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Investigating the Antioxidant Properties of Some Herbal Infusions During In Vitro Digestion

Bazı Bitkisel İnfüzyonların In Vitro Sindirim Sırasında Antioksidan Özelliklerinin İncelenmesi

Gulay OZKAN[1*], Aliye ARAS[2], Esra CAPANOGLU GUVEN[1]

[1] Istanbul Technical University, Faculty of Chemical and Metallurgical Engineering, Department of Food Engineering, Maslak, Istanbul, Turkey.

*gulayozkann@gmail.com, ORCID: 0000-0002-6375-1608

capanogl@itu.edu.tr, ORCID: 0000-0003-0335-9433

[2] Istanbul Technical University, Faculty of Science, Department of Botany, Istanbul, Turkey.

aaras@istanbul.edu.tr, ORCID: 0000-0002-2188-4031

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***Corresponding author** /Yazışılan yazar

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Abstract

In the present study, the content and antioxidant potential of phenolic compounds from five herbal infusions including *Aspalathus linearis, Sambucus nigra, Rhamnus frangula, Plantago lanceolata and Equisetum arvense* were analyzed. Additionally, simulated in vitro gastrointestinal digestion were conducted to predict the bioaccessibility of infusion phenolics. Accordingly, Rooibos infusion had the highest total phenolic (2191.18±93.39 mg GAE/100 g dw) and flavonoid content (1226.14±93.07 mg CE/100 g dw) as well as antioxidant capacity measured by CUPRAC (3124.12 mg TE/100 g dw) and FRAP (2634.95 mg TE/100 g dw) assays. On the other hand, the highest ABTS (2351 mg TE/100 g dw) and DPPH (475.19 mg TE/100 g dw) antioxidant capacity values were recorded for elderflower infusion. Based on the gastrointestinal digestion, retention of phenolics in the infusions was found to be variable througout mouth to intestine. It can be deduced from the results that those herbs can be considered as ~~a~~ valuable beverage alternatives with their high nutritional value.

Keywords: Herbal infusions, Antioxidant capacity, Bioaccessibility, Phenolic compounds

Özet

Bu çalışmada, *Aspalathus linearis, Sambucus nigra, Rhamnus frangula, Plantago lanceolata* ve *Equisetum arvense* olmak üzere beş bitkisel infüzyonun fenolik içeriği ile antioksidan potansiyeli analiz edilmiştir. Ayrıca, infüzyonlarda bulunan fenolik bileşiklerin biyoerişilebilirliklerini belirlemek amacıyla simüle edilmiş in vitro gastrointestinal sindirim gerçekleştirilmiştir. Sonuçlara göre, Rooibos infüzyonunun en yüksek toplam fenolik (2191.18±93.39 mg GAE/100 g ka), toplam flavonoid (1226.14±93.07 mg CE/100 g ka) ve antioksidan kapasiteye (CUPRAC, 3124.12 mg TE/100 g ka; FRAP, 2634.95 mg TE/100 g ka) sahip olduğu bulunmuştur. Diğer yandan, en yüksek ABTS (2351 mg TE/100 g ka) ve DPPH (475.19 mg TE/100 g ka) antioksidan kapasite değerleri mürver çiçeği infüzyonu ile elde edilmiştir. Gastrointestinal sindirim sonuçlarına göre, infüzyonlarda bulunan fenoliklerin elde edilme oranının ağızdan bağırsağa değişken olduğu bulunmuştur. Çalışmada elde edilen sonuçlara göre, bu bitkilerin yüksek besinsel içerikleri nedeniyle değerli bir içecek alternatifi olduğu sonucuna varılabilmektedir.

Anahtar Kelimeler: Bitkisel infüzyonlar, Antioksidan kapasite, Biyoerişilebilirlik, Fenolik bileşikler

Abbreviations: GAE, gallic acid equivalents; TE, Trolox equivalents; Trolox, 6-hydroxy-2,5,7,8 tetramethyl chroman-2-carboxylic acid; CE, catechin equivalents; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ABTS, 2,2 azinobis (3 ethlybenzothiazoline-6-sulfonic acid) diammonuium salt); TPTZ, Triphenyltetrazolium; CUPRAC, cupric ion reducing antioxidant capacity; FRAP, ferric reducing antioxidant power; ORAC, oxygen radical absorbance capacity; TRAP, total radical-trapping antioxidant parameter; dm, dry matter.

