ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

COMPARING THE ANTIOXIDANT PROPERTIES AND *IN VITRO* BIOACCESSIBILITY OF PHENOLIC COMPOUNDS IN PLAIN AND MILK ADDED GREEN TEA AND SEA BUCKTHORN LEAF TEA

M.Sc. THESIS

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Department of Food Engineering

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4 MAY 2015

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

SADE VE SÜT EKLENMİŞ YEŞİL ÇAY VE YALANCI İĞDE YAPRAĞI ÇAYLARININ ANTİOKSİDAN ÖZELLİKLERİNİN VE FENOLİK BİLEŞİKLERİN *İN VİTRO* BİYOERİŞİLEBİLİRLİKLERİNİN KARŞILAŞTIRILMASI

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Fatma Duygu Ceylan, a **M.Sc.** student of ITU **Graduate School of Science Engineering and Technology** student ID 506121534, successfully defended the **thesis** entitled "**COMPARING THE ANTIOXIDANT PROPERTIES AND *IN VITRO* BIOACCESSIBILITY OF PHENOLIC COMPOUNDS IN PLAIN AND MILK ADDED GREEN TEA AND SEA BUCKTHORN LEAF TEA**" which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my family,

FOREWORD

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May 2015 Fatma Duygu Ceylan

(Food Engineer)

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ABBREVIATIONS

ABTS	: 2,2- azinobis 3-ethylbenzothiazoline-6-sulfonic acid diammonium Salt
CE	: Catechin Equivalent
CHA	: Chlorogenic acid
CUPRAC	: Copper Reducing Antioxidant Capacity
DPPH	: 1,1-Diphenyl-2- picrylhydrazyl
DW	: Dry weight
ES	: Ethanolic extracts of sea buckthorn leaf tea
EG	: Ethanolic extracts of green tea
FRAP	: Ferric Reducing Antioxidant Capacity
FW	: Fresh weight
GA	: Gallic acid
GAE	: Gallic Acid Equivalent
GI	: Gastrointestinal
HPLC	: High Performance Liquid Chromatography
SBT	: Sea buckthorn
SM	: Skimmed milk added
SPSS	: Statistical Package for the Social Sciences
TAC	: Total antioxidant capacity
TEAC	: Trolox Equivalent Antioxidant Capacity
TF	: Total flavonoid content
TG	: Tea infusion form of green tea
TP	: Total phenolic content
TS	: Tea infusion form of sea buckthorn leaf tea
WM	: Whole milk added

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COMPARING THE ANTIOXIDANT PROPERTIES AND *IN VITRO* BIOACCESSIBILITY OF PHENOLIC COMPOUNDS IN SIMPLE AND MILK ADDED GREEN TEA AND SEA BUCKTHORN LEAF TEA

SUMMARY

Green tea is a commonly recommended beverage for its strong antioxidant properties. On the other hand, sea buckthorn leaf tea also contains abundant amount of bioactive compounds. Present study focused on comparing the antioxidant properties and *in vitro* bioaccessibility of green tea and sea buckthorn leaf tea extracts. Green tea and sea buckthorn leaf tea samples were extracted with ethanol and also prepared in forms of tea infusions. Skimmed and whole milk (at room temperature) was added to get 20% and 40% (v/v) to tea infusions. *In vitro* gastrointestinal digestion model was used for investigating the bioaccessibility of samples. All samples were analyzed for their contents of total phenolics (TP) and total flavonoids (TF) as well as their total antioxidant capacity by using four different methods including 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis(3-ethylbenzo-thiazoline-6-sulphonic acid) diammonium salt (ABTS), CUPRAC (Copper Reducing Antioxidant Capacity), and FRAP (Ferric Reducing Antioxidant Power) methods.

According to TP the results, ethanolic extaction of green tea (EG) was richer in phenolic material (153.5 mg of GAE/ g sample) compared to ethanolic extraction of Sea buckthorn leaf tea (ES) (114.8 mg of GAE/g sample). On the contrary, sea buckthorn leaf tea (TS) had higher phenolic content than green tea (TS) considering that the tea infusion forms. In fact, the highest TP content among all fractions was observed in Ethanolic green tea extract (GE) ($p<0.05$). TP results of milk added samples were ranged between 43.6 to 58.4 mg GAE/g for SBT leaf tea and 37.8 to 44.9 mg GAE/g for green tea. Comparing the control group, whole and skimmed milk added samples, results showed that 20% whole milk added samples of SBT were increased compared to the 20% control group. However there were a decrease in 40% whole milk added samples. On the other hand there were a decrease in 20% skimmed milk added SBT and there were a little increase in 40% skimmed milk added samples of SBT. Considering the green tea samples, there were no significant variation between 20% of whole milk and control group samples while slight decreases were observed between control groups and 40% whole milk, 20% skimmed milk and 40% skimmed milk added samples. There were no significant difference between the TP of skimmed and whole milk added both green tea and SBT leaf tea samples, however, control groups were significantly higher than milk added samples ($p<0.05$).

Total flavonoid (TF) of plain and milk added tea samples were measured colorimetrically in terms of both rutin and catechin equivalent and found to be higher rutin in EG (115.2 mg of Rutin eq./g) compared to ES (66.7 mg of RE/g). Also, the flavonoid content in TG (43.8 mg of RE/g) was higher than TS (39.9 mg of RE/g). Contrary the rutin content results, ES (35.0 mg of Catechin eq./g) and TS (14.0 mg of

CE/g) were higher catechin content than EG (21.6 mg of CE/g) and TG (12.3 mg of CE/g). There was no significant difference between the TF of tea infusion forms both in terms of rutin and catechin, however, EG was significantly higher than ES in terms of rutin and ES was significantly higher than EG in terms of catechin ($p<0.05$). TF of milk added tea samples were observed range between 19.0 to 107.8 mg RE/g for SBT leaf tea and 30.8 to 88.4 mg RE/g for green tea. Also catechin equivalent results were observed range between 5.9 to 33.5 mg CE/g for SBT leaf tea and 9.6 to 27.5 mg CE/g for green tea. Both rutin and catechin results showed similar variation between control group and milk added samples of SBT leaf tea and green tea. There were only slight differences among the skimmed milk added sample and control group results. On the other hand, rapid increases were detected on the whole milk added samples.

TAC was found to be higher in EG, ranging from 89.2 mg of TE/g for ABTS to 622.9 mg of TE/g for CUPRAC. For both SBT leaf tea and green tea, ethanolic extractions were more efficient to the total antioxidant capacity compared to tea infusions, having ~1.5-2.5 fold higher capacity. Thus, TAC for TG varied from 55.9-227.0 mg of TE/g, 89.1-456.1 mg of TE/g for ES and 55.7-251.3 mg of TE/g for TS. According to all four analyses carried out, TAC was decreased in 20% whole milk added samples both SBT leaf tea and green tea, respectively. Similarly TAC of 20% skimmed milk added samples decreased both SBT leaf tea and green tea except ABTS assay results. Considering the 40% milk added samples results, there were slight increase in both whole and skimmed milk added SBT leaf tea in DPPH assay. There were also slight increases in skimmed milk added green tea in ABTS assay.

After the simulation of *in vitro* gastrointestinal digestion system, 18.1% and 8.3% of the antioxidant compounds were present in the dialyzed fraction for Sea buckthorn leaf tea and Green tea, respectively. Milk addition caused an increase in the dialyzed fractions for both tea samples.

For milk added samples, percent recovery of total phenolics and total antioxidant capacity after *in vitro* GI digestion were significantly higher from control group samples whereas total flavonoid was found to be significantly lower except skimmed milk added samples of green tea.

SADE VE SÜT EKLENMİŞ YEŞİL ÇAY VE YALANCI İĞDE YAPRAĞI ÇAYLARININ ANTİOKSİDAN ÖZELLİKLERİNİN VE FENOLİK BİLEŞİKLERİN *İN VİTRO* BİYOERİŞİLEBİLİRLİKLERİNİN KARŞILAŞTIRILMASI

ÖZET

Çay, *Camellia sinensis* olarak bilinen bir bitkinin yapraklarından elde edilen ve dünyada sudan sonra 2. sırada yer alan en yaygın içecektir. Üç farklı şekilde elde edilen çay; yeşil çay, siyah çay ve oolong çay olarak bilinmektedir. Yeşil çay, polifenol bileşenleri oksidasyona uğratılmadan *C. sinensis* yapraklarının dehidretasyonundan elde edilmektedir. Bu nedenle, yeşil çay, kateşin grubundan monomerik polifenollerin yüksek düzeyde konsantrasyonunu içerir. Yeşil çay kateşinleri yeşil çay infüzyonunda bulunan polifenollerin ana bileşenleridir. Çaydaki polifenollerin biyolojik etkilerine olan ilgi yapılan in vitro ve in vivo çalışmalarla gün geçtikçe artmakta ve antioksidan özelliklerine vurgu yapılmaktadır. Son yıllarda yeşil çayın sağlık üzerine etkilerini inceleyen çok sayıda çalışma yapılmıştır. Çaydaki polifenolik bileşiklerin, mide, kolon, meme, prostat ve pankreas kanserleri gibi değişik kanser türlerini, kalp damar hastalıklarını, iltihabi ve nörodejeneratif hastalıkları önleyici ve tedaviyi destekleyici etki gösterebileceği vurgusu yapılmaktadır. Bu sebeplerden ötürü, yeşil çay tüketimi yaygın bir şekilde tavsiye edilen bir içecektir.

Öte yandan, yalancı iğde, ülkemizde, çıçırgan ya da yer iğdesi (*Hippophae rhamnoides*) olarak da bilinen iğdegiller ailesine mensup bir bitki çeşididir.10m yüksekliğinde, dikenli bir ağaçtır. Antik kaynaklarda da ismine rastlanan bitki, günümüzde Kuzey Avrupa ve ABD'de alternatif bir tarım ürünü olarak incelenirken, Çin Halk Cumhuriyetinde büyük miktarlarda üretilmektedir. Fakat ülkemizde kullanımı çok yaygın değildir. Bitkinin meyvesi ve meyve yağı, özellikle tıp ve kozmetik alanlarında geniş bir kullanım alanına sahiptir. Yapılan çalışmalarda bitkinin yüksek fenolik bileşen içeriğine sahip olmasıyla doğru orantılı olarak, iltihaba karşı, mikrop öldürücü, ağrı kesici, yaraları tedavi edici özelliklere sahip olduğu görülmüştür. Bitkinin meyvesinin olduğu Kadar dal ve yapraklarının da yüksek fenolik içeriğe sahip olduğu yapılan çalışmalarda kanıtlanmıştır. Bu nedenle özellikle Uzak Doğu'da yalancı iğde yaprağı çayı tüketimi son derece yaygındır. Yalancı iğde yaprağı çayı da yeşil çay gibi bol miktarda biyoaktif bileşen içermektedir. Çalışmada yeşil çay ve yalancı iğde yaprağı çayı örneklerinin antioksidan özellikleri açısından karşılaştırarak, ülkemizde çok bilinmeyen yalancı iğde yaprağı çayının antioksidan özellikleri hakkında fikir oluşturmak amaçlanmıştır.

Son yıllarda sağlıklı beslenmeye yönelik artan ilgiyle birlikte, fenolik bileşenlerce zengin ve güçlü antioksidan özelliklere sahip yiyecek ve içecek tüketimi pek çok çevrece tavsiye edilmektedir. Fakat gıda, ne kadar yüksek antioksidan özelliğe sahip olursa olsun, eğer biyoerişilebilirliği düşükse, vücudumuza tahmin ettiğimizden çok

daha az antioksidan özellikte madde geçişi sağlanmış demektir. Bu nedenle tükettiğimiz gıdaların içerdiği fenolik bileşenlerin miktarlarının çokluğunun yanı sıra, bu bileşenlerin biyoerişilebilirlikleri de büyük önem arzetmektedir. Fenolik maddece zengin gıdaların, proteince veya yağca zengin gıdalarla birlikte tüketildiğinde biyoerişilebilirliklerinde meydana gelen değişimlerle ilgili yapılan çalışmalarda tartışmalı sonuçlar bulunduğu görülmektedir. Böyle gıdaların birlikte tüketildiklerinde, fenolik bileşiklerle protein ve yağların nasıl bir etkileşim göstereceklerine dair bir bakış açısı sunması açısından, özellikle İngiltere'de yaygın şekilde çaya süt ilave edilerek tüketiminden yola çıkarak, çalışmamızda yeşil çay ve yalancı iğde yaprağı çay örneklerine protein ve yağca zengin olan süt eklenmiştir.

Bu çalışmada sade ve süt eklenmiş yeşil çay ve yalancı iğde yaprağı çaylarının antioksidan özelliklerinin ve fenolik bileşiklerin *in vitro* biyoerişilebilirliklerinin karşılaştırılmasına odaklanılmıştır. Bu amaçla yeşil çay ve yalancı iğde yaprağı çay örnekleri etanol ile ekstrakte edilmiş ayrıca sıcak su ile demleme işlemi uygulanmıştır. Demleme çay örneklerine oda sıcaklığında hacimce %20 ve %40 oranlarında olacak şekilde yağsız ve tam yağlı süt eklenmiştir. Örneklerin biyoerişilebilirliklerini araştırmak için *in vitro* gastrointestinal (GI) sindirim simülasyonu kullanılmıştır. Bütün örneklerin, toplam fenolik (TP), toplam flavanoid (TF) madde içerikleri analizlenmiş, toplam antioksidan kapasiteleri ise 4 farklı analiz yöntemi (DPPH, CUPRAC, FRAP, ABTS) kullanılarak belirlenmiştir.

Çalışmada elde edilen sonuçlara bakıldığında, etanolik yeşil çay (EG) ekstraktının (153.5 mg GAE/g) fenolik maddece, etanolik yalancı iğde yaprağı çay (ES) ekstraktına (114.8 mg GAE/g) göre daha zengin olduğu görülmüştür. Demleme formlarında ise etanolik ekstraktların aksine, yalancı iğde yaprağı çayı (TS), yeşil çaya (TG) göre daha yüksek fenolik madde içeriğine sahiptir. Bütün fraksiyonlar arasında en yüksek fenolik madde içeriği etanolik yeşil çay ekstraktında (EG) gözlemlenmiştir. Süt eklenmiş örneklerin fenolik madde miktarı sonuçları yalancı iğde çayı için 43.6 mg GAE/g ila 58.4 mg GAE/g arasında, yeşil çay için ise 37.8 mg GAE/g ila 44.9 mg GAE/g arasında değişmektedir. Tam yağlı ve yağsız süt eklenmiş örneklerin fenolik madde miktarı sonuçlarını kontrol grubu ile karşılaştırdığımızda, %20 tam yağlı süt eklenmiş yalancı iğde yaprağı çayı örneklerinde artış görülmüştür. Buna karşın %40 tam yağlı süt eklenmiş yalancı iğde yaprağı çayı örneklerinde ise düşüş gözlemlenmiştir. Diğer taraftan %20 yağsız süt eklenmiş yalancı iğde yaprağı çayı örneklerinde düşüş ve %40 yağsız süt eklenmiş örneklerde ise bir miktar artış görülmüştür. Yeşil çay örnekleri göz önüne alındığında ise %20 tam yağlı süt eklenmiş örnekler ile kontrol grubu arasında önemli bir fark görülmüyorken , %40 tam yağlı, %20 yağsız ve %40 yağsız süt eklenmiş örneklerde bir miktar düşüş görülmüştür. Hem yağlı hem yağsız süt eklenmiş yeşil çay ve yalancı iğde yaprağı çayı örneklerinin toplam fenolik miktarları arasnda istatistiksel olarak önemli bir fark bulunmamışken, kontrol grubu örneklerinin toplam fenolik madde miktarlarının, süt eklenmiş örneklerin toplam fenolik madde miktarlarından anlamlı derecede yüksek olduğu görülmüştür ($p<0.05$)

Sade ve süt eklenmiş çay örnekleri için toplam flavanoid miktarları kolorimetrik olarak hem rutin hem de kateşin eşdeğeri cinsinden ölçülmüştür. Etanolik yeşil çay örnekleri (115.2 mg Rutin/g), etanolik yalancı iğde çayı örneklerine (66.7 mg RE/g) göre rutince daha zengin bulunmuştur. Ayrıca demleme yeşil çay örneklerinin (43.8 mg RE/g) rutin cindinden flavanoid içerikleri demleme yalancı iğde yaprağı çayları örneklerine (39.9 mg RE/g) göre daha yüksektir. Rutin miktarı sonuçlarının tersine, etanolik (35.0 mg CE/g) ve demleme çayı (14.0 mg CE/g) yalancı iğde yaprağı örneklerinin kateşin miktarları etanolik (21.6 mg CE/g) ve demleme (12.3 mg CE/g) yeşil çay örneklerinden daha yüksektir ($p<0.05$). Süt ilave edilmiş çay örneklerinin flavanoid miktarları rutin cinsinden yalancı iğde yaprağı çayı için 19.0 ila 107.8 mg RE/g arasında, yeşil çay için ise 30.8 ila 88.4 mg RE/g arasında değiştiği gözlemlenmiştir. Ayrıca kateşin eşdeğeri cinsinden yalancı iğde yaprağı çayı için 5.9 ila 33.5 mg CE/g arasında, yeşil çay için ise 9.6 ila 27.5 mg CE/g arasında değiştiği gözlemlenmiştir. Süt eklenmiş yalancı iğde yaprağı çayı ve yeşil çay örnekleri ile kontrol grubu arasında, hem rutin hem de kateşin sonuçları benzer değişiklikler göstermiştir. Yağsız süt eklenmiş örneklerle kontrol grubu arasında küçük bir fark görülmüştür. Diğer taraftan tam yağlı süt eklenmiş örneklerde hızlı bir artış saptanmıştır ($p<0.05$).

