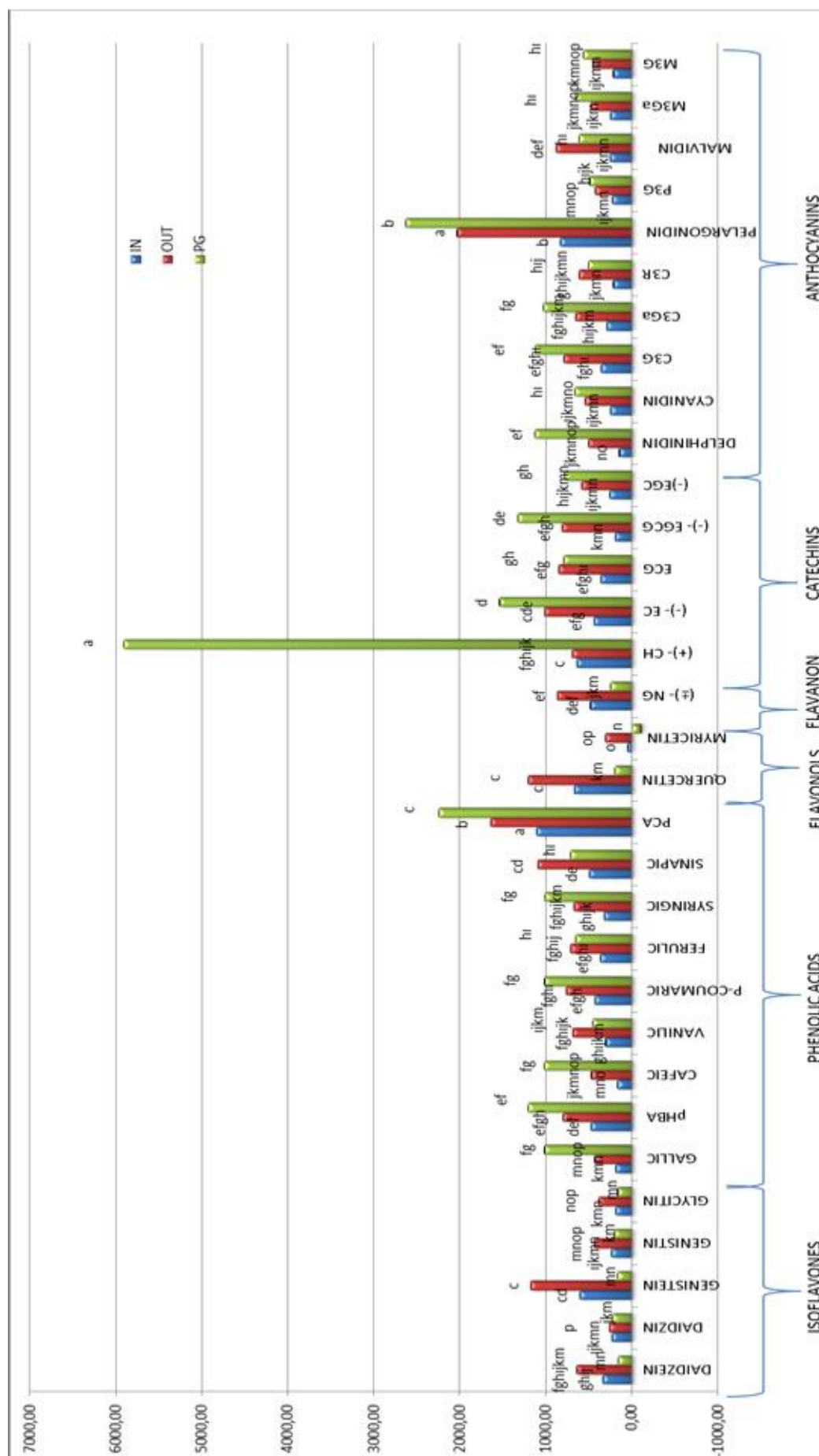


Figure 4.10 : Total phenolic contents of samples after *in-vitro* digestion.

Table 4.2: Total phenolic content of phenolic compounds after *in vitro* digestion

		FOLIN			
	SAMPLE	INITIAL	IN	OUT	PG
ISOFLAVONES	DAIDZEIN	356.9±7.8 β	335.8±2.8 β	639.7±147.2 α	158.9±27.6 β
	DAIDZIN	289.3±14.2 α	229.8±109.3 α	258.9±238.0 α	224.3±49.6 α
	GENISTEIN	1521.5±27.5 α	601.8±77.3 δ	1173.8±66.0 β	164.3±18.1 θ
	GENISTIN	333.9±8.9 β	233.2±27.3 δ	434.8±20.0 α	200.0±9.5 δ
	GLYCITIN	178.2±2.4 β	187.8±25.5 β	381.4±60.1 α	163.0±2.9 β
PHENOLIC ACIDS	GALLIC ACID	1104.1±11.3 α	183.2±7.5 δ	430.6±73.1 β	1011.5±156.3 α
	Phba	617.1±5.8 $\beta\delta$	473.1±112.2 δ	800.5±23.6 β	1204.2±93.4 α
	CAFEIC ACID	1047.4±32.2 α	163.2±7.5 δ	468.9±35.4 β	1015.5±83.9 α
	VANILIC ACID	1249.7±1.7 α	299.8±0.9 δ	683.9±96.6 β	452.8±58.1 δ
	P-COUMARIC ACID	839.7±5.4 β	426.5±21.7 θ	760.5±9.4 δ	1012.1±41.0 α
	FERULIC ACID	1052.9±28.7 α	361.1±108.4 β	708.9±143.8 $\alpha\beta$	648.9±293.6 $\alpha\beta$
	SYRINGIC ACID	699.6±44.6 β	319.1±98.0 δ	668.9±87.2 β	1003.4±38.1 α
	SINAPIC ACID	1429.0±55.8 α	491.1±139.5 β	1088.8±443.0 $\alpha\beta$	712.9±368.9 $\alpha\beta$
	PCA	1570.6±50.6 β	1103.0±96.2 δ	1637.8±170.9 β	2244.1±130.6 α
FLAVONOLS	QUERCETIN	1643.3±10.7 α	664.4±181.0 δ	1204.6±258.0 β	193.3±11.4 θ
	MYRICETIN	1842.7±32.2 α	45.9±47.1 θ	307.3±4.7 δ	(-107.97)±1.0 β
	(±)- NG	1192.5±100.8 α	477.8±148.9 β	862.2±160.3 α	243.8±27.6 β
FLAVANONE	(+)- CH	777.8±29.2 β	634.4±138.6 β	689.8±210.9 β	5906.14±692.0 α
CATECHINS	(-)- EC	1576.1±102.9 α	437.1±0.9 δ	1010.5±9.4 β	1535.8±72.4 α
	ECG	1491.2±96.5 α	357.8±45.3 δ	846.3±74.2 β	786.4±131.5 β
	(-)- EGCG	1166.7±90.1 α	190.5±4.7 δ	808.0±62.5 β	1324.8±149.6 α
	(-)EGC	1242.5±61.1 α	252.5±12.9 θ	583.9±13.2 δ	776.9±14.2 β
	DELPHINIDIN	1784.5±139.4 α	141.8±55.5 θ	500.6±37.9 δ	1127.4±42.6 β
ANTHOCYANINS	CYANIDIN	1294.7±21.4 α	244.5±33.5 θ	542.2±11.5 δ	662.4±14.6 β
	C3G	1901.2±51.5 δ	352.5±10.1 δ	785.5±27.5 β	1119.3±7.0 α
	C3Ga	1751.1±53.6 α	288.5±6.1 θ	650.5±16.1 δ	1029.0±9.3 β
	C3R	1497.9±8.6 α	211.2±10.6 θ	610.6±5.8 δ	503.3±22.3 β
	PELARGONIDIN	852.1±19.7 α	828.4±8.3 θ	2028.6±36.1 δ	2626.2±45.5 β
	P3G	1071.8±87.9 α	227.2±10.6 θ	423.9±5.0 δ	487.1±6.2 β
	MALVIDIN	1469.1±87.9 α	249.8±22.7 δ	880.5±32.5 β	611.1±2.3 β
	M3Ga	1091.5±64.3 α	244.5±2.3 θ	472.2±30.5 δ	647.5±10.2 β
	M3G	1408.4±109.4 α	211.2±4.0 θ	453.9±18.0 δ	562.6±22.3 β

* $\alpha, \beta, \delta, \theta$ shown that statistical evaluation between the Initial, IN, OUT, PG fractions.

(pHBA:P-Hydroxybenzoic acid, PCA:Protocatechuic acid, (±)- NG:Naringenin, (+)- CH:Catechin hydrate, (-)- EC:Epicatechin, ECG:Epicatechin gallate, (-)-EGCG:Epigallocatechin gallate, (-)-EGC:Epigallocatechin, C3G:Cyanidin-3-O-glucoside, C3Ga:Cyanidin-3-O-galactoside, C3R:Cyanidin-3-O-rutinoside, P3G:Pelargonidin 3-O-glucoside, M3Ga:Malvidin-3-O-galactoside, M3G:Malvidin-3-O-glucoside).

Table 4.3. Total phenolic content distribution (recovery %) between PG, IN and OUT fractions.

		INITIAL	IN	OUT	PG
ISOFLAVONES	DAIDZEIN	100.0	94.1	179.2	44.5
	DAIDZIN	100.0	79.4	89.5	77.5
	GENISTEIN	100.0	39.6	77.2	10.8
	GENISTIN	100.0	69.8	130.2	59.9
	GLYCITIN	100.0	105.4	214.0	91.4
PHENOLIC ACIDS	GALLIC ACID	100.0	16.6	39.0	91.6
	pHBA	100.0	76.7	129.7	195.1
	CAFEIC ACID	100.0	15.6	44.8	97.0
	VANILIC ACID	100.0	24.0	54.7	36.2
	P-COUMARIC ACID	100.0	50.8	90.6	120.5
	FERULIC ACID	100.0	34.3	67.3	61.6
	SYRINGIC ACID	100.0	45.6	95.6	143.4
	SINAPIC ACID	100.0	34.4	76.2	49.9
	PCA	100.0	70.2	104.3	142.9
FLAVONOLS	QUERCETIN	100.0	40.4	73.3	11.8
	MYRICETIN	100.0	2.5	16.7	-5.9
FLAVANONE	(±)- NG	100.0	81.6	88.7	379.7
CATECHINS	(+)- CH	100.0	81.6	88.7	379.7
	(-)- EC	100.0	27.7	64.1	97.4
	ECG	100.0	24.0	56.7	52.7
	(-)- EGCG	100.0	16.3	69.3	113.6
	(-)-EGC	100.0	20.3	47.0	62.5
ANTHOCYANINS	DELPHINIDIN	100.0	8.0	28.1	63.2
	CYANIDIN	100.0	18.9	41.2	51.2
	C3G	100.0	18.5	41.3	58.9
	C3Ga	100.0	16.5	37.2	58.8
	C3R	100.0	14.1	40.7	33.6
	PELARGONIDIN	100.0	97.2	238.1	308.2
	P3G	100.0	21.2	39.6	45.5
	MALVIDIN	100.0	17.0	59.9	41.6
	M3Ga	100.0	22.4	43.3	59.3
	M3G	100.0	15.0	32.2	39.0

(pHBA:P-Hydroxybenzoic acid, PCA:Protocatechuic acid, (±)- NG:Naringenin, (+)- CH:Catechin hydrate, (-)- EC:Epicatechin, ECG:Epicatechin gallate, (-)-EGCG:Epigallocatechin gallate, (-)-EGC:Epigallocatechin, C3G:Cyanidin-3-O-glucoside, C3Ga:Cyanidin-3-O-galactoside, C3R:Cyanidin-3-O-rutinoside, P3G:Pelargonidin 3-O-glucoside, M3Ga:Malvidin-3-O-galactoside, M3G:Malvidin-3-O-glucoside).

4.2.2 Total antioxidant activity by DPPH method after GI digestion

The antioxidant capacity was determined by DPPH method in all samples collected from fractions (PG, IN and OUT) after digestion. Standard calibration curve was prepared by using Trolox and it is given in Figure 4.11. The results were expressed as mg TEAC/g standard. The standard calibration curve was prepared between 0.01-0.25 mg/ml concentrations and the equation obtained from the curve was used to calculate the antioxidant activity of the sample measured by UV-VIS spectrophotometer. The results are shown in Table 4.4.

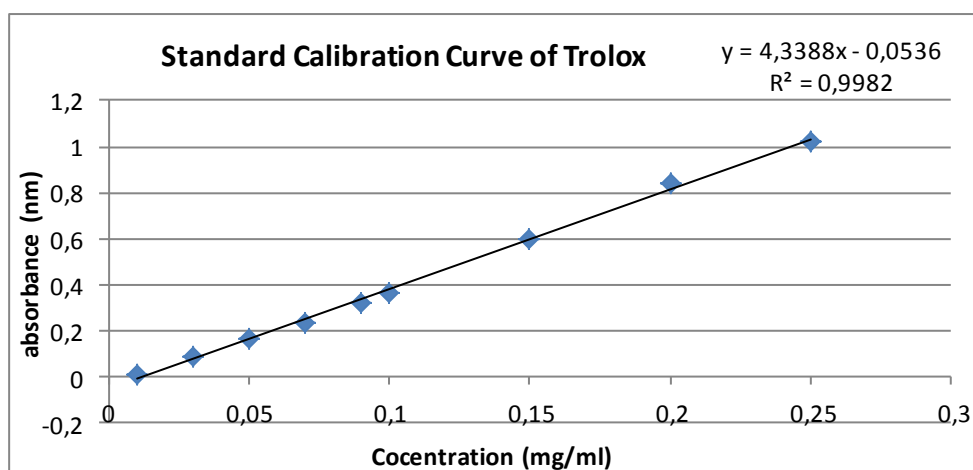


Figure 4.11: Calibration curve for Trolox standard by DPPH method.

All the samples were evaluated according to their total antioxidant activity results by DPPH as shown in Figure 4.12. (+)- Catechin in PG fraction showed the highest and PCA in IN fraction had the lowest values 5414.3 ± 555.3 and 527.3 ± 13.1 mg TEAC/g standard, respectively, as presented in Table 4.4.

When IN fractions of all standards were compared with each other, it showed that anthocyanins and catechins were close to each other and they had higher levels than the other phenolic groups. In respect to OUT fractions, anthocyanins, catechins and isoflavones had the highest results. When PG values were examined, catechins had the highest value between the phenolic groups.

Within the isoflavones, OUT fractions had the highest value between IN, OUT and PG fractions. This might be due to the increase in the extractability of phenolics and

flavonoids by the effect of their release as a result of enzyme application and incubation (2 hours) during the intestinal phase (Bouayed et al., 2011). Generally, in regard to phenolic acids, catechins and anthocyanins an increasing order of $IN < OUT < PG$ was obtained.

Antioxidant activity results of phenolic compounds after *in vitro* digestion with their statistical evaluation at INITIAL, IN, OUT and PG fractions for each sample are shown in Table 4.4. Antioxidant activity distribution (recovery %) between PG, IN and OUT fractions by DPPH method for each sample are presented in Table 4.5.

For isoflavones, IN, OUT and PG % values were higher than INITIAL % values. OUT % values were very high. For daidzin and glycitin there was no difference between the IN, OUT and PG % values. On the other hand, there was no difference between the IN and PG % values, statistically. Also the difference between the OUT and PG % values of genistein was found to be insignificant ($p < 0.05$).

For pHBA, vanilic acid and p-coumaric acid all fractions were very high in comparison to other phenolic acids.

In respect to catechins, PG % values were high except for ECG. But the OUT and PG % values of this standard was insignificant ($p < 0.05$).

Similarly for anthocyanins, PG % values were found to be high. When IN % values of malvidin compared with glycosides, it was obtained that $malvidin > M3Ga > M3G$. In respect to cyanidin, it was shown that order was $Cyanidin > C3G > C3R > C3Ga$. Besides, pelargonidin was greater than P3G. According to these results, the chemical structure of phenolics also plays a role in the free radical-scavenging activity, which is mainly depending on the number and position of hydrogen donating hydroxyl groups on the aromatic rings of the phenolic molecules (Rice-Evans, Miller, & Paganga, 1996). In this sense, it is well known that aglycones display an antioxidant power higher than their glycosides (Lee et al., 2003).

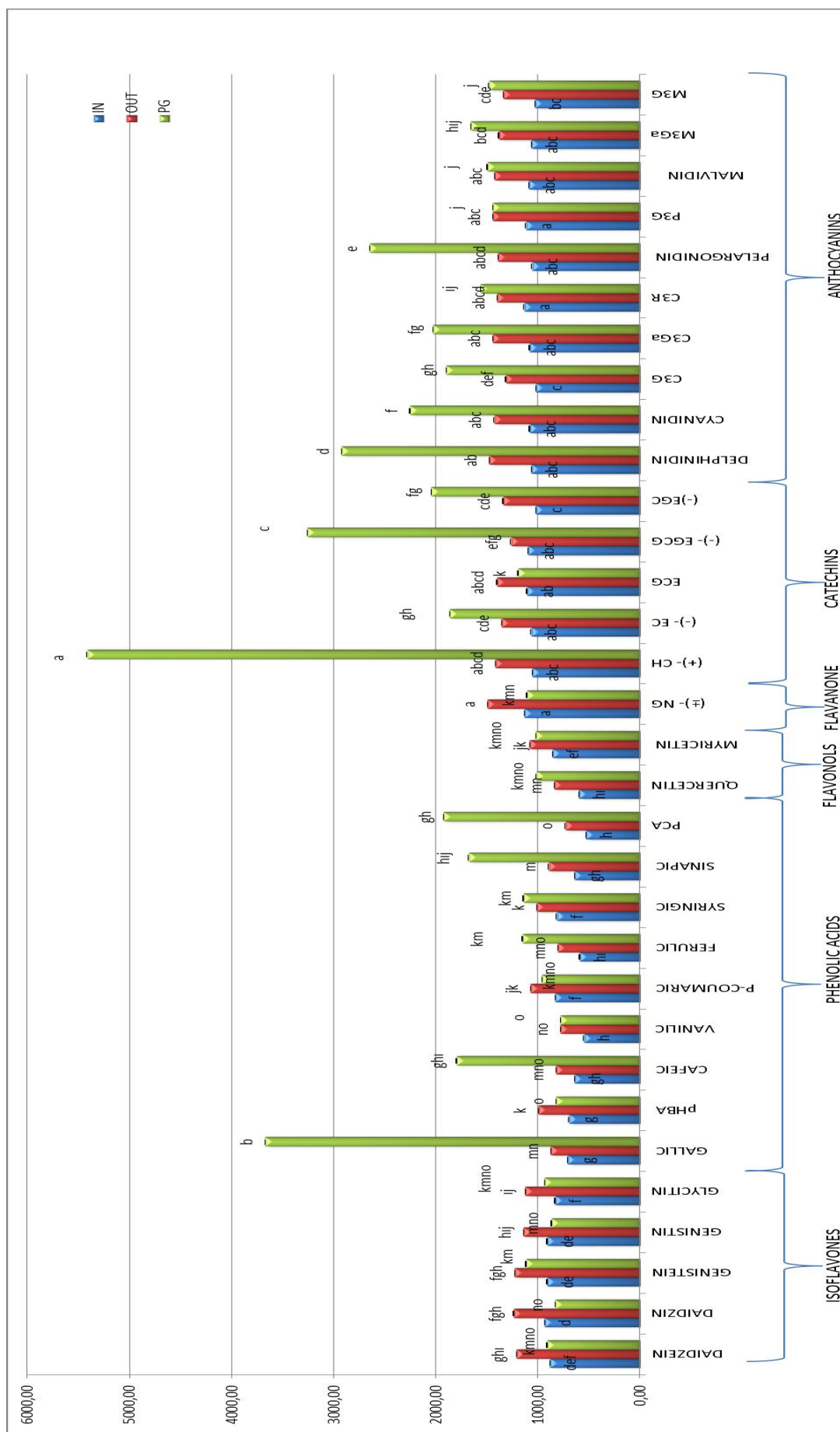


Figure 4.12 : Total antioxidant activity of samples after *In-vitro* digestion by DPPH method.

Table 4.4: Total antioxidant activity of phenolic compounds by DPPH method after *in vitro* digestion.

		DPPH			
	SAMPLE	INITIAL	IN	OUT	PG
ISOFLAVONES	DAIDZEIN	47.8±0.5β	879.4±21.5α	1207.9±123.9α	907.9±200.4α
	DAIDZIN	45.4±5.0δ	932.6±22.7β	1235.3±55.2α	829.2±82.1β
	GENISTEIN	11.0±1.2δ	908.1±50.2β	1223.7±59.7α	1115.4±27.8α
	GENISTIN	43.2±0.8δ	908.1±9.6β	1137.2±6.0α	866.1±32.6β
	GLYCITIN	43.0±2.2β	830.4±11.9α	1121.4±61.2α	928.4±214.9α
	GALLIC ACID	4022.4±186.1α	706.3±20.3δ	870.2±19.4δ	3667.7±1.2β
PHENOLIC ACIDS	Phba	10.9±0.9δ	695.3±40.6β	992.6±64.2α	820.0±119.5αβ
	CAFEIC ACID	1455.2±107.6β	637.9±50.2δ	819.6±31.3δ	1796.5±56.7α
	VANILIC ACID	18.8±0.9δ	548.4±7.2β	777.3±7.5α	776.5±16.9α
	P-COUMARIC ACID	11.5±0.8θ	829.6±6.0δ	1070.7±10.5θ	958.3±10.9β
	FERULIC ACID	932.4±13.0β	590.6±9.6δ	803.7±3.0δ	1149.5±206.4α
	SYRINGIC ACID	572.1±20.4δ	815.2±19.1θ	1010.6±11.9β	1140.1±55.5α
	SINAPIC ACID	2263.1±28.5α	637.9±62.1β	893.4±117.9β	1676.2±403.2α
	PCA	2367.8±173.8α	527.3±13.1δ	728.8±13.4δ	1921.2±6.0β
	QUERCETIN	1517.9±148.0α	593.2±8.4δ	834.3±103.0βδ	1013.8±24.1β
FLAVONOLS	MYRICETIN	2165.2±34.0α	852.4±19.1δ	1079.2±10.5β	1014.6±78.5β
	(±)- NG	10.2±0.2δ	1129.3±9.6β	1492.8±25.4α	1106.0±26.6β
FLAVANONE	(+)- CH	909.1±83.1β	1053.3±59.7β	1412.6±117.9β	5414.7±555.3α
CATECHINS	(-)- EC	991.3±59.2δ	1065.9±13.1δ	1352.5±17.9β	1858.9±147.3α
	ECG	2716.4±172.5α	1107.3±66.9β	1400.0±94.0β	1192.2±13.3β
	(-)- EGCG	3472.2±236.3α	1094.7±146.9β	1263.8±3.0β	3255.4±166.6α
	(-)-EGC	2163.2±12.2α	1016.1±41.9δ	1339.8±6.3θ	2043.2±29.1β
	DELPHINIDIN	1794.5±31.2β	1058.3±30.5θ	1472.8±12.7δ	2919.1±52.3α
ANTHOCYANINS	CYANIDIN	838.9±16.3δ	1080.3±16.3δ	1426.4±3.7β	2251.5±36.9α
	C3G	995.5±23.1θ	1014.4±20.5δ	1312.4±20.4β	18923.0±13.6α
	C3Ga	1397.9±51.6θ	1080.3±19.2δ	1439.0±24.0β	2026.2±15.4α
	C3R	1193.3±9.5δ	1134.3±7.7δ	1396.8±44.3β	1560.1±13.6α
	PELARGONIDIN	365.2±4.9β	1056.7±8.8δ	1388.4±3.7β	2644.2±33.3α
	P3G	981.1±35.3δ	1117.4±35.1θ	1436.9±67.7β	1437.2±62.1α
	MALVIDIN	822.6±28.5δ	1087.1±22.1β	1422.1±11.0α	1493.5±22.3α
	M3Ga	970.5±4.1θ	1061.7±35.5δ	1382.0±36.0β	1654.0±5.9α
	M3G	1036.8±8.2δ	1026.3±10.1δ	1337.7±9.7β	1478.1±5.1α

*α,β,δ,θ shown that statistical evaluation between the Initial, IN,OUT,PG fractions.