1. INTRODUCTION

A great number of fruits, vegetables, aromatic, spicy, medicinal and other plants are natural sources of phytochemicals including phenolic compounds, nitrogen compounds, vitamins, terpenoids and other endogenous metabolites (Kamiloglu et al., 2014). Epidemiological studies have shown that many of the phytochemicals possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, or antiviral activities (Owen et al., 2000; Zielinski et al., 2014). They are also associated with reduced risks of cancer, cardiovascular disease, diabetes and lower mortality rates of several human diseases (Sun et al., 2002). Therefore, there has been a great interest on the bioactivity phenolic compounds present in the herb extracts as well as infusion/tea prepared by these herbs (Ozkan et al., 2016; Ozkan et al., 2021).

The response of antioxidants may differ according to radical or oxidant sources; therefore, multi assays such as ORAC assay, total TRAP assay, β-carotene or crocin-bleaching assay, total phenol assay by using the Folin-Ciocalteu reagent, ABTS assay, DPPH radical scavenging capacity assay, FRAP assay, and CUPRAC assay are prefered to reflect the

mechanism of action of all radical sources or all antioxidants in a complex system (Karadag et al., 2009).

In the recent years, more attention has been given to the studies about bioaccessibility and bioavailability of polyphenols to predict the release of these phytochemicals from the food matrix and absorption of their metabolites in the throughout the gut epithelium (Kamiloglu et al., 2017; Ozkan et al., 2021, 2022a, 2022b; Wu et al., 2017). In order to estimate the bioaccessibility and bioavailability, several in vitro digestion methods, including static (Minekus et al., 2014) and dynamic (Menard et al., 2014) methods, have been proposed as an alternative to *in vivo* methods due to their lower cost, time and energy saving properties and their independence from ethical concerns and physiological factors (Tang et al., 2006). Minekus et al. (2014) proposed a standardized and practical static digestion model due to the diversity of in vitro digestion model conditions has limited the ability to compare results across different studies.

Due to the strong relationship between benefial effects of the phenolic compounds and their bioaccessibilities/bioavailiabilities, it is a necessity to conduct in vitro digestion study in order to predict their metabolic fates and final bioactivities. In the study described here, the herbs were selected owing to their widely consumption and health promoting effects. Therefore, the objective of the present study was to evaluate the phenolic contents and the antioxidant potentials of the herbal infusions prepared by *Aspalathus linearis* (rooibos leaves), *Plantago lanceolata* (narrow leaf plantain), *Sambucus nigra* (elderberry flowers), *Rhamnus frangula* (alder buckthorn bark) and *Equisetum arvense* (field horsetail leaves). In addition to these, the retention of phenolics and their antioxidant capacities in herbal infusions were evaluated after in vitro gastrointestinal digestion.

2. MATERIALS and METHODS

2.1. Plant Materials

Herbs including *Aspalathus linearis* (rooibos), *Plantago lanceolata* (narrow leaf plantain), *Sambucus nigra* (elderberry), *Rhamnus frangula* (alder buckthorn) and *Equisetum arvense* (field horsetail) were obtained as three independent biological replicates from Arifoğlu brand in Istanbul, Türkiye.

2.2. Chemicals

For simulated in vitro digestion, α-amylase (EC 3.2.1.1, from human saliva), pepsin (EC 3.4.23.1, from porcine gastric mucosa), pancreatin (EC 232.468.9, from porcine pancreas, contains trypsin, amylase and lipase) and bile were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

The Folin–Ciocalteu reagent, gallic acid, catechin, neocuproine, DPPH (1,1-diphenyl-2-picrylhydrazyl), Trolox (6-hydroxy-2,5,7,8 tetramethyl chroman-2-carboxylic acid), ABTS (2,2 azinobis (3 ethlybenzothiazoline-6-sulfonic acid) diammonuium salt), TPTZ (Triphenyltetrazolium) were purchased from the Sigma Chemical Co. (St. Louis, Mo, USA). All other reagents used were of analytical grade.

2.3. Preparation of the Infusion

Herbal infusions were prepared according to ISO Standard of "Preparation of a liquor of tea for use in sensory tests". For this purpose, 2 grams of herb was weighed and placed into a beaker and mixed with 100 mL of boiling water. Then, it was allowed to brew for 6 minutes. After, each infusion was filtered and left for cooling (BSI, 1980).