Toplam antioksidan kapasitesi sonuçları etanolik yeşil çay örneklerinde ABTS için 89.2 mg TE/g ve CUPRAC için 622.9 mg TE/g arasında değişen değerlerle diğer örneklerden daha yüksek bulunmuştur. Hem yalancı iğde yaprağı çayı hem de yeşil çay için etanolik ekstraksiyonun, demleme ile karşılaştırıldığında toplam antioksidan kapasitesi üzerine yaklaşık 1.5-2.5 kat daha fazla etkili olduğu görülmüştür. Böylelikle, demleme yeşil çayın toplam antioksidan kapasitesi sonuçları 55.9 ila 227.0 mg TE/g arasında, etanolik yalancı iğde yaprağı çayının sonuçları 89.1 ila 456.1 mg TE/g arasında, demleme yalancı iğde yaprağı çayının sonuçaları da 55.7 ila 251.3 mg TE/g arasında değişmektedir. %20 tam yağlı süt eklenmiş yalancı iğde yaprağı çayı ve yeşil çay örneklerinin tümünde kontrol grubu örneklerine göre, uygulanan dört analizin sonuçlarında da düşüş gözlemlenmiştir. Yine benzer biçimde %20 yağsız süt eklenmiş yalancı iğde yaprağı çayı ve yeşil çay örneklerinde kontrol grubu ile kaşılatırıldıklarında ABTS analizi dışında düşüş gözlemlenmiştir. %40 süt eklenmiş örneklerin sonuçlarını göz önünde bulundurduğumuzda ise, hem tam yağlı hem de yağsız süt eklenmiş yalancı iğde yaprağı çayı örneklerinin DPPH analizi sonuçlarında küçük bir artış görülmüştür. Yine yağsız süt eklenmiş yeşil çay örneklerinin ABTS sonuçlarında da benzer şekilde küçük bir artış gözlemlenmiştir.

In vitro mide-bağırsak sistemi simülasyonu sonucunda Yalancı iğde yaprağı çayı ve Yeşil çay örneklerinin diyalize edilmiş toplam antioksidan kapasitesi sırasıyla %18.1 ve %8.3 olarak bulunmuştur. Süt ekleme, her iki çay çeşidi içinde diyalize edilmiş toplam antioksidan kapasitesinde artış sağlamıştır.

Süt eklenmiş örnek için, in vitro mide-bağırsak sistemi simülasyonunun ardından tespit edilen toplam fenolik madde miktarlarının ve toplam antioksidan kapasitelerinin yüzde geri kazanımı kontrol grubu örneklerine göre önemli ölçüde yüksekken, toplam flavanoid madde miktarları, yağsız süt eklenmiş yeşil çay örnekleri hariç önemli ölçüde düşüktür ($p<0.05$).

1. INTRODUCTION

Sea buckthorn (SBT) (*Hippophaë rhamnoides* L.) is a deciduous shrub with good adaptability to various climate conditions and extensive genetic variability with numerous greenish-yellow flowers and bright orange, globular, ellipsoid fruit. It is native to Europe and Asia and has been domesticated in several countries (India, China, Nepal, Pakistan, Myanmar, Russia, Britain, Germany, Finland, Romania, France, etc.) (Khan et al., 2010; Yogendra Kumar et al., 2011; Pop et al., 2013). In Turkey, SBT is widely distributed throughout North and East Anatolia and known locally as 'Yalancı igde, Cıcırgan, Dijırgan, Cıcılık' or 'Sincan Çalısı' (Aras - Tayhan, 1997). All parts of the plant are considered to be good source of large number of bioactive substances like vitamin, carotenoids, and flavonoids, organic acids, sterols and some essential amino acids. For instance, berries and seeds of SBT are well known for their antioxidative properties, attributed to hydrophilic and lipophilic compounds including ascorbic acid, flavonoids, proanthocyanidins and carotenoids. The leaves of SBT are also considered for their antioxidant potential correlated to flavonoids and phenolic acids derivatives, and they have been used in some countries to make extracts, tea, animal feed, pharmaceuticals and cosmetics (Michel et al., 2012; Wani et al., 2013).

Tea is originating from China and it has gained the world's taste in the past 2000 years. From past to present tea is one of the most popular beverages consumed worldwide, well ahead of coffee, beer, and wine and carbonated soft drinks. Green tea is a 'non-fermented' form of the tea, and contains more catechins, than black tea. The Assan type (*Camellia sinensis* var. assamica) has a too high content of polyphenols, which would make green tea taste excessively bitter. Green tea contains polyphenols, which include flavanols, flavandiols, flavonoids, and phenolic acids; these compounds may account for up to 30% of the dry weight. Green tea can be considered an important dietary source of polyphenols, particularly flavonoids. Flavonoids are phenol derivatives synthesized in substantial amounts (0.5–1.5%) and variety (more than 4000 identified), and widely distributed among plants (Vinson et al., 1995). Green tea might be regarded as a basic functional food, as it is naturally rich in active antioxidants. It could be incorporated into the diet in the form of a beverage for favorable oral and systemic health of older adults (Gaur & Agnihotri, 2014).

Flavonoids are the widest group of secondary metabolites involved in many biological functions in plants. They are classified into flavonols, flavones, flavanones, catechins, anthocyanidins and chalcones (Gupta et al., 2011). Most of the green tea polyphenols (GTPs) are flavonols, commonly known as catechins. Products derived from green tea are mainly extracts of green tea in liquid or powder form that vary in the proportion of polyphenols (45-90%) and caffeine content (0.4-10%). The composition of tea catechins, phenolic acids and caffeine in commercial teas varies with species, season, horticultural conditions and particularly with degree of fermentation during the manufacturing process (Zuo et al., 2002). Rutin is one of the bioactive flavonoid compounds, which are present in substantial amounts in various plants (Attanassova & Bagdassarian, 2009). Grapes and buckwheat are the most important rutin containing foods between fruits, vegetables and grain crops (Kreft et al., 2006). It is expected that seabuckthorn has a high rutin content. Rutin, a naturally occurring flavonol consisting of aglycone quercetin and a rutinoside moiety in position 3 of the C ring, is found in many food substances. It has been reported to exhibit beneficial effects against several types of liver diseases (Pan et al., 2014). Within the group of flavonoids, many studies have been conducted on rutin, since this flavonoid is of great therapeutic importance.

The interactions of phenolic compounds with proteins may lead to changes in physicochemical properties of proteins such as digestibility, solubility and thermal stability. In addition to nutritional properties of proteins may be affected as a result of the modification of essential amino acids and through the inhibition of proteases. In addition to this, the interactions of other compounds including lipids, other proteins, vitamins with proteins are also known to change the characteristics of those compounds. Polyphenols may interact with proteins both reversibly and irreversibly (Ozdal et al., 2013). Tea is commonly consumed with the addition of milk in some countries. Thus, interaction between phenolic compounds with milk protein in beverage systems has been the focus of various studies. Due to the rich contents in proteins and lipids, milk or milk isolates have been used as carriers for bioactive compounds by means of ligand binding in order to enhance the sensation, water solubility or bioavailability of bioactives. The interactions between individual catechins and pure proteins or milk proteins occurred with the formation of catechin–protein complexes (Ye et al., 2013).

Different extraction techniques used to obtain plant extracts rich in natural antioxidants have been widely investigated. Extraction with organic solvents presents major restrictions such as the presence of solvent residues in the extracts, which are often toxic, and the obtainment of undesirable compounds in the product (Do Prado et al., 2014). Thus, aqueous extraction techniques such as infusion and softening might be preferable. The aim of the present study is to investigate and compare the antioxidant properties and *in vitro* bioaccessibility of green tea and seabuckthorn leaf tea extracts and milk added samples.

2. LITERATURE REVIEW

2.1. Sea buckthorn (*Hippophae rhamnoides* L.)

Sea buckthorn (SBT) (*Hippophaë rhamnoides* L.) is a deciduous, dioecious plant. It is good adaptability to various climate conditions and extensive genetic variability with numerous greenish-yellow flowers and bright orange, globular, ellipsoid fruit. It is native to Europe and Asia and has been domesticated in several parts of the world. (India, China, Nepal, Pakistan, Myanmar, Russia, Britain, Germany, Finland, Romania, France, etc.) (Khan et al., 2010; Kumar et al., 2011; Pop et al., 2013). In Turkey, SBT is widely distributed throughout North and East Anatolia and known locally as 'Yalancı igde, Cıcırgan, Dijırgan, Cıcılık' or 'Sincan Çalısı' (Aras-Tayhan, 1997). The sea buckthorn plant is shown in Figure 2.1.

Figure 2.1: Sea buckthorn plant.

2.1.1. Composition and health benefits of sea buckthorn

All parts of the plant are considered to be good source of large number of bioactive substances like vitamins, carotenoids, and flavonoids, organic acids, sterols and some essential amino acids. For instance, berries and seeds of SBT are well known for their antioxidative properties, attributed to hydrophilic and lipophilic compounds including ascorbic acid, flavonoids, proanthocyanidins and carotenoids.

The leaves of SBT are also considered for their antioxidant potential correlated to flavonoids and phenolic acids derivatives, and they have been used in some countries to make extracts, tea, animal feed, pharmaceuticals and cosmetics (Michel et al., 2012; Wani et al., 2013). Also it has been used in traditional Chinese medicine going back more than 1000 years. Indepth survey and documentation of indigenous ethnobotanical knowledge of SBT reveal that this plant was traditionally utilized by local people of Asia, Nordic countries and the Baltic region in multidimensional aspects of food, fuel, medicine, veterinary, agricultural tools and bio-fencing (Yang et al., 2000; Dhyani et al., 2010). Sea buckthorn has been used extensively in oriental traditional system of medicine for treatment of asthma, skin diseases, gastric ulcers and lung disorders. A wide spectrum of pharmacological effects of SBT have been recently reported by numerous reserchers, including antioxidant, immunomodulatory, anti-atherogenic, anti-stress, hepatoprotective, radioprotective and tissue repair (Suryakumar & Gupta, 2011).

Sea buckthorn (*Hippophae rhamnoides* L.) is a unique plant currently being domesticated in several parts of the world. Fruit characteristics, Asiatic geographical distribution, and cultural practices, insofar as known, have been reviewed recently by Li & Schroeder (1996). The plant is reputed to have considerable medicinal value being useful for the treatment of skin disorders resulting from bed confinement, stomach and duodenal ulcers, cardiovascular diseases, and perhaps growth of some tumors. These beneficial effects have made sea buckthorn products, especially its oils, desirable for medicinal and cosmetic purposes. Products on the market from sea buckthorn range from oil, juice, and food additives to candies, jellies and cosmetics. The literature describing the formation of these products is both scattered and limited.

Nevertheless, the utilization of the sea buckthorn fruits and seeds, presently

Being grown in Saskatchewan and British Columbia, requires that processing be undertaken to capture the value added possibilities. What follows is a detailed

Summary of the available literature on sea buckthorn related to the processing and composition of products by utilizing juice, oil, and leaves. (Beveridge et al., 1999)

Flavonoid compounds existed in all part of sea buckthorn— root, stem, leaf, flower and fruit. According to the former Soviet Union researcher, in fresh fruit, the highest contents is 854 mg/100g, dried leaf is 3888 mg/100g; according to Chinese researcher, the average in fresh fruit is 354 mg/100g, leaf is 867 mg/100g. Studies also showed that in sea buckthorn from high sea level area, the flavonoids content was higher (Yuzhen & Fuheng, 1997).

The leaves of the plant are rich in flavonoids, tannins and triterpenes, and they have been used in certain countries to make extracts, tea, pharmaceuticals and cosmetics (Beveridge et al., 1999). Sea buckthorn has been shown to have many other benefits, including preventive effects against influenza infection, cardiovascular disease, mucosal injuries, skin disorders and the adaption to the effects of stress. Especially, extracts from sea buckthorn leaves exhibited antioxidant, anti-viral, anti-bacterial, anti-tumor and immunomodulatory properties. Gallic and ellagic acids of sea buckthorn leaf extracts are anti-diabetic and radio-protective. The phenolic rich fraction of sea buckthorn leaves has hepatoprotective action against oxidative damage. Taken together, sea buckthorn leaf extracts have important potential as natural antioxidants and in disease prevention. (Wani et al., 2013)

2.2. Green Tea

Tea is originating from China and it has gained the world's taste in the past 2000 years. From past to present tea is one of the most popular beverages consumed worldwide, well ahead of coffee, beer, wine and carbonated soft drinks.

Tea is a product made up from leaf and gemma of the plant *Camellia sinensis*. Green tea is a 'non-fermented' form of the tea, and includes more catechins, than black tea.

The Assan type (*Camellia sinensis* var. assamica) has a too high content of polyphenols, which would make green tea taste extremely bitter. Green tea leaves are shown in Figure 2.2.

Figure 2.2: Green tea leaves.

Depending upon the manufacturing process, teas are classified into three major types: 'non-fermented' green tea (produced by drying and steaming the fresh leaves to inactivate the polyphenol oxidase and by this way, non oxidation occurs); 'semi-fermented' oolong tea (produced when the fresh leaves are subjected to a partial fermentation stage before drying); and 'fermented' black and red (Pu-Erh) teas which undergo a post-harvest fermentation stage before drying and steaming, despite the fermentation of black tea is due to an oxidation catalyzed by polyphenol oxidase, and that of Pu-Erh tea is attained by using microorganisms (Cabrera et al., 2006). Figure 2.2. shows the principal differences between green and black tea processing.

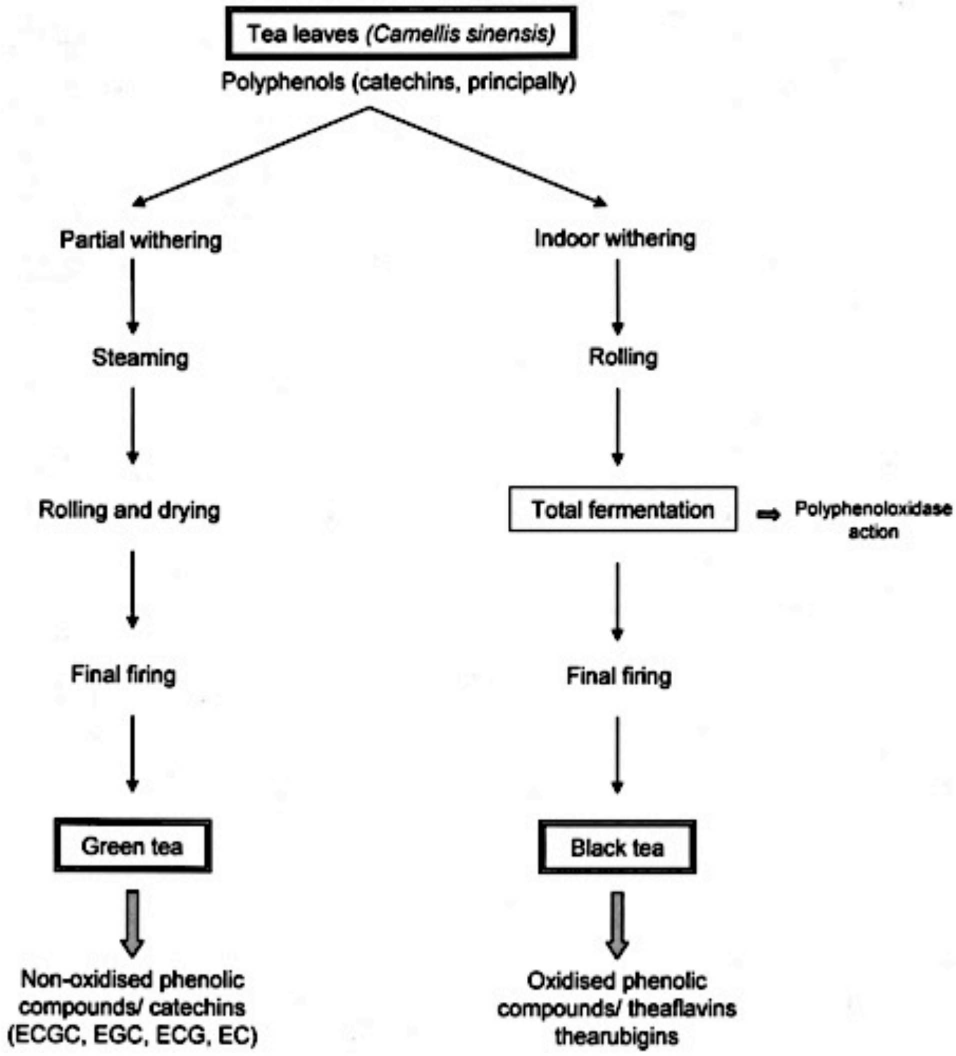

Figure 2.3: Principal differences between green and black tea processing (Cabrera et al., 2006).

The production of green tea is characterized by an initial heating process, which kills the enzyme polyphenol oxidase, which is responsible for the conversion of the flavanols in the leaf into the dark polyphenolic compounds that colour black tea.

The other significant process is rolling, in which leaves are cut and twisted. The ultimate form of green tea baseds on the appropriate variant being produced. The rolling stage is very similar to the operation with the same name in black tea production (Zuo et al., 2002)

2.2.1. Composition of green tea

The chemical composition of green tea is complex: proteins (15-20% dry weight), whose enzymes constitute an significant fraction; amino acids (1-4% dry weight) such as theanine or 5-N-ethylglutamine, tryptophan, glycine, serine, glutamic acid, aspartic acid, tyrosine, valine, leucine, threonine, arginine, and lysine; carbohydrates (5-7% dry weight) just as cellulose, glucose, fructose, pectins and sucrose; minerals and trace elements (5% dry weight) such as calcium, magnesium, chromium, manganese, iron, copper, zinc, molybdenum, selenium, sodium, phosphorus, cobalt, strontium, nickel, potassium, fluorine, and aluminum; and trace amounts of lipids (linoleic and a-linolenic acids), sterols (stigmasterol), vitamins (B, C, E), xanthic bases (caffeine, theophylline), pigments (chlorophyll, carotenoids), and volatile compounds (aldehydes, alcohols, esters, lactones, hydrocarbons. Fresh leaves comprise, on average, 3-4% of alkaloids known as methylxanthines, such as caffeine, theobromine, and theophylline (Chacko et al., 2010).

Due to the great importance of the mineral presence in tea, many studies have been performed to identify their levels in green tea leaves and their infusions. For instance, Costa et al. (2002) observed large variations of the mineral content (Al, Ca, Mg and Mn) in green tea from different origins.

Fernández-Cáceres et al. (2001) determined the content of Al, Ba, Ca, Cu, Fe, K, Mg, Mn, Na, Sr, Ti, and Zn in 46 tea samples, and no clear differences were found between mineral content of green and black teas.

Shu et al. (2003) observed the great variations among different tea varieties in accumulating fluoride and aluminum. Fung et al. (2003) pointed out that black tea had higher Al and F concentrations than green tea. Xu et al. (2003) reported that the content of Se in green teas was exceedingly increased by foliar application of Se-enriched ferilizers; furthermore, the selenium-enriched green tea exhibited significantly higher antioxidant activity than regular green tea.

Green tea contains polyphenols, which include flavanols, flavandiols, flavonoids, and phenolic acids; these compounds may account for up to 30% of the dry weight.