(pHBA:P-Hydroxybenzoic acid, PCA:Protocatechuic acid, (±)- NG:Naringenin, (+)- CH:Catechin hydrate, (-)- EC:Epicatechin, ECG:Epicatechin gallate, (-)-EGCG:Epigallocatechin gallate, (-)-EGC:Epigallocatechin, C3G:Cyanidin-3-O-glucoside, C3Ga:Cyanidin-3-O-galactoside, C3R:Cyanidin-3-O-rutinoside, P3G:Pelargonidin 3-O-glucoside, M3Ga:Malvidin-3-O-galactoside, M3G:Malvidin-3-O-glucoside).

Table 4.5: Antioxidant activity distribution (recovery %) between PG, IN and OUT fractions by DPPH method.

		INITIAL	IN	OUT	PG
ISOFLAVONES	DAIDZEIN	100.0	1840.2	2527.7	1900.0
	DAIDZIN	100.0	2054.8	2721.9	1827.4
	GENISTEIN	100.0	8228.2	11088.5	10106.4
	GENISTIN	100.0	2103.2	2633.8	2005.9
	GLYCITIN	100.0	1931.9	2608.8	2159.9
PHENOLIC ACIDS	GALLIC ACID	100.0	17.6	21.6	91.2
	pHBA	100.0	6400.7	9137.4	7548.3
	CAFEIC ACID	100.0	43.8	56.3	123.5
	VANILIC ACID	100.0	2920.8	4140.0	4135.3
	P-COUMARIC ACID	100.0	7239.6	9344.2	8363.0
	FERULIC ACID	100.0	63.4	86.2	123.3
	SYRINGIC ACID	100.0	142.5	176.6	199.3
	SINAPIC ACID	100.0	28.2	39.5	74.1
	PCA	100.0	22.3	30.8	81.1
FLAVONOLS	QUERCETIN	100.0	39.1	55.0	66.8
	MYRICETIN	100.0	39.4	49.8	46.9
FLAVANONE	(±)- NG	100.0	115.9	14676.2	10872.8
CATECHINS	(+)- CH	100.0	115.9	155.4	595.6
	(-)- EC	100.0	107.5	136.4	187.5
	ECG	100.0	40.8	51.5	43.9
	(-)- EGCG	100.0	31.5	36.4	93.8
	(-)EGC	100.0	47.0	61.9	94.5
ANTHOCYANINS	DELPHINIDIN	100.0	59.0	82.1	162.7
	CYANIDIN	100.0	128.8	170.0	268.4
	C3G	100.0	101.9	131.8	190.2
	C3Ga	100.0	77.3	102.9	145.0
	C3R	100.0	95.1	117.1	130.7
	PELARGONIDIN	100.0	289.4	380.2	724.1
	P3G	100.0	113.9	146.5	146.5
	MALVIDIN	100.0	132.2	172.9	181.6
	M3Ga	100.0	109.4	142.4	170.4
	M3G	100.0	99.0	129.0	142.6

(pHBA:P-Hydroxybenzoic acid, PCA:Protocatechuic acid, (±)- NG:Naringenin, (+)- CH:Catechin hydrate, (-)- EC:Epicatechin, ECG:Epicatechin gallate, (-)-EGCG:Epigallocatechin gallate, (-)-EGC:Epigallocatechin, C3G:Cyanidin-3-O-glucoside, C3Ga:Cyanidin-3-O-galactoside, C3R:Cyanidin-3-O-rutinoside, P3G:Pelargonidin 3-O-glucoside, M3Ga:Malvidin-3-O-galactoside, M3G:Malvidin-3-O-glucoside).

4.2.3 Total antioxidant activity by CUPRAC method after GI digestion

Trolox standard calibration curve was prepared for CUPRAC method as shown in Figure 4.13 and the results were expressed as mg Trolox equivalents (TEAC)/g standard for each sample. The standard calibration curve was prepared between 0.004-0.4 mg/ml and the equation was used to calculate the antioxidant activity of the samples measured by spectrophotometer. The results are shown in Table 4.6.

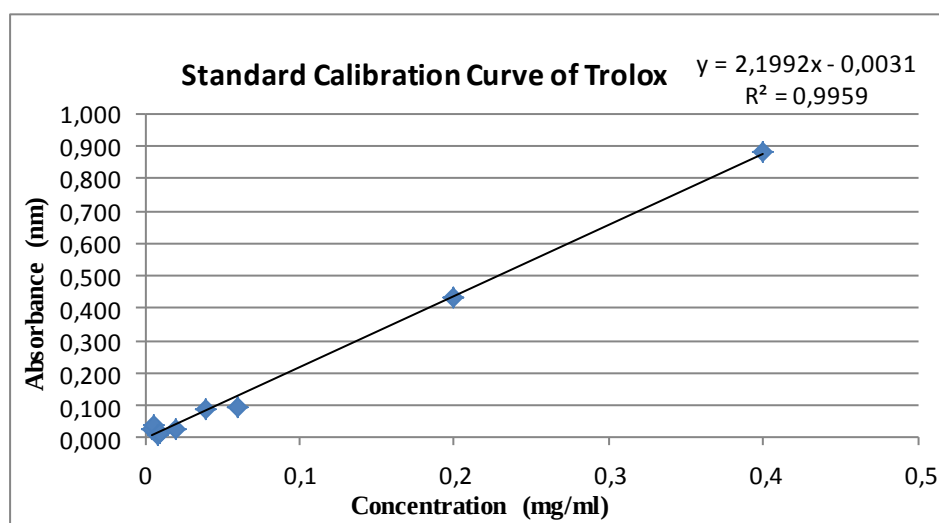


Figure 4.13 : Standard calibration curve of Trolox for CUPRAC method.

Figure 4.14 illustrates changes in the antioxidant activity of phenolic compounds after GI digestion in each fraction by CUPRAC method. Accordingly, PG fraction of (+)-catechin showed the highest value as 5876.2 ± 1000.3 and PG fraction of genistein showed the lowest as 71.7 ± 19.1 mg TEAC/g standard.

When PG fractions of all pure compounds were evaluated, it was obtained that catechins had the highest PG results in comparison to the other groups. In respect to OUT results, flavanols had the highest values.

When IN fractions of all standards compared with each other, it was obtained that order was as follows: phenolic acids > catechins > flavonols > anthocyanins > isoflavones. The low recovery of anthocyanins may have two main causes. At the pH of the small intestine (pH 7.5-8.0), anthocyanins (which exist in equilibrium of four molecular species; the colored basic flavylum cation and three secondary structures

the quinoidal bases, the carbinol pseudobase, and the chalcone pseudobase forms) are predominantly in the colorless chalcone pseudobase molecular form. The formation of the chalcone and its subsequent fission between the B and C rings is also favored by elevated temperatures and results in the destruction of the anthocyanin chromophore (Markakis, 1982). For example the main anthocyanins in blackcurrant were increasingly unstable at pH>4.5 (Nielsen et al., 2003). In addition, anthocyanins can form insoluble complexes with particulates (Bridle et al., 1997) and anthocyanins bound to components of the pancreatin/bile salts mixture in the IN and the OUT samples.

When results were examined within the groups, it showed that for isoflavones and flavonols OUT fractions had the highest antioxidant activity. On the other hand, phenolic acids, anthocyanins and catechins had the highest PG fractions.

Antioxidant activity results of phenolic compounds after *in vitro* digestion with their statistical evaluation at INITIAL, IN, OUT and PG fractions for each sample are shown in Table 4.6. Antioxidant activity distribution (recovery %) between PG, IN and OUT fractions by CUPRAC method for each sample are presented in Table 4.7.

According to those results, OUT % values were high for isoflavones. Among the phenolic acids, pHBA had the high recovery % values in all fractions compared with other acids. Besides, phenolic acids and catechins had high PG recovery % values. On the other hand, flavonols had high OUT recovery% values similar to naringenin which was a flavanone. Anthocyanins had the high PG recovery% values than IN and OUT % values in this study.

The transition from the gastric acidic to the mild alkaline intestinal environment caused a decrease in the amount of bioaccessible phenolic acids, catechins and especially anthocyanins in this study. Sensitivity of anthocyanins to neutral or slightly alkaline pH has already been pointed out in several studies. Anthocyanins, are highly unstable at intestinal pH (Bouayed et al., 2011). Also there is the impact of bile acids and pancreatin. Furthermore, Tagliazucchi et al. (2010) have also shown that radical-scavenging activities of polyphenols may be pH-dependent, suggesting greater scavenging capacity in the intestine than in the stomach. For instance, 80% of bioaccessible grape anthocyanins during gastric digestion were lost after intestinal digestion (Tagliazucchi et al., 2010).

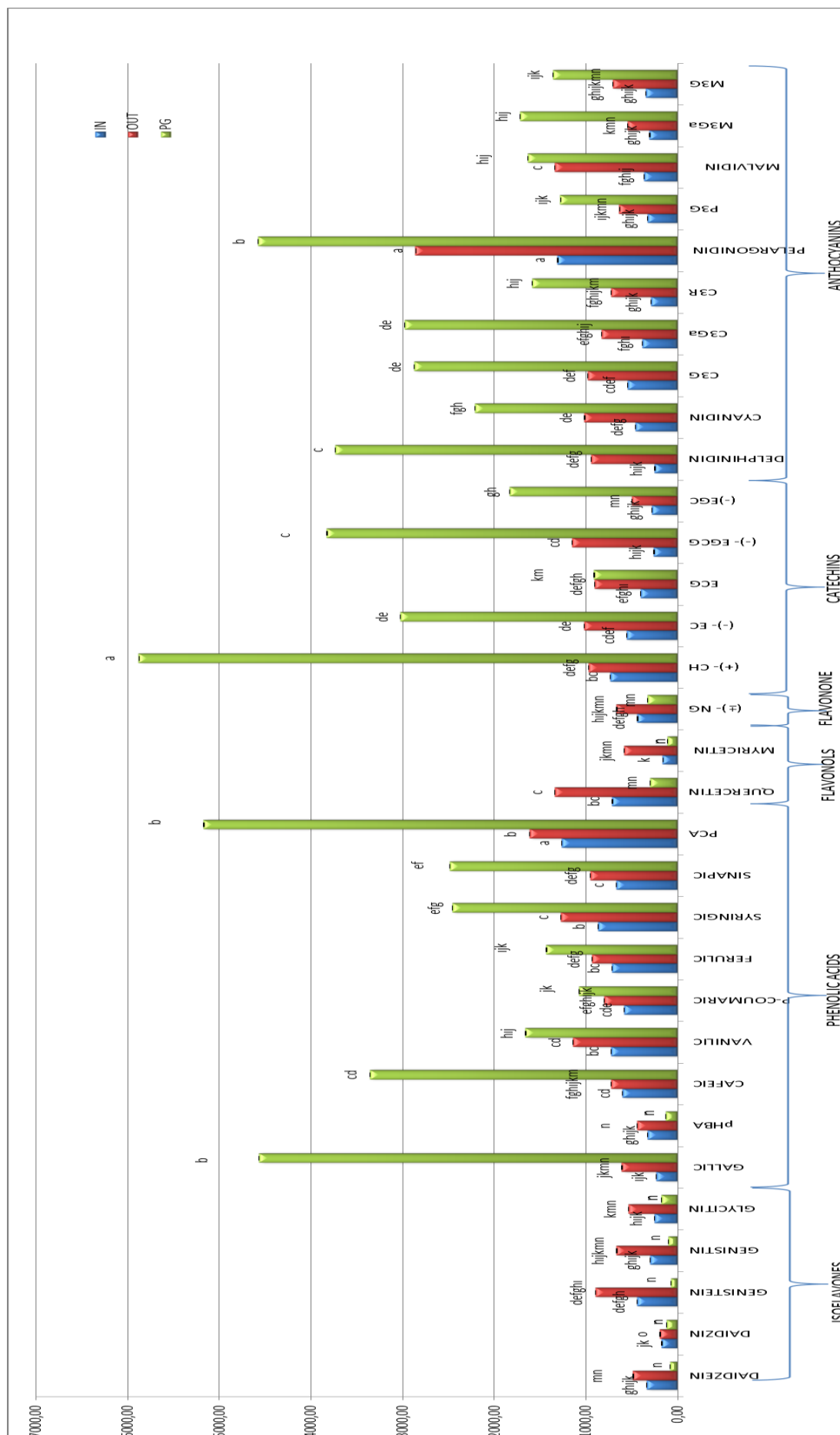


Figure 4.14 : Total antioxidant activity of samples after *In-vitro* digestion by CUPRAC method.

Table 4.6: Antioxidant activity of phenolic compounds after *in vitro* digestion.

	SAMPLE	CUPRAC			
		INITIAL	IN	OUT	PG
ISOFLAVONES	DAIDZEIN	326.9±1.1 α	337.5±4.7 α	484.3±135.4 α	80.2±16.7 β
	DAIDZIN	217.4±4.8 α	172.6±40.0 α	190.7±173.7 α	120.6±7.2 α
	GENISTEIN	573.9±13.9 β	440.7±9.4 δ	896.5±58.9 α	71.7±19.1 θ
	GENISTIN	255.1±65.7 $\beta\delta$	302.5±96.6 β	663.3±5.9 α	102.1±19.1 δ
	GLYCITIN	127.2±41.3 δ	252.5±54.2 β	534.2±23.6 α	177.8±45.3 $\beta\delta$
	GALLIC ACID	4397.8±53.6 α	230.9±4.7 β	607.1±138.4 β	4563.3±421.6 α
PHENOLIC ACIDS	Phba	37.6±2.6 β	325.8±63.6 α	442.6±70.7 α	130.7±35.7 β
	CAFEIC ACID	3418.3±345.6 α	602.3±280.3 β	727.9±144.3 β	3357.5±240.6 α
	VANILIC ACID	1698.2±59.0 α	725.5±25.9 δ	1138.0±123.7 β	1656.5±111.9 α
	P-COUMARIC ACID	1011.4±84.4 α	584.0±0.0 δ	798.7±55.9 β	1075.5±9.5 α
	FERULIC ACID	1712.0±13.40 α	712.2±190.8 δ	934.0±229.7 $\beta\delta$	1430.8±345.4 $\alpha\beta$
	SYRINGIC ACID	1475.5±38.9 β	868.8±157.8 δ	1273.4±238.5 $\beta\delta$	2456.5±157.2 α
	SINAPIC ACID	1629.8±122.2 $\alpha\beta$	667.2±183.7 β	954.8±318.0 β	2485.1±821.7 α
	PCA	3165.2±12.9 β	1261.9±101.3 δ	1616.9±58.9 δ	5167.9±347.7 α
	QUERCETIN	3392.5±294.7 α	710.6±155.5 $\beta\delta$	1337.9±347.4 β	302.5±21.4 δ
FLAVONOIS	MYRICETIN	3345.2±294.7 α	160.9±9.4 δ	582.1±44.2 β	110.5±40.5 δ
	(±)- NG	323.1±174.2 β	437.4±141.3 $\alpha\beta$	663.3±117.8 α	324.4±4.8 β
FLAVANONE	(+)- CH	3306.5±85.7 β	733.9±150.8 δ	973.6±226.7 δ	5876.2±1000.3 α
CATECHINS	(-)- EC	2369.5±150.1 β	554.0±14.1 θ	1021.4±82.4 δ	3023.0±235.8 α
	ECG	2876.5±16.1 α	400.8±56.5 δ	904.8±41.2 β	908.8±31.0 β
	(-)- EGCG	2472.6±94.3 $\alpha\beta$	255.8±2.4 δ	1148.4±55.9 $\beta\delta$	3824.0±1335.8 α
	(-)EGC	2182.8±22.8 α	277.5±66.5 θ	496.8±26.0 δ	1833.3±21.0 β
	DELPHINIDIN	2836.6±96.5 β	247.5±11.5 θ	942.3±134.7 δ	3733.0±88.3 α
ANTHOCYANINS	CYANIDIN	1309.6±198.3 β	460.7±17.3 θ	1013.1±78.0 δ	2207.2±30.9 α
	C3G	2795.0±37.5 θ	544.0±20.8 δ	979.8±47.3 β	2874.1±57.5 α
	C3Ga	2435.0±42.9 $\alpha\beta$	384.1±83.2 δ	829.9±106.2 β	2978.5±32.5 α
	C3R	2086.4±32.2 α	290.8±113.5 δ	725.8±45.0 β	1587.5±10.1 α
	PELARGONIDIN	1061.5±32.2 β	1306.8±35.1 θ	2857.8±40.2 δ	4578.4±35.0 α
	P3G	1014.0±80.4 α	330.8±20.0 θ	634.2±38.2 δ	1277.6±30.9 β
	MALVIDIN	1449.8±64.3 β	367.4±55.0 θ	1337.9±78.0 δ	1634.6±226.0 α
	M3Ga	1055.7±32.2 β	304.1±30.5 θ	542.6±38.2 δ	1718.8±148.9 α
	M3G	1241.4±5.4 β	344.1±15.3 θ	705.0±26.0 δ	1361.8±40.8 α

* α , β , δ , θ shown that statistical evaluation between the Initial, IN,OUT,PG fractions.

(pHBA:P-Hydroxybenzoic acid, PCA:Protocatechuic acid, (±)- NG:Naringenin, (+)- CH:Catechin hydrate, (-)- EC:Epicatechin, ECG:Epicatechin gallate, (-)-EGCG:Epigallocatechin gallate, (-)-EGC:Epigallocatechin, C3G:Cyanidin-3-O-glucoside, C3Ga:Cyanidin-3-O-galactoside, C3R:Cyanidin-3-O-rutinoside, P3G:Pelargonidin 3-O-glucoside, M3Ga:Malvidin-3-O-galactoside, M3G:Malvidin-3-O-glucoside).

Table 4.7: Antioxidant activity distribution (%) between PG, IN and OUT fractions by CUPRAC method.

		INITIAL	IN	OUT	PG
ISOFLAVONES	DAIDZEIN	100.0	103.2	148.2	24.5
	DAIDZIN	100.0	79.4	87.7	55.5
	GENISTEIN	100.0	76.8	156.2	12.5
	GENISTIN	100.0	118.6	260.1	40.0
	GLYCITIN	100.0	198.6	420.1	139.9
PHENOLIC ACIDS	GALLIC ACID	100.0	5.3	13.8	103.8
	pHBA	100.0	866.2	1176.8	347.5
	CAFEIC ACID	100.0	17.6	21.3	98.2
	VANILIC ACID	100.0	42.7	67.0	97.6
	P-COUMARIC ACID	100.0	57.7	79.0	106.3
	FERULIC ACID	100.0	41.6	54.6	83.6
	SYRINGIC ACID	100.0	58.9	86.3	166.5
	SINAPIC ACID	100.0	40.9	58.6	152.5
	PCA	100.0	39.9	51.1	163.3
FLAVONOLS	QUERCETIN	100.0	20.9	39.4	8.9
	MYRICETIN	100.0	4.8	17.4	3.3
FLAVANONE	(±)- NG	100.0	22.2	205.3	88.9
CATECHINS	(+)- CH	100.0	22.2	29.4	88.9
	(-)- EC	100.0	23.4	43.1	127.6
	ECG	100.0	13.9	31.5	31.6
	(-)- EGCG	100.0	10.4	46.5	154.7
	(-)-EGC	100.0	12.7	22.8	131.6
ANTHOCYANINS	DELPHINIDIN	100.0	8.7	33.2	168.5
	CYANIDIN	100.0	35.2	77.4	102.8
	C3G	100.0	19.5	35.1	102.8
	C3Ga	100.0	15.8	34.1	122.3
	C3R	100.0	13.9	34.8	76.1
	PELARGONIDIN	100.0	123.1	269.2	431.3
	P3G	100.0	32.6	62.5	126.0
	MALVIDIN	100.0	25.3	92.3	112.8
	M3Ga	100.0	28.8	51.4	162.8
	M3G	100.0	27.7	56.8	109.7

(pHBA:P-Hydroxybenzoic acid, PCA:Protocatechuic acid, (±)- NG:Naringenin, (+)- CH:Catechin hydrate, (-)- EC:Epicatechin, ECG:Epicatechin gallate, (-)-EGCG:Epigallocatechin gallate, (-)-EGC:Epigallocatechin, C3G:Cyanidin-3-O-glucoside, C3Ga:Cyanidin-3-O-galactoside, C3R:Cyanidin-3-O-rutinoside, P3G:Pelargonidin 3-O-glucoside, M3Ga:Malvidin-3-O-galactoside, M3G:Malvidin-3-O-glucoside).

4.2.4 Total antioxidant activity by ABTS method after GI digestion

Standard calibration curve for ABTS was prepared by using Trolox as shown in Figure 4.15 and the results were expressed as mg TEAC/g standard for each sample. The standard calibration curve was obtained between 0.001-0.01 mg/ml and the equation was used to evaluate the antioxidant activity of the samples measured by spectrophotometer. The results are shown in Table 4.8.

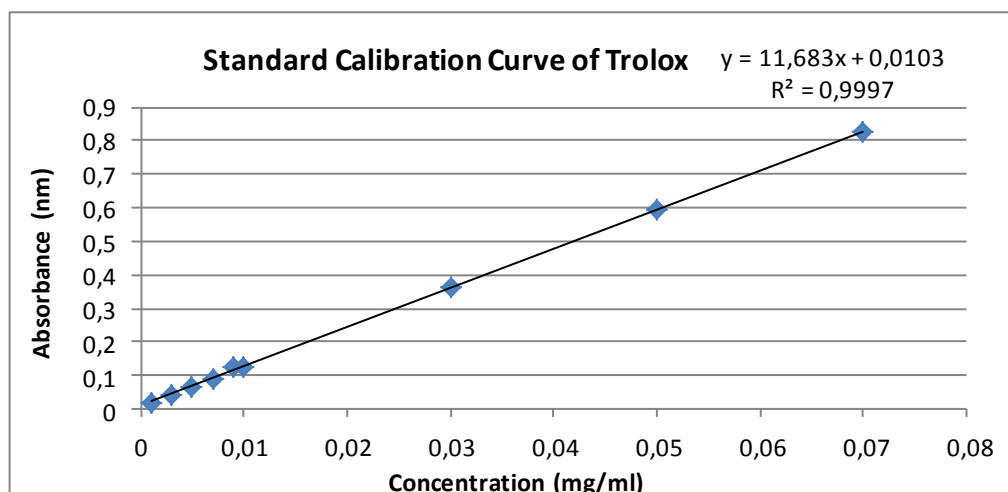


Figure 4.15 : Calibration curve of Trolox standard for ABTS method.

