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Effect of brewing material and various additives on polyphenolic composition and antioxidant bioactivity of commercial *Tilia platyphyllos* Scop. infusions

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ABSTRACT: Herbal infusions have become very popular due to their pleasant flavor as well as their positive influence on health. The compositions of such infusions are affected by the extraction technique, duration, additives as well as container materials. This study implements comparison of antioxidant activities and phenolic contents of the infusions commercially purchased *Tilia platyphyllos* Scop. samples, one of the most preferred herbal infusions worldwide, prepared by using teapots with different materials. Antioxidant potencies of the samples were examined using tests with different mechanisms such as free radical scavenging test (DPPH), metal-related activity tests (CUPRAC, FRAP). On account of assessing the phenolic profile, total phenol, phenolic acid and flavonoid contents were estimated spectrophotometrically. In addition, the presence of protocatechuic acid in the extracts was investigated by HPTLC densitometry (between 0.762-1.037 w/w%). Besides, antioxidant activities (DPPH, CUPRAC and TOAC) of the extracts were re-calculated after addition of natural/synthetic sweeteners, brown and white sugar, lemon, flower and pine honeys to the infusions. Results showed that the highest total antioxidant capacity was seen on Tiliae infusions prepared in ceramic teapot (672.80±1.40 mg AAE/g DE). Moreover, stevioside addition enhanced DPPH radical scavenging of Tiliae extracts (2781.76±44.38 EC_{50} in µg/mL). This is the first report related with comparison of these brewing materials and additives in respect to their phenolic content and antioxidant activity of herbal teas.

KEYWORDS: *Tilia platyphyllos;* infusion; antioxidant activity; HPTLC; protocatechuic acid.

1. INTRODUCTION

Even though the antioxidants were once known only as food preservatives, it is now evidential that they inhibit the oxidation processes in human body as well as in foods [1]. Reactive oxygen species (ROS) are the intermediates generated during the normal metabolic activity, which are effectively neutralized by the antioxidant system of the human body. Any imbalance in this normal metabolism leads ROS to damage cell membranes, enzymes and DNA, which has been one of the major causes of various diseases including neurodegenerative diseases, cancer, and inflammatory condition [2]. Antioxidants are believed to prevent the damage caused by oxidative stress. They show this activity via different mechanisms like chelation of metallic ions (Fe^{2+}, Fe^{3+}, Cu^{2+} and Cu^{+}) and activation of antioxidant enzymes [3].

In phytotherapy, Tiliae flowers are commonly used for numerous indications, such as for the common cold treatment, nervous tension, migraine, liver and gall bladder disorders as well as due to its expectorant, diuretic, antispasmodic, sedative, stomachic and diaphoretic activities [4-6]. European Medicines Agency published the traditional use of *Tilia cordata* and *Tilia platyphyllos* in relief of the symptoms of common cold, chronic cough and mental stress in various countries [7]. Due to its medicinal purposes and its pleasant taste, it is frequently used in Turkey as herbal tea. In the light of previous studies on composition of *Tilia* spp. it was determined that these species contain essential oil, mucilaginous polysaccharides, condensed tannins, procyanidin dimers, flavonoids, phenolic acids, amino acids, carbohydrates and saponins [7,8]. Besides, protocatechuic acid was already found to be the most abundant phenolic acid found in *T. plathyphyllos* [9].

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Among them, medicinal properties of *Tilia* spp. have been referred to its flavonoid, volatile oil and mucilage composition [6.10].

Although various extraction/preparation techniques are present for herbal teas such as maceration, decoction, soxhlet extraction, linden tea is chosen to be prepared as infusion. In literature, there are many publications related with the bioavailability of the herbal teas and their extraction technique, extraction solvent even brewing duration while those parameters appear to influence the composition of herbal teas [11-13]. Teapot materials are as important as brewing conditions such as solvent selection and extraction duration; while they can release heavy metals into teas that people use for healing purposes [14,15]. It also brings the question if the other container materials have a role on the activities of herbal teas. Along with the extraction material, addition of flavoring substances like lemon, sugar, milk and honey also affects the bioactivity. The way of brewing tea differs among societies. People add various materials in order to give flavor and sweeten their teas. Pękal et al. examined the antioxidant activities of flavored black teas and stated the difference in bioactivity between flavored teas and standard black tea [16]. Sharma et al. and Toydemir et al. conducted similar studies related with the influence of milk, sugar and honey addition in various herbal teas on their antioxidant activities [17,18].

This study evaluates the antioxidant activities, phenolic and protocatechuic acid contents of *Tilia platyphyllos* infusions prepared by using different brewing materials like copper, stainless steel, ceramic and glass pots, which are usual members of households and aluminum pots which are generally preferred by campers as well as addition of several flavors such as natural sweetener, synthetic sweetener, brown sugar, white sugar, lemon, flower honey and pine honey to the infusions, since the extraction techniques and flavoring compounds influence the phytochemical compositions and