Green tea can be considered an important dietary source of polyphenols, particularly flavonoids. Flavonoids are phenol derivatives synthesized in considerable amounts (0.5–1.5%) and variety (more than 4000 identified), and relatively distributed between plants (Vinson et al., 1995). The United States Department of Agriculture (USDA) has recently published a Database for the Flavonoid Content of Selected Foods (Bhagwat et al., 2011).

Greater parts of the green tea polyphenols (GTPs) are flavonols, widely known as catechins. Products derived from green tea are mostly extracts of green tea in liquid or powder form that vary in the proportion of polyphenols (45-90%) and caffeine content (0.4-10%).

The composition of tea catechins, phenolic acids and caffeine in commercial teas varies with species, season, horticultural conditions and notably with degree of fermentation throughout the manufacturing process (Zuo et al., 2002). The structures of catechins, gallic acid and caffeine are shown in Figure 2.2.3.

There are four kinds of catechins mainly find in green tea: epicatechin, epigallocatechin, epicatechin-3-gallate, and EGCG. The preparation methods affect the catechins both quantitatively and qualitatively; the amount of catechins as well as varies in the original tea leaves due to differences in variety, origin, and growing conditions (Khokhar & Magnusdottir, 2002). The overview of green tea composition is shown in Table 2.1.

The relative catechin content of green tea subjected to how the leaves are processed before drying a certain grade of fermentation and heating of tea leaves during the manufacturing process can result in polymerization of monopolyphenolic compounds such as the catechins, leading to conformational changes and thus modifying its properties. Other factors influencing catechin content are the geographical location and growing conditions (soil, climate, agricultural practices, fertilizers), the type of green tea (e.g., blended, decaffeinated, instant…) and the preparation of the infusion (e.g., amount of the product used, brew time, temperature) (Hakim et al., 2000).

McKay & Blumberg (2002) stated that decaffeinating reduces merely the tea catechin content; also, instant preparations and iced and ready-to drink teas present fewer content of catequins. The production of bottled green tea beverage has encountered a browning problem mainly caused by the oxidation of catechins (L. Wang & Kim, 2003).

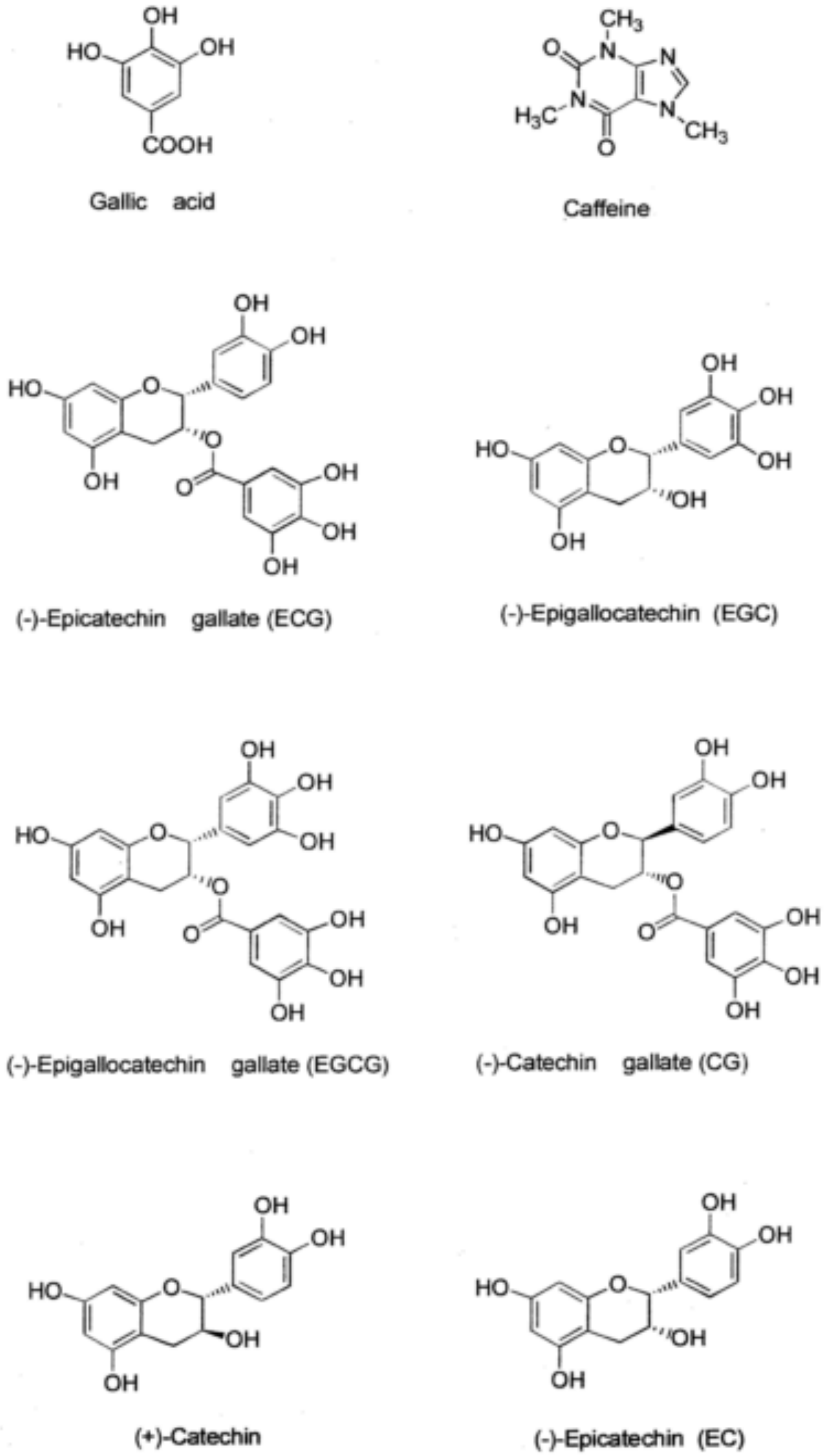

Figure 2.2.3: The structures of catechins, gallic acid and caffeine (Zuo et al., 2002).

Table 2.1 Composition of Green Tea (Gaur & Agnihotri, 2014).

Components	Concentration
Proteins	• 15–20% dry weight • Mainly enzymes
Carbohydrates	• 5–7% dry weight • Cellulose, pectins, glucose, fructose, sucrose
Lipids	• Linoleic and linolenic acids
Sterols	• Stigmasterol
Vitamins	• Vitamins B,C,E
Xanthic bases	• Caffeine • Theophylline
Pigments	• Chlorophyll • Carotenoids
Volatile compounds	• Aldehydes, alcohols, esters, lactones, hydrocarbons
Minerals and trace elements	• 5% dry weight • Calcium, magnesium, chromium, manganese, iron, copper, zinc, molybdenum, selenium, sodium, phosphorus, cobalt, strontium, nickel, potassium, fluoride and aluminium
Polyphenols	• Catechins: Epigallocatechin-3-gallate (59% of the total of catechins), epigallocatechin (19%), epicatechin-3-gallate (13.6%), epicatechin (6.4%)
Other components	• Gallic, chlorogenic and caffeic acids, kaempferol, myricetin, quercetin

Lin et al. (2003) analyzed 31 commercial teas, and determined that the levels of EGCG and total catechins were in the following order: green tea (old leaves) > green tea (young leaves) and oolong tea > black tea and Pu-Erh tea. Fernández et al. (2002) specified the contents of GA, EGCG, EGC, EC and ECG, in a set of 45 tea samples, comprising black and green teas from different geographical origins (i.e. China, Japan, Kenya, Sri Lanka, and India); the GA levels were always higher in black tea because the amount of GA increases throughout the fermentation process because of its liberation from catechin gallates (Xie et al., 1998). The amount of catechins were always higher in green tea; EGCG and EGC were the major catechins present with average contents of 7.358% and 3.955%, alternately; ECG presented values ranging between 0.910 and 3.556%. For black tea, EGCG and ECG were the catechins found in higher percentages, with average contents of 1.583 and 0.706%, respectively. Cabrera et al. (2003) stated the mean content of the four major catechins (EGCG, EGC, ECG and EC) and gallic acid in 45 samples of different types of tea including black, red, oolong and green teas; the higher levels of EGCG appeared in green tea samples.

2.2.2. Health benefits of green tea

It has long been a quest for the scientific community to describe food substances with benefits that extend besides their basic nutritional properties. The literature demonstrate that the physical, chemical, and physiological properties of food substances influence the oral tissues and organs. A diet rich in fermentable carbohydrates, acidic and soft foods is hazardous to oral health. Recent advances have led to the development of foods and beverages with "*functional ingredients*" that prevent the improvement or advancement of oral diseases. These contain non-fermentable sugars (polyols), antioxidant rich beverages, probiotics, prebiotics and foods with remineralizing properties (Wang & Lussi, 2012).

There are three categories of functional foods: basic foods with natural bioactives (e.g. carrots that contain beta carotenes), processed foods with added bioactives (e.g. milk supplemented with omega-3 fatty acids) and foods that are developed to have more bioactives (e.g. tomatoes with increased levels of lycopenes). Green tea might be declared a basic functional food, as it is naturally rich in active antioxidants. It could be included the diet in the form of a beverage for profitable oral and systemic health of older adults (Gaur & Agnihotri, 2014).

Studies using animal models indicate that green tea polyohenols supply some protection against degenerative diseases. Certain studies showed that green tea has an antiproliferative activity on hepatoma cells and a hypolipidemic activity in hepatoma-treated rats, besides the prevention of hepatoxicity and as a preventive agent against mammary cancer postinitiation (Crespy & Williamson, 2004). Green tea catechins could also act as antitumorigenic agents (Roomi et al*.,* 2005) and as immune modulators in immunodysfunction caused by transplanted tumors or by carcinogen treatment. In addition, green tea, its extract, and its isolated constituents were also found to be efficient in counteracting oxidative stress (Babu et al*.,* 2006) and neurological problems (Unno et al., 2007). Green tea consumption has also been related to the prevention of many types of cancer, containing lung, colon, mouth, stomach, small intestine, kidney, pancreas, and mammary glands (Koo & Cho, 2004). Several epidemiological studies and clinical trials showed that green tea (and black and Oolong teas to a lesser extent) may reduce the risk of many chronic diseases (Zaveri, 2006). This beneficial effect has been based on the presence of high amounts of polyphenols, which are potent antioxidants. Specifically, green tea may lower blood pressure and thereby reduce the risk of stroke and coronary heart disease. Some animal's studies suggested that green tea might protect against the improvement of coronary heart diseasebyreducingblood glucoselevelsand body weight (Tsuneki et al., 2004). However, all these data are founded on middle-aged animals' populations, not the elderly populations, which nutritional status tends to be more adversely influenced by age-related biological and socio-economic factors (Chacko et al., 2010).

3. MATERIALS AND METHODS

3.1. Plant material

Wild Sea buckthorn (*H. rhamnoides* L.) were harvested from Central Anatolia, particularly Sivas region (Figure 3.1 and Table 3.1).

Figure 3.1. Location of *H. rhamnoides* L. samples from Turkey

Table 3.1. Some characteristics of the sampling sites

Sampling site	Latitude/ Altitude Longitude	Annual precipitation (Mm)	Annual temperature (°C)	Climate type	Sampling dates
Sivas Sincan stream	39054'N– 2000m 37059'E	794	2.8	Humid	June 2013 August 2014

The leaves and branches of sea buckthorn were separated from each other and ventilated. In order to inhibit the activation of oxidation enzyme, leaves were subjected to shocking procedure. For this purpose plant materials were shocked with 90-100 °C vapor in rotary evaporator. Output materials of the shocking unit were cooled with dry air. Cooled materials were subjected to folding procedure for 25-30 min. After folding unit, leaves are transferred into the drying chamber and dried at 110 °C. At the end of this treatment sea bucktorn leaf tea were obtained. These materials stored in the dark at room temperature before use.

Green tea samples were purchased from local market in Istanbul, Turkey. The brand name of green tea was 'Çaykur Organik Zümrüt Yeşil Çay'.

Sea buckthorn leaf tea and green tea samples were extracted with ethanol as described below and also prepared in forms of tea infusions and further analyzed. All samples were analyzed for their contents of total phenolics and total flavonoids as well as their total antioxidant capacity by using four different methods including 1.1-diphenyl-2-picrylhydrazyl (DPPH), CUPRAC (Copper Reducing Antioxidant Capacity), FRAP (Ferric Reducing Antioxidant Power) and ABTS (2,2- azinobis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt).

3.2. Extraction Procedure

3.2.1. Ethanolic extraction

Two independent extractions were carried out for dried sea buckthorn leaf tea and green tea samples as described previously by Maheshwari et al. (2011) with slight modifications. 2.5 ± 0.01 g of each sample was extracted with 30 ml of 70% aqueous-ethanol in a cooled ultrasonic bath (Azakli, Turkey) for 15 min. After 24 h incubation, the treated samples were centrifuged (Hettich Zentrifugen Universal 32R, UK) for 10 min at 4000 rpm at 4 °C and the supernatant were collected. Then 10 ml 70% aqueous-ethanol was added to the pellet and this extraction procedure was repeated one more times. All three supernatants were combined and adjusted to a final volume of 50 mL. Prepared extracts were stored at -20 °C until analysis.

3.2.2. Tea infusion

For the preparation of tea infusions, 2±0.01 g of samples were weighed and dissolved in 100 ml of pure water at 90 °C. After waiting for 5 minutes, the leaves were removed and the infusion was filtered, and further analyzed.

3.2.3. Milk addition

Tea solutions with milk were prepared in the following way: the same quantity of tea infusion (2 g) was prepared in distilled water (100 ml at 90 °C), then water (control), skimmed and whole milk (at room temperature) was added to get 20% and 40% (v/v) milk concentration in final solutions. Samples were centrifuged (Hettich Zentrifugen Universal 32R, UK) for 10 min at 4000 rpm at 4 °C All solutions were prepared freshly and subjected to in vitro digestion immediately after preparation.

3.3. Determination of Total Phenolic Content (TPC)

The TP of extracts was determined using Folin-Ciocalteu reagent according to the method modified from Velioglu et al. (2006) using gallic acid as a standard. A mixture of 100 µl of the extract, 900 µl of distilled water and 1.5 ml of 0.2 N Folin-Ciocalteu reagents were prepared and allowed to react for 5 minutes. Then 1.2 ml 7.5% Na_2CO_3 solutions added into the reaction mixture. After incubation for 90 minutes at room temperature, the absorbance was measured at 765 nm using Optima SP-3000 nano spectrophotometer. The TP of extracts was expressed as mg of gallic acid equivalent (GAE) per g sample.

3.4. Determination of Total Flavonoid Content (TFC)

The TF was measured colorimetrically as described by Kim et al. (2003) a mixture of 0.25 ml of the extract, 1.25 ml of distilled water and 75 µl of 5% $NaNO_2$ were prepared and allowed to react for 6 minutes. Then 150 µl $AlCl_3.6H_2O$ was added and mixed througly. After 5 minutes 0.5 ml of 1M NaOH was added. The total volume was adjusted to 2.5 ml with distilled water. Absorbance of the mixture was measured at 510 nm versus prepared blank. The TF of extracts was determined by a (+)-rutin standard curve and expressed as mg of (+)-rutin equivalent (RE) per g sample.

3.5. Determination of Total Antioxidant Capacity (TAC)

Antioxidant activity of plant extract cannot be evaluated by only a single method due to the complex nature of phytochemicals. Therefore, in this study the TAC was estimated by two different assays. The DPPH and CUPRAC assays were performed according to Kumaran and Karunakaran (2006) and Apak et al. (2005) respectively. Both two assays, Trolox was used as a standard and results were expressed in terms of mg of Trolox equivalent (TE) per g sample.

3.5.1. DPPH-radical scavenging activity assay

The free radical scavenging activity of sea buckthorn leaves and branches aqueous and ethanolic extracts on DPPH radical were determined according to the method introduced by Kumaran et al. (2006) and Rai et al. (2006) 0.1 mM of DPPH was prepared by dissolving 3.943 mg DPPH with 100 ml ethanol. 100 µl of each extract was mixed with 2 ml ethanolic solution of DPPH (0.1 mM). Extracts were substituted by methanol and distilled water blanks. Decolourisation of purple free radical DPPH solution was measured at 517 nm after 30 min incubation in the dark and at room temperature. A trolox calibration curve was done between 0.01 and 0.2 mg/ml. Results were expressed in mg of trolox equivalents/g of extract (mg TE/g).

3.5.2. The Copper Reducing Antioxidant Capacity (CUPRAC) assay

The Cuprac assay was carried out according to Apak et al. (2005). $CuCl_2$ solution, 10^{-2} mM, was prepared by dissolving 0.4262 g $CuCl_2.2H_2O$ in distilled water, and diluted to 250 mL. Ammonium acetate buffer at pH=7.0, 1.0 M, was prepared by dissolving 19.27 g NH_4Ac in distilled water and diluted to 250 mL. Neocuproine (Nc) solution, 7.510^{-3} M, was prepared daily by dissolving 0.078 g Nc in 96% ethanol, and diluted to 50 mL with ethanol. 100 µl of extract was mixed with 1 ml of $CuCl_2$ solution, 1 ml of Nc solution, 1 ml of Ammonium acetate buffer and 1 ml of distilled water. After 30 minutes absorbances was measured at 450 nm against reagent blank. Results were expressed in mg of Trolox equivalents/g of sample.

3.6. Simulated *in vitro* gastrointestinal (GI) digestion

In order to simulate the *in vitro* GI digestion conditions, and to determine the amount of free soluble polyphenols potentially available for further uptake, the procedure adapted from McDougall et al. (2005) was followed. To follow the release of phytochemicals (phenolics, flavonoids and anthocyanins) from tea samples, at different stages of digestion, aliquots from gastric digesta, GI digesta and dialyzable content were analyzed, respectively. Antioxidant activity was also determined.

3.6.1. Gastric phase

In a 250 mL glass beaker, 5 g of homogenized tea infusions were mixed with 20 mL stomach solution (7.5 ml of 10 mg pepsin in 10 ml 0.1N HCl + 0.2 g NaCl + 100 ml distilled water). The pH was adjusted to 2 by adding 5 M HCl and the beaker was sealed with parafilm. The mixture was incubated for 2 h in a Memmert shaking water bath (Nürnberg, Germany) at 37 °C and 100 rpm. After 2 h, 2 mL aliquots of the postgastric digestion were collected and stored at -20 ^{0}C until further analysis. A blank was prepared with identical chemicals but without food matrix, and underwent the same conditions as the samples.