IN, OUT and PG values of all standards was compared with each other statistically in Figure 4.16. PG fraction of gallic acid showed the highest value as 5781.4 ± 383.3 and OUT fraction of P3G had the lowest as 40.1 ± 21.9 mg TEAC/g standard. Besides, PG fraction of (+)- catechin, pelargonidin and (-)- epicatechin had the highest amounts in respect to antioxidant activity after gallic acid.

Generally PG values of all standards were higher than IN and OUT values. When PG results were evaluated according to Figure 4.16, it was obtained that isoflavones had very low antioxidant activity in comparison to the other groups. On the other hand, IN values of isoflavones were higher than other phenolic compounds. In respect to OUT fractions, the phenolic group having the lowest antioxidant activity was catechins.

Between the isoflavones, the order of antioxidant activity in IN fractions was as follows: daidzein > daidzin and genistin > genistein. But the difference was not statistically significant between the genistin and genistein ($p < 0.05$).

Within the catechins, it was obtained that $ECG < EC$ and $EGCG < EGC$ in respect to antioxidant activity of both PG and IN values. However, there is no difference between the gallate forms of Catechins and their aglycones ($p < 0.05$).

Within the anthocyanins, when glycoside and aglycone forms were compared with each other, it was obtained that the values of aglycones were higher than the values of glycosides in PG fractions.

Antioxidant activity results of phenolic compounds after *in vitro* digestion with their statistical evaluation at INITIAL, IN, OUT and PG fractions for each sample are shown in Table 4.8. Antioxidant activity distribution (recovery %) between PG, IN and OUT fractions by ABTS method for each sample are presented in Table 4.9.

Accordingly, for isoflavones generally IN recovery % values were higher than PG recovery% values. In fact, from the literature it is well known that the radical scavenger activity of polyphenols is strongly pH-dependent with higher pH values which significantly increase this capacity. This increase in the radical scavenger activity has been attributed to the deprotonation of the hydroxyl moieties present on the aromatic rings of the phenolic compounds (Mukai, Oka, Watanabe, Egawa, & Nagaoka, 1997; Tyrakowska et al., 1999). The transition from the stomach to the intestinal environment may induce structural changes in the phenolic molecules that could be attributed to the ionisation of the hydroxyl groups (Tagliazucchi et al., 2010).

In respect to phenolic acids, PG recovery % values were high except for PCA and caffeic acid. This two standards had the high OUT recovery % values. There was no difference between the INITIAL and PG values of all phenolic acids except for pHBA and caffeic acid. Similarly, the difference between IN and OUT values of all phenolic acids except for coumaric acid and caffeic acid was statistically insignificant ($p < 0.05$).

OUT recovery % values of flavonols and ($\pm$)- naringenin were high. In contrast to this, catechins had the highest PG recovery % values.

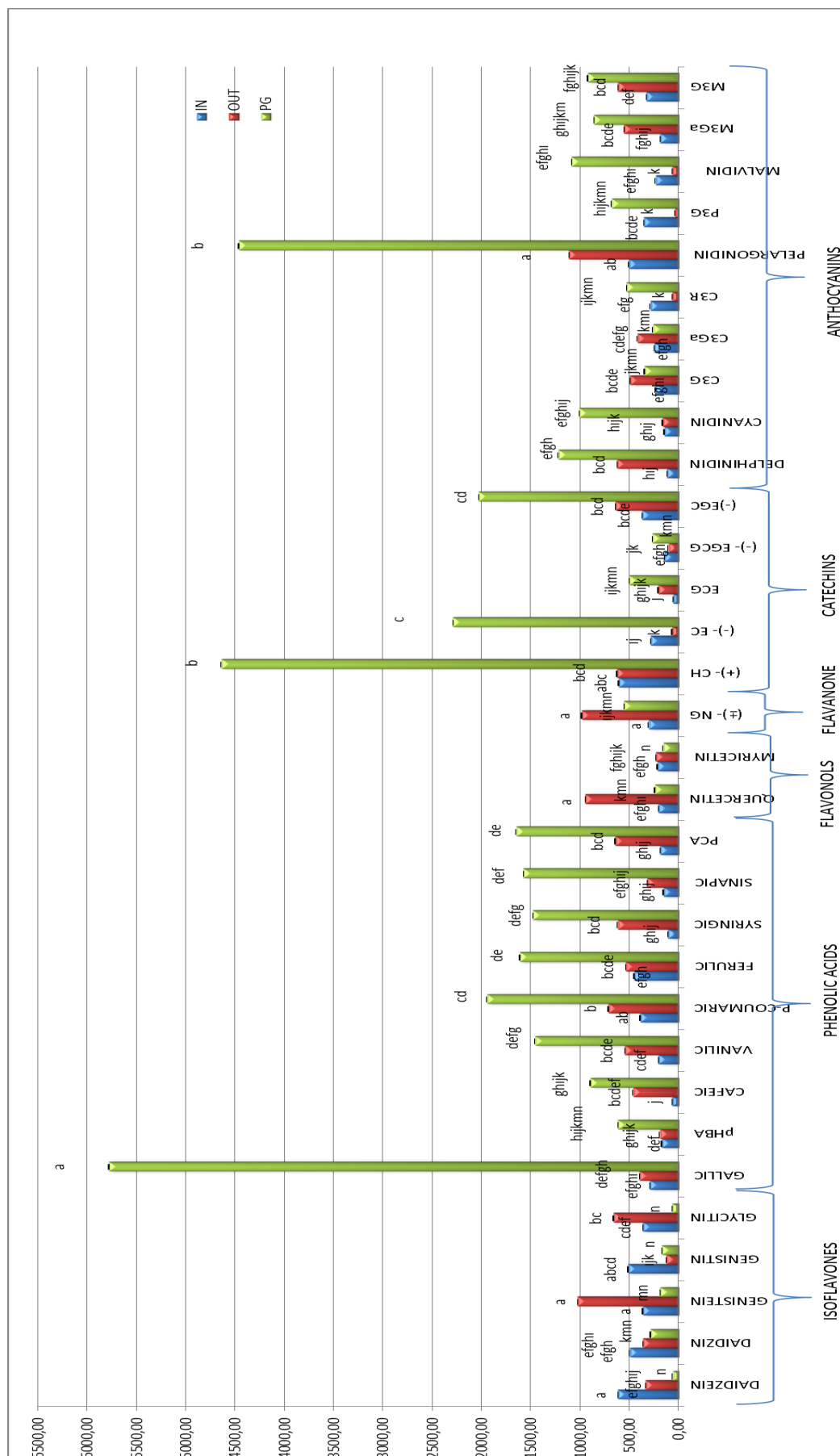


Figure 4.16 : Total antioxidant activity of samples after *In-vitro* digestion by ABTS method.

Table 4.8: Total phenolic content and antioxidant activity of phenolic Compounds After *in vitro* digestion.

	SAMPLE	ABTS			
		INITIAL	IN	OUT	PG
ISOFLAVONES	DAIDZEIN	1110.3±69.1 α	614.0±0.0 β	336.4±267.2 $\beta\delta$	60.6±4.9 δ
	DAIDZIN	985.1±2.0 α	497.6±180.0 β	359.5±328.7 β	285.1±19.3 β
	GENISTEIN	2829.0±34.3 α	363.8±62.1 θ	1023.1±87.6 δ	185.5±18.4 β
	GENISTIN	838.5±27.7 α	513.7±54.1 β	124.0±115.3 δ	170.0±2.7 δ
	GLYCITIN	911.3±25.7 α	362.8±67.4 δ	661.2±68.2 β	64.1±21.5 θ
	GALLIC ACID	5646.7±40.4 α	291.3±20.8 β	394.7±94.8 β	5781.4±383.3 α
PHENOLIC ACIDS	Phba	275.4±6.2 β	173.5±4.4 δ	193.0±88.7 $\beta\delta$	616.0±37.2 α
	CAFEIC ACID	1308.3±76.7 α	66.9±18.2 θ	462.9±2.8 δ	897.5±125.5 β
	VANILIC ACID	1625.7±128.1 α	206.4±33.7 β	542.6±22.2 β	1456.5±119.3 α
	P-COUMARIC ACID	2035.2±132.1 θ	391.4±42.6 δ	712.7±18.8 β	1944.7±18.8 α
	FERULIC ACID	2013.8±116.0 α	451.0±172.0 β	534.0±54.3 β	1612.5±449.2 α
	SYRINGIC ACID	785.2±12.9 β	108.0±103.8 δ	619.4±352.5 $\beta\delta$	1477.6±13.5 α
	SINAPIC ACID	1545.8±5.5 α	155.0±69.2 β	317.6±207.3 β	1572.7±677.0 α
	PCA	1304.7±19.2 α	184.8±60.8 β	643.6±78.7 β	1649.1±430.4 α
	QUERCETIN	2645.7±77.7 α	202.9±90.9 δ	945.4±5.5 δ	243.2±8.5 β
FLAVONOLS	MYRICETIN	2970.3±155.4 α	215.8±8.9 β	228.3±63.2 β	157.6±2.2 β
	(±)- NG	1802.6±75.7 α	305.5±252.7 δ	984.7±95.3 δ	552.9±21.5 β
FLAVANONE	(+)- CH	2445.4±149.3 $\alpha\beta$	608.4±16.9 β	625.6±51.0 β	4641.8±1614.0 α
CATECHINS	(-)- EC	2078.8±296.6 α	281.9±11.1 β	67.6±46.6 β	2287.6±316.1 α
	ECG	2735.0±353.1 α	54.0±19.5 β	207.1±104.2 β	502.3±278.0 β
	(-)- EGCG	2586.7±123.1 α	142.4±28.4 β	105.5±5.0 β	263.5±30.3 β
	(-)-EGC	2906.8±35.3 α	368.8±32.2 θ	641.3±48.9 δ	2025.2±71.8 β
	DELPHINIDIN	2456.0±77.7 α	118.6±76.9 θ	624.8±86.1 δ	1221.3±11.6 β
ANTHOCYANINS	CYANIDIN	1062.2±25.2 α	146.8±36.0 β	164.8±81.8 β	1008.4±99.0 α
	C3G	1894.3±40.4 θ	236.5±25.3 δ	492.3±120.9 β	347.5±66.3 α
	C3Ga	1694.9±6.1 α	249.6±46.2 δ	417.9±66.2 θ	261.3±17.7 β
	C3R	1525.9±1.0 α	287.9±55.6 δ	62.8±12.4 β	526.9±47.4 $\beta\delta$
	PELARGONIDIN	176.6±57.0 α	505.5±65.7 δ	1109.2±74.3 β	4464.2±151.0 δ
	P3G	970.9±53.5 α	353.7±12.5 δ	40.1±21.9 θ	681.7±28.9 β
	MALVIDIN	1371.1±46.4 α	235.2±38.8 θ	62.9±38.3 δ	1085.6±21.0 β
	M3Ga	876.8±9.1 α	186.9±43.4 δ	551.1±42.4 δ	859.88±90.53 α
	M3G	1016.6±39.3 θ	324.9±48.2 δ	615.4±67.1 β	922.0±32.4 α

* $\alpha, \beta, \delta, \theta$ shown that statistical evaluation between the Initial, IN, OUT, PG fractions.

(pHBA: P-Hydroxybenzoic acid, PCA: Protocatechuic acid, (±)- NG: Naringenin, (+)- CH: Catechin hydrate, (-)- EC: Epicatechin, ECG: Epicatechin gallate, (-)-EGCG: Epigallocatechin gallate, (-)-EGC: Epigallocatechin, C3G: Cyanidin-3-O-glucoside, C3Ga: Cyanidin-3-O-galactoside, C3R: Cyanidin-3-O-rutinoside, P3G: Pelargonidin 3-O-glucoside, M3Ga: Malvidin-3-O-galactoside, M3G: Malvidin-3-O-glucoside).

Generally PG recovery % values of anthocyanins were higher than the other fractions. Cyanidin and C3R had the high PG recovery % values, on the other hand, C3G and C3Ga had the high OUT recovery % values. Although PG recovery % values were higher than IN recovery % values, there was no statistical difference between these two fractions for pelargonidin.

Tagliazucchi et al. (2010) reported that the results obtained with pure flavonoids like catechin and quercetin which were only slightly degraded in the mild alkaline environment of the intestine. But in our work the results were found to be different from this study. These differences could be explained by using Vitamin C standard curve in the study of Tagliazucchi et al. who correlated the concentration of Vitamin C and the results were calculated as milligrams of Vitamin C equivalent antioxidant capacity/ L in contrast to our work where Trolox standard curve was used. Besides, in their study ABTS method were different from the one in our study. Results obtained for catechin stability have shown a loss of catechin of less than 8% in simulated intestinal fluid (Tagliazucchi et al., 2010). In contrast, Bermúdez-Soto et al. (2007) found a loss of 58% of catechin similar to our work when incubated in simulated intestinal fluid. These differences could be explained by considering the interaction between catechin and digestive enzymes that could mask catechin and make it undetectable. Laurent et al. (2007) found a recovery of catechin after intestinal digestion of about 59% that increases to about 98% after extraction with acetonitrile that unmasks the flavanol bound with digestive enzymes.

Table 4.9 : Antioxidant activity distribution (%) between PG, IN and OUT fractions by ABTS method.

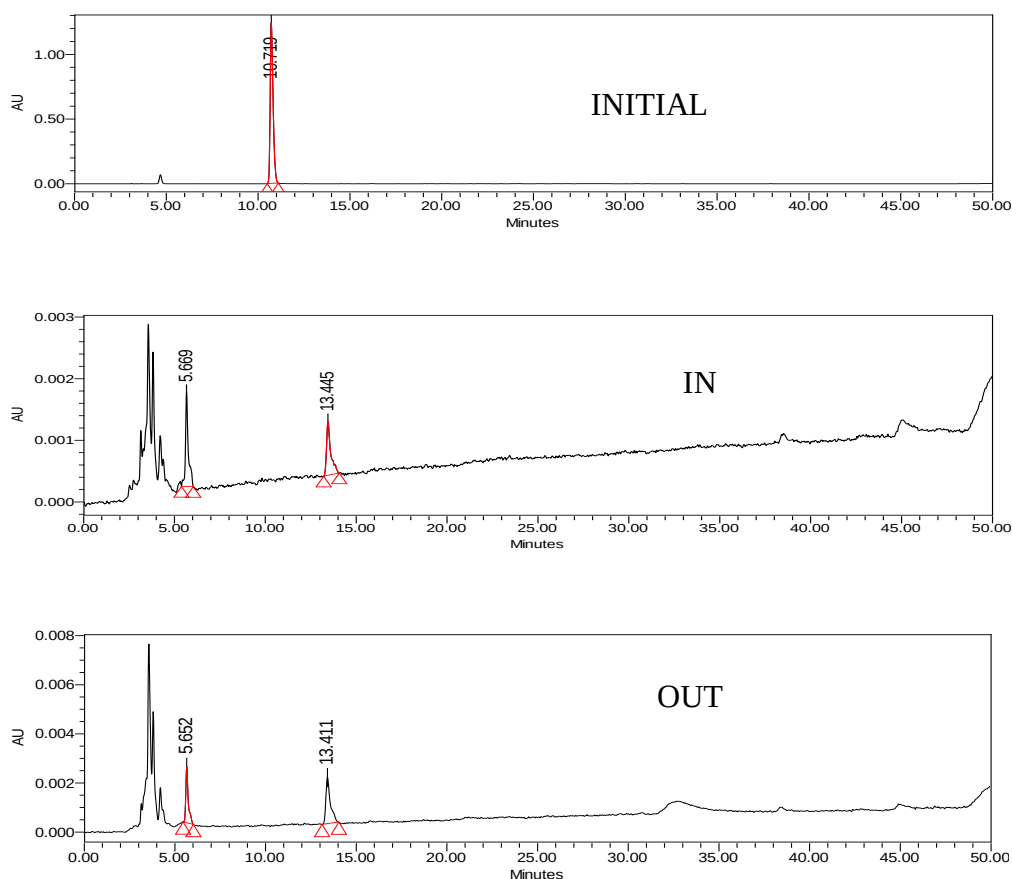
		INITIAL	IN	OUT	PG
ISOFLAVONES	DAIDZEIN	100.0	55.3	30.3	5.5
	DAIDZIN	100.0	50.5	36.5	28.9
	GENISTEIN	100.0	12.9	36.2	6.6
	GENISTIN	100.0	61.3	14.8	20.3
	GLYCITIN	100.0	39.8	72.6	7.0
PHENOLIC ACIDS	GALLIC ACID	100.0	5.2	14.0	102.4
	pHBA	100.0	63.0	70.1	223.7
	CAFEIC ACID	100.0	5.1	70.8	68.6
	VANILIC ACID	100.0	12.7	33.4	89.6
	P-COUMARIC ACID	100.0	19.2	35.0	95.6
	FERULIC ACID	100.0	22.4	26.5	80.1
	SYRINGIC ACID	100.0	13.8	78.9	188.2
	SINAPIC ACID	100.0	10.0	20.6	101.7
	PCA	100.0	14.2	246.7	126.4
FLAVONOOLS	QUERCETIN	100.0	7.7	178.7	9.2
	MYRICETIN	100.0	7.3	7.7	5.3
FLAVANONE	(±)- NG	100.0	17.0	54.6	30.7
CATECHINS	(+)- CH	100.0	24.9	25.6	189.8
	(-)- EC	100.0	13.6	3.3	110.0
	ECG	100.0	2.0	7.6	18.4
	(-)- EGCG	100.0	5.5	4.1	10.2
	(-)-EGC	100.0	12.7	22.1	69.7
ANTHOCYANINS	DELPHINIDIN	100.0	4.8	25.4	49.7
	CYANIDIN	100.0	13.8	15.5	94.9
	C3G	100.0	12.5	26.0	18.3
	C3Ga	100.0	14.7	24.7	15.4
	C3R	100.0	18.9	4.1	34.5
	PELARGONIDIN	100.0	286.3	628.1	2527.7
	P3G	100.0	36.4	4.1	70.2
	MALVIDIN	100.0	17.2	4.6	79.2
	M3Ga	100.0	21.3	62.9	98.1
	M3G	100.0	32.0	60.5	90.7

(pHBA:P-Hydroxybenzoic acid, PCA:Protocatechuic acid, (±)- NG:Naringenin, (+)- CH:Catechin hydrate, (-)- EC:Epicatechin, ECG:Epicatechin gallate, (-)-EGCG:Epigallocatechin gallate, (-)-EGC:Epigallocatechin, C3G:Cyanidin-3-O-glucoside, C3Ga:Cyanidin-3-O-galactoside, C3R:Cyanidin-3-O-rutinoside, P3G:Pelargonidin 3-O-glucoside, M3Ga:Malvidin-3-O-galactoside, M3G:Malvidin-3-O-glucoside).

4.3 Evaluating Bioavailability of Phenolic Compounds After GI Digestion By HPLC-PDA

Phenolic compounds were also evaluated by HPLC-PDA after GI digestion for PG, IN and OUT fractions and compared with the initial values to determine the bioavailability of samples. Data obtained are presented in Table 4.10.

Glycitin had the highest values for IN and OUT fractions when compared with IN and OUT fractions of other compounds (96.3 ± 3.2 ; 158.1 ± 14.1 $\mu\text{g/ml}$, respectively). In respect to PG fractions; (+)-catechin hydrate had the highest value as 109.3 ± 17.7 $\mu\text{g/ml}$. Generally PG values were found to be higher than IN and OUT fractions. On the other hand, epigallocatechin had very low value as 0.3 ± 0.1 ; 0.2 ± 0.0 $\mu\text{g/ml}$, for the IN and OUT fractions, respectively. When PG results were examined value of myricetin was found to be lower than other compounds as 0.8 ± 0.2 $\mu\text{g/ml}$. Moreover, compounds such as delphinidin, cyanidin, cyanidin-3-O-glucoside, cyanidin-3-galactoside, pelargonidin and malvidin could not be determined in IN, OUT and PG fractions. Besides, for quercetin, caffeic acid, myricetin, catechin hydrate, EGC, C3R, P3G, M3Ga and M3G compounds, the peaks could only be detected at PG fraction.



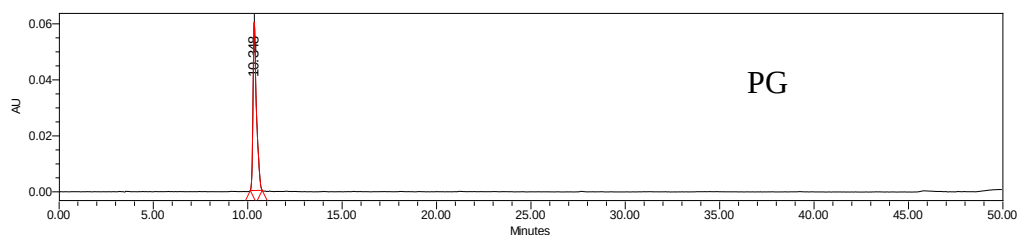
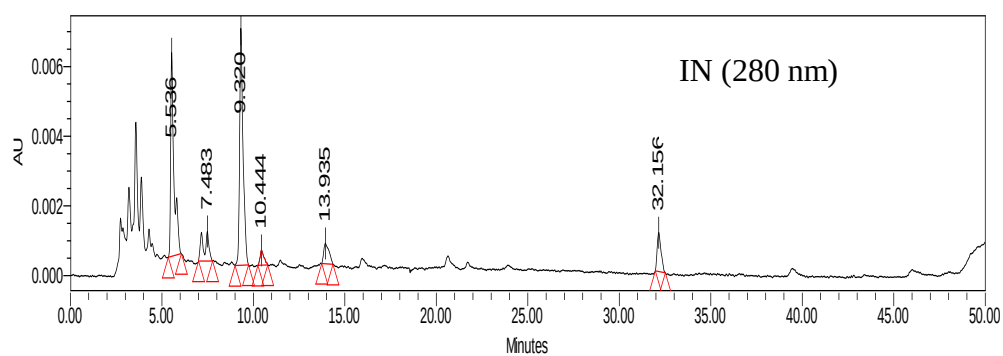
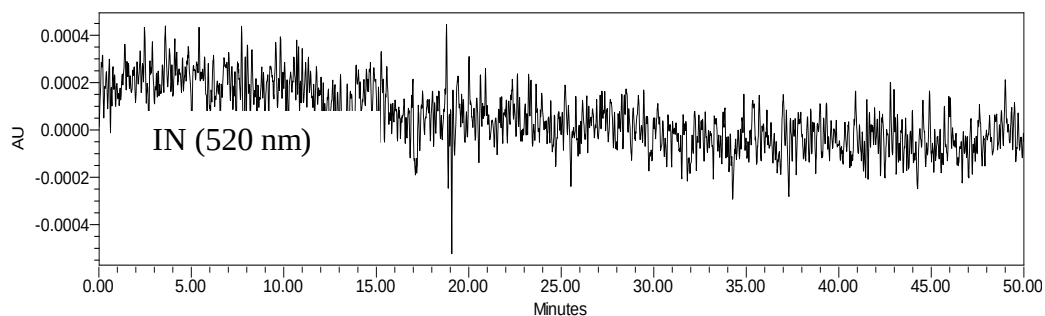
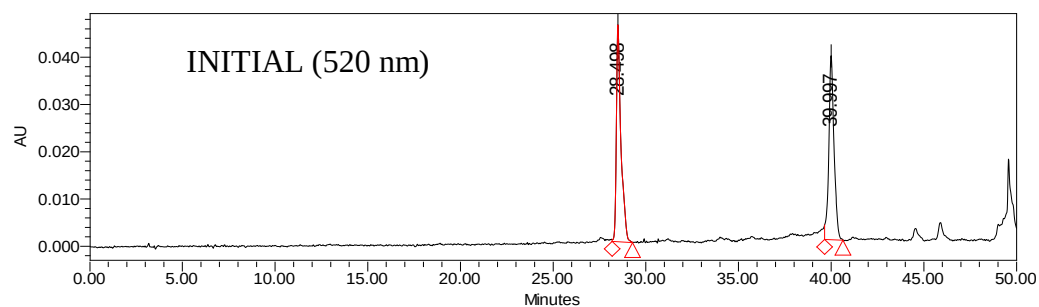


Figure 4.17: Chromatograms obtained by HPLC for catechin hydrate compound after digestion.