2.4. In Vitro Gastrointestinal Digestion Model

The in vitro gastrointestinal digestion protocol was conducted based on Minekus et al. (2014). This method comprises sequentially simulated oral, gastric and intestinal digestion steps.

The oral digestion was simulated by mixing 5 mL of each herbal infusion with 3.5 mL of salivary juice, 0.5 mL of α-amylase solution (25 µkat/mL), 25 µL of 0.3 mol/L $CaCl_2$, and 0.975 µL of distilled water to obtained a final volume of 10 mL. This mixture was incubated at 37 °C in a shaking water bath (Memmert SV 1422, Memmert GmbH & Co. Nürnberg, Germany) for 2 min. After simulated oral digestion, 2 mL samples were collected for each infusion.

The gastric digestion was simulated by adding 8 mL of gastric juice, 1.28 mL of pepsin solution (417 µkat/mL), 4 µL of 0.3 mol/L $CaCl_2$ into the remaining oral bolus and the pH was adjusted to 3.0 using 1 mol/L HCl. The total volume of this mixture was adjusted to 16 mL using distilled water. Then, the mixture was incubated in a shaking water bath at 37 °C for 2 h. After simulated gastric digestion, 2 mL aliquots were seperated from each infusion.

The intestinal digestion was simulated bymixing the gastric chyme with 7.7 mL of intestinal juice, 3.5 mL of pancreatin (13 µkat/mL), 1.75 mL 160 mmol/L bile and 28 µL 0.3

mol/L $CaCl_2$. The pH of the mixture was adjusted to 7.0 using 1 mol/L NaOH. The total volume of this mixture was completed to 28 mL using distilled water. The mixture was incubated in a shaking water bath at 37 °C for 2 h.

A blank (use of same amount of water instead of samples) was also incubated under the same conditions in order to correct the any interference from the simulated digestive fluids.

All samples obtained from the simulated oral, gastric and intestinal digestion steps were centrifuged (Hettich, Tuttlingen, Germany) at 23000 g and 4 °C for 5 min. Supernatants were kept at -20 °C until further analysis.

2.5. Determination of Total Phenolics and Antioxidant Capacity

Total phenolic content was analyzed using Folin – Ciocalteu reagent (Singleton and Rossi, 1965). The results are showed as mg gallic acid equivalents (GAE) per 100 g dw sample (Singleton & Rossi, 1965).

Total flavonoid content assay was conducted based on Dewanto et al. (2002). The results are stated as mg catechin equivalents (CE) per 100 g dw sample (Dewanto et al., 2002).

Total antioxidant capacity of the samples was determined by FRAP, CUPRAC, ABTS and DPPH assays (Apak et al., 2004; Benzie & Strain, 1996; Kumaran & Joel Karunakaran, 2006; Miller & Rice-Evans, 1997). In all protocols, the results are given as mg Trolox equivalents (TE) per 100 g dw of sample.

2.6. Statistical Analysis

All analyses were performed at least in three replicates. Results were stated as mean ± standard deviation. Statistical analysis was carried out using SPSS software (version 20.0, SPSS Inc. Chicago, IL, USA). Results were compared using one-way analysis of variance (ANOVA) followed by a Tukey post hoc test ($p < 0.05$). The correlation coefficients (R) were calculated using Microsoft Office Excel 2011 software (Microsoft Co. Redmond, WA, USA).

3. RESULTS and DISCUSSION

3.1. Recovery of Phenolic Compounds During In Vitro Gastrointestinal Digestion

The effect of in vitro digestion on total phenolic and total flavonoid content of herbal infusions was shown in Table 1.