3.6.2. Intestinal phase

4.5 mL of 4 mg/mL pancreatin and 25 mg/mL bile salt mixture were added to the remainder in the glass beaker. Segments of dialysis bags were cut to a specified length, and filled with sufficient $NaHCO_3$ (20 mL) to neutralize the sample's titratable acidity and then the beaker was again sealed with parafilm. After 2 h of incubation in Memmert shaking water bath (Nürnberg, Germany) at 37 °C and 100 rpm, the solution in the dialysis tubing was taken as the IN sample representing the material that entered the serum and the solution outside the dialysis bags were taken as the OUT sample representing material that remained in the gastrointestinal tract. IN and OUT samples were stored at -20 ^{0}C until further analysis.

3.6.3. Further sample preparation for analysis

Prior to analysis, post gastric, IN and OUT samples were thawed and centrifuged (Hettich Zentrifugen Universal 32R Tuttlingen, Germany) at 18000 rpm and the supernatants were assayed for total phenolic (TP), flavonoid (TF) and antioxidant capacity (TAC). The calibration curves obtained by TP, TF and TAC are shown in the Appendix, Figures A.1-A.14.

3.7. Statistical Analysis

All the experiments were replicated three times and the data were represented as mean ± SD. For multiple comparisons, data were subjected to statistical analysis using SPSS software (version 16.0 for Windows, SPSS Inc.) for the analysis of variance (ANOVA). Duncan's new multiple range test was used to analyze differences between treatments ($p<0.05$).

4. RESULTS AND DISCUSSION

4.1. Total Phenolic Content (TP)

TP of all fractions are expressed on fresh weight basis in Table 4.1. and 4.2. Comparing the extraction methods, results showed that ethanolic extacts of green tea (EG) was richer in phenolic material (153.5 mg of GAE/ g sample) compared to ethanolic extracts of sea buckthorn leaf tea (ES) (114.8 mg of GAE/g sample). On the contrary, sea buckthorn leaf tea (TS) had higher phenolic content than green tea (TG) considering that the tea infusion forms. In fact, the highest TP content among all fractions was observed in Ethanolic green tea extract (EG) ($p<0.05$).

Table 4.1: The contents of total phenolics of Green tea and Sea buckthorn leaf tea extracts.

Sample	Extraction method	Total phenolics (mg GAE/g)
Green Tea	**Ethanolic extraction (EG)**	153.5 ± 14.4^{a}
	Tea infusion (TG)	65.7 ± 9.7^{d}
Sea buckthorn Leaf Tea	**Ethanolic extraction (ES)**	114.8 ± 8.9^{b}
	Tea infusion (TS)	85.0 ± 4.8^{c}

[1]Data represent average quantities ± standard deviation of 3 independent samples. Different letters in columns represent statistically significant differences ($p<0.05$).

Several authors have determined the profile of major phenolic compounds of SBT (Arimboor & Arumughan, 2012; Arimboor et al., 2008; Pop et al., 2013; Upadhyay et al., 2010; Zu et al., 2006). In these studies, compounds belonging to groups of phenolic acids, flavonols, and flavones were identified. In the group of phenolic acids, gallic acid (GA) was the dominant phenolic acid for SBT leaves. Arimboor et al. (2008) estimated the polyphenols content of parts of the SBT plant (seed kernel, leaves, seed coat and pulp). According to their results highest amount of polyphenols was found in seed kernel (11.4 g/100 g of dry mass) and leaves (10.9 g/100 g of dry mass) followed by pulp (2.6 g/100 g of dry mass) and seed coat (1.2 g/100 g of dry mass). Similarly Bittová et al. (2014) summarized the data about polyphenolic profiles in various plant parts (leaves, shoots, berries) of SBT (*Hippophaë rhamnoides* L.). According to their results TPC values increased in the following order: fruits < shoots < leaves. As it is seen from results of these studies, SBT leaves have non-negligible amounts of phenolic compounds. Within this scope SBT leaves are gradable as herbal tea in the food industry. Green tea is known for its high phenolic content for ages. Regarding this matter plenty of studies were performed (Carloni et al., 2013; Giménez et al., 2013; Jeszka-Skowron et al., 2015; Vinson et al., 1995). Carloni et al. (2013) compared the antioxidant properties of teas (white, green and black tea) coming from a single cultivar but processed differently. TPC was found to be higher in green and low-caffeine green teas (23.6 and 22.6 mM GAE) followed by the white (18.1 mM GAE) and black and Orthodox teas (10.7 and 14.9 mM GAE). Cho et al. (2014) compared the antioxidant activities of SBT leaf tea and green tea similarly with our study. In their study, the polyphenol contents of ethanolic and tea infusion forms of SBT leaf tea were 47.8 and 22.9 mg GAE/ g while green tea results were 45.7 and 100.2 mg GAE/ g respectively. Our results higher than their ethanolic extract results and tea infusion of SBT leaf tea results. Also our results were differ from theirs as tea infusion of SBT leaf tea had higher phenolic content than green tea.

TP results of milk added samples were ranged between 43.6 to 58.4 mg GAE/g for SBT leaf tea and 37.8 to 44.9 mg GAE/g for green tea. Comparing the control group, whole and skimmed milk added samples, results showed that %20 whole milk added samples of SBT were increased compared to the 20% control group. However there were a decrease in 40% whole milk added samples. On the other hand there were a decrease in 20% skimmed milk added SBT and there were a little increase in 40% skimmed milk added samples of SBT. Considering the green tea samples, there were no significant variation between 20% of whole milk and control group samples while slight decreases were observed between control groups and 40% whole milk, 20% skimmed milk and 40% skimmed milk added samples. There were no significant difference between the TP of skimmed and whole milk added both green tea and SBT leaf tea samples, however, control groups were significantly higher than milk added samples ($p<0.05$).

Table 4.2: The contents of total phenolics of milk added green tea and sea buckthorn leaf tea extracts.

Sample	Total phenolics (mg GAE/g)
SBT+ 20% H_2O	54.8 ± 7.4[a]
SBT+ 20% WM	58.4 ± 16.5[b]
SBT+ 20% SM	44.4 ± 4.1[b]
SBT+ 40% H_2O	44.8 ± 4.7[a]
SBT+ 40% WM	43.6 ± 5.4[b]
SBT+ 40% SM	46.8 ± 6.1[b]
GT+ 20% H_2O	44.9 ± 9.4[a]
GT+ 20% WM	44.0 ± 3.1[b]
GT+ 20% SM	41.3 ± 6.8[b]
GT+ 40% H_2O	41.5 ± 8.9[a]
GT+ 40% WM	38.2 ± 4.2[b]
GT+ 40% SM	37.8 ± 4.9[b]

[1]The terms represent; SBT, sea buckthorn leaf tea; GT, green tea; WM, whole milk; SM, skimmed milk. Data represent average values ± standard deviation of 3 independent samples. Different letters in columns represent statistically significant differences ($p<0.05$).

In some countries, milk is commonly added to tea, which may lead to the formation of polyphenol-milk protein complexes. Some authors have proposed that the formation of these complexes may decrease the bioavailability and the antioxidant potential of polyphenols in vivo. In this context several studies were performed (Moser et al., 2014; Ryan & Petit, 2010; Sharma et al., 2008; Xie et al., 2013). Sharma et al. (2008) investigated the effect on antioxidant and radical scavenging activities of black tea on addition of milk and sugar. Their results showed that the black tea extracts (B) possesses maximum amount of polyphenol (%) followed by black tea with sugar (BS) and black tea with milk and sugar (BMS). But the free total polyphenols were found to be low in black tea with milk BM similar to our results. They interpreted these results as it could be due to interaction between plant phenolics and proteins either covalently or non-covalently. Both ways may lead to precipitation of proteins, via either multisite interactions or multidentate interactions. This could lead to the masking of polyphenols i.e., catechins and the flavonoids on the milk proteins. The types of interaction depend on the molar ratio of phenolic compounds to proteins.

4.2. Total Flavonoid Content (TF)

TF of plain and milk added tea samples were measured colorimetrically in terms of both rutin and catechin equivalent (Table 4.3 and 4.4) and found to be higher rutin in EG (115.2 mg of Rutin eq./g) compared to ES (66.7 mg of RE/g). Also, the flavonoid content in TG (43.8 mg of RE/g) was higher than TS (39.9 mg of RE/g). Contrary the rutin content results, ES (35.0 mg of Catechin eq./g) and TS (14.0 mg of CE/g) were higher catechin content than EG (21.6 mg of CE/g) and TG (12.3 mg of CE/g). There was no significant difference between the TF of tea infusion forms both in terms of rutin and catechin, however, EG was significantly higher than ES in terms of rutin and ES was significantly higher than EG in terms of catechin ($p<0.05$).

Table 4.3 : The contents of total flavonoids of Green tea and Sea buckthorn leaf tea extracts.

Sample	Extraction Method	Total flavonoids (mg RE/ g)	Total flavonoids (mg CE/g)
Green Tea	Ethanolic extraction (EG)	115.2 ± 10.5^{a}	21.6 ± 3.7^{b}
	Tea infusion (TG)	43.8 ± 6.6^{c}	12.3 ± 0.8^{c}
Sea buckthorn Leaf Tea	Ethanolic extraction (ES)	66.7 ± 5.1^{b}	35.0 ± 4.0^{a}
	Tea infusion (TS)	39.9 ± 2.9^{c}	14.0 ± 2.5^{c}

[1]Data represent average quantities ± standard deviation of 3 independent samples. Different letters in columns represent statistically significant differences ($p<0.05$).

Many researchers performed studies about flavonoid contents of green tea and sea buckthorn leaves (Bittová et al., 2014; Carloni et al., 2013; Chi et al., 2009; Cho et al., 2014; Michel et al., 2012; Pop et al., 2013; Vinson et al., 1995; Yuzhen & Fuheng, 1997; Zu et al., 2006; Zuo et al., 2002). Bittová et al., (2014) monitored the HPLC profiles of polyphenolic compounds in different SBT plant parts during annual growth cycle and estimation of their antioxidant potential. Their results showed that catechin, epicatechin and gallic acid were the most abundant analytes in annual green shoots and leaves, and their content varied significantly during the studied period. Besides, according to the result of Pop et al. (2013) leaves had higher levels of flavonol glycosides than berries, at average 1118 mg/100 g dry weight. On the other hand, isorhamnetin were the predominant flavonoid for berries, but rutin, quercetin-3-glucoside and kaempferol were found to be predominant in leaves. Generally green tea is known as its rich catechin content. Besides studies showed that rutin is significant flavonoid compund for SBT leaves. Therefore we determined our TF results as both catechin and rutin equivalent.

Table 4.4: The contents of total flavonoids of milk added green tea and sea buckthorn leaf tea extracts.

Sample	Total flavonoids (mg RU eq./g)	Total flavonoids (mg CA eq./g)
SBT+ 20% H_2O	29,5 ± 3.5[b]	9,2 ± 1,1[b]
SBT+ 20% WM	95,2 ± 19,8[a]	29,6 ± 6,2[a]
SBT+ 20% SM	22,6 ± 3,0[c]	7 ,0 ± 0.9[b]
SBT+ 40% H_2O	20,5 ± 4.2[b]	6,4 ± 1,3[b]
SBT+ 40% WM	107,8 ± 7,1[a]	33,5 ± 2.2[a]
SBT+ 40% SM	19 ± 4,0[c]	5,9 ± 1.3[b]
GT+ 20% H_2O	32,2 ± 9,6[b]	10,0 ± 3,0[b]
GT+ 20% WM	78,5 ± 12,0[a]	24,4 ± 3.7[a]
GT+ 20% SM	33,4 ± 8,1[c]	10,4 ± 2.5[b]
GT+ 40% H_2O	32,7 ± 10.4[b]	10,1 ± 3,2[b]
GT+ 40% WM	88,4 ± 8,4[a]	27,5 ± 2.6[a]
GT+ 40% SM	30,8 ± 4,3[c]	9,6 ± 1.3[b]

[1]The terms represent; SBT, sea buckthorn leaf tea; GT, green tea; WM, whole milk; SM, skimmed milk. Data represent average values ± standard deviation of 3 independent samples. Different letters in columns represent statistically significant differences ($p<0.05$).

TF of milk added tea samples were observed range between 19.0 to 107.8 mg RE/g for SBT leaf tea and 30.8 to 88.4 mg RE/g for green tea. Also catechin equivalent results were observed range between 5.9 to 33.5 mg CE/g for SBT leaf tea and 9.6 to 27.5 mg CE/g for green tea. Both rutin and catechin results showed similar variation between control group and milk added samples of SBT leaf tea and green tea. There were only slight differences among the skimmed milk added sample and control group results. On the other hand, rapid increases were detected on the whole milk added samples ($p<0.05$). These increases were not expected under normal conditions. In our study we didn't apply removing process of fat from whole milk added samples. Therefore milk fat might interact with flavonoid compounds in tea samples and consequently this unexpected increase might be consisted.

4.3. Total Antioxidant Capacity (TAC)

To investigate the antioxidant activities of SBT leaf tea and green tea, four different *in vitro* assays (DPPH, CUPRAC, FRAP, ABTS) were used. Results shown in table 4.5 and 4.6. As it is seen from the results, TAC was found to be higher in EG, ranging from 89,2 mg of TE/g for ABTS to 622,9 mg of TE/g for CUPRAC. For both SBT leaf tea and green tea, ethanolic extractions were more efficient to the total antioxidant capacity compared to tea infusions, having ~1.5-2.5 fold higher capacity. Thus, TAC for TG varied from 55.9-227.0 mg of TE/g, 89.1-456.1 mg of TE/g for ES and 55.7-251.3 mg of TE/g for TS.

Table 4.5: The total antioxidant capacities of sea buckthorn leaf tea and green tea extracts

Sample	**Extraction Method**	**DPPH (mg TE/g)**	**CUPRAC (mg TE/g)**	**FRAP (mg TE/g)**	**ABTS (mg TE/g)**
Green Tea	**Ethanolic extraction (EG)**	185.6 ±2.8[a]	622.9 ±32.3[a]	111.4 ±2.0[a]	89.2 ± 0.5[a]
	Tea infusion (TG)	108.6 ±8.0[c]	227.0 ±27.9[c]	61.8 ± 8.2[c]	55.9 ± 0.4[b]
Sea buckthorn Leaf Tea	**Ethanolic extraction (ES)**	184.5 ±2.0[a]	456.1 ±36.6[b]	96.4 ± 5.9[b]	89.1 ± 0.7[a]
	Tea infusion (TS)	115.4 ±1.1[b]	251.3 ±31.2[c]	66.8 ± 7.3[c]	55.7 ± 0.3[b]

[1]Data represent average quantities ± standard deviation of 3 independent samples Different letters in columns represent statistically significant differences ($p<0.05$).

According to all four analyses carried out, TAC was decreased in 20% whole milk added samples both SBT leaf tea and green tea, respectively. Similarly TAC of 20% skimmed milk added samples decreased both SBT leaf tea and green tea except ABTS assay results. Considering the 40% milk added samples results, there were slight increase in both whole and skimmed milk added SBT leaf tea in DPPH assay. There was also slight increase in skimmed milk added green tea in ABTS assay ($p<0.05$).

In literature, there are numerous study were performed about antioxidant capacity of both SBT leaves, green tea and milk addition of tea samples (Anissi et al., 2014; Geetha et al., 2002; Kusmita et al., 2015; Ryan & Petit, 2010; Upadhyay et al., 2010; Wani et al., 2013). Our results are parallel with these study results.

Table 4.6: The total antioxidant capacities of milk added sea buckthorn leaf tea and green tea extracts

Sample	DPPH (mg TE/g)	CUPRAC (mg TE/g)	FRAP (mg TE/g)	ABTS (mg TE/g)
SBT+ 20% H_2O	116.8 ± 14.0[a]	194.2 ± 24.6 [a]	67.2 ± 10.6[a]	104.4 ± 30.6[b]
SBT+ 20% WM	113.2 ± 6.3[b]	190.3 ± 32.3 [a]	44.3 ± 10.5[b]	125..2 ± 2.7[a]
SBT+ 20% SM	113.6 ± 12.4[b]	180.2 ± 25.8 [a]	35.2 ± 8.5[b]	115.2 ± 0.4[a]
SBT+ 40% H_2O	96.1 ± 12.7[a]	183.9 ± 34.6 [a]	60.0 ± 13.4[a]	117.0 ± 16.9[b]
SBT+ 40% WM	98.6 ± 11.2[b]	156.4 ± 22.8 [a]	33.2 ± 5.3[b]	97.9 ± 10.5[a]
SBT+ 40% SM	100.9 ± 12.6 [b]	134.4 ± 16.7 [a]	32.8 ± 7.8[b]	111.5 ± 7.7[a]
GT+ 20% H_2O	113.6 ± 16.2[a]	175.2 ± 42.5 [a]	58.9 ± 16.3[a]	117.6 ± 14.8[b]
GT+ 20% WM	93.6 ± 19.6[b]	160.1 ± 30.6 [a]	48.7 ± 14.1[b]	114.3 ± 8.8[a]
GT+ 20% SM	99.4 ± 13.8[b]	142.0 ± 28.4 [a]	43.2 ± 12.1[b]	121.5 ± 6.7[a]
GT+ 40% H_2O	102.9 ± 20.3[a]	141.6 ± 19.9 [a]	49.3 ± 18.8[a]	90.4 ± 0.8[b]
GT+ 40% WM	79.3 ± 12.3[b]	125.7 ± 21.3 [a]	33.1 ± 9.4[b]	89.3 ± 7.2[a]
GT+ 40% SM	85.9 ± 18.3[b]	123.3 ± 32.1 [a]	34.5 ± 9.3[b]	102.3 ± 16.6[a]

[1]The terms represent; SBT, sea buckthorn leaf tea; GT, green tea; WM, whole milk; SM, skimmed milk. Data represent average values ± standard deviation of 3 independent samples. Different letters in columns represent statistically significant differences ($p<0.05$).

Many *in vitro* and *in vivo* antioxidant capacity assays have been developed, but only a few are rapid and reliable methods that are applicable for a large number of plant extract samples (Cai et al., 2004). Yet, the measurement of antioxidant activities, in the case of multifunctional or complex multiphase systems, like herbs and spices, cannot be evaluated satisfactorily by a single method. The principles of the methods vary exceedingly depending upon the radical that is generated or the time of reaction. Even the methods based on the same principle such as ABTS and DPPH can show several important differences in their response to antioxidants. Furthermore, various different mechanisms may contribute to oxidative processes, such as in Fenton reactions, where transition metal ions play a vital role, different reactive oxygen species might be generated and various target structures such as lipids, proteins and carbohydrates, can be influenced (Hinneburg et al., 2006). For this reason, it is highly advised to apply several test procedures to estimate antioxidant activities to obtain the full picture. All antioxidant assays applied in this study have been widely used to investigate the antioxidant capacities of plant extracts as they require relatively standard equipment and deliver fast and reproducible results. The ABTS assay is specifically interesting in plant extracts due to the wavelength absorption at 734 nm eliminates color interference (Dudonne et al., 2009).