Catechin hydrate were detected in initial and PG samples but could not be detected for IN and OUT samples but two peaks were detected (RT:5.6 and RT:13.4 min) (Figure 4.17).



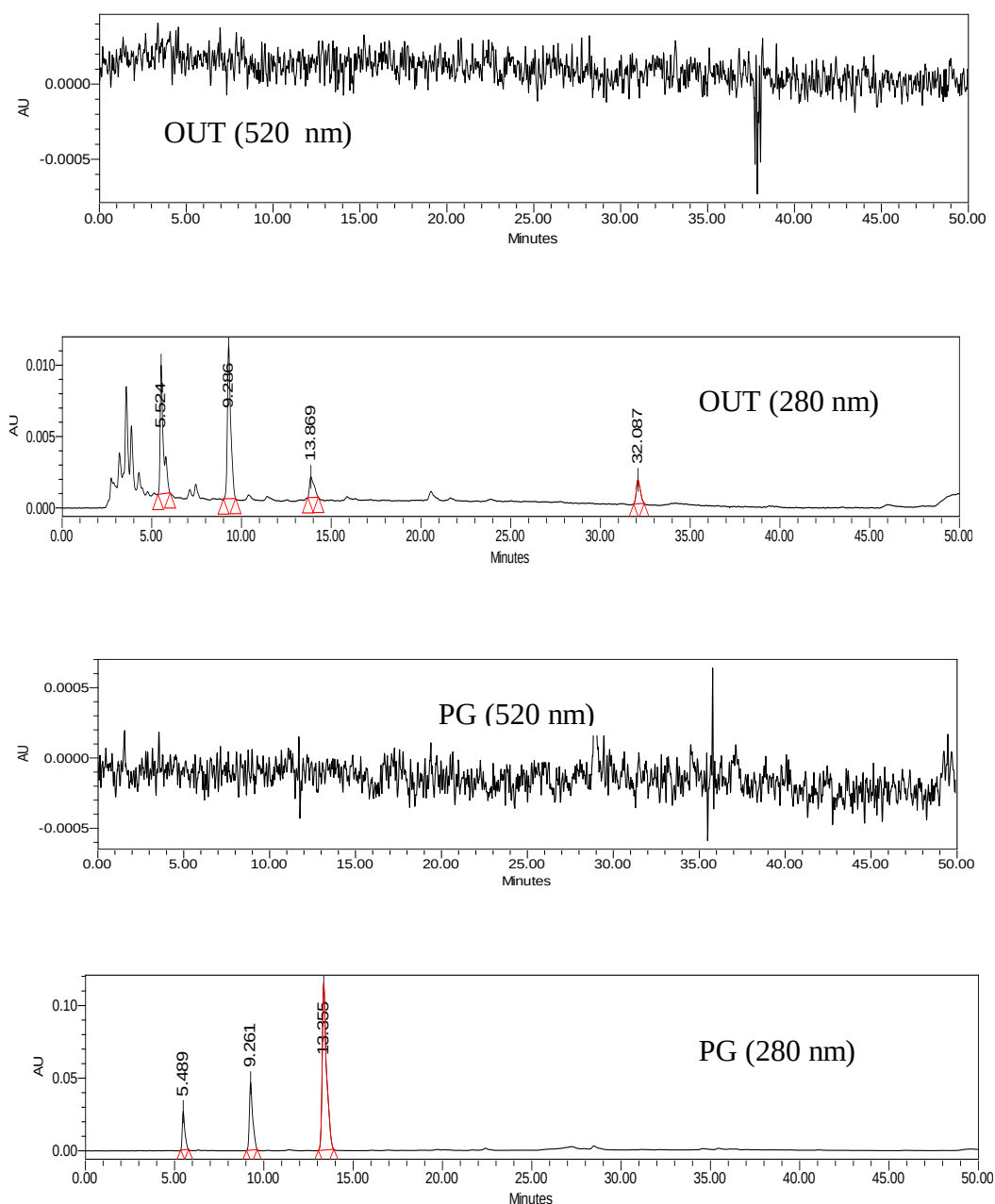
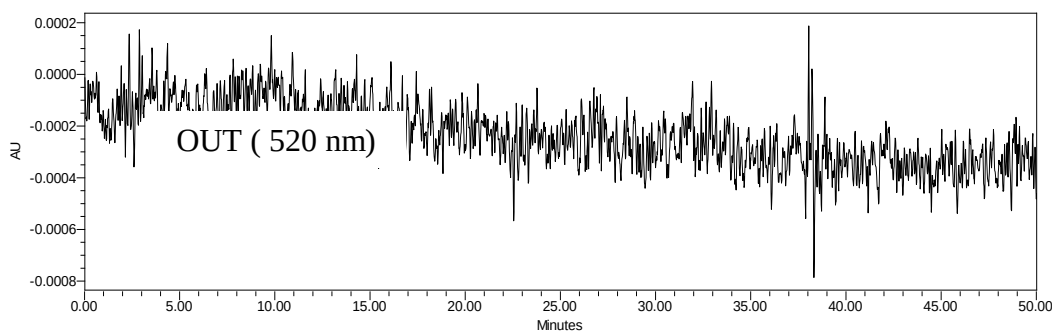
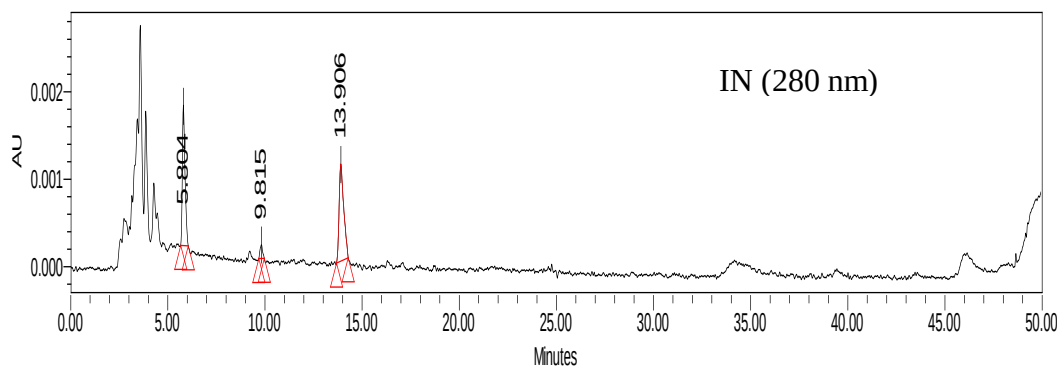
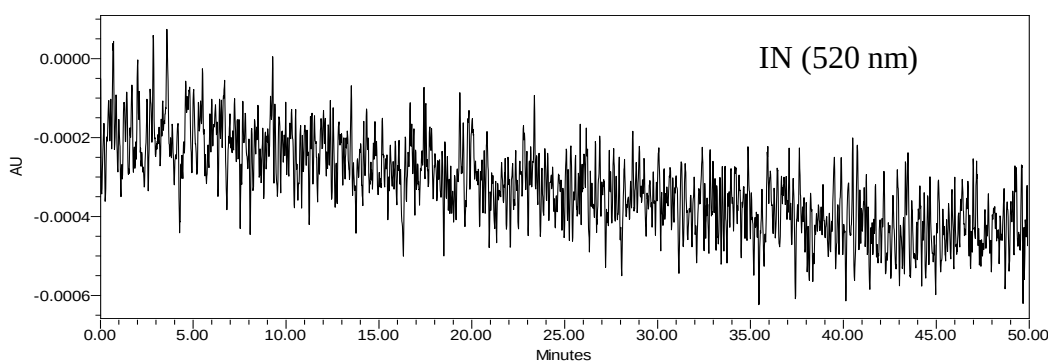
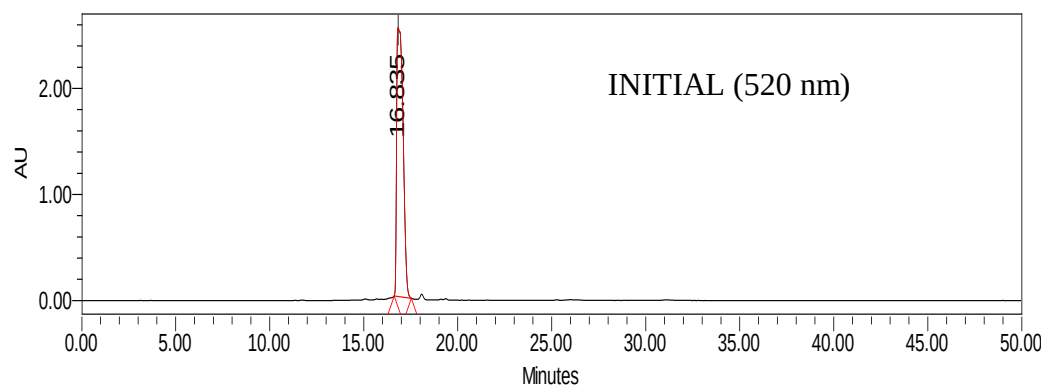


Figure 4.18: Chromatograms obtained by HPLC for pelargonidin compound after digestion.

According to HPLC results; generally anthocyanins showed that they could not be detected in IN, OUT and PG samples at 520 nm. But the peaks were detected when the samples were examined at 280 nm (Figure 4.18). The reason for not determining any peak at 520 nm may be the conversion of anthocyanins to other phenolic compounds. Similar to the results of pelargonidin; delphinidin, cyanidin, cyanidin-3-O-glucoside and malvidin could not be determined in IN, OUT and PG fractions at 520 nm but determined at 280 nm as well.



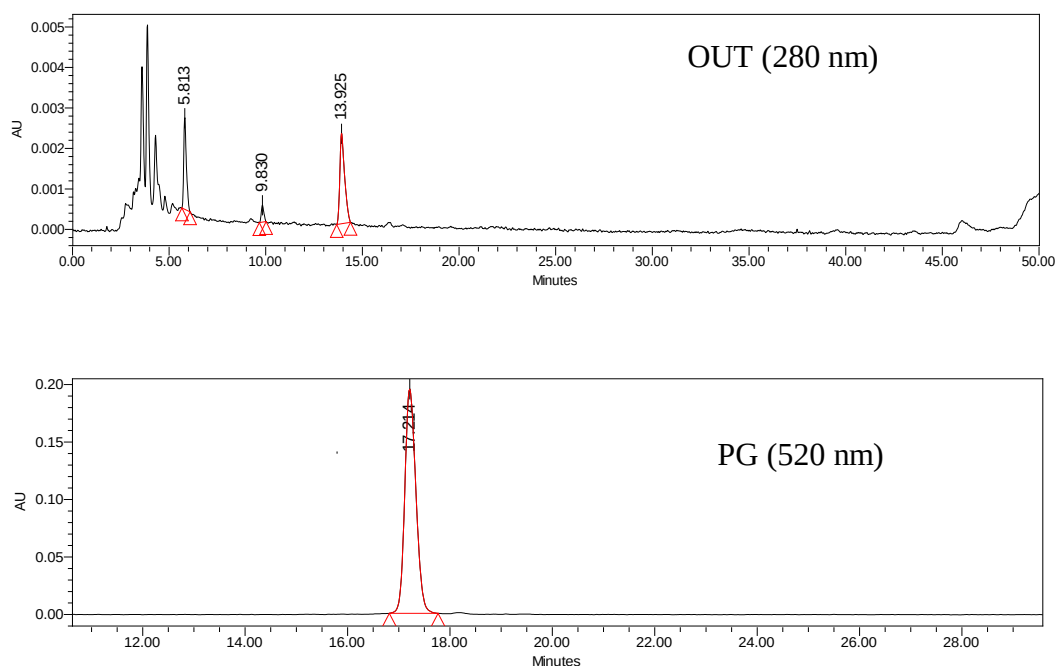


Figure 4.19: Chromatograms obtained by HPLC for cyanidin-3-galactoside compound after digestion.

In contrast to pelargonidin; cyanidin-3-galactoside could be detected in PG samples (58.7 $\mu\text{g/ml}$) at 520 nm but not determined in IN and OUT fractions. On the other hand, at 280 nm peaks were detected in IN and OUT samples (Rt: 5.8; 9.815; 13.9 min) (Figure 4.19).

According to Table 4.10, the recovery values obtained for each fraction was really low for the target phenolic compounds. But the important point is that those compounds are converted to some other phenolic compounds, still having some bioavailability as understood from the results obtained in the previous sections. In other words; total phenolic and antioxidant activities are available after digestion but not in the real form of the compound. As a future study it is necessary to identify the compounds formed during digestion especially using LC-MS/MS techniques.

If assumptions are to be made by just looking at the UV spectrum and retention times of the peaks obtained we can say that pelargonidin might be converted into catechin hydrate, to epigallocatechin and to epigallocatechin gallate. Besides, catechin hydrate might be converted into epigallocatechin gallate and to epicatechin. Moreover, cyanidin 3-O-galactoside might be converted into epigallocatechin and to epigallocatechin gallate.

Table 4.10: Phenolic profiles and contents analyzed by HPLC at each fraction after GI digestion.

				OTHER IDENTIFIED PEAKS				
	IN ($\mu\text{g/ml}$)	OUT ($\mu\text{g/ml}$)	PG ($\mu\text{g/ml}$)	PEAKS	RT (min)	IN (Area)	OUT (Area)	PG (Area)
DAIDZEIN	22.3 $\pm$ 1.9	22.8 $\pm$ 8.9	1.9 $\pm$ 0.6					
DAIDZIN	13.7 $\pm$ 0.8	16.1 $\pm$ 2.3	11.9 $\pm$ 1.6					
GENISTEIN	18.7 $\pm$ 2.4	20.0 $\pm$ 5.7	1.8 $\pm$ 0.1	UP1 (280nm)	13.8	17599	42692.5	ND
GENISTIN	7.6 $\pm$ 0.3	9.4 $\pm$ 0.1	5.4 $\pm$ 0.2	UP1 (280nm)	13.8	17934.5	39596	ND
GLYCITIN	96.3 $\pm$ 3.2	158.1 $\pm$ 14.1	26.1 $\pm$ 0.3	UP1 (280 nm)	13.5	20062	47079	ND
				UP2 (280 nm)	5.6	19650	28559	ND
GALLIC ACID	0.4 $\pm$ 0.0	0.8 $\pm$ 0.1	58.7 $\pm$ 1.8	UP1 (280 nm)	5.5	14158	21800	ND
				UP2 (280 nm)	13.3	9281	30514	ND
pHBA	20.2 $\pm$ 3.9	23.0 $\pm$ 4.7	65.4 $\pm$ 5.3					
CAFEIC ACID	ND	ND	42.9 $\pm$ 0.5	UP1 (280 nm)	5.6	8038	17296.5	ND
				UP2 (280 nm)	13.4	5771	21876	ND
				UP3 (280 nm)	44.8	10818	ND	ND
VANILIC ACID	15.4 $\pm$ 0.6	16.8 $\pm$ 1.4	70.3 $\pm$ 11.0	UP1 (280 nm)	5.8	14156	25951	ND
				UP2 (280 nm)	13.9	15249.5	43715	ND
P-COUMARIC ACID	14.8 $\pm$ 0.9	16.1 $\pm$ 1.5	76.5 $\pm$ 4.4					
FERULIC ACID	12.2 $\pm$ 2.6	13.5 $\pm$ 2.7	72.8 $\pm$ 0.0					
SYRINGIC ACID	21.1 $\pm$ 0.6	23.5 $\pm$ 0.5	65.2 $\pm$ 1.4					
SINAPIC ACID	2.1 $\pm$ 0.2	3.2 $\pm$ 0.1	45.6 $\pm$ 1.2	UP1 (280 nm)	5.8	15463.5	25335.5	ND
				UP2 (280 nm)	13.9	16558	43577	ND
				UP3 (280 nm)	28.5	23298	17348.5	ND
PCA	0.9 $\pm$ 0.2	0.5 $\pm$ 0.0	64.8 $\pm$ 4.9					
QUERCETIN	ND	ND	2.4 $\pm$ 0.0	UP1 (280 nm)	13.3	13349.5	24988	ND
				UP2 (280 nm)	5.6	14509	15367.5	ND
				UP3 (312 nm)	25.1	3795	3651	ND
				UP4 (280 nm)	11.5	ND	ND	4162
				UP5 (280 nm)	14.4	ND	ND	5763.5
				UP6 (280 nm)	32.4	ND	ND	6248
				UP7 (360 nm)	22.2	ND	ND	5030
				UP8 (312 nm)	32.4	ND	ND	9039.5
MYRICETIN	ND	ND	0.8 $\pm$ 0.2	UP9 (312 nm)	14.4	ND	ND	4429
				UP1 (280 nm)	5.9	12619	22546.5	ND
				UP2 (280 nm)	7.2	8487	22521.5	ND
				UP3 (280 nm)	14.1	11674.5	34896.5	ND
(±)- NG	4.2 $\pm$ 1.1	5.6 $\pm$ 0.4	8.0 $\pm$ 0.4	UP4 (312 nm)	10.2	ND	ND	15616
				UP1 (280 nm)	14.0	11786	42202	ND
(+) - CH	ND	ND	109.3 $\pm$ 17.7	UP2 (280 nm)	5.6	ND	25229	ND
				UP1 (280 nm)	14.0	14003.5	32791	ND
(-) - EC	2.0 $\pm$ 0.3	5.3 $\pm$ 0.0	52.4 $\pm$ 6.8	UP2 (280 nm)	5.6	16756	22051	ND
ECG	0.3 $\pm$ 0.1	0.2 $\pm$ 0.0	8.4 $\pm$ 0.7	UP1 (280 nm)	5.8	12026	19889.5	ND
				UP1 (280 nm)	5.8	17329	23432	ND
(-) - EGCG	1.2 $\pm$ 0.1	2.8 $\pm$ 0.2	37.0 $\pm$ 0.8	UP2 (280 nm)	14.0	19858.5	37777	ND
				UP1 (280 nm)	5.8	14490.5	22207.5	ND
(-)EGC	ND	ND	20.9	UP1 (280 nm)	5.8	18598	27370	ND
				UP2 (280 nm)	5.8	18598	27370	ND
				UP3 (280 nm)	14.0	19927	42791	ND

DELPHINIDIN	ND	ND	ND	UP1 (280 nm)	5.8	14516	25726.5	367160.5
CYANIDIN	ND	ND	ND	UP1 (280 nm)	5.8	15103	20922	104367
				UP2 (280 nm)	13.9	16770	33330	238660
C3G	ND	ND	ND	UP1 (280 nm)	10.0	4099	2812	ND
				UP2 (280 nm)	13.3	17371	39116	ND
				UP3 (280 nm)	18.1	ND	ND	1852486
C3Ga	ND	ND	70.3	UP1 (280 nm)	5.8	15155	21654	ND
				UP2 (280 nm)	9.8	1408	3904	ND
				UP3 (280 nm)	13.9	18375	38343	ND
C3R	ND	ND	46.0	UP1 (280 nm)	5.7	15726	24856	ND
				UP2 (280 nm)	12.1	8821	12951	ND
PELARGONIDIN	ND	ND	ND	UP1 (280 nm)	5.5	73059	113009	265053
				UP2 (280 nm)	9.3	88569	138786	596798
				UP3 (280 nm)	13.9	11786	25491	1916589
				UP4 (280 nm)	32.1	16484	26174	ND
P3G	ND	ND	49.7	UP1 (280 nm)	5.7	29590	40301	ND
				UP2 (280 nm)	9.2	16232	18973	ND
				UP3 (280 nm)	13.8	16710	32284	ND
MALVIDIN	ND	ND	ND	UP1 (280 nm)	5.7	15225	23632	ND
				UP2 (280 nm)	13.0	48762	35706	738388
				UP3 (280 nm)	13.8	13147	97261	ND
M3Ga	ND	ND	58.7	UP1 (280 nm)	11.2	18245	22653	ND
				UP2 (280 nm)	13.3	19675	38711	ND
M3G	ND	ND	37.4	UP1 (280 nm)	5.6	18388	25604	ND
				UP2 (280 nm)	8.1	3141	4867	ND
				UP3 (280 nm)	8.9	5281	9013	ND
				UP4 (280 nm)	12.6	14888	20117	ND
				UP5 (280 nm)	13.4	20655	41176	ND

Table 4.11: Phenolic content profile distribution (%) between PG, IN AND OUT fractions by HPLC-PDA method.

		INITIAL	% IN	% OUT	% PG
ISOFLAVONES	DAIDZEIN	100.0	1.1	1.1	0.1
	DAIDZIN	100.0	0.7	0.8	0.6
	GENISTEIN	100.0	0.9	1.0	0.1
	GENISTIN	100.0	0.4	0.5	0.3
	GLYCITIN	100.0	4.8	7.9	1.3
PHENOLIC ACIDS	GALLIC ACID	100.0	0.0	0.0	2.9
	pHBA	100.0	1.0	1.2	3.2
	CAFEIC ACID	100.0	ND	ND	2.1
	VANILIC ACID	100.0	0.8	0.8	3.5
	P-COUMARIC ACID	100.0	0.7	0.8	3.8
	FERULIC ACID	100.0	0.6	0.7	3.6
	SYRINGIC ACID	100.0	1.1	1.2	3.3
	SINAPIC ACID	100.0	0.1	0.2	2.3
	PCA	100.0	0.0	0.0	3.2
FLAVANOLS	QUERCETIN	100.0	ND	ND	0.1
	MYRICETIN	100.0	ND	ND	0.0
FLAVANONE	(±)- NG	100.0	0.2	0.3	0.4
CATECHINS	(+)- CH	100.0	ND	ND	5.5
	(-)- EC	100.0	0.1	0.3	2.6
	ECG	100.0	0.0	0.0	0.4
	(-)- EGCG	100.0	0.1	0.1	1.9
	(-)EGC	100.0	ND	ND	1.0
ANTHOCYANINS	DELPHINIDIN	100.0	ND	ND	ND
	CYANIDIN	100.0	ND	ND	ND
	C3G	100.0	ND	ND	ND
	C3Ga	100.0	ND	ND	3.5
	C3R	100.0	ND	ND	2.3
	PELARGONIDIN	100.0	ND	ND	ND
	P3G	100.0	ND	ND	2.5
	MALVIDIN	100.0	ND	ND	ND
	M3Ga	100.0	ND	ND	2.9
	M3G	100.0	ND	ND	1.9

(ND: Not Detected).