Table 1. Changes in the total phenolic and total flavonoid contents of herbal infusions during simulated in vitro digestion[a]

Sample	Undigested	Simulated oral digestion	Simulated gastric digestion	Simulated intestinal digestion
Total phenolic content (mg GAE/100 g dw)				
Equisetum arvense (field horsetail)	781.60±37^{cC}	1074.75±13^{bC}	737.81±48^{cE}	4313.73±39^{aC}
Rhamnu sfrangula (alder buckthorn)	1198.60±57^{dB}	1753.72±94^{cB}	2614.77±10^{bB}	4012.77±30^{aD}
Sambucu snigra (elderberry)	1890.88±67^{dA}	2268.72±19^{cA}	3314.07±35^{bA}	6646.55±40^{aA}
Aspalathus linearis (rooibos)	2191.18±93^{bA}	2343.19±21^{bA}	2097.89±21^{bC}	5875.84±55^{aB}
Plantago lanceolata (narrow leaf plantain)	783.70±79^{cC}	787.35±74^{cC}	1230.36±14^{bD}	4229.33±38^{aCD}
Total flavonoid content (mg CE/100 g dw)				
Equisetum arvense (field horsetail)	342.14±24^{aC}	373.82±52^{aC}	226.80±45^{aD}	243.22±24^{aC}
Rhamnu sfrangula (alder buckthorn)	838.05±63^{bB}	1280.43±67^{aB}	554.41±10^{cC}	1289.65±24^{aAB}
Sambucus nigra (elderberry)	1163.40±77^{cAB}	1917.54±19^{aA}	1972.82±20^{aA}	1408.43±13^{bA}
Aspalathu slinearis (rooibos)	1226.14±93^{bA}	1669.15±20^{aA}	1144.81±13^{bB}	1204.80±36^{bB}
Plantago lanceolata (narrow leaf plantain)	357.31±19^{aC}	396.17±47^{aC}	350.40±39^{aD}	67.88±24^{bD}

[a] The data presented in this table consist of average values ± standard deviation of three independent batches. Total phenolic and total flavonoid content is expressed as mg of gallic acid equivalents (GAE), and catechin equivalents (CE) per 100 g of dry weight (dw) of sample, respectively. Different capital letters in the columns or small letters in the rows represent statistically significant differences ($p < 0.05$).

The difference in total phenolic and total flavonoid content was found to be statistically significant ($p < 0.05$) for undigested, simulated oral digested, simulated gastric digested and simulated intestinal digested samples. For undigested samples, the highest total phenolic and total flavonoid value were obtained with *Aspalathus linearis* as 2191.18 ± 93 mg GAE/100g dw and 1226.14±93 mg CE/100 g dw, respectively.

Total phenolic content of each herbal infusion used in this study was found to be higher than those of *Zingiber officinale* (Ginger; 710±90 mg GAE/100g dw), *Chamomillae romanae* (Daisy; 730±170 mg GAE/100g dw) and *Tilia platyphyllos* (Linden; 860±70 mg GAE/100g dw) which are the most widely known infusions throughout the world. Total phenolic content of those infusons were reported as 710±90, 730±170, 860±70 mg GAE/100g dry sample, respectively (Toydemir et al., 2015). On the other hand, it has been reported that brewing time is an effective factor on the total phenolic content of the infusions. For instance; previous studies reported that the total phenolic content of *Aspalathus linearis* was found to be 2350±246 mg GAE/100g dw with 5 min (Joubert & de Beer, 2012), while 3750±235 mg GAE/100g dw was found with an hour brewing duration (Chan et al., 2010).

After simulated oral digestion, total phenolic content of *Aspalathus linearis* and *Plantago lanceolate*; total flavonoid content of *Equisetum arvense* and *Plantago lanceolata* did not change significantly ($p > 0.05$), whereas the results of other herbal infusions were obtained significantly higher compared to undigested samples ($p < 0.05$). After the simulated gastric digestion, there was a significant increase in only total phenolic content of *Rhamnu sfrangula*, *Sambucu snigra* and *Plantago lanceolate* ($p < 0.05$); while there were no any increase in total flavonoid content of all herbal infusions. After simulated intestinal digestion further significant increases in total phenolic content were determined ($p < 0.05$). On the other hand, total flavonoid content remained stable with the exception of *Rhamnu sfrangula*, *Sambucus nigra* and *Plantago lanceolate*.

Correlations between total phenolic and total flavonoid content assays were differ depending on whether the samples is undigested or digested as well as the stage of digestion. The highest correlation coefficient was found to be R = 0.95 for simulated oral digested samples, followed by undigested samples, simulated gastric digested samples and simulated intestinal digested samples with 0.94, 0.70 and 0.37 correlation coefficients, respectively. In the study of Pirbalouti et al. (2013), correlation coefficient between total phenolic and total flavonoid contents was determined as 0.954 which is highly compatible with the correlation coefficient of simulated oral digested samples and undigested samples in the present experiment. It can be concluded from the results that infusions with high levels of phenolic content were also rich in flavonoids (Oh et al., 2013).