4.4. *In Vitro* Gastrointestinal (GI) Digestion

4.4.1. Total Phenolic Content (TP)

The impact of *in vitro* GI digestion on TP is shown in Figure 4.1, 4.2 and Table 4.7. For both SBT leaf tea and green tea samples, the significant decrease in TP were evident when the amounts before and after pepsin digestion (85 from 26.3 and 65.7 from 29.2 mg of GAE/ g sample for SBT leaf ta and green tea, respectively) were compared. When the amount after intestinal digestion was analyzed, 18.8 and 15.2% of the compounds were present in the dialyzed fraction (IN) for SBT leaf ta and green tea, respectively, whereas the rest remained in the non-dialyzed fraction (OUT).

Table 4.7 : Total phenolic content of *in vitro* digested Sea buckthorn leaf tea and Green tea samples, expressed as gallic acid equivalent (GAE) per g weight.

Sample	Initial	PG	IN	OUT
Sea bucthorn tea	85.0 ± 4.8^{a}	26.3 ±9.0 ^{c}	15.3 ±1.4^{a}	119.5 ±14.3 ^{a}
SBT+ 20% H_2O	54.8 ± 7.4^{b}	55.8 ±12.1^{c}	6.8 ±2.6 ^{ab}	23.7±6.0^{c}
SBT+ 20% WM	58.4 ± 16.5^{b}	79.5 ±5.9 ^{a}	12.3 ±2.2 ^{ab}	33.5±8.2 ^{c}
SBT+ 20% SM	44.4 ± 4.1^{b}	91.2 ±11.8 ^{b}	11.5 ±3.5 ^{ab}	35.0 ±7.7 ^{c}
SBT+ 40% H_2O	44.8 ± 4.7^{c}	41.0 ±15.6 ^{c}	5.9 ±2.5 ^{ab}	27.8 ±9.5 ^{c}
SBT+ 40% WM	43.6 ± 5.4^{c}	108.2 ±24.1^{a}	16.8 ±5.5 ^{ab}	34.1 ±7.8 ^{c}
SBT+ 40% SM	46.8 ± 6.1^{c}	80.1 ±7.4^{b}	18.0 ±5.4 ^{ab}	27.4 ±7.5 ^{c}
Green tea	65.7 ± 9.7^{a}	29.2 ±6.0^{c}	10.0 ±2.6^{b}	80.9 ±14.1^{b}
GT+ 20% H_2O	44.9 ± 9.4^{b}	50.3 ±8.6 ^{c}	5.5 ±2.1 ^{b}	17.0 ±9.6 ^{c}
GT+ 20% WM	44.0 ± 3.1^{b}	67.8 ±8.7 ^{a}	8.5 ±4.1 ^{b}	38.3 ±12.1 ^{c}
GT+ 20% SM	41.3 ± 6.8^{b}	71.1±15.3 ^{b}	12.4 ±2.5 ^{b}	33.3 ±11.3 ^{c}
GT+ 40% H_2O	41.5 ± 8.9^{c}	49.4 ± 14.0 ^{c}	5.5 ±2.1 ^{b}	17.7 ±8.1 ^{c}
GT+ 40% WM	38.2 ± 4.2^{c}	92.4 ±19.9 ^{a}	13.3 ±2.8 ^{b}	38.4 ±9.2 ^{c}
GT+ 40% SM	37.8 ± 4.9^{c}	66.0 ±15.5 ^{b}	16.4 ±1.8 ^{b}	42.3 ±14.5 ^{c}

[1]The terms represent; Initial, as initially determined from tea samples using tea infusion method; PG, phenolics remaining after gastric digestion; IN, dialyzed fraction after intestinal digestion; OUT, non-dialyzed fraction after intestinal digestion, SBT, sea buckthorn leaf tea; GT, green tea; WM, whole milk; SM, skimmed milk. Data represent average values ± standard deviation of 3 independent samples. Different letters in columns represent statistically significant differences ($p<0.05$).

For both tea samples, IN values were higher in milk added samples compared to control group samples. The increases in IN value as a result of milk addition were found to be 55.11 and 34.8% for whole milk added samples, 58.9 and 32.8% for skimmed milk added samples for SBT leaf tea; on the other hand an increase by 64.3 and 41% for whole milk added samples and 68.4 and 81.3% for skimmed milk added samples were observed for Green tea. For all fractions (plain, control group, whole milk added, skimmed milk added) in each tea sample, IN values were significantly lower compared to the initial TP values ($p<0.05$). TP of plain tea samples after gastric phase, showed significant losses in comparison to initial values whereas an increase in TP of milk added samples were observed. Milk is commonly added to fruit-based beverages in order to improve their sensorial and nutritional properties (Zulueta et al., 2007) but it contains high amounts of proteins. Although complexes can be formed reversibly or irreversibly, depending on factors such as the protein type, phenolic concentration, pH and temperature (Roura et al., 2008), they interfere in the bioaccessibility of phenolic constituents.

Results demonstrate that the complexity of the foodmatrix in which bioactive compounds are contained can affect their degree of digestibility and therefore, their bioaccessibility. In addition to this, gastrointestinal digestion could afford either the transformation of phenolic compounds into other phenolic derivatives or the interaction with other dietary constituents. Phenolic substances could interact with dietary proteins, iron or fiber, leading to the formation of complexes (Rodríguez-Roque et al*.,* 2014).

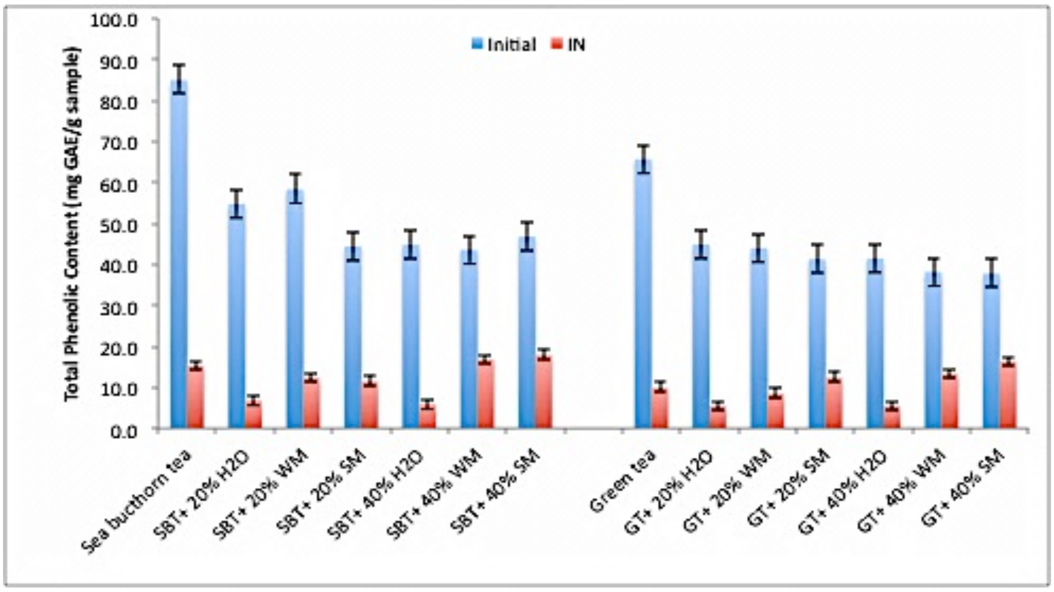

Figure 4.1: Total phenolic content of Sea buckthorn leaf tea and Green tea samples, expressed as gallic acid equivalent (GAE) per g weight. The terms represent; Initial, as initially determined from tea samples using tea infusion method; IN, dialyzed fraction after intestinal digestion; SBT, sea buckthorn leaf tea; GT, green tea; WM, whole milk; SM, skimmed milk. Data represent average values ± standard deviation of 3 independent samples**.**

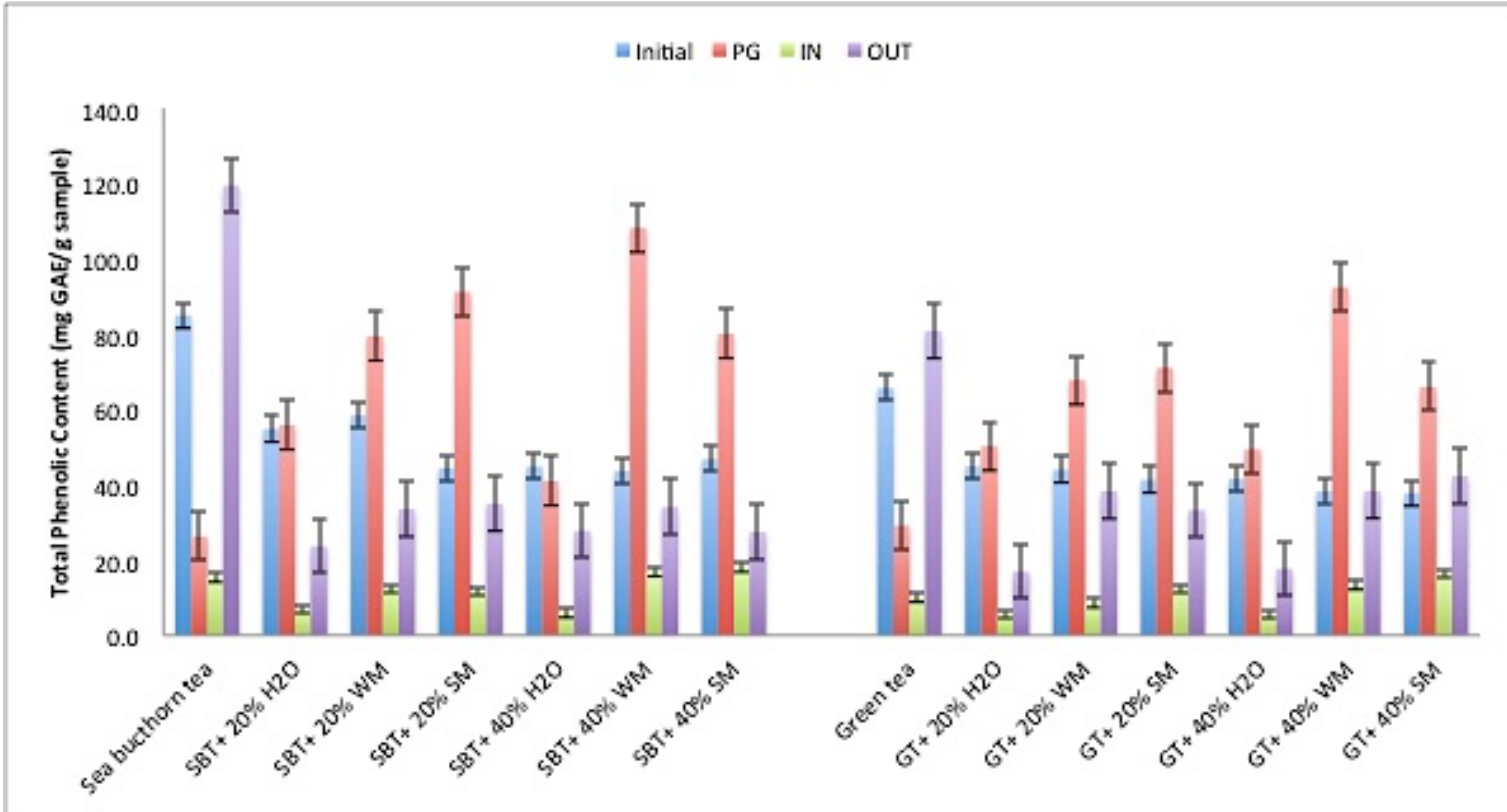

Figure 4.2: Total phenolic content of Sea buckthorn leaf tea and Green tea samples, expressed as gallic acid equivalent (GAE) per g weight. The terms represent; Initial, as initially determined from tea samples using tea infusion method; PG, phenolics remaining after gastric digestion; IN, dialyzed fraction after intestinal digestion; OUT, non-dialyzed fraction after intestinal digestion, SBT, sea buckthorn leaf tea; GT, green tea; WM, whole milk; SM, skimmed milk. Data represent average values ± standard deviation of 3 independent samples.

4.4.2. Total Flavonoid Content (TF)

Total flavonoid content of initial samples, PG (post gastric), IN (dialyzed) and OUT (non-dialyzed) fractions and their comparison with TF of extracts are given in Figure 4.3, 4.4 and Table 4.8.

During the in vitro simulation of GI digestion, for both tea samples, an increase in TF was observed after pepsin digestion except whole milk added samples. The dialyzed flavonoid fraction represented 26.6% for plain SBT leaf tea, 8.4 and 4.1% for whole milk added SBT leaf tea, 17.3 and 24.7% for skimmed milk added SBT leaf tea and 20% for plain green tea, 12.4 and 7.9% for whole milk added green tea, 24.3 and 22.1% for skimmed milk added green tea of the initial TF values for SBT leaf tea and green tea, respectively. In contrast to TP, IN values were lower in both whole and skimmed milk added samples compared to plain tea samples ($p<0.05$).

Table 4.8 : Total flavonoid content of *in vitro* digested Sea buckthorn leaf tea and Green tea samples, expressed as Rutin equivalent (RE) per g weight.

Sample	Initial	PG	IN	OUT
Sea bucthorn tea	39.9 ±2.9^{b}	57.6 ±9.2^{a}	10.6 ±2.8^{a}	39.0 ±3.7^{b}
SBT+ 20% H_2O	29,5 ± 3.5^{b}	46.2 ±16.2 ^{ab}	5.7 ±2.8^{c}	20.4 ±4.0 ^{b}
SBT+ 20% WM	95,2 ± 19,8^{a}	57.9 ±6.3 ^{ab}	8.0 ±6.2 ^{c}	76.4 ±15.0^{a}
SBT+ 20% SM	22,6 ± 3,0^{c}	37.7 ±14.2 ^{ab}	3.9 ±1.7 ^{c}	22.3 ±5.3 ^{b}
SBT+ 40% H_2O	20,5 ± 4.2^{b}	36.8 ±11.1 ^{ab}	4.0 ±0.7 ^{c}	17.8 ±2.6 ^{b}
SBT+ 40% WM	107,8 ± 7,1^{a}	61.1 ±11.3 ^{ab}	4.4 ±1.5 ^{c}	78.0 ±38.5^{a}
SBT+ 40% SM	19 ± 4,0 ^{c}	44.5 ±9.5 ^{ab}	4.7 ±2.5 ^{c}	20.7 ±8.1 ^{b}
Green tea	43.8 ±6.6 ^{b}	40.5 ±6.6^{b}	8.7 ±2.6^{ab}	43.3 ±4.4 ^{b}
GT+ 20% H_2O	32,2 ± 9,6 ^{b}	48.0 ±11.9^{a}	6.5 ±1.5^{bc}	28.1 ±6.8 ^{b}
GT+ 20% WM	78,5 ± 12,0^{a}	64.7 ±4.7 ^{a}	9.7 ±1.9 ^{bc}	82.0 ±21.8 ^{a}
GT+ 20% SM	33,4 ± 8,1 ^{c}	55.8 ±17.5 ^{a}	8.1 ±2.4 ^{bc}	36.3 ±4.3 ^{b}
GT+ 40% H_2O	32,7 ±10.4 ^{b}	42.1 ±7.7 ^{a}	6.2 ±2.9 ^{bc}	25.6 ±8.3 ^{b}
GT+ 40% WM	88,4 ± 8,4^{a}	76.0 ±24.9 ^{a}	7.0 ±2.1 ^{bc}	91.7 ±11.2 ^{a}
GT+ 40% SM	30,8 ± 4,3 ^{c}	43.7 ±6.9 ^{a}	6.8 ±2.5 ^{bc}	28.3± 3.7 ^{b}

[1]The terms represent; Initial, as initially determined from tea samples using tea infusion method; PG, flavonoids remaining after gastric digestion; IN, dialyzed fraction after intestinal digestion; OUT, non-dialyzed fraction after intestinal digestion, SBT, sea buckthorn leaf tea; GT, green tea; WM, whole milk; SM, skimmed milk. Data represent average values ± standard deviation of 3 independent samples. Different letters in columns represent statistically significant differences ($p<0.05$).