4.4 The Relations between Total Phenolic and Total Antioxidant Activity

Methods

The relations between all total antioxidant activity methods (CUPRAC, DPPH and ABTS) and total phenolic content analysis were evaluated by basic linear regression analysis. The correlation coefficients are given in Table 4.12. And ANOVA table of regression variance analysis is presented in APPENDIX B.

Table 4.12: Regression analysis for total phenolic content and total antioxidant activity methods.

Methods	Folin Ciocalteu method	CUPRAC	DPPH	ABTS
FOLIN	-	-	-	-
CUPRAC	0.763**	-	-	-
DPPH	0.552**	0.736**	-	-
ABTS	0.652**	0.728**	0.566**	-

** The regression variance analysis for the results is significant, statistically ($p < 0.01$).

According to the Table 4.12, there was an important relation between total phenolic content and all of total antioxidant activity methods, CUPRAC ($r=0.763$), DPPH ($r=0.552$) and ABTS ($r=0.652$), ($p < 0.01$). The relation between CUPRAC and DPPH, CUPRAC and ABTS were also statistically significant ($p < 0.01$). There was weak relation (0.566) between ABTS and DPPH, but according to statistical evaluation it was significant again.

Between the antioxidant analysis, CUPRAC method gave higher values for antioxidant activity in many phenolic standards. The reason for this situation; the redox reaction producing colored species is carried out at nearly physiological pH (pH 7 of ammonium acetate buffer) as opposed to the unrealistic acidic conditions (pH 3.6) of FRAP. Besides, the method can simultaneously measure hydrophilic as well as lipophilic antioxidants (e.g., R-tocopherol) (Apak et al., 2004).

5. CONCLUSIONS AND RECOMMENDATIONS

In this study, bioavailability and antioxidant capacity of anthocyanins, isoflavones, phenolic acids and flavanoids were compared with each other after digestion by using *in vitro* models. By the data and knowledge obtained throughout this research hopefully it has been a useful study for the food science area.

For pure phenolic standards; total phenolic contents and total antioxidant activities were determined successfully. Moreover, *in vitro* digestion was performed for phenolic compounds. Total phenolic content and total antioxidant activity by CUPRAC, DPPH, ABTS assays were carried out for digested samples to evaluate bioavailability. The results showed that anthocyanins, phenolic compounds, and their antioxidant activity changed at different conditions of digestion.

Phenolic compounds and anthocyanins were found to be stable in gastric digestion due to acidic conditions. Anthocyanins presented lower bioavailability than phenolic compounds in IN condition representing the compound passed into serum.

Total phenolic compounds ranged between 1901.2-178.2 mg GAE/g standard. The results approximately showed that the order of phenolic content is in the following order: flavonols > anthocyanins > catechins > naringenin > phenolic acids > isoflavones due to the chemical structure.

When total antioxidant activity was evaluated by DPPH, CUPRAC and ABTS methods, gallic acid showed the highest activity. Within the phenolic groups the general order of antioxidant capacity by DPPH method was as: Catechins > Flavanols > Phenolic Acids > Anthocyanins > Isoflavones > Naringenin.

In CUPRAC method, highest antioxidant capacities were observed for epicatechin gallate, epigallocatechin gallate, quercetin, epigallocatechin, catechin, caffeic acid, epicatechin, gallic acid. While pHBA showed the lowest antioxidant activity. In

accordance with theoretical expectations, the number and position of the hydroxyl groups, as well as the degree of conjugation of the whole molecule are important for easy electron transfer.

Total antioxidant activity by ABTS method for phenolic standards is in the order of Flavonols > Catechins > Phenolic Acids > Isoflavones > Anthocyanins and pelargonidin had the lowest value as observed similarly in other total antioxidant activity methods.

When the antioxidant activity method performances are evaluated between the phenolic compounds it was evident that CUPRAC method had given higher responses for anthocyanins. On the other hand, ABTS method gave better results for isoflavones while DPPH was really unsuccessful for isoflavones.

When the samples are evaluated after GI digestion, isoflavones except for genistein gave the lowest responses for total phenolic content, while the responses of delphinidin, myricetin and cyanidin-3-glucoside was highest. For antioxidant assays; DPPH method gave lower responses especially for isoflavones while CUPRAC method was found to give higher responses for wider range of polyphenols. After GI digestion, for all polyphenols except for isoflavones, PG had higher total phenolic content and antioxidant analysis, while for isoflavones OUT and IN fractions were higher than PG. For all analysis after digestion, pelargonidin and (+)catechin gave highest results (2 to 20-fold increase), especially in PG fraction, showing that they are converted into different compounds with higher responses. Among isoflavones; genistein, among phenolic acids; protocatechuic acid, among flavonoids; (+)catechin and among anthocyanins; pelargonidin was found to be more bioavailable after digestion.

As a result, antioxidant activity and bioavailability differs greatly between polyphenols (PP) due to structure, stability and solubility. As method responses against targeted phenolic compounds are determined, proper method selection will be possible and useful knowledge about more bioavailable forms of PP is obtained.

The correlation between total phenolic and total antioxidant activity methods by CUPRAC, DPPH, ABTS analysis were also evaluated statistically. There was an

important relation between total phenolic content and all of the total antioxidant activity methods performed.

When phenolic contents and profiles were investigated by HPLC-PDA for PG, IN and OUT fractions obtained after GI digestion recovery values for calculated for each fraction to understand bioavailability. Glycitin had the highest values in IN and OUT fractions when compared with the other compounds' values in IN and OUT fractions. In respect to PG fractions; (+)- catechin hydrate had the highest value. Generally PG values were a little bit higher than IN and OUT fractions. Moreover, for some compounds such as delphinidin, cyanidin, cyanidin-3-O-glucoside, cyanidin-3-galactoside, pelargonidin and malvidin no peak could be detected in IN, OUT and PG fractions. Besides, for quercetin, caffeic acid, myricetin, catechin hydrate, EGC, C3R, P3G, M3Ga and M3G compounds peaks could be obtained only in PG fractions. However interestingly at other wavelengths than the targeted compound's spectrum new peaks were observed possibly showing that conversions during digestion might have taken place. For further evaluation; HPLC-MS/MS detections should be performed to see the conversions or metabolites that are formed during digestion of phenolic compounds.

As the future study, further work has to be carried out to determine the bioavailability of anthocyanins and other phenolic compounds and their protective effect in humans health. Because, metabolites formed during digestion may exert different bioavailability than expected.

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APPENDICES

APPENDIX A: Calibration Curves

APPENDIX B: Anova Tables

APPENDIX A

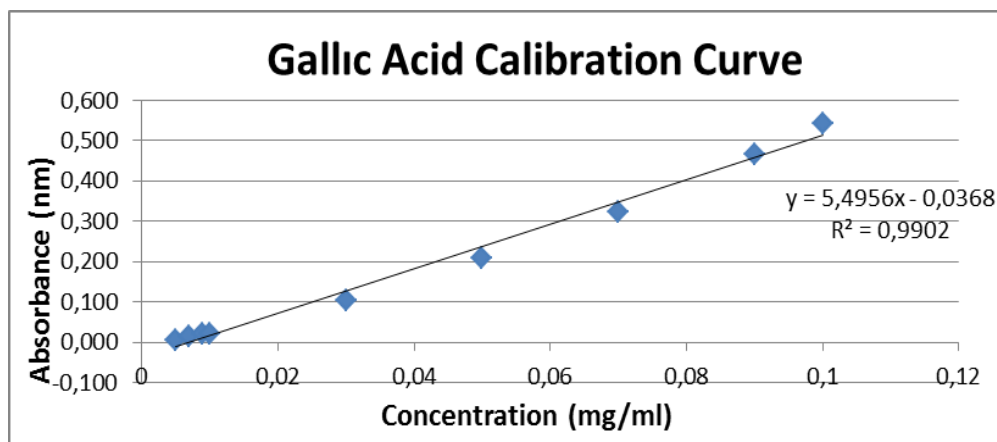


Figure A.1: Standard calibration curve of gallic acid.

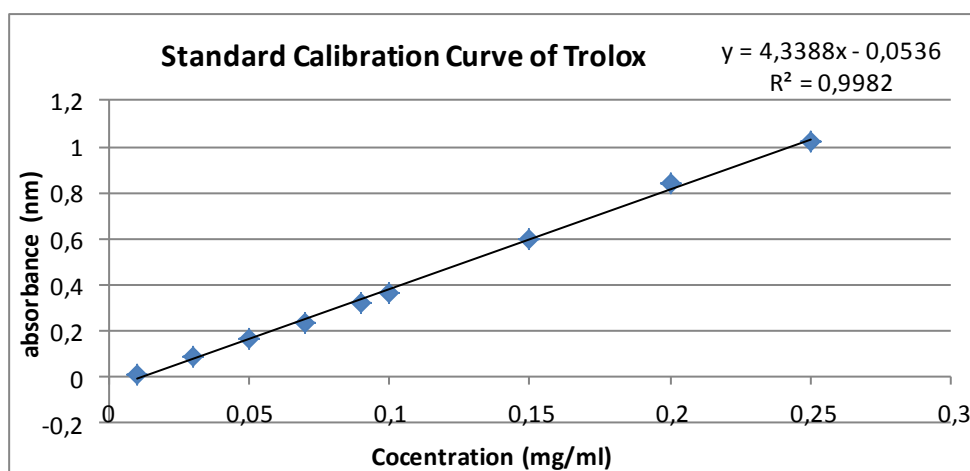


Figure A.2 : Standard calibration curve of Trolox for DPPH method.

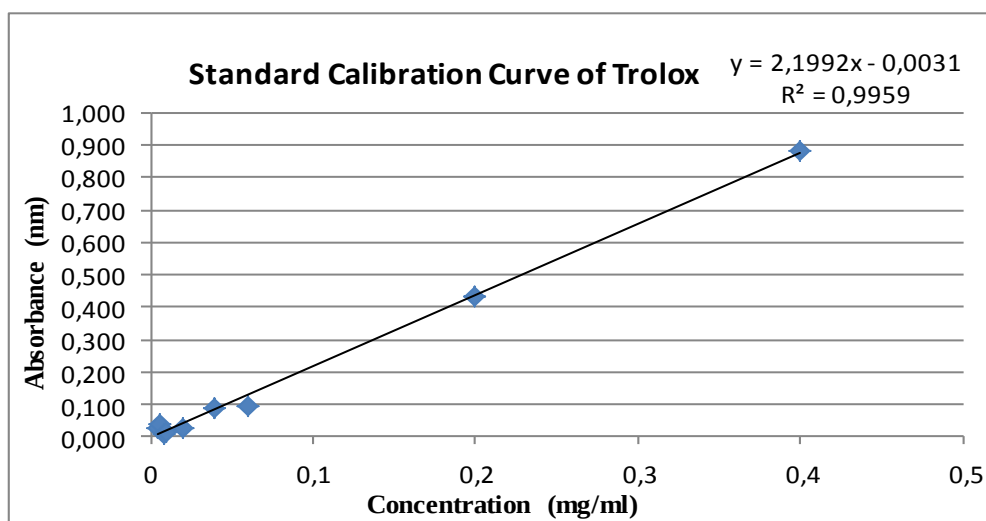


Figure A.3 : Standard calibration curve of Trolox for CUPRAC method.

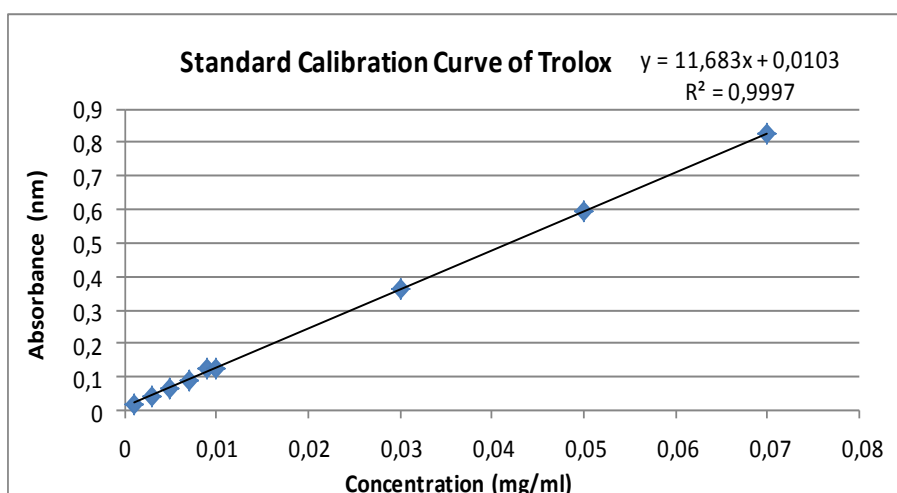


Figure A.4 : Standard calibration curve of Trolox for ABTS method.

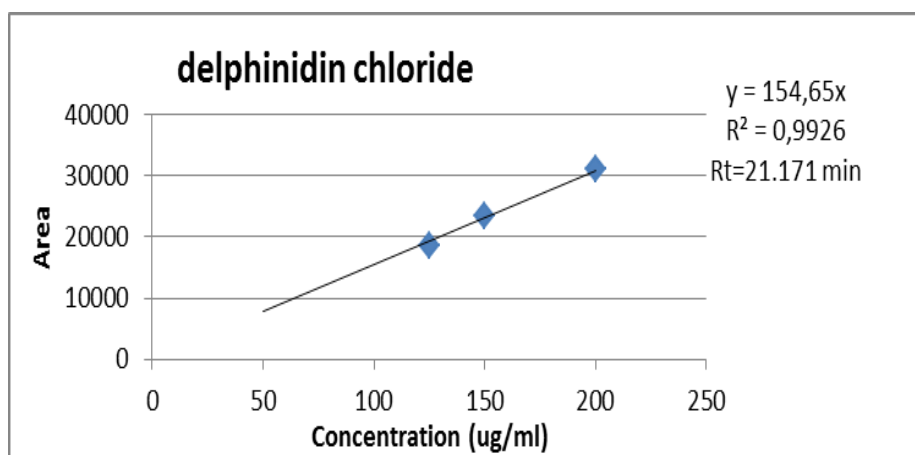


Figure A.5: Standard calibration curve of delphinidin chloride for HPLC.

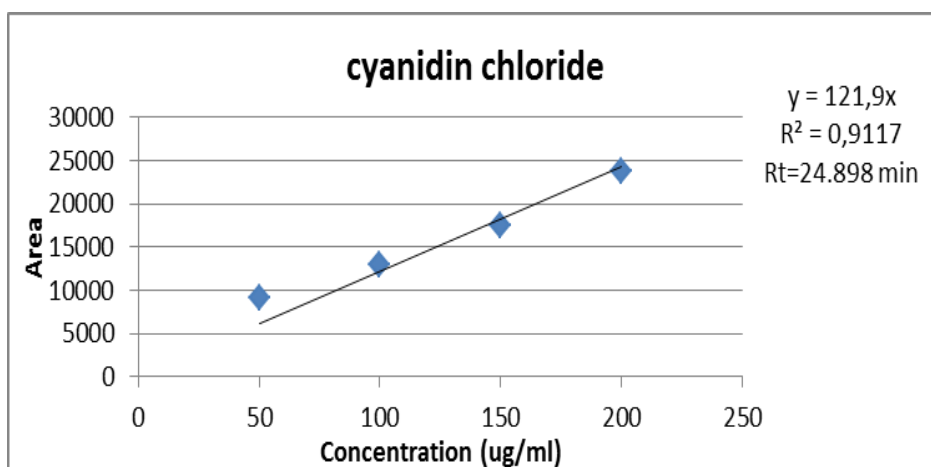


Figure A.6: Standard calibration curve of cyanidin chloride for HPLC.

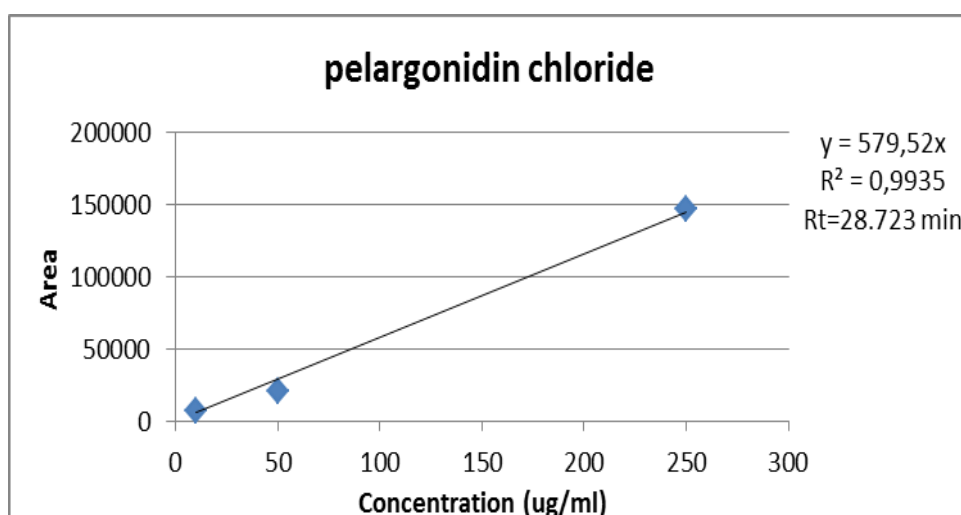


Figure A.7: Standard calibration curve of pelargonidin chloride for HPLC.

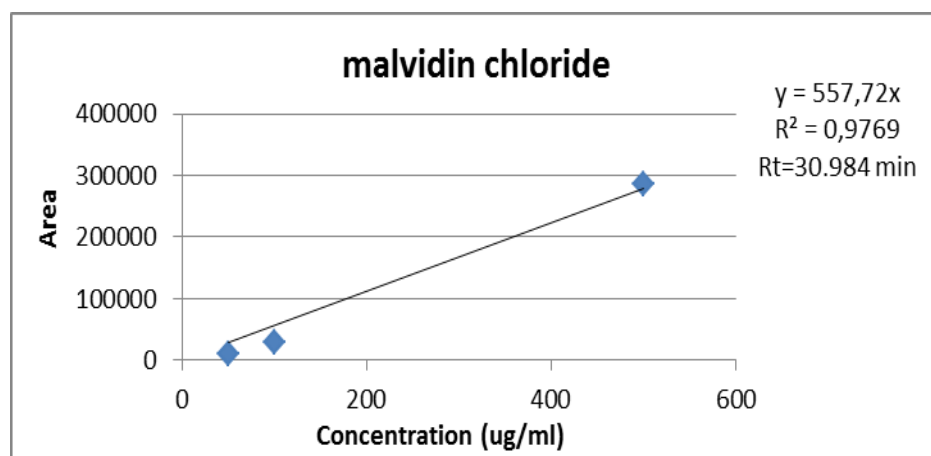


Figure A.8: Standard calibration curve of malvidin chloride for HPLC.

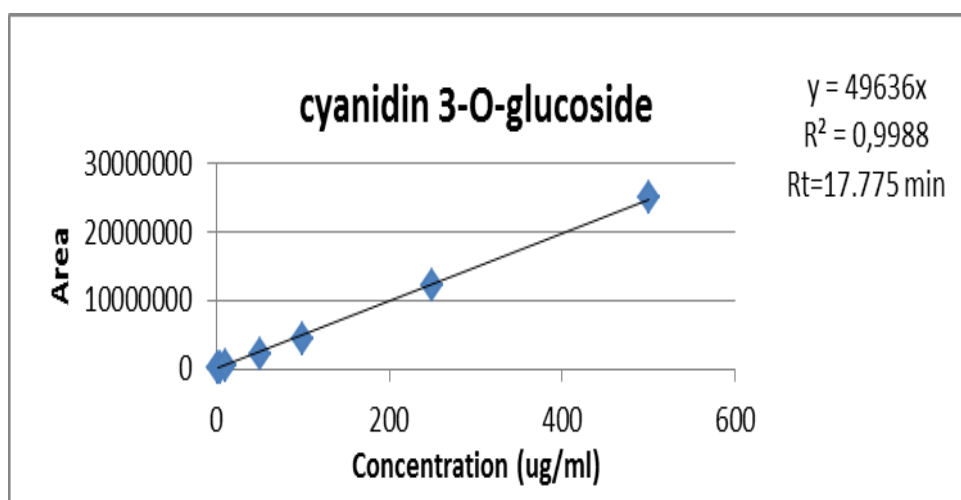


Figure A.9: Standard calibration curve of cyanidin3-O-glucoside for HPLC.

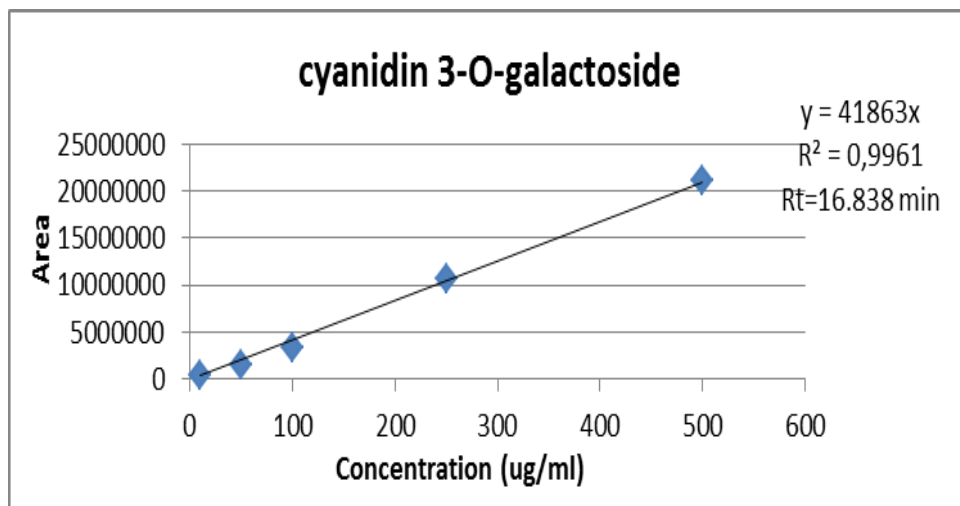


Figure A.10: Standard calibration curve of cyanidin 3-O-galactoside for HPLC.