3.2. Antioxidant Capacity of Digested Polyphenols

The effect of in vitro digestion on total antioxidant capacity of herbal infusions was shown in Table 2.

The variation in total antioxidant capacity of infusions was found to be statistically significant ($p < 0.05$) for undigested, simulated oral digested, simulated gastric digested and simulated intestinel digestinal digested samples. For undigested samples, the highest total antioxidant capacity values were obtained with *Sambucus nigra* and *Aspalathus linearis*. In a study of Wong et al. (2015), total antioxidant capacity of rooibos herbal infusion was found to be 2496 mg TE/100 g dw by FRAP assay which is similar to the that of present study as 2634.95 mg TE/100 g dw. On the other hand, according to Oh et al. (2013), total antioxidant capacity of rooibos by using ABTS assay was found to be 3931 mg AAE/100 g dw (AAE; ascorbic acid equivalent) which was higher than that of the present study as 2342.91 mg TE/100 g dw,

probably due to the difference in the standard compound. It has been indicated that the variety of standards affect the chemical properties including redox potential, stoichiometries, kinetics, solubility and auto-oxidation susceptibility (Poljšak & Raspor, 2008).

Table 2. Changes in the total antioxidant capacity of herbal infusions during simulated in vitro digestion[a]

Sample	Undigested	Simulated oral digestion	Simulated gastric digestion	Simulated intestinal digestion
2,2-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS)				
Equisetum arvense (field horsetail)	1884.31±15^{cAB}	1129.56±0.02^{dE}	2508.41±0.01^{bD}	3165.49±0.15^{aD}
Rhamnu sfrangula (alder buckthorn)	2114.73±12^{cAB}	2091.36±0.02^{cC}	3217.22±0.001^{bC}	3460.93±0.27^{aC}
Sambucu snigra (elderberry)	2351±11^{cA}	3057.23±0.03^{cB}	5579.93±0.22^{bA}	8584.64±0.32^{aA}
Aspalathus linearis (rooibos)	2342.91±11^{dAB}	3612.84±0.03^{cA}	4285.69±0.23^{bB}	7854.56±0.38^{aB}
Plantago lanceolata (narrow leaf plantain)	1287.67±12^{dB}	1361.86±0.03^{cD}	2434.90±0.11^{bE}	3035.56±0.19^{aE}
Cupric ion reducing antioxidant capacity (CUPRAC)				
Equisetum arvense (field horsetail)	1100.12±11^{dB}	1914.72±0.15^{cE}	2027.37±0.02^{bE}	4425.04±0.6^{aD}
Rhamnu sfrangula (alder buckthorn)	1189.35±113^{dB}	2542.64±0.16^{cC}	3250.79±0.15^{bD}	5495.99±0.8^{aC}
Sambucus nigra (elderberry)	2981.28±155^{dA}	14209.30±0.36^{cA}	15714.67±0.23^{bA}	16664.53±0.5^{aA}
Aspalathus slinearis (rooibos)	3124.12±135^{dA}	3883.72±0.37^{cB}	5048.47±0.17^{bB}	6743.48±0.11^{aB}
Plantago lanceolata (narrow leaf plantain)	1104.96±112^{dC}	2405.68±0.24^{cD}	3255.78±0.20^{bC}	4154.36±0.56^{aE}
2,2-diphenyl-1-picrylhydrazyl (DPPH)				
Equisetum arvense (field horsetail)	447.14±14^{cAB}	1204.37±0.04^{aD}	1221.29±0.16^{aC}	690.39±0.17^{bC}
Rhamnu sfrangula (alder buckthorn)	240.42±23^{dB}	797.88±0.3^{bE}	1180.08±0.7^{aD}	690±0.25^{cC}
Sambucus nigra (elderberry)	475.19±52^{dA}	1546.03±0.4^{cA}	3515.23±0.9^{aA}	1968.12±0.03^{bA}
Aspalathus slinearis (rooibos)	439.48±39^{dAB}	1395.17±0.01^{cB}	2588.33±0.11^{aB}	1810.12±0.7^{bB}
Plantago lanceolata (narrow leaf plantain)	413.60±56^{cAB}	1231.52±0.01^{aC}	1090.04±0.9^{aE}	632±0.03^{bD}
Ferric reducing antioxidant power (FRAP)				
Equisetum arvense (field horsetail)	626.01±57^{aD}	417.89±0.03^{cE}	308.79±0.01^{dE}	546.01±0.51^{bE}
Rhamnu sfrangula (alder buckthorn)	625.26±55^{bD}	542.20±0.05^{bC}	587.39±0.3^{bD}	855.37±0.31^{aC}
Sambucus nigra (elderberry)	2309.15±12^{aB}	2190.53±0.02^{bA}	2169.76±0.16^{bA}	1503.47±0.33^{cA}
Aspalathus slinearis (rooibos)	2634.95±13^{aA}	1746.99±0.16^{bB}	1663.09±0.7^{cB}	727±0.62^{dD}
Plantago lanceolata (narrow leaf plantain)	836.56±58^{aC}	512.48±0.01^{bD}	609.38±0.3^{bC}	883.97±0.22^{aB}