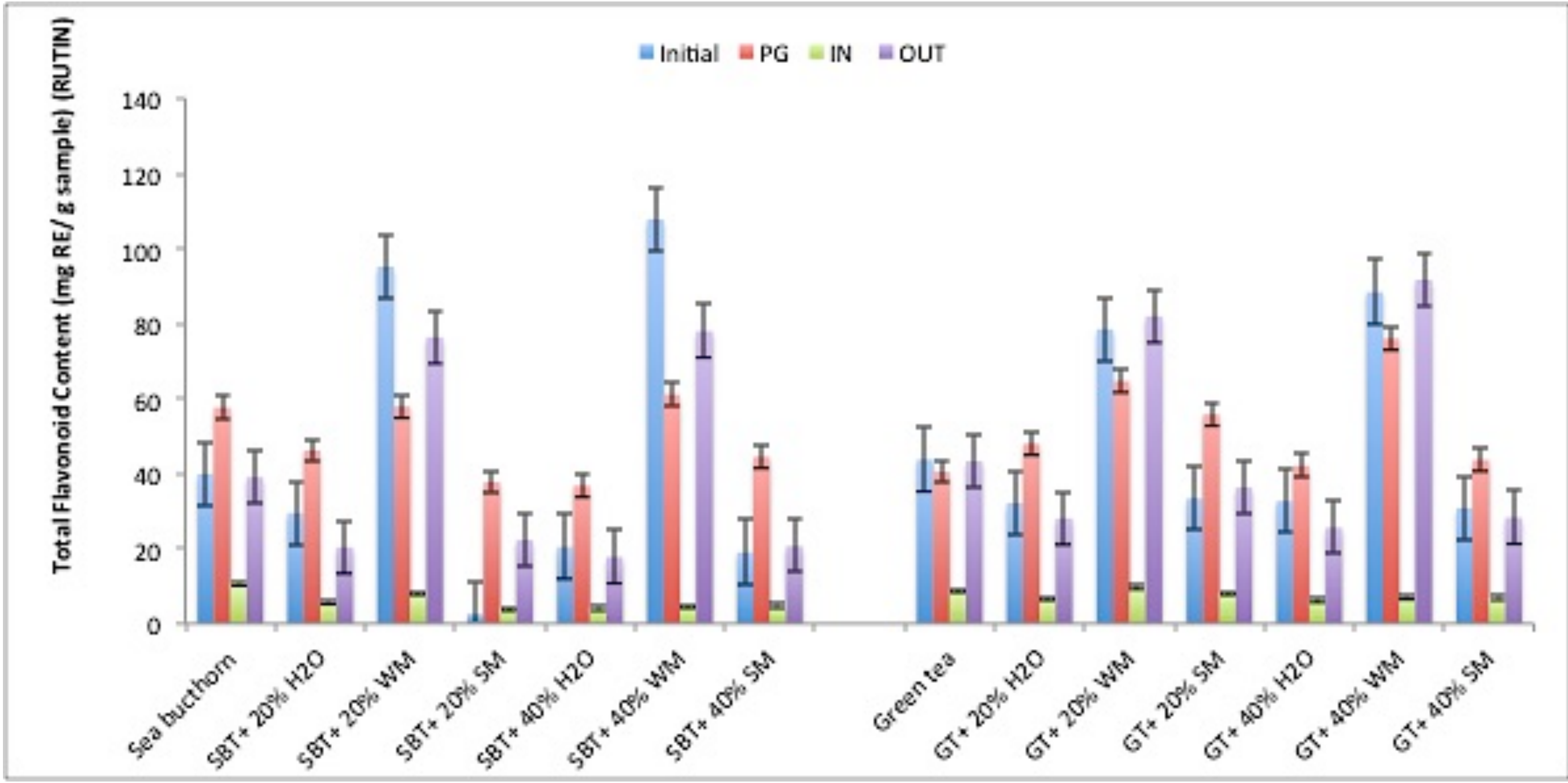

Figure 4.3: Total flavonoid content of Sea buckthorn leaf tea and Green tea samples, expressed as Rutin equivalent (RE) per g weight. The terms represent; Initial, as initially determined from tea samples using tea infusion method; PG, phenolics remaining after gastric digestion; IN, dialyzed fraction after intestinal digestion; OUT, non-dialyzed fraction after intestinal digestion, SBT, sea buckthorn leaf tea; GT, green tea; WM, whole milk; SM, skimmed milk. Data represent average values ± standard deviation of 3 independent samples.

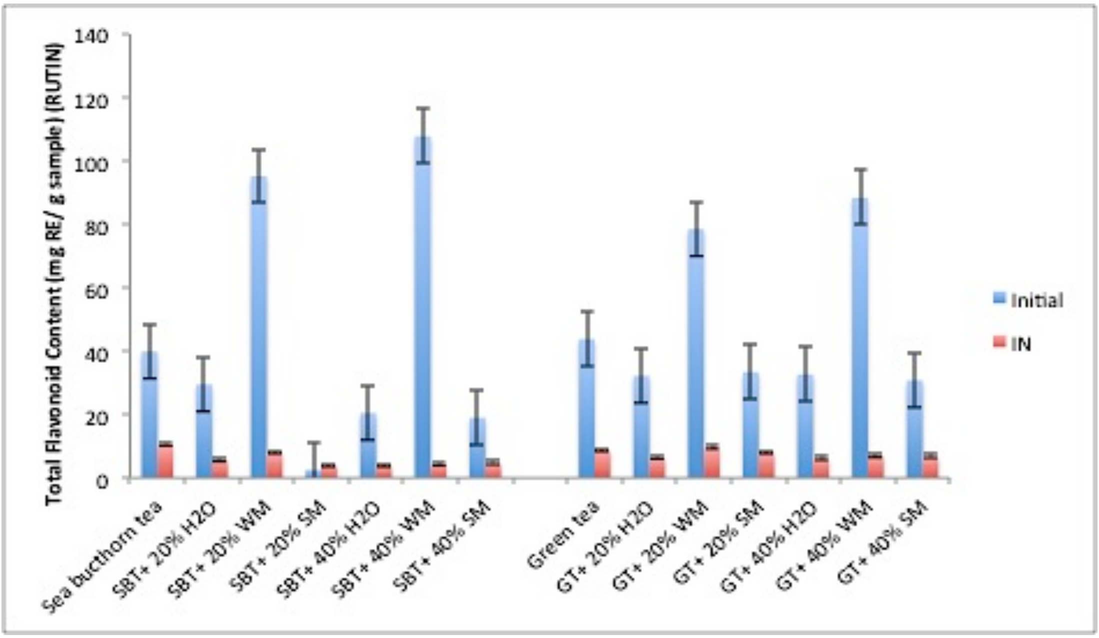

Figure 4.4: Total flavonoid content of Sea buckthorn leaf tea and Green tea samples, expressed as Rutin equivalent (RE) per g weight. The terms represent; Initial, as initially determined from tea samples using tea infusion method; IN, dialyzed fraction after intestinal digestion; SBT, sea buckthorn leaf tea; GT, green tea; WM, whole milk; SM, skimmed milk. Data represent average values ± standard deviation of 3 independent samples.

Previous studies have reported that milk proteins all have the capacity to interact with polyphenols, binding non-specifically with various affinities primarily through hydrophobic interactions, hydrogen bonding interactions and, to a lesser extent, electrostatic interactions (Bohin et al. 2012; Hasni et al., 2011; Kanakis et al., 2011; Tantoush et al., 2012; von Staszewski et al., 2012). Interaction between specific milk proteins and GT flavan-3-ols may provide some level of protection for individual flavan-3-ols from oxidative degradation, like circumstances that may be encountered in the upper small intestine (Neilson et al., 2007). Both oxidative protection and the potential for delayed release following digestion of protein may increase availability for intact polyphenols to be afterwards absorbed in the upper small intestinal epithelia (bioaccessibility). Further, evidence exists to support the notion that intestinal uptake may be altered by the presence of protein (Keogh et al., 2007). However, strong binding without release may serve tomake these phenolics less available for absorption (bioavailability). Therefore understanding the extent and implications of such interactions is needed in order to optimize stability but also the availability of polyphenols for delivery of health benefit.

Previous studies have shown that the addition of milk to polyphenol-rich foods effects flavan-3-ol bioaccessibility and bioavailability (Green et al., 2007; Reddy et al., 2005; Serafini et al., 2003, 2009). While this effect is typically attributed to the presence of milk protein, milk minerals may have an effect on both polyphenol and protein structure and stability.

4.4.3. Total Antioxidant Capacity (TAC)

In order to determine the effect of in vitro GI digestion on TAC, CUPRAC assay was performed, because among all four TAC assays the highest correlation was observed between either TP or TF and CUPRAC assays. For both tea samples, an increase in TAC was observed after pepsin digestion (Figure 4.6). 18.1 and 8.3% of the compounds were present in the dialyzed fraction for SBT leaf tea and green tea, respectively (Figure 4.5 and Figure 4.7). Similar to TP and TF, IN values were lower in skimmed milk added samples (36.3 and 39.9 mg of TE/g for SBT leaf and 30.8 and 28.0 mg of TE/g for green tea, respectively) compared to whole milk added samples (49.6 and 43.4 mg of TE/ g for SBT leaf and 34.3 and 34.3 mg of TE/g for green tea, respectively), but statistically there was no significant difference between these samples ($p<0.05$).

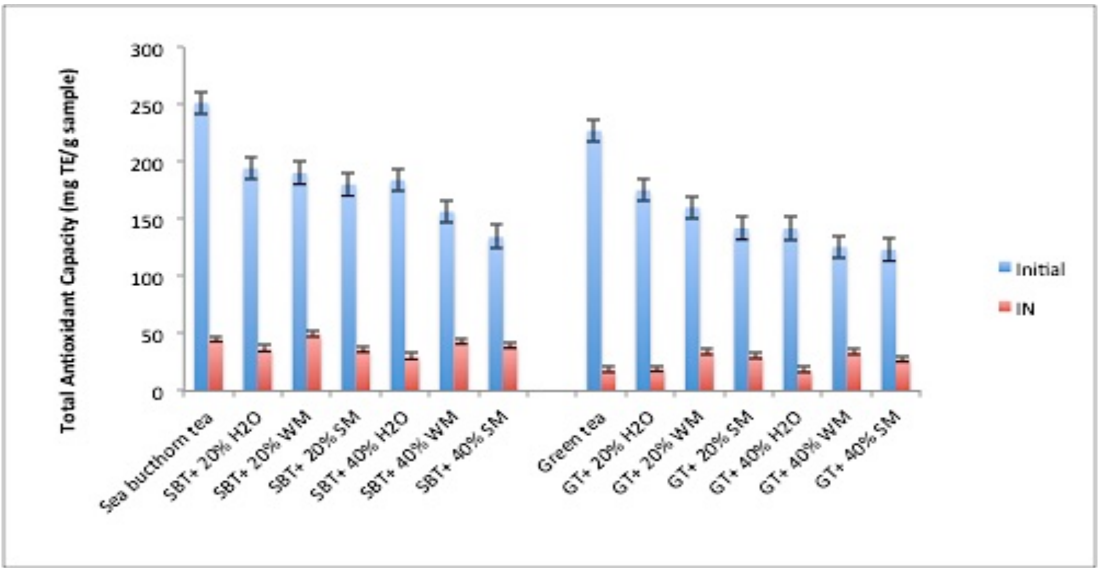

Figure 4.5: Total antioxidant capacity of Sea buckthorn leaf tea and Green tea samples, expressed as Trolox equivalent (TE) per g weight. The terms represent; Initial, as initially determined from tea samples using tea infusion method; IN, dialyzed fraction after intestinal digestion; SBT, sea buckthorn leaf tea; GT, green tea; WM, whole milk; SM, skimmed milk. Data represent average values ± standard deviation of 3 independent samples.

Table 4.9: Total antioxidant capacity of Sea buckthorn leaf tea and Green tea samples, expressed as Trolox equivalent (TE) per g weight.

Sample	Initial	PG	IN	OUT
Sea bucthorn tea	251.3 ±31.2^{a}	283.4 ±48.4^{ab}	45.5 ±4.4^{a}	145.0 ±62.1^{a}
SBT+ 20% H_2O	194.2 ± 24.6^{b}	323.2 ±89.4^{a}	36.9 ±8.5^{ab}	109.0±11.6^{ab}
SBT+ 20% WM	190.3 ± 32.3^{b}	330.4±130.7^{a}	49.6 ±18.3^{ab}	139.1 ±8.6^{ab}
SBT+ 20% SM	180.2 ± 25.8^{b}	357.0 ±50.5^{a}	36.3 ±8.0^{ab}	118.2±22.4^{ab}
SBT+ 40% H_2O	183.9 ± 34.6^{b}	271.9 ±97.0^{a}	30.0 ±4.6^{ab}	93.9 ±21.3^{ab}
SBT+ 40% WM	156.4 ± 22.8^{b}	329.3 ±46.7^{a}	43.4 ±6.9^{ab}	144.6±14.6^{ab}
SBT+ 40% SM	134.4 ± 16.7^{b}	317.8 ±28.6^{a}	39.9 ±7.5^{ab}	100.3±21.9^{ab}
Green tea	227.0 ±27.9^{b}	237.2 ±45.8^{b}	18.8 ±7.6^{c}	101.3±28.0^{ab}
GT+ 20% H_2O	175.2 ± 42.5^{b}	314.1 ±63.1^{a}	19.5 ±4.8^{b}	71.8 ±20.1^{b}
GT+ 20% WM	160.1 ± 30.6^{b}	331.5 ±38.7^{a}	34.3 ±4.2^{b}	115.9 ±15.2^{b}
GT+ 20% SM	142.0 ± 28.4^{b}	328.8 ±49.3^{a}	30.8 ±9.4^{b}	110.0 ±6.4^{b}
GT+ 40% H_2O	141.6 ± 19.9^{b}	296.1 ±36.1^{a}	18.1 ±5.7^{b}	67.4 ±21.1^{b}
GT+ 40% WM	125.7 ± 21.3^{b}	276.1 ±48.9^{a}	34.3 ±2.6^{b}	108.5 ±11.8^{b}
GT+ 40% SM	123.3 ± 32.1^{b}	287.9 ±73.5^{a}	28.0 ±6.6^{b}	99.0 ±6.8^{b}

[1]The terms represent; Initial, as initially determined from tea samples using tea infusion method; PG, flavonoids remaining after gastric digestion; IN, dialyzed fraction after intestinal digestion; OUT, non-dialyzed fraction after intestinal digestion, SBT, sea buckthorn leaf tea; GT, green tea; WM, whole milk; SM, skimmed milk. Data represent average values ± standard deviation of 3 independent samples. Different letters in columns represent statistically significant differences ($p<0.05$).

As shown in Figure 4.7, recovery of TP and TAC were significantly higher for milk added samples. Contrary to this recovery of TF was significantly lower for milk added samples except skimmed milk added samples.

As shown in Figure 4.7, SBT leaf tea samples were found to be more bioavailable than green tea samples in terms of TP, TF and TAC.

It has been reported that phenolic substances possess a high affinity for proteins, including caseins (Luck et al., 1994). In fact, Jobstl et al., (2006) stated a non-covalent cross-link between epigallocatechin gallate and casein. Vitamin C also can interact with proteins andminerals from milk (Claeys et al., 2013), reducing its antioxidant power (Rodríguez-Roque et al., 2014).

In vitro digestion results suggest that milk protein–phenolic interactions are: depended upon structure of both protein and polyphenol, altered by gastrointestinal digestion and possibly modified by milk minerals. These interactions have some effect on polyphenol bioaccessibility, especially through gastric and small intestinal digestion.

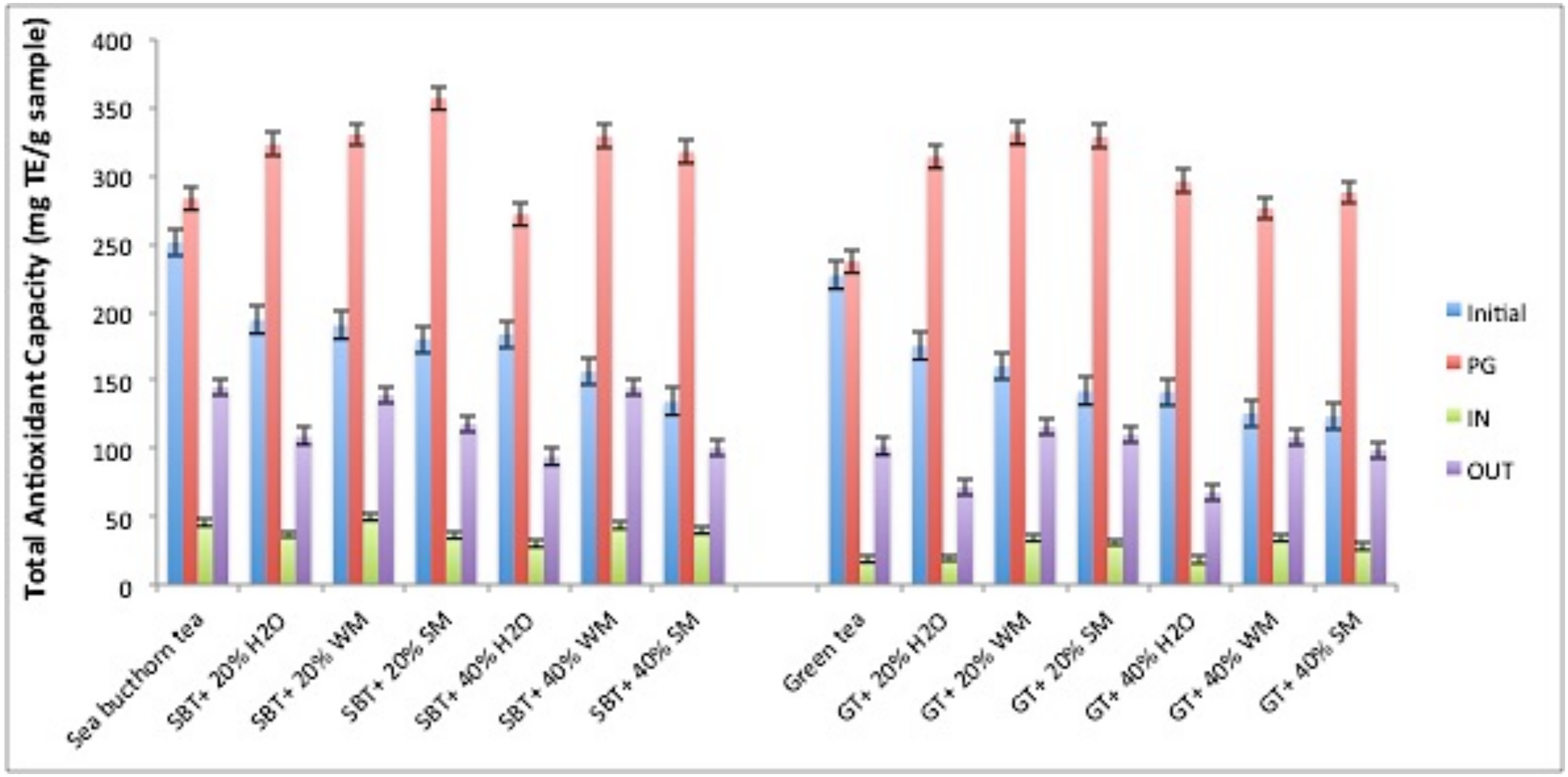

Figure 4.6: Total antioxidant capacity of Sea buckthorn leaf tea and Green tea samples, expressed as Trolox equivalent (TE) per g weight. The terms represent; Initial, as initially determined from tea samples using tea infusion method; PG, phenolics remaining after gastric digestion; IN, dialyzed fraction after intestinal digestion; OUT, non-dialyzed fraction after intestinal digestion, SBT, sea buckthorn leaf tea; GT, green tea; WM, whole milk; SM, skimmed milk.