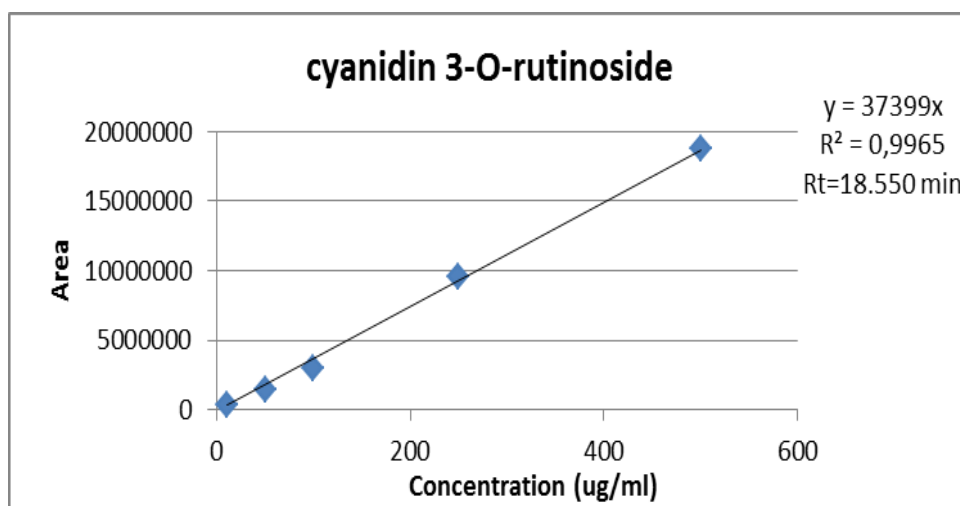


Figure A.11: Standard calibration curve of cyanidin 3-O-rutinoside for HPLC.

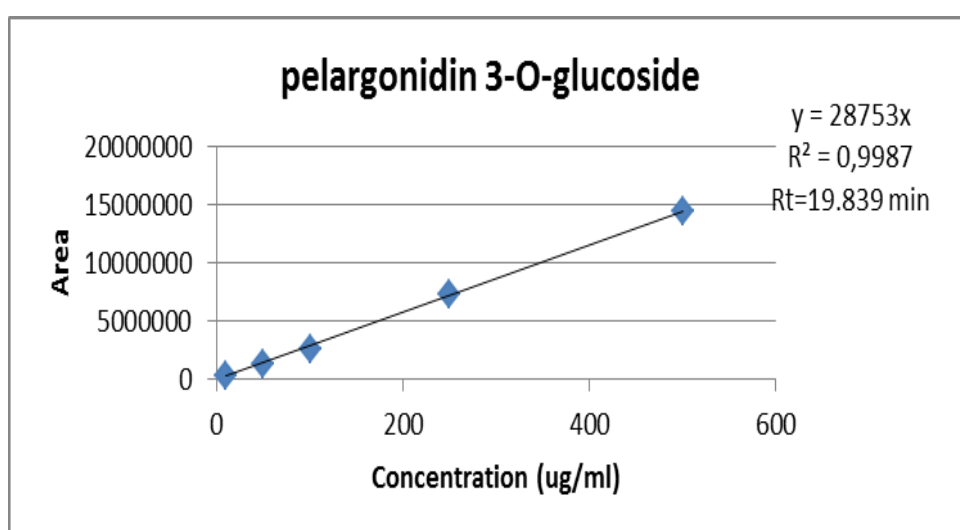


Figure A.12: Standard calibration curve of pelargonidin 3-O-glucoside for HPLC.

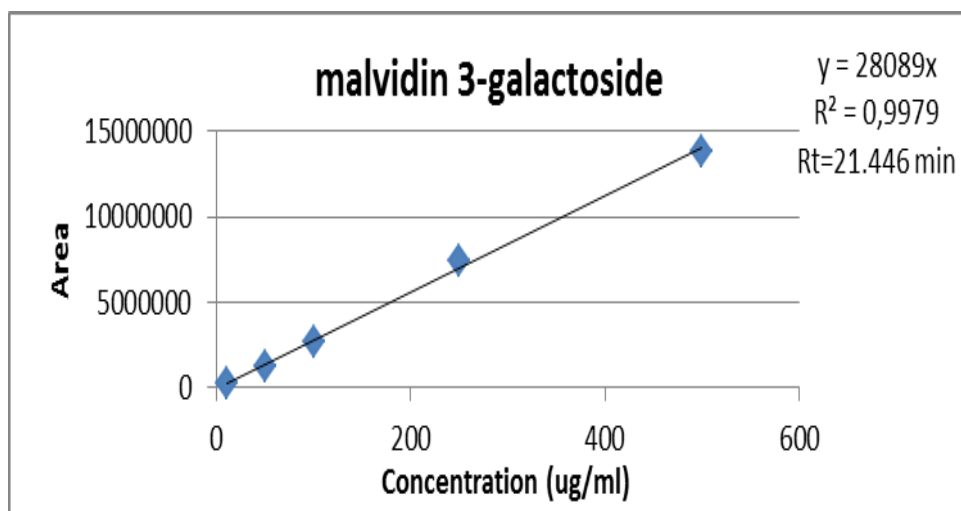


Figure A.13: Standard calibration curve of malvidin 3-O-galactoside for HPLC.

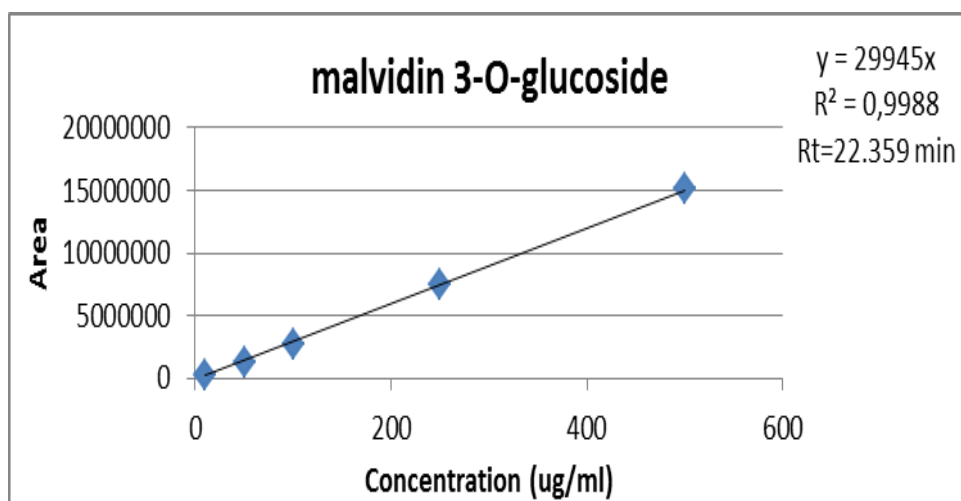


Figure A.14: Standard calibration curve of malvidin 3-O-glucoside for HPLC.

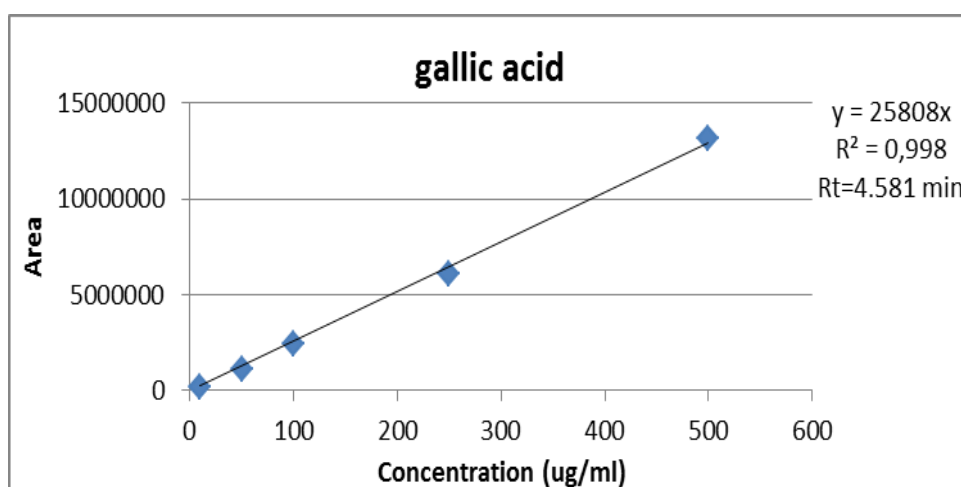


Figure A.15: Standard calibration curve of gallic acid for HPLC.

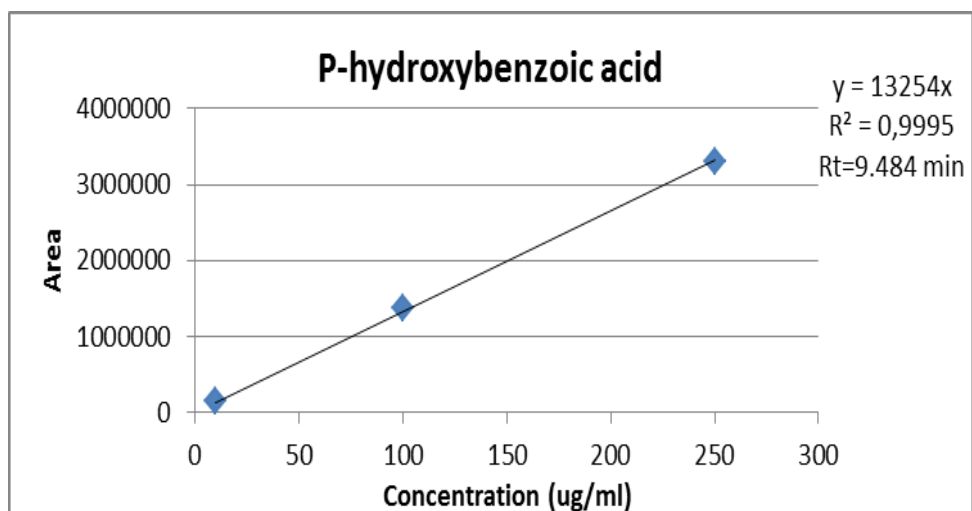


Figure A.16: Standard calibration curve of P-hydroxybenzoic acid for HPLC.

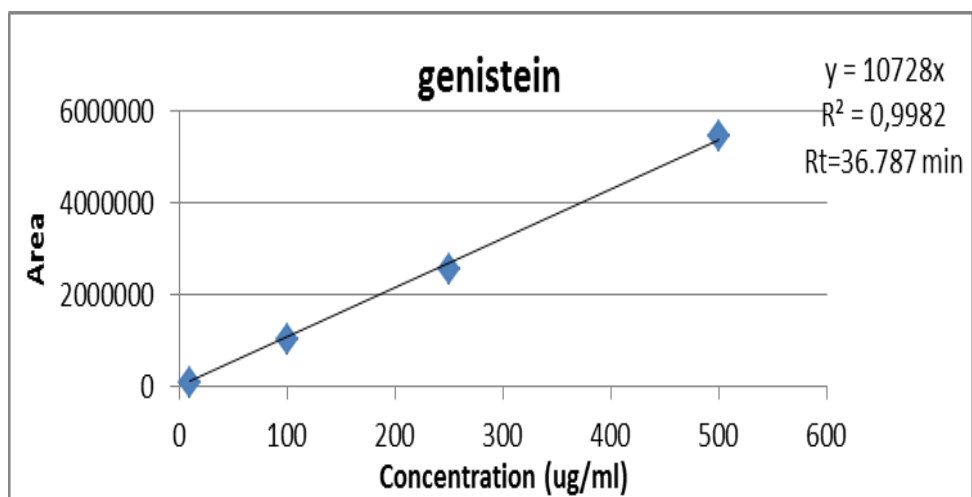


Figure A.17: Standard calibration curve of genistein for HPLC.

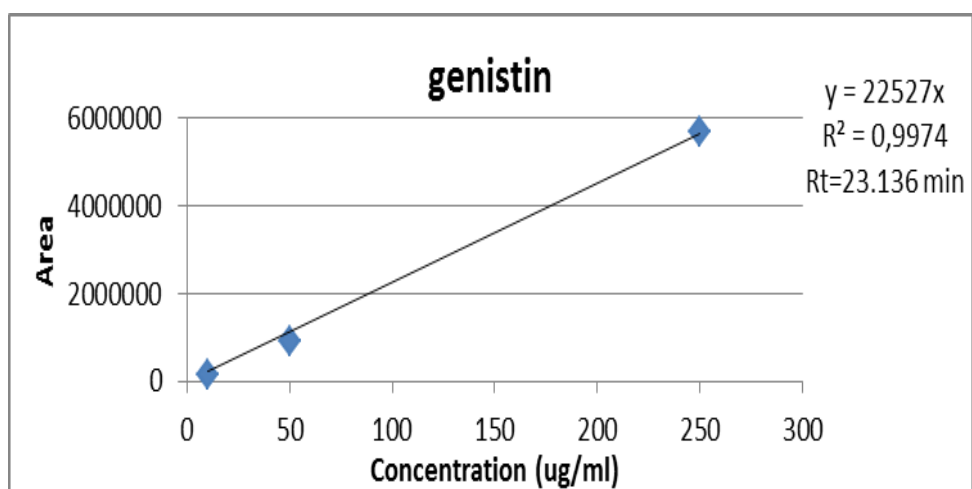


Figure A.18: Standard calibration curve of genistin for HPLC.

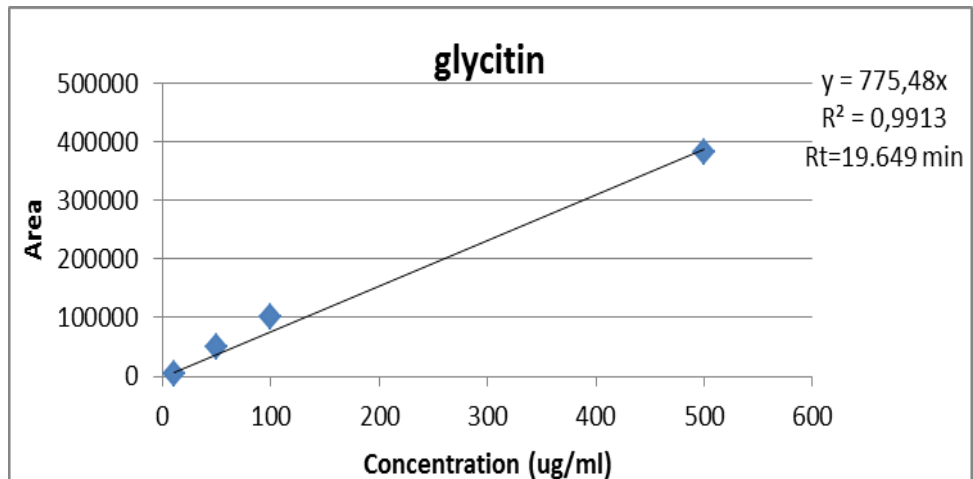


Figure A.19: Standard calibration curve of glycitin for HPLC.

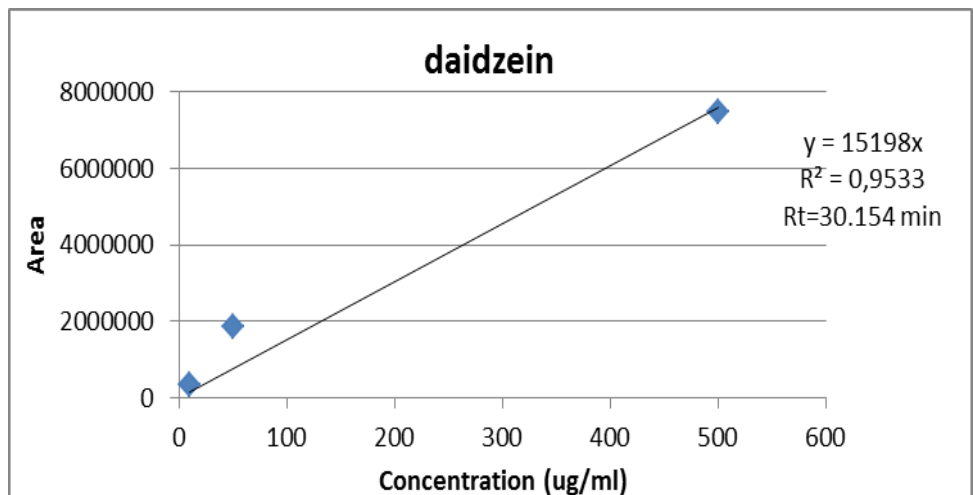


Figure A.20: Standard calibration curve of daidzein for HPLC.

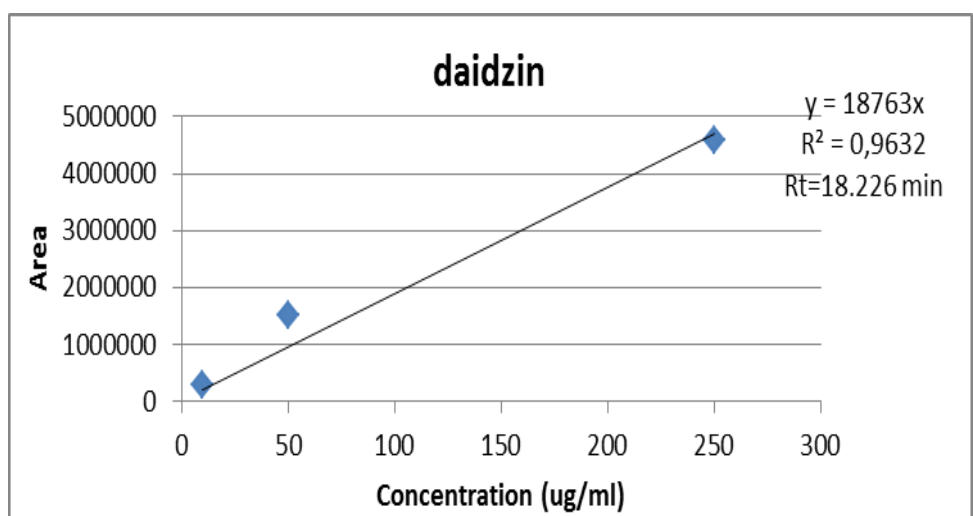


Figure A.21: Standard calibration curve of daidzin for HPLC.

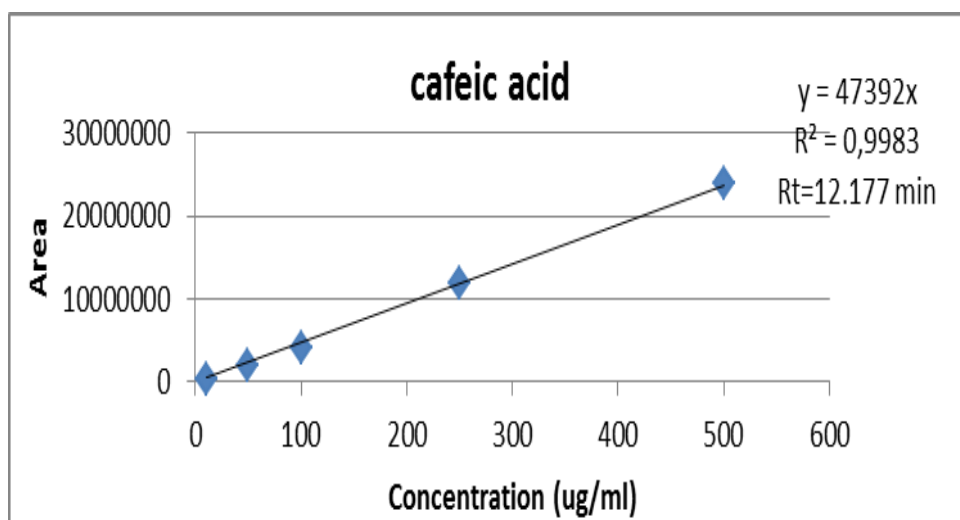


Figure A.22: Standard calibration curve of caffeic acid for HPLC.

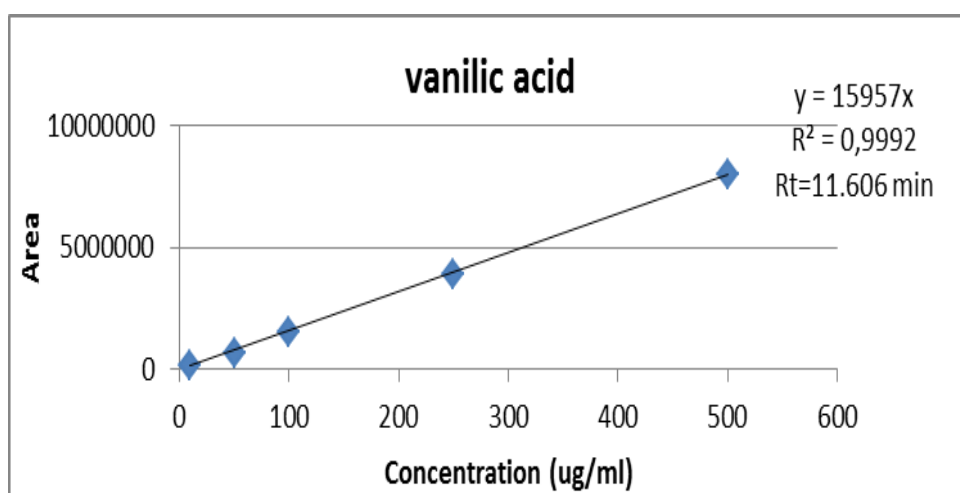


Figure A.23: Standard calibration curve of vanillic acid for HPLC.

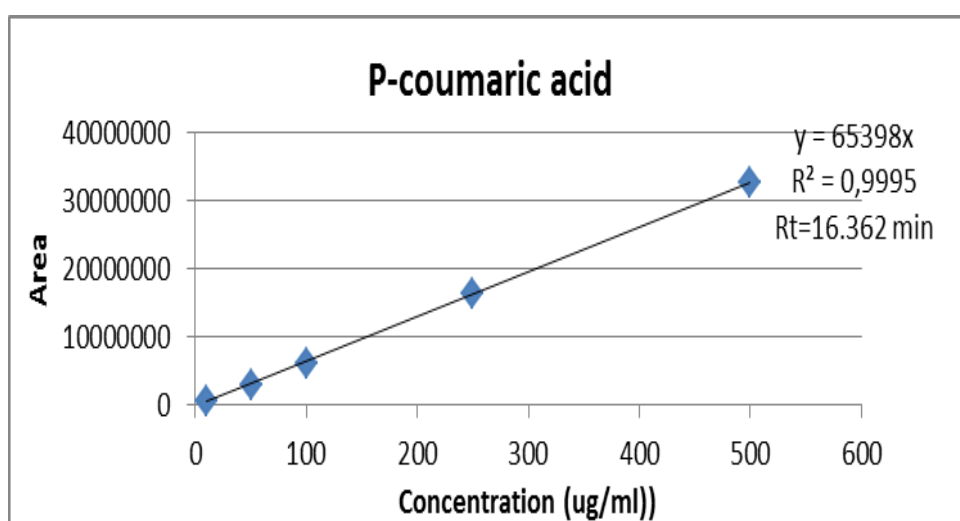


Figure A.24: Standard calibration curve of P-coumaric acid for HPLC.