[a] The data presented in this table consist of average values ± standard deviation of three independent batches. Total antioxidant capacity is expressed as mg Trolox equivalents (TE) per 100 g of dw of sample. Different capital letters in the columns or small letters in the rows represent statistically significant differences ($p < 0.05$).

After simulated oral digestion, total antioxidant capacity values of herbal infusions changed significantly ($p < 0.05$). After simulated gastric digestion, significant increases in total antioxidant capacity (in case of ABTS and CUPRAC) were obtained in comparison with the results obtained after simulated oral digestion ($p < 0.05$). After simulated intestinal digestion, further significant increases in total antioxidant capacity (in case of ABTS and CUPRAC) were obtained ($p < 0.05$). On the other hand, total antioxidant capacity measured by DPPH assay showed a significant decrease in comparison with the results obtained after simulated gastric digestion ($p < 0.05$).

Due to the fact that the radical scavenging capacity of phenolic compounds many depend on pH of the environment, the antioxidant potential may be variable during digestion. Besides, there may be structural alterations in the phenolic substances by ionizing the hydroxyl groups due to passing from gastric to intestinal conditions, resulting in improved antioxidant capacity at higher pH values. Similarly, Tagliazucchi et al. (2010) indicated that the free radical scavenging activities of some phenolic compounds such as gallic and caffeic acids, catechin, quercetin and resveratrol were enhanced after intestinal digestion.

Furthermore, it has been recommended that antioxidant potential of food products could be assessed by using multiple assays because of their complex multiphase structures that may have an impact on the results (Apak et al., 2016). Thereby, correlations between total antioxidant capacity assays were also investigated. For both the undigested and digested samples, from moderate to high correlations were determined; the highest (R = 0.6938-0.9874) correlation was indicated between FRAP and CUPRAC assays among others. Similarly, Kamiloglu et al. (2017) found the highest correlations between FRAP and CUPRAC assays changing between 0.7492 and 0.9704 (Kamiloglu et al., 2014). It can also be deduced from the results that ABTS and DPPH assays could be more appropriate for evaluating total antioxidant capacity after simulated gastric digestion in accordance with the pH conditions.

4. CONCLUSION

The present study focused on the evaluation of total phenolics, total flavonoids, total antioxidant capacities as well as examination of digestive stability and bioaccessibility of the phenolics in herbal infusions using the standardized static in vitro digestion protocol. It was found that these infusions can be consumed as an alternative to most known herbal teas with compatible phenolic and flavonoid contents. Besides, total phenolic content and total antioxidant capacity increased significantly as a result of digestion, whereas the loss of total flavonoid content of

some of the infusions were obtained. Moreover, it is clear that the measurement of antioxidant capacity of food products cannot be evaluated satisfactorily using a single antioxidant assay due to their complex multiphase systems, which can influence the results. Therefore, more than one assay with different mechanisms should be applied to evaluate total antioxidant capacities of food products. As a future aspect, it can be suggested that conducting in vitro Caco-2 cell culture method to assess the final absorption of phenolic compounds may be helpful to evaluate the fate of the infusion phenolics in human digestive system.

DECLARATIONS

The authors declare that they have no conflicts of interest.

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