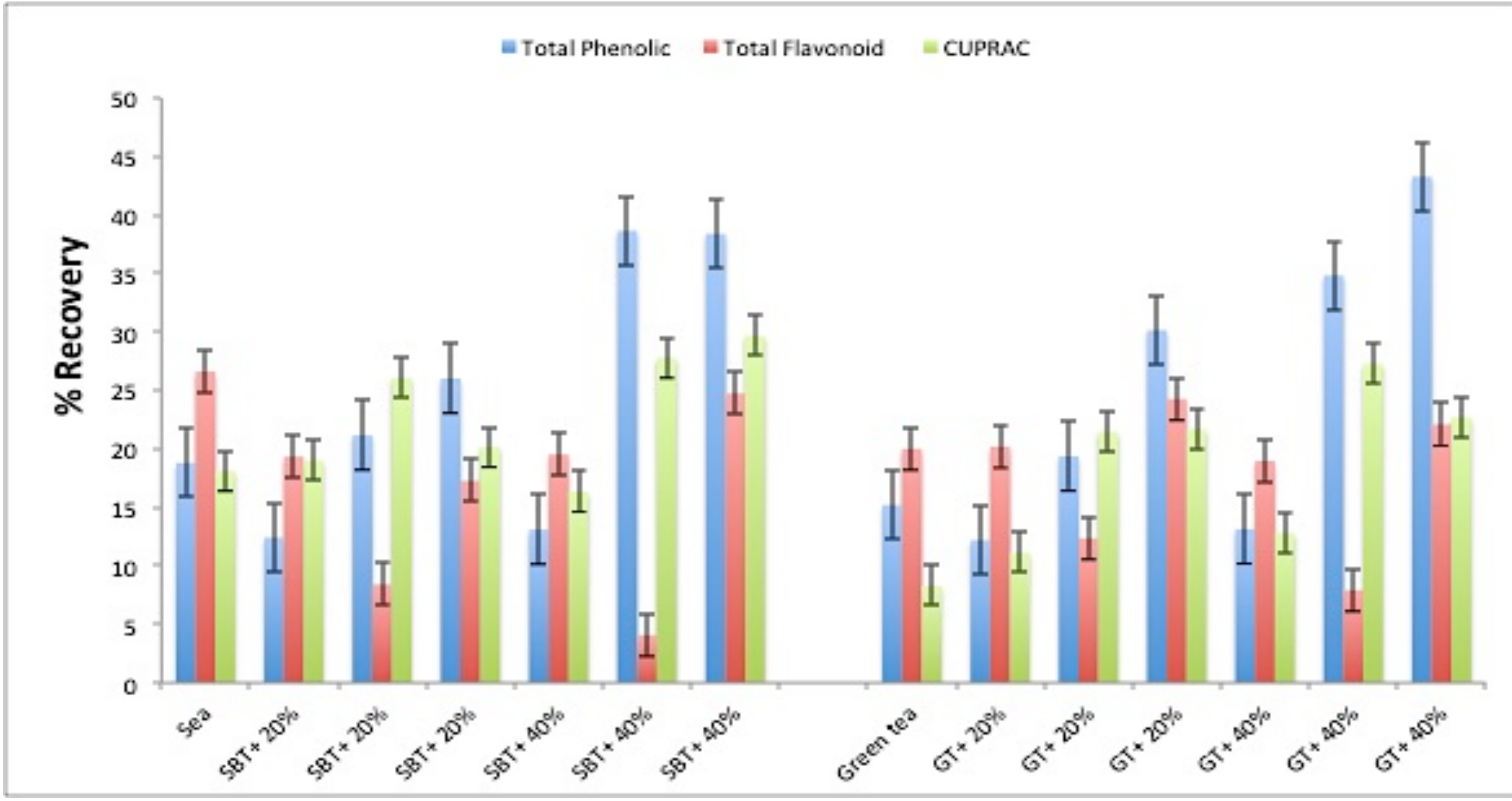

Figure 4.7 : Recovery of total phenolics (TP), total flavonoids (TF) and total antioxidant capacities (TAC) of Sea buckthorn leaf tea and Green tea samples after in vitro intestinal digestion, dialyzed fractions of the intestinal digestion (IN), expressed as percentage. Data represent average values ± standard deviation of three independent samples.

However, subsequent digestion of protein during duodenal phase may disturb these interactions, restoring availability of polyphenols for absorption resulting in a modest increase in absolute bioaccessibility in the small intestine. Overall while these data implement evidence that milk protein interactions may modulate of polyphenol bioaccessibility, they commonly agree with prior reports of the minimal overall effect of “milk” as a complete matrix on polyphenol bioavailability from tea beverages (Keogh et al., 2007; Kyle et al., 2007; Roura et al., 2007). However, milk mineral–flavonoid interactions do merit further investigation as these interactions may influence both protein structure and polyphenol stability during digestion.

5. CONCLUSIONS AND RECOMMENDATIONS

Tea is commonly consumed with the addition of protein-rich milk in some countries. Thus, interaction between phenolic compuonds with milk protein in beverage systems has been the focus of several studies. However, the ability of protein to influence phenol bioavailability remains controversial. In this study, the impact of milk addition, on digestive stability and ultimate bioaccessibility of sea buckthorn leaf tea and green tea polyphenols were investigated.

According to the results, SBT leaf, green tea and their milk added forms acquire high amounts of phenolics, flavonoids and also present some degree of antioxidant activities. Green tea has consumed for many years due to its high antioxidant capacity and positive health effect on health all over the world. Our results showed that SBT leaf tea has high antioxidant capacity even more than green tea. Taking the high antioxidant and rutin content of leaves into account and with respect to their positive health effects, consumption of SBT in forms of a herbal tea should be recommended in Turkey.

According to TP and TF analyses, it is obvious that recovery of polyphenols were close to 80% of the amount of extracts, which is consistent with the study of McDougall et al. (2005). In this study, the reason for highest TP, TF and TEAC values of OUT samples was explained as only a little amount of polyphenols enters IN samples and the majority of polyphenols passes intact of the colon fraction and may be degraded to phenolic compounds, by the way the values for OUT samples are higher than that of IN samples. In TP or TF analyses, it is possible to only detect some polyphenols, but in TAC analyses, a general view to antioxidant capacity may be provided which includes the effects of most polyphenols and by the way, increase in TAEC values after *in vitro* digestion is observed much more than other analyses.

In the light of the experimental results, changes in TF, TP and TAC values may be related to interactions between phenolic compounds from tea extracts and proteins from milk.

Certainly, interactions between proteins and phenolics continue to get attention via scientists. More studies should be done in this area, especially for plant proteins which consider not only protein characteristics but also polyphenol activities and contents, and also effects on rheology of blends to have desired products with best sensory and nutritional quality.

In conclusion, this study was investigated to examine effects of interactions between milk proteins and polyphenols of Sea buckthorn leaf and green tea. Although the results obtained with simulated *in vitro* GI digestion do not directly predict the human *in vivo* conditions, still this model is considered as helpful for investigating the bioavailability of polyphenols. In further studies, the consequences of interactions in different fields would be interesting to focus on.