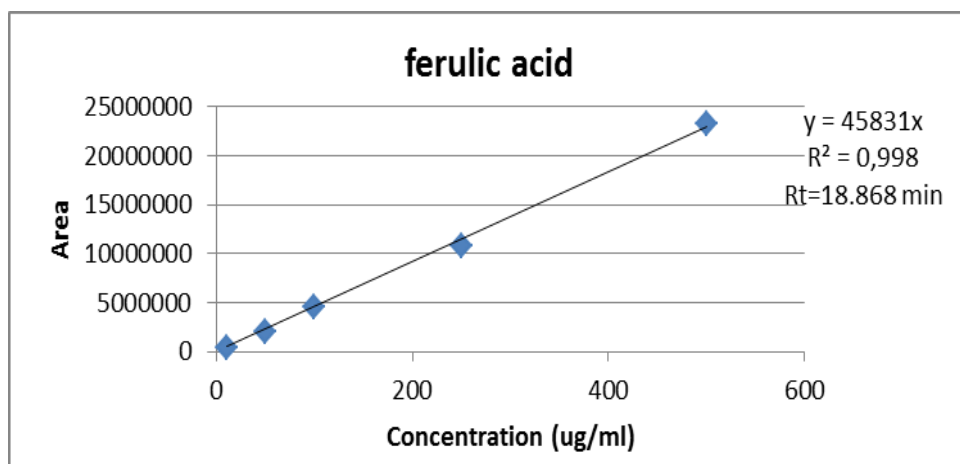


Figure A.25: Standard calibration curve of ferulic acid for HPLC.

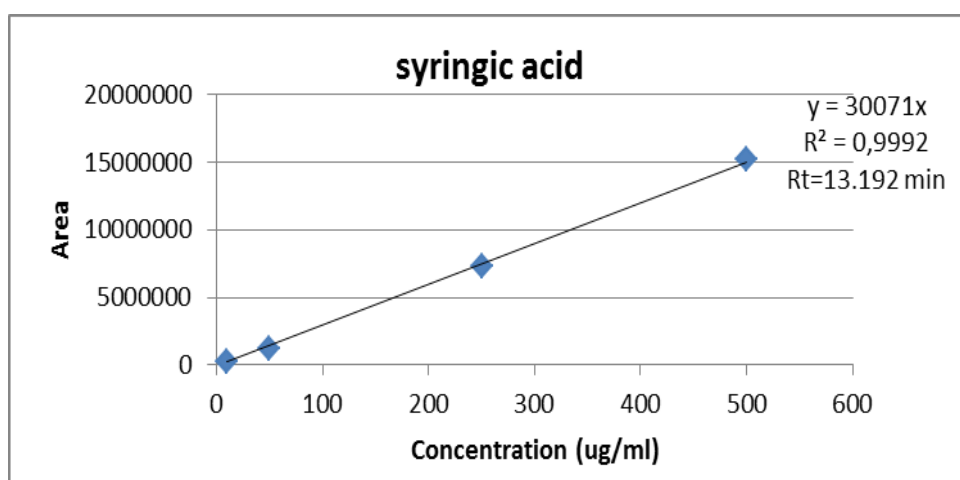


Figure A.26: Standard calibration curve of syringic acid for HPLC.

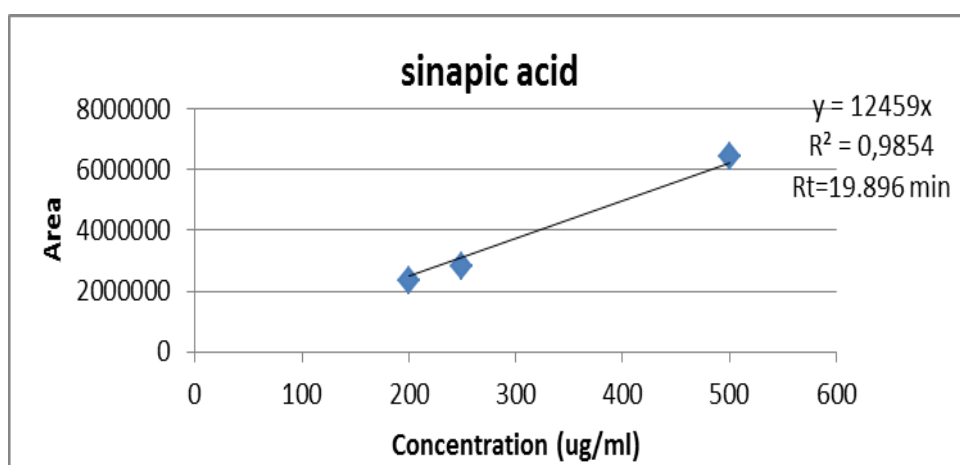


Figure A.27: Standard calibration curve of sinapic acid for HPLC.

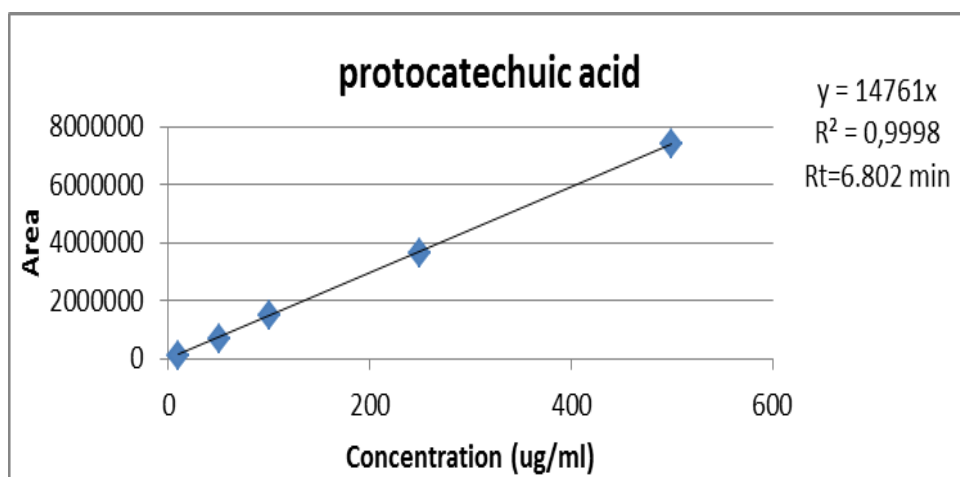


Figure A.28: Standard calibration curve of protocatechuic acid for HPLC.

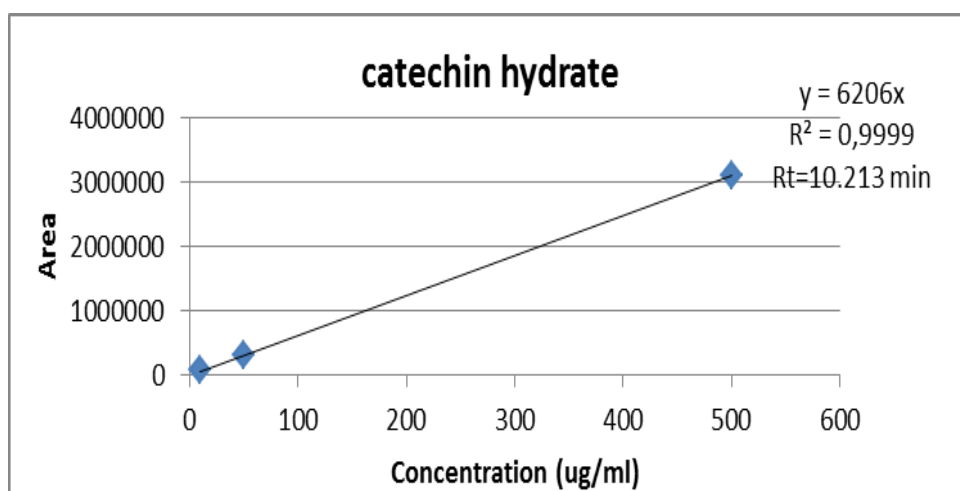


Figure A.29: Standard calibration curve of catechin hydrate for HPLC.

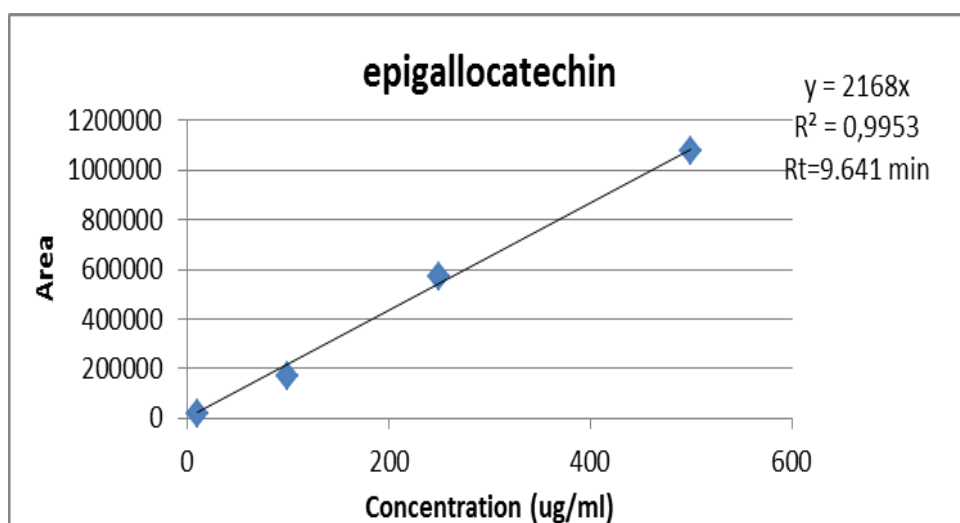


Figure A.30: Standard calibration curve of epigallocatechin for HPLC.

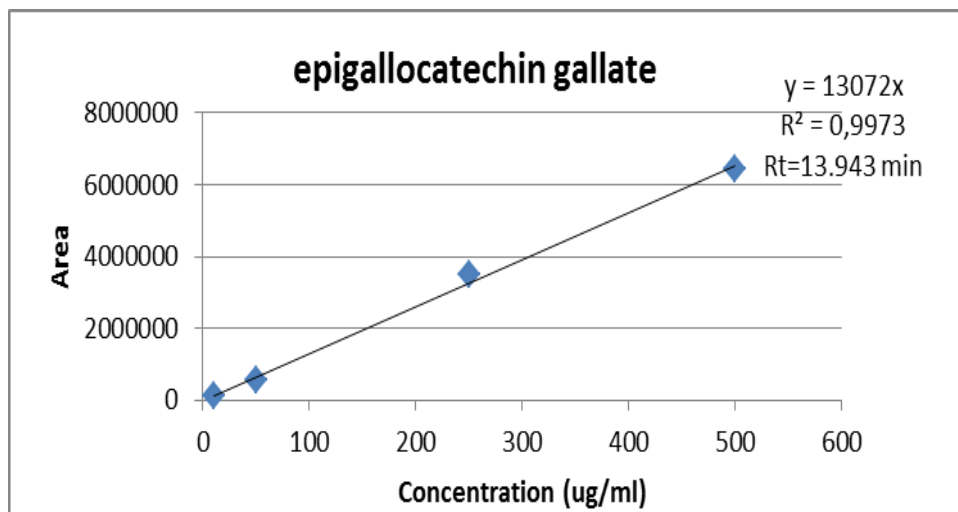


Figure A.31: Standard calibration curve of epigallocatechin gallate for HPLC.

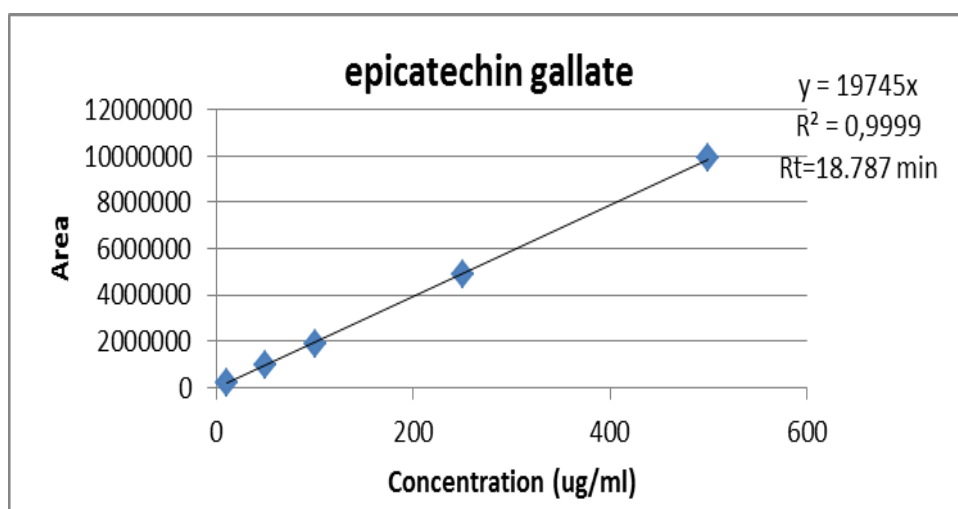


Figure A.32: Standard calibration curve of epicatechin gallate for HPLC.

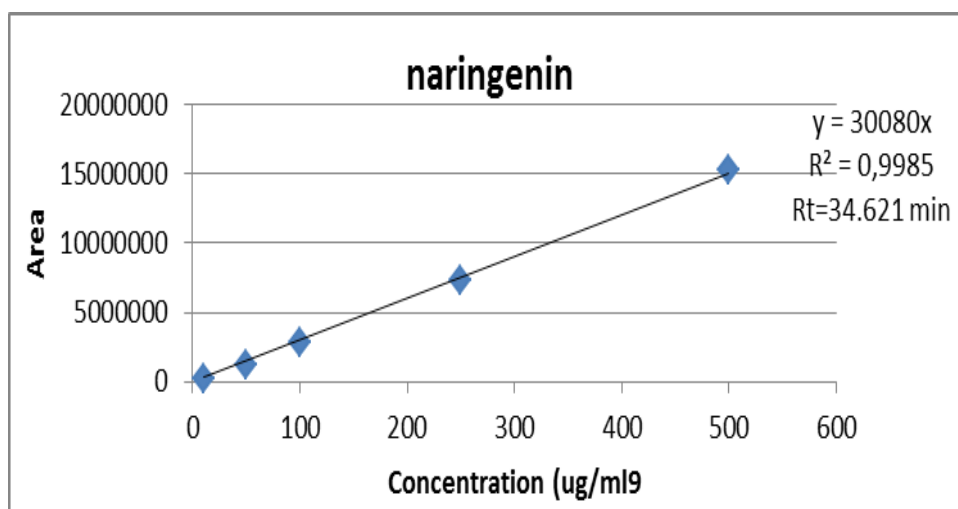


Figure A.33: Standard calibration curve of naringenin for HPLC.

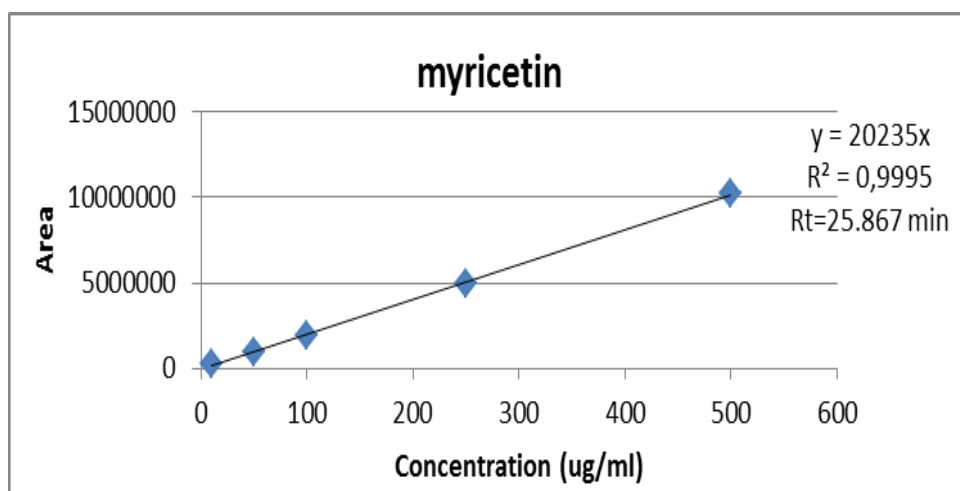


Figure A.34: Standard calibration curve of myricetin for HPLC.

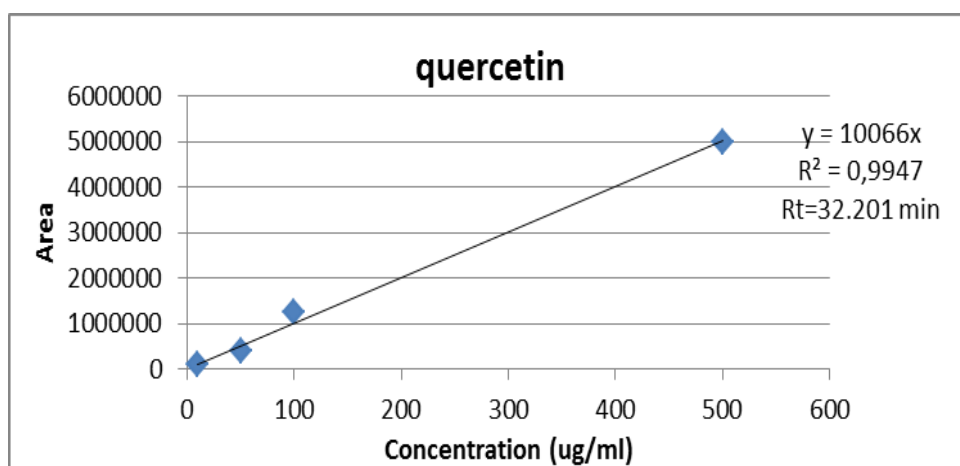


Figure A.35: Standard calibration curve of quercetin for HPLC.

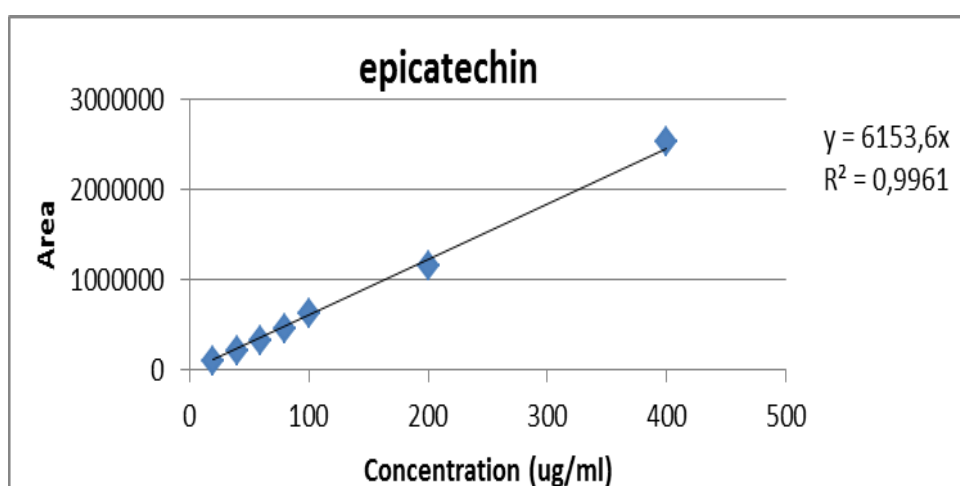


Figure A.36: Standard calibration curve of epicatechin for HPLC.

Table A.1: Wavelength and retention time of phenolic compounds by HPLC-PDA

	WAVELENGTH	RETENTION TIME
DELPHINIDIN	520 nm	21.171
CYANIDIN	520 nm	24.898
PELARGONIDIN	520 nm	28.723
MALVIDIN	520 nm	30.984
CYANIDIN 3-O-GLUCOSIDE	520 nm	17.775
CYANIDIN 3-O-GALACTOSIDE	520 nm	16.838
CYANIDIN 3-O-RUTINOSIDE	520 nm	18.550
PELARGONIDIN 3-O-GLUCOSIDE	520 nm	19.839
MALVIDIN 3-GALACTOSIDE	520 nm	21.446
MALVIDIN 3-GLUCOSIDE	520 nm	22.359
GALLIC ACID	280 nm	4.581
P-HYDROXY BENZOIC ACID	280 nm	9.484
GENISTEIN	280 nm	36.787
GENISTIN	280 nm	23.136
GLYCITIN	280 nm	19.649
DAIDZEIN	280 nm	30.154
DAIDZIN	280 nm	18.226
CAFEIC ACID	312 nm	12.177
VANILIC ACID	280 nm	11.606
P-COUMARIC ACID	312 nm	16.362
FERULIC ACID	312 nm	18.868
SYRINGIC ACID	280 nm	13.192
SINAPIC ACID	280 nm	19.896
PROTOCATECHUIC ACID	280 nm	6.802
CATECHIN HYDRATE	280 nm	10.213
EPIGALLOCATECHIN	280 nm	9.641
EPICATECHIN GALLATE	280 nm	18.787
EPIGALLOCATECHIN GALLATE	280 nm	13.943
NARINGENIN	280 nm	34.621
MYRICETIN	360 nm	25.867
QUERCETIN	360 nm	32.201
EPICATECHIN	280 nm	14.489

APPENDIX B

Table B.1 : Each analysis of initial compound.

methods		Sum of Squares	df	Mean Square	F	Sig.
ABTS	Between Groups	68088049.091	31	2196388.68	189.307	.000
	Within Groups	371272.708	32	11602.272		
	Total	68459321.8	63			
CUPRAC	Between Groups	85747955.189	31	2766063.071	232.676	.000
	Within Groups	380417.85	32	11888.058		
	Total	86128373.039	63			
DPPH	Between Groups	68617827.245	31	2213478.298	347.622	.000
	Within Groups	203759.36	32	6367.48		
	Total	68821586.605	63			
FOLIN	Between Groups	13771728.975	31	444249.322	126.74	.000
	Within Groups	112166.864	32	3505.214		
	Total	13883895.839	63			

Table B.2 : PG, IN and OUT of bioavailability samples for ABTS analysis.

		Sum of Squares	df	Mean Square	F	Sig.
IN	Between Groups	1642133.467	31	52972.047	10.414	.000
	Within Groups	218717.964	43	5086.464		
	Total	1860851.431	74			
OUT	Between Groups	6612253.460	31	213298.499	17.416	.000
	Within Groups	526634.324	43	12247.310		
	Total	7138887.784	74			
PG	Between Groups	131520311.498	31	4242590.693	46.542	.000
	Within Groups	3919690.622	43	91155.596		
	Total	135440002.119	74			

Table B.3 : PG, IN and OUT of bioavailability samples for CUPRAC analysis.

		Sum of Squares	df	Mean Square	F	Sig.
IN	Between Groups	5749997.502	31	185483.790	23.821	.000
	Within Groups	334827.981	43	7786.697		
	Total	6084825.483	74			
OUT	Between Groups	18101101.654	31	583906.505	39.648	.000
	Within Groups	633270.414	43	14727.219		
	Total	18734372.069	74			
PG	Between Groups	181007554.536	31	5838953.372	59.605	.000
	Within Groups	4212283.085	43	97960.072		
	Total	185219837.621	74			

Table B.4 : PG, IN and OUT of bioavailability samples for DPPH analysis.

		Sum of Squares	df	Mean Square	F	Sig.
IN	Between Groups	2634592.809	31	84986.865	64.103	.000
	Within Groups	57008.789	43	1325.786		
	Total	2691601.598	74			
OUT	Between Groups	4028265.049	31	129944.034	56.676	.000
	Within Groups	98587.617	43	2292.735		
	Total	4126852.666	74			
PG	Between Groups	64287578.685	31	2073792.861	125.822	.000
	Within Groups	708722.236	43	16481.912		
	Total	64996300.920	74			

Table B.5: PG, IN and OUT of bioavailability samples for FOLIN-CIOCALTEU analysis.

		Sum of Squares	df	Mean Square	F	Sig.
IN	Between Groups	3341914.153	31	107803.682	27.003	.000
	Within Groups	171669.607	43	3992.316		
	Total	3513583.761	74			
OUT	Between Groups	10786507.664	31	347951.860	28.988	.000
	Within Groups	516140.176	43	12003.260		
	Total	11302647.840	74			
PG	Between Groups	76000448.777	31	2451627.380	127.722	.000
	Within Groups	825383.637	43	19194.968		
	Total	76825832.414	74			

Table B.6: Initial, PG, IN and OUT of bioavailability samples for DPPH analysis.

		Sum of Squares	df	Mean Square	F	Sig.
DAIDZEIN	Between Groups	1487978.538	3	495992.846	35.449	.002
	Within Groups	55966.703	4	13991.676		
	Total	1543945.240	7			
DAIDZIN	Between Groups	1542416.077	3	514138.692	199.100	.000
	Within Groups	10329.249	4	2582.312		
	Total	1552745.326	7			
GENISTEIN	Between Groups	1824576.500	3	608192.167	355.110	.000
	Within Groups	6850.754	4	1712.689		
	Total	1831427.254	7			
GENISTIN	Between Groups	1374929.425	3	458309.808	1540.839	.000
	Within Groups	1189.767	4	297.442		
	Total	1376119.192	7			
PBHA	Between Groups	1110366.592	3	370122.197	73.835	.001
	Within Groups	20051.381	4	5012.845		
	Total	1130417.973	7			

Table B.6 (Continued): Initial, PG, IN and OUT of bioavailability samples for DPPH analysis.

QUERCETIN	Between Groups	921892.965	3	307297.655	37.056	.002
	Within Groups	33170.727	4	8292.682		
	Total	955063.692	7			
NARINGENIN	Between Groups	2466874.714	3	822291.571	2284.363	.000
	Within Groups	1439.861	4	359.965		
	Total	2468314.576	7			
EGCG	Between Groups	9619981.620	3	3206660.540	121.955	.000
	Within Groups	105174.972	4	26293.743		
	Total	9725156.592	7			
ECG	Between Groups	3390648.886	3	1130216.295	104.562	.000
	Within Groups	43236.135	4	10809.034		
	Total	3433885.021	7			
EC	Between Groups	927950.874	3	309316.958	48.162	.001
	Within Groups	25689.700	4	6422.425		
	Total	953640.574	7			
CH	Between Groups	27871363.054	3	9290454.351	111.683	.000
	Within Groups	332745.088	4	83186.272		
	Total	28204108.142	7			
MYRICETIN	Between Groups	2154265.555	3	718088.518	369.018	.000
	Within Groups	7783.767	4	1945.942		
	Total	2162049.322	7			
PROTOCATECHUIC	Between Groups	4839103.217	3	1613034.406	210.801	.000
	Within Groups	30607.766	4	7651.941		
	Total	4869710.983	7			
SINAPIC	Between Groups	3308889.957	3	1102963.319	24.355	.005
	Within Groups	181144.212	4	45286.053		
	Total	3490034.169	7			
SYRINGIC	Between Groups	367256.210	3	122418.737	122.212	.000
	Within Groups	4006.781	4	1001.695		
	Total	371262.991	7			
FERULIC	Between Groups	328875.342	3	109625.114	10.225	.024
	Within Groups	42884.663	4	10721.166		
	Total	371760.004	7			
COUMARIC	Between Groups	1387559.993	3	462519.998	7022.962	.000
	Within Groups	263.433	4	65.858		
	Total	1387823.426	7			
VANILIC	Between Groups	767214.735	3	255738.245	2600.808	.000
	Within Groups	393.321	4	98.330		
	Total	767608.056	7			

Table B.6 (Continued): Initial, PG, IN and OUT of bioavailability samples for DPPH analysis.

		Sum of Squares	df	Mean Square	F	Sig.
EGC	Between Groups	2449122.441	3	816374.147	1065.258	.000
	Within Groups	5364.538	7	766.363		
	Total	2454486.979	10			
DELP	Between Groups	5726510.083	3	1908836.694	1547.313	.000
	Within Groups	8635.521	7	1233.646		
	Total	5735145.604	10			
CY	Between Groups	3085789.842	3	1028596.614	2027.551	.000
	Within Groups	3551.170	7	507.310		
	Total	3089341.011	10			
C3G	Between Groups	7107353.535	3	2369117.845	6837.477	.000
	Within Groups	2425.431	7	346.490		
	Total	7109778.966	10			
M3Ga	Between Groups	783158.988	3	261052.996	351.832	.000
	Within Groups	5193.876	7	741.982		
	Total	788352.864	10			
M3G	Between Groups	418277.648	3	139425.883	1908.908	.000
	Within Groups	511.277	7	73.040		
	Total	418788.925	10			
PEL	Between Groups	1390612.979	3	463537.660	646.408	.000
	Within Groups	5019.683	7	717.098		
	Total	1395632.662	10			
MAL	Between Groups	404077.806	3	134692.602	45.794	.000
	Within Groups	20588.864	7	2941.266		
	Total	424666.669	10			
P3G	Between Groups	324356.603	3	108118.868	167.963	.000
	Within Groups	4505.938	7	643.705		
	Total	328862.541	10			
C3Ga	Between Groups	710886.336	3	236962.112	548.179	.000
	Within Groups	3025.900	7	432.271		
	Total	713912.236	10			
C3R	Between Groups	1474326.759	3	491442.253	1340.045	.000
	Within Groups	2567.149	7	366.736		
	Total	1476893.909	10			
GALLIC	Between Groups	18840793.551	3	6280264.517	709.464	.000
	Within Groups	35408.478	4	8852.119		
	Total	18876202.029	7			
CAFEIC	Between Groups	1759270.911	3	586423.637	128.275	.000
	Within Groups	18286.430	4	4571.608		
	Total	1777557.341	7			
GLYCITIN	Between Groups	1349196.778	3	449732.259	35.932	.002
	Within Groups	50065.466	4	12516.367		
	Total	1399262.244	7			

Table B.7: Initial, PG, IN and OUT of bioavailability samples for FOLIN-CIOCALTEU analysis.

		Sum of Squares	df	Mean Square	F	Sig.
DAIDZEIN	Between Groups	237211.529	3	79070.510	14.041	.014
	Within Groups	22525.649	4	5631.412		
	Total	259737.177	7			
DAIDZIN	Between Groups	5384.174	3	1794.725	.101	.955
	Within Groups	71266.263	4	17816.566		
	Total	76650.437	7			
GENISTEIN	Between Groups	2173103.657	3	724367.886	253.960	.000
	Within Groups	11409.148	4	2852.287		
	Total	2184512.805	7			
CAFEIC	Between Groups	1118182.017	3	372727.339	159.028	.000
	Within Groups	9375.154	4	2343.788		
	Total	1127557.171	7			
VANILIC	Between Groups	1041011.302	3	347003.767	109.128	.000
	Within Groups	12719.146	4	3179.786		
	Total	1053730.448	7			
EGCG	Between Groups	1520881.220	3	506960.407	58.905	.001
	Within Groups	34425.917	4	8606.479		
	Total	1555307.137	7			
ECG	Between Groups	1311586.487	3	437195.496	51.179	.001
	Within Groups	34169.651	4	8542.413		
	Total	1345756.138	7			
MYRICETIN	Between Groups	4827775.597	3	1609258.532	1963.187	.000
	Within Groups	3278.870	4	819.718		
	Total	4831054.467	7			
EC	Between Groups	1715296.058	3	571765.353	143.551	.000
	Within Groups	15932.018	4	3983.005		
	Total	1731228.076	7			
CH	Between Groups	4066671.813	3	1355557.271	99.797	.000
	Within Groups	543323.275	4	135830.819		
	Total	41209995.088	7			
NARINGENIN	Between Groups	1052308.106	3	350769.369	23.869	.005
	Within Groups	58781.544	4	14695.386		
	Total	1111089.650	7			
SYRINGIC	Between Groups	470156.965	3	156718.988	30.348	.003
	Within Groups	20656.429	4	5164.107		
	Total	490813.394	7			
QUERCETIN	Between Groups	2394831.089	3	798277.030	32.063	0,003
	Within Groups	99587.953	4	24896.988		
	Total	2494419.041	7			

Table B.7 (Continued): Initial, PG, IN and OUT of bioavailability samples for FOLIN-CIOCALTEU analysis.

PROTOCATECHUIC	Between Groups	1316253.015	3	438751.005	30.235	.003
	Within Groups	58045.052	4	14511.263		
	Total	1374298.067	7			
SINAPIC	Between Groups	1027999.253	3	342666.418	3.862	.112
	Within Groups	354895.448	4	88723.862		
	Total	1382894.701	7			
COUMARIC	Between Groups	362353.768	3	120784.589	213.092	.000
	Within Groups	2267.271	4	566.818		
	Total	364621.039	7			
FERULIC	Between Groups	483729.378	3	161243.126	5.401	.068
	Within Groups	119412.305	4	29853.076		
	Total	603141.683	7			
GENISTIN	Between Groups	67535.702	3	22511.901	68.299	.001
	Within Groups	1318.441	4	329.610		
	Total	68854.143	7			
GLYCITIN	Between Groups	63718.031	3	21239.344	19.881	.007
	Within Groups	4273.355	4	1068.339		
	Total	67991.387	7			
PHBA	Between Groups	601831.935	3	200610.645	36.650	.002
	Within Groups	21894.523	4	5473.631		
	Total	623726.458	7			
GALLIC	Between Groups	1197574.820	3	399191.607	53.306	.001
	Within Groups	29954.921	4	7488.730		
	Total	1227529.741	7			
EGC	Between Groups	1234852.507	3	411617.502	597.851	.000
	Within Groups	4819.469	7	688.496		
	Total	1239671.975	10			
DELP	Between Groups	3827771.765	3	1275923.922	278.428	.000
	Within Groups	32078.159	7	4582.594		
	Total	3859849.924	10			
CY	Between Groups	1355704.310	3	451901.437	930.085	.000
	Within Groups	3401.096	7	485.871		
	Total	1359105.406	10			
C3G	Between Groups	6587368.050	3	2195789.350	2117.695	.000
	Within Groups	7258.139	7	1036.877		
	Total	6594626.189	10			
MAL	Between Groups	894734.138	3	298244.713	258.371	.000
	Within Groups	8080.288	7	1154.327		
	Total	902814.426	10			

Table B.7 (Continued): Initial, PG, IN and OUT of bioavailability samples for FOLIN-CIOCALTEU analysis.

		Sum of Squares	df	Mean Square	F	Sig.
M3G	Between Groups	1827903.871	3	609301.290	312.803	.000
	Within Groups	13635.117	7	1947.874		
	Total	1841538.988	10			
M3Ga	Between Groups	908464.208	3	302821.403	340.670	.000
	Within Groups	6222.303	7	888.900		
	Total	914686.511	10			
PEL	Between Groups	2785060.141	3	928353.380	1785.304	.000
	Within Groups	3639.982	7	519.997		
	Total	2788700.123	10			
P3G	Between Groups	2081803.160	3	693934.387	3581.611	.000
	Within Groups	1356.245	7	193.749		
	Total	2083159.404	10			
C3Ga	Between Groups	1892858.620	3	630952.873	405.504	.000
	Within Groups	10891.799	7	1555.971		
	Total	1903750.419	10			
C3R	Between Groups	3046528.045	3	1015509.348	1591.937	.000
	Within Groups	4465.356	7	637.908		
	Total	3050993.401	10			

Table B.8: Initial, PG, IN and OUT of bioavailability samples for ABTS analysis.

		Sum of Squares	df	Mean Square	F	Sig.
DAIDZEIN	Between Groups	1203184.894	3	401061.631	21.063	.007
	Within Groups	76165.310	4	19041.327		
	Total	1279350.204	7			
DAIDZIN	Between Groups	716822.475	3	238940.825	6.788	.048
	Within Groups	140807.846	4	35201.961		
	Total	857630.321	7			
GENISTEIN	Between Groups	8234402.218	3	2744800.739	842.209	.000
	Within Groups	13036.200	4	3259.050		
	Total	8247438.418	7			
GENISTIN	Between Groups	654317.603	3	218105.868	51.334	.001
	Within Groups	16995.058	4	4248.764		
	Total	671312.661	7			
GLYCITIN	Between Groups	824048.731	3	274682.910	106.527	.000
	Within Groups	10314.127	4	2578.532		
	Total	834362.858	7			

Table B.8 (Continued): Initial, PG, IN and OUT of bioavailability samples for
ABTS analysis.

GALLIC	Between Groups	58577757.853	3	19525919.284	494.382	.000
	Within Groups	157982.299	4	39495.575		
	Total	58735740.151	7			
PHBA	Between Groups	292401.289	3	97467.096	41.887	.002
	Within Groups	9307.635	4	2326.909		
	Total	301708.924	7			
COUMARIC	Between Groups	3896453.290	3	1298817.763	259.971	.000
	Within Groups	19984.043	4	4996.011		
	Total	3916437.334	7			
VANILIC	Between Groups	2485659.115	3	828553.038	102.729	.000
	Within Groups	32261.845	4	8065.461		
	Total	2517920.960	7			
NARINGENIN	Between Groups	2100268.346	3	700089.449	35.379	.002
	Within Groups	79154.115	4	19788.529		
	Total	2179422.461	7			
EGCG	Between Groups	8538751.855	3	2846250.618	672.324	.000
	Within Groups	16933.792	4	4233.448		
	Total	8555685.647	7			
ECG	Between Groups	9510516.626	3	3170172.209	58.023	.001
	Within Groups	218545.093	4	54636.273		
	Total	9729061.719	7			
EC	Between Groups	8924563.465	3	2974854.488	62.580	.001
	Within Groups	190147.258	4	47536.815		
	Total	9114710.723	7			
CH	Between Groups	22712748.458	3	7570916.153	11.514	.019
	Within Groups	2630097.511	4	657524.378		
	Total	25342845.969	7			
MYRICETIN	Between Groups	11412910.719	3	3804303.573	539.421	.000
	Within Groups	28210.300	4	7052.575		
	Total	11441121.019	7			
QUERCETIN	Between Groups	7732670.318	3	2577556.773	716.018	.000
	Within Groups	14399.397	4	3599.849		
	Total	7747069.714	7			
PROTOCATECHUIC	Between Groups	2700648.681	3	900216.227	18.419	.008
	Within Groups	195498.369	4	48874.592		
	Total	2896147.050	7			
SINAPIC	Between Groups	3605026.425	3	1201675.475	9.441	.028
	Within Groups	509131.561	4	127282.890		
	Total	4114157.986	7			

Table B.8 (Continued): Initial, PG, IN and OUT of bioavailability samples for
ABTS analysis.

SYRINGIC	Between Groups	1799738.271	3	599912.757	17.727	.009
	Within Groups	135368.550	4	33842.138		
	Total	1935106.822	7			
FERULIC	Between Groups	4264885.388	3	1421628.463	22.947	.006
	Within Groups	247808.220	4	61952.055		
	Total	4512693.608	7			
CAFEIC	Between Groups	1810639.961	3	603546.654	109.865	.000
	Within Groups	21974.084	4	5493.521		
	Total	1832614.045	7			
EGC	Between Groups	10609878.335	3	3536626.112	1344.233	.000
	Within Groups	18416.737	7	2630.962		
	Total	10628295.072	10			
DELPHINIDIN	Between Groups	7136336.200	3	2378778.733	505.628	.000
	Within Groups	32932.190	7	4704.599		
	Total	7169268.390	10			
CYANIDIN	Between Groups	2088072.918	3	696024.306	134.591	.000
	Within Groups	36199.937	7	5171.420		
	Total	2124272.855	10			
C3G	Between Groups	32890510.156	3	10963503.385	1119.948	.000
	Within Groups	68525.062	7	9789.295		
	Total	32959035.217	10			
M3G	Between Groups	791338.607	3	263779.536	106.839	.000
	Within Groups	17282.562	7	2468.937		
	Total	808621.170	10			
P3G	Between Groups	2812321.472	3	937440.491	597.247	.000
	Within Groups	10987.210	7	1569.601		
	Total	2823308.682	10			
M3Ga	Between Groups	874616.789	3	291538.930	85.599	.000
	Within Groups	23840.975	7	3405.854		
	Total	898457.764	10			
MAL	Between Groups	1231359.437	3	410453.146	495.908	.000
	Within Groups	5793.756	7	827.679		
	Total	1237153.193	10			
C3Ga	Between Groups	3153471.858	3	1051157.286	820.374	.000
	Within Groups	8969.199	7	1281.314		
	Total	3162441.057	10			
PEL	Between Groups	3193294.148	3	1064431.383	543.597	.000
	Within Groups	13706.882	7	1958.126		
	Total	3207001.030	10			
C3R	Between Groups	3958742.666	3	1319580.889	225.672	.000
	Within Groups	40931.448	7	5847.350		
	Total	3999674.114	10			

Table B.9: Initial, PG, IN and OUT of bioavailability samples for CUPRAC analysis.

		Sum of Squares	df	Mean Square	F	Sig.
DAIDZEIN	Between Groups	168404.049	3	56134.683	12.042	.018
	Within Groups	18646.807	4	4661.702		
	Total	187050.855	7			
DAIDZIN	Between Groups	10014.968	3	3338.323	.419	.75
	Within Groups	31857.071	4	7964.268		
	Total	41872.039	7			
GENISTEIN	Between Groups	699055.329	3	233018.443	226.579	.000
	Within Groups	4113.687	4	1028.422		
	Total	703169.016	7			
GENISTIN	Between Groups	338874.046	3	112958.015	32.196	.003
	Within Groups	14033.598	4	3508.400		
	Total	352907.644	7			
GLYCITIN	Between Groups	197984.097	3	65994.699	36.459	.002
	Within Groups	7240.451	4	1810.113		
	Total	205224.549	7			
SINAPIC	Between Groups	3921328.469	3	1307109.490	6.338	.053
	Within Groups	824979.125	4	206244.781		
	Total	4746307.594	7			
CH	Between Groups	34600882.938	3	11533627.646	42.634	.002
	Within Groups	1082111.157	4	270527.789		
	Total	35682994.095	7			
EC	Between Groups	7935745.325	3	2645248.442	124.329	.000
	Within Groups	85105.085	4	21276.271		
	Total	8020850.411	7			
EGCG	Between Groups	14590006.748	3	4863335.583	10.862	.022
	Within Groups	1790906.407	4	447726.602		
	Total	16380913.155	7			
ECG	Between Groups	7200528.367	3	2400176.122	1570.595	.000
	Within Groups	6112.782	4	1528.196		
	Total	7206641.149	7			
NARINGENIN	Between Groups	153786.453	3	51262.151	5.697	.063
	Within Groups	35992.055	4	8998.014		
	Total	189778.508	7			
MYRICETIN	Between Groups	14319670.012	3	4773223.337	561.341	.000
	Within Groups	34012.999	4	8503.250		
	Total	14353683.010	7			
QUERCETIN	Between Groups	11297546.679	3	3765848.893	64.868	.001
	Within Groups	232215.339	4	58053.835		
	Total	11529762.017	7			

Table B.9: (Continued): Initial, PG, IN and OUT of bioavailability samples for CUPRAC analysis.

PROTOCATECHUIC	Between Groups	19011571.146	3	6337190.382	188.037	.000
	Within Groups	134807.447	4	33701.862		
	Total	19146378.593	7			
FERULIC	Between Groups	1248148.359	3	416049.453	7.978	.037
	Within Groups	208593.582	4	52148.395		
	Total	1456741.941	7			
SYRINGIC	Between Groups	2727643.684	3	909214.561	33.672	.003
	Within Groups	108007.556	4	27001.889		
	Total	2835651.240	7			
VANILIC	Between Groups	1283546.669	3	427848.890	53.530	.001
	Within Groups	31970.807	4	7992.702		
	Total	1315517.477	7			
COUMARIC	Between Groups	298160.447	3	99386.816	38.432	.002
	Within Groups	10344.112	4	2586.028		
	Total	308504.559	7			
CAFEIC	Between Groups	14846671.094	3	4948890.365	71.535	.001
	Within Groups	276727.121	4	69181.780		
	Total	15123398.215	7			
GALLIC	Between Groups	33161493.284	3	11053831.095	221.349	.000
	Within Groups	199754.019	4	49938.505		
	Total	33361247.302	7			
PHBA	Between Groups	202391.648	3	67463.883	26.148	.004
	Within Groups	10320.364	4	2580.091		
	Total	212712.012	7			
EGC	Between Groups	7078754.803	3	2359584.934	1422.397	.000
	Within Groups	11612.157	7	1658.880		
	Total	7090366.960	10			
DELPH	Between Groups	22758901.149	3	7586300.383	863.968	.000
	Within Groups	61465.368	7	8780.767		
	Total	22820366.517	10			
CY	Between Groups	4792416.772	3	1597472.257	207.107	.000
	Within Groups	53992.911	7	7713.273		
	Total	4846409.683	10			
C3G	Between Groups	21687406.624	3	7229135.541	5516.493	.000
	Within Groups	9173.211	7	1310.459		
	Total	21696579.835	10			

Table B.9: (Continued): Initial, PG, IN and OUT of bioavailability samples for CUPRAC analysis.

C3Ga	Between Groups	2820797.601	3	940265.867	52.851	.000
	Within Groups	124537.134	7	17791.019		
	Total	2945334.734	10			
P3G	Between Groups	5041872.384	3	1680624.128	378.746	.000
	Within Groups	31061.345	7	4437.335		
	Total	5072933.730	10			
MAL	Between Groups	1518599.512	3	506199.837	293.376	.000
	Within Groups	12078.003	7	1725.429		
	Total	1530677.515	10			
M3G	Between Groups	1910795.578	3	636931.859	860.231	.000
	Within Groups	5182.938	7	740.420		
	Total	1915978.516	10			
M3Ga	Between Groups	3507453.951	3	1169151.317	163.281	.000
	Within Groups	50122.670	7	7160.381		
	Total	3557576.621	10			
C3R	Between Groups	12097990.803	3	4032663.601	2114.662	.000
	Within Groups	13349.011	7	1907.002		
	Total	12111339.814	10			
PEL	Between Groups	13307750.550	3	4435916.850	769.113	.000
	Within Groups	40373.009	7	5767.573		
	Total	13348123.559	10			

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