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APPENDIX

APPENDIX A. CALIBRATION CURVES

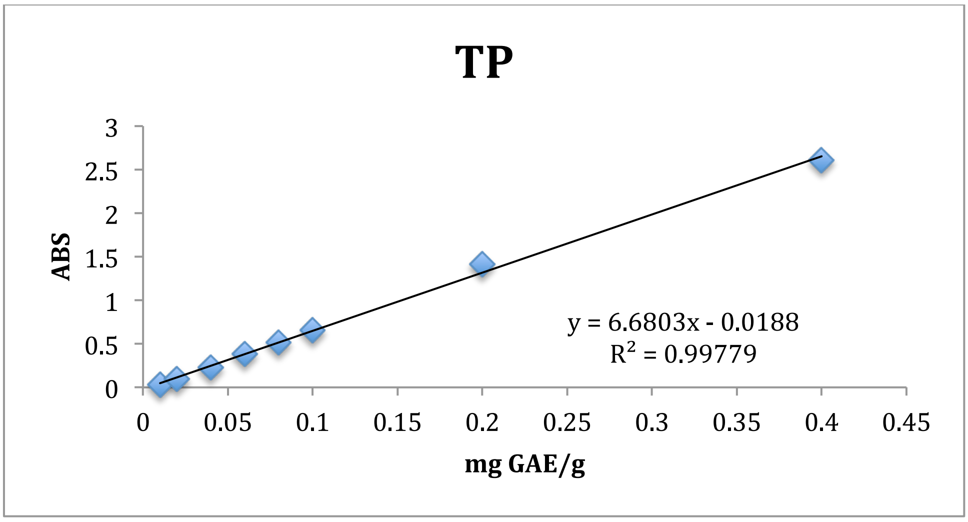

Figure A.1: Calibration curve for total phenolics in 70% aqueous-ethanol

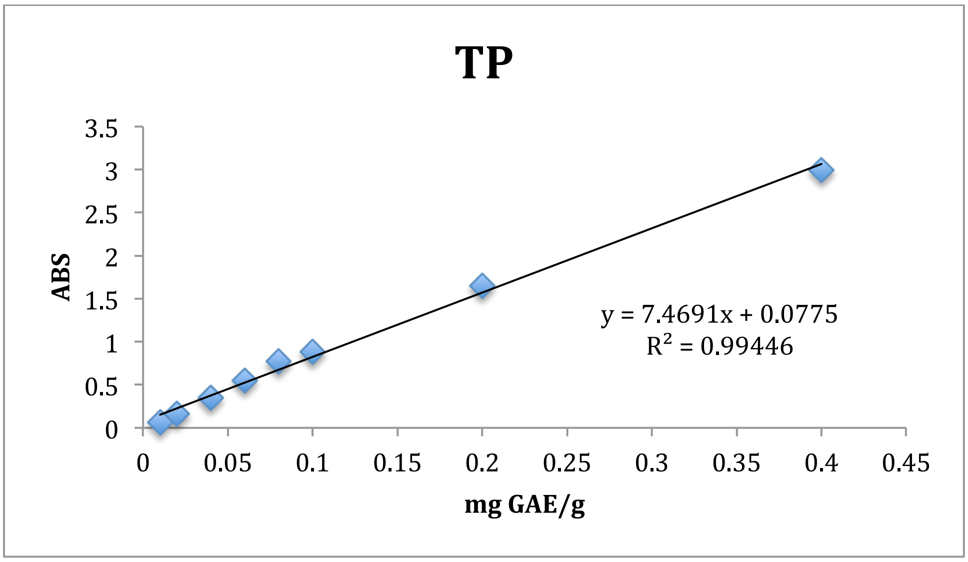

Figure A.2: Calibration curve for total phenolics in water

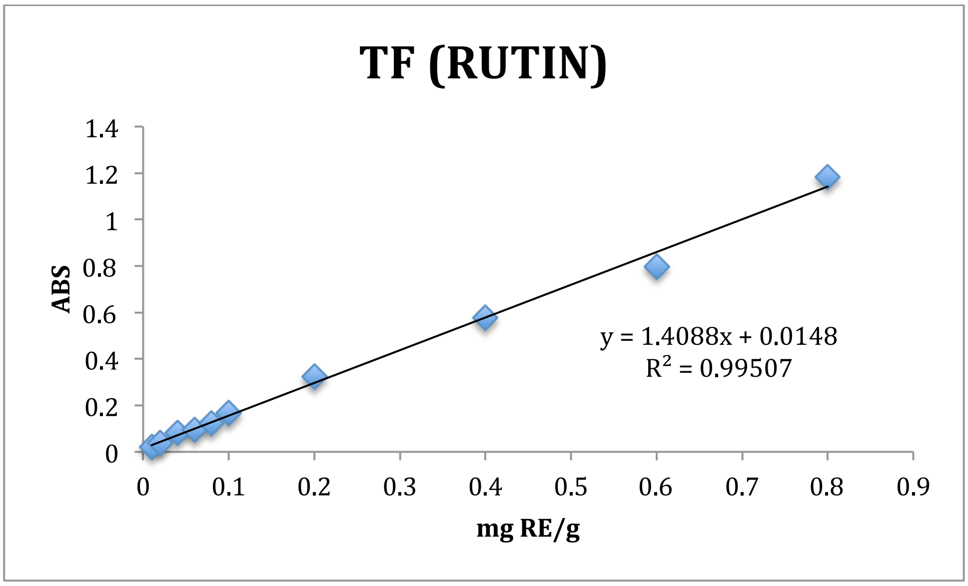

Figure A.3: Calibration curve for total flavonoids in 70% aqueous-ethanol

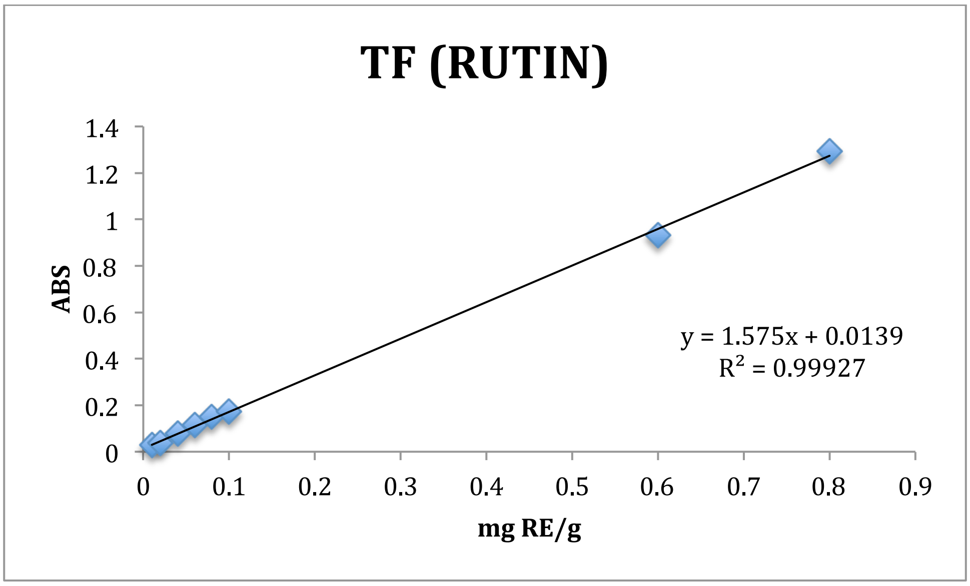

Figure A.4: Calibration curve for total flavonoids in water

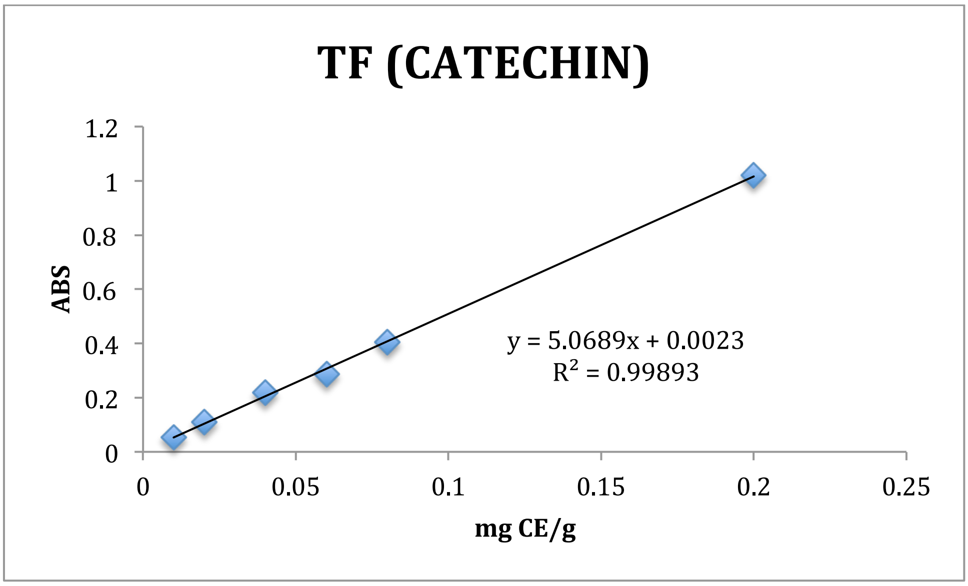

Figure A.5: Calibration curve for total flavonoids in 70% aqueous-ethanol

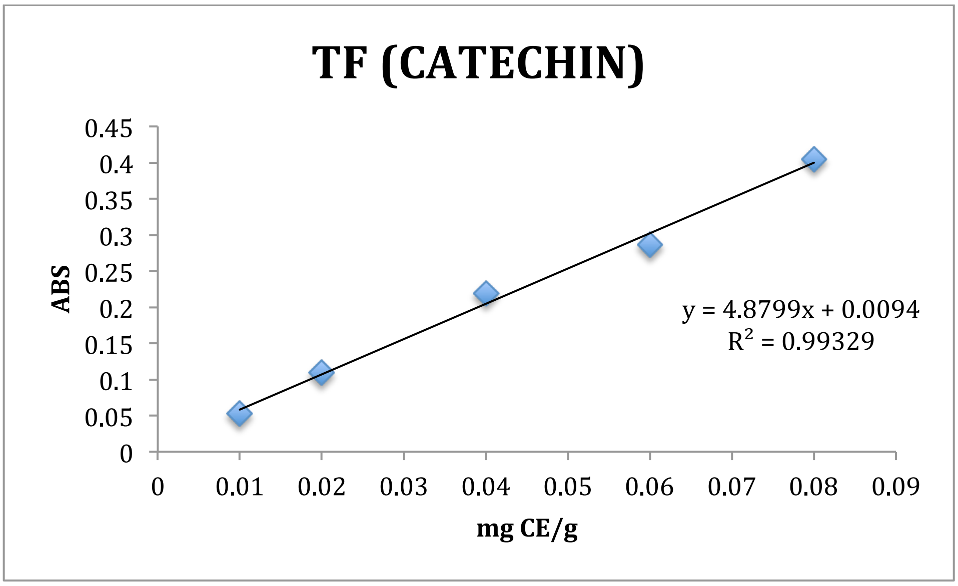

Figure A.6: Calibration curve for total flavonoids in water

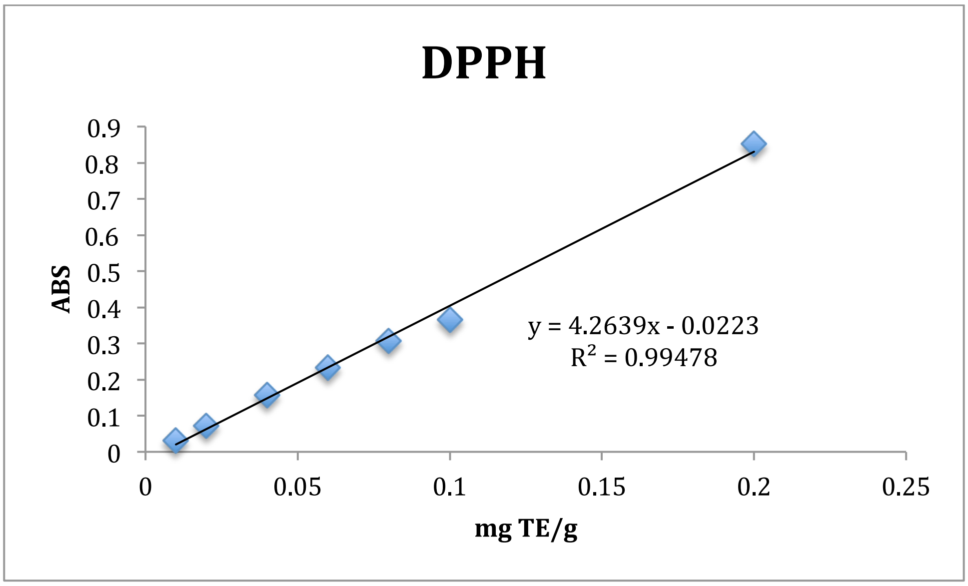

Figure A.7: Calibration curve for DPPH assay in 70% aqueous-ethanol

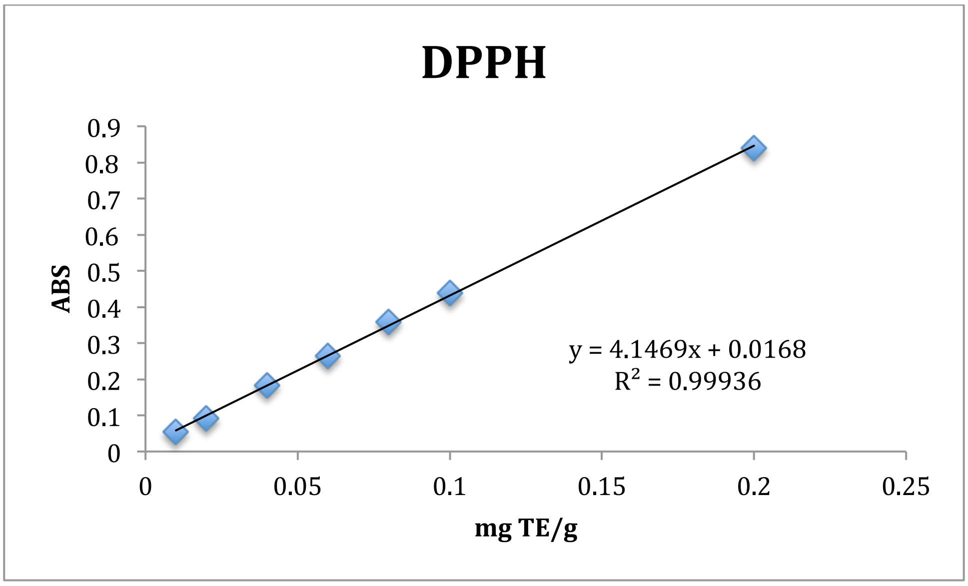

Figure A.8: Calibration curve for DPPH assay in water

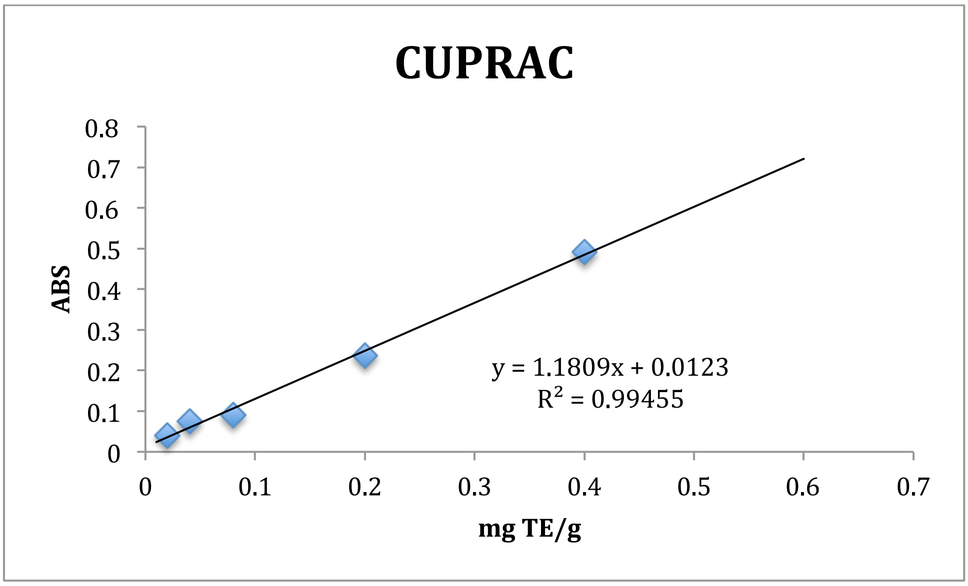

Figure A.9: Calibration curve for CUPRAC assay in 70% aqueous-ethanol

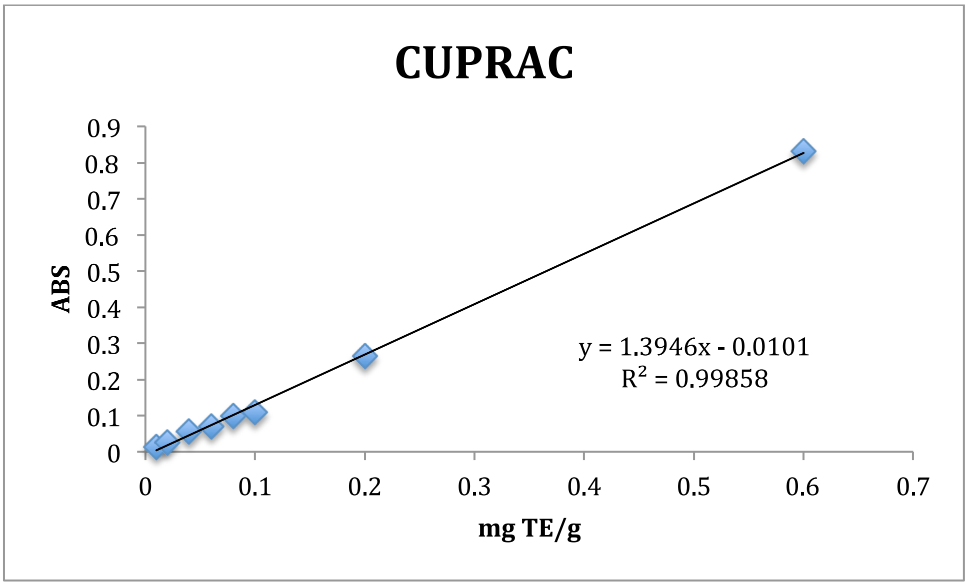

Figure A.10: Calibration curve for CUPRAC assay in water

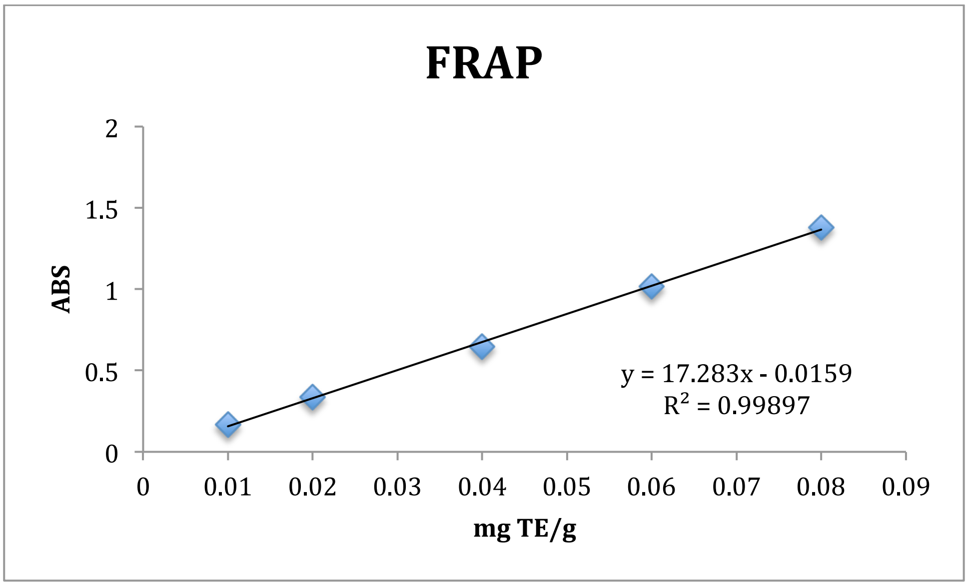

Figure A.11: Calibration curve for FRAP assay in 70% aqueous-ethanol

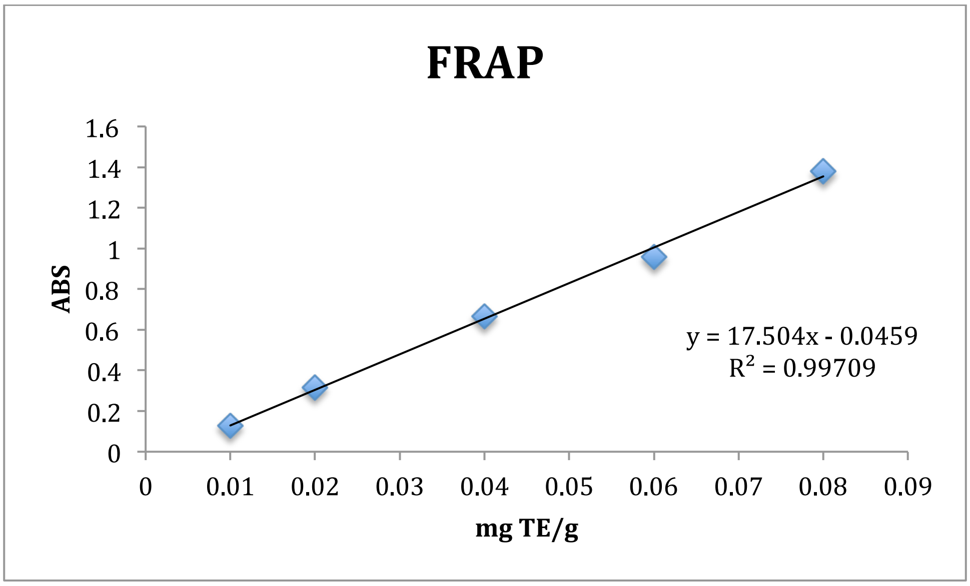

Figure A.12: Calibration curve for FRAP assay in water

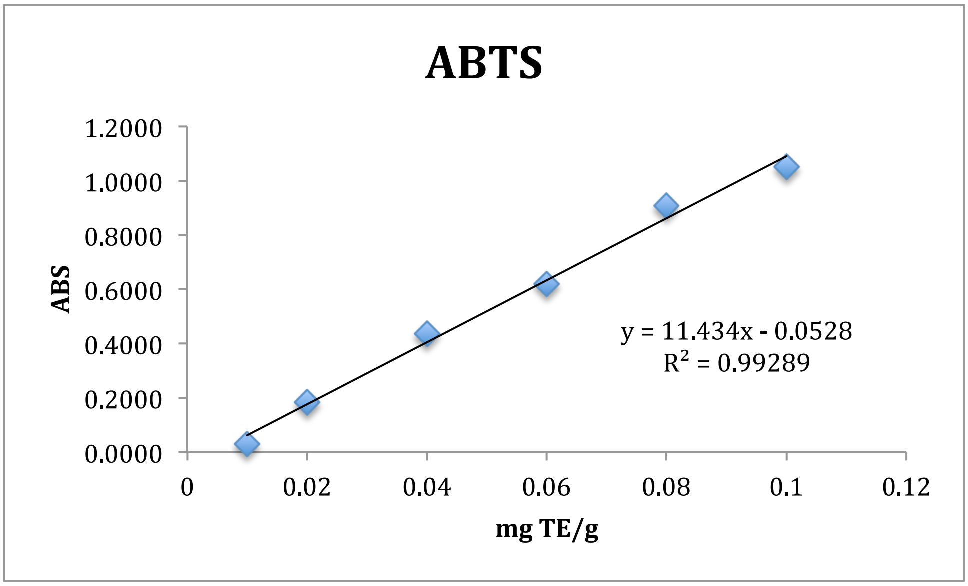

Figure A.13: Calibration curve for ABTS assay in 70% aqueous-ethanol

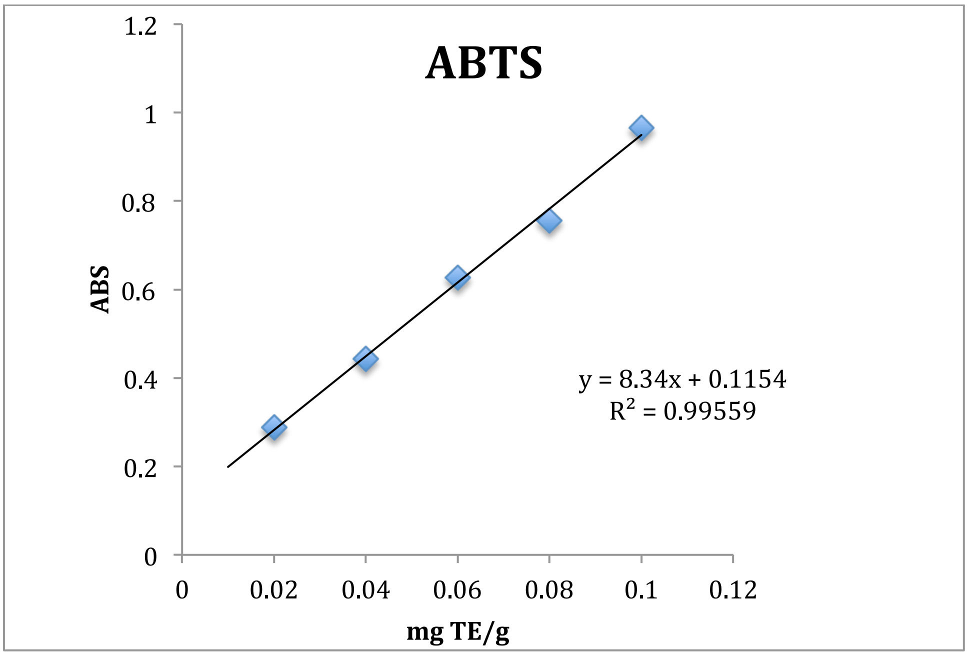

Figure A.14: Calibration curve for ABTS assay in water

APPENDIX B. ANOVA TABLES

Table B.1: Statistical analysis results of Green tea and Sea buckthorn leaf tea extracts.

Extraction Type	Sum of Squares	df	Mean Square	F	Sig.
Total_phenolic BetweenGroups Within Groups Total	 39606.248 3229.529 42835.776	3 32 35	13202.083 100.923	130.814	.000
Total_flavonoid_rutin Between Groups Within Groups Total	32385.763 1326.576 33712.339	3 32 35	10795.254 41.456	260.406	.000
Total_flavonoid_catechin Between Groups Within Groups Total	2868.223 288.440 3156.663	3 32 35	956.074 9.014	106.069	.000
DPPH Between Groups Within Groups Total	48206.392 617.194 48823.586	3 32 35	16068.797 19.287	833.128	.000
CUPRAC Between Groups Within Groups Total	939781.268 29629.120 969410.389	3 32 35	313260.423 925.910	338.327	.000
FRAP Between Groups Within Groups Total	15215.388 1089.018 16304.407	3 32 35	5071.796 34.032	149.031	.000
ABTS Between Groups Within Groups Total	10012.737 6.829 10019.566	3 32 35	3337.579 .213	15639.016	.000
Milk Addition	Sum of Squares	df	Mean Square	F	Sig.
Total_phenolic Between Groups Within Groups Total	10802.458 5374.019 16176.477	3 80 83	3600.819 67.175	53.603	.000
Total_flavonoid_rutin Between Groups Within Groups Total	547.941 76722.940 77270.880	3 80 83	182.647 959.037	.190	.903

Table B.1: Statistical analysis results of Green tea and Sea buckthorn leaf tea extracts (continuing).

Total_flavonoid_catechin Between Groups Within Groups Total	128.918 8012.792 8141.710	3 80 83	42.973 100.160	.429	.733
DPPH Between Groups Within Groups Total	3489.779 20069.052 23558.831	3 80 83	1163.260 250.863	4.637	.005
CUPRAC Between Groups Within Groups Total	43338415.8 477100077 520438493	3 80 83	14446138.6 5963750.96	2.422	.072
FRAP Between Groups Within Groups Total	3161.110 16815.802 19976.913	3 80 83	1053.703 210.198	5.013	.003
ABTS Between Groups Within Groups Total	29688.622 10764.547 40453.169	3 80 83	9896.207 134.557	73.547	.000
Bioavailability	Sum of Squares	df	Mean Square	F	Sig.
TP_Initial Between Groups Within Groups Total	10802.458 5374.019 16176.477	3 80 83	3600.819 67.175	53.603	.000
TP_PG Between Groups Within Groups Total	22575.594 37492.987 60068.582	3 80 83	7525.198 468.662	16.057	.000
TP_IN Between Groups Within Groups Total	155.982 2046.143 2202.125	3 80 83	51.994 25.577	2.033	.116
TP_OUT Between Groups Within Groups Total	47831.673 11448.311 59279.984	3 80 83	15943.891 143.104	111.415	.000
TF_RUtin_Initial Between Groups Within Groups Total	547.941 76722.940 77270.880	3 80 83	182.647 959.037	.190	.903

Table B.1: Statistical analysis results of Green tea and Sea buckthorn leaf tea extracts (continuing).

TF_Rutin_PG Between Groups Within Groups Total	1984.822 19241.311 21226.133	3 80 83	661.607 240.516	2.751	.048
TF_Rutin_IN Between Groups Within Groups Total	221.768 640.537 862.305	3 80 83	73.923 8.007	9.233	.000
TF_Rutin_OUT Between Groups Within Groups Total	1743.940 65884.540 67628.480	3 80 83	581.313 823.557	.706	.551
CUPRAC_Initial Between Groups Within Groups Total	18443818.7 265722726 284166544	3 80 83	6147939.56 3321534.07	1.851	.145
CUPRAC_PG Between Groups Within Groups Total	65728.698 335178.876 400907.574	3 80 83	21909.566 4189.736	5.229	.002
CUPRAC_IN Between Groups Within Groups Total	3791.874 8921.809 12713.684	3 80 83	1263.958 111.523	11.334	.000
CUPRAC_OUT Between Groups Within Groups Total	10502.511 52736.273 63238.784	3 80 83	3500.837 659.203	5.311	.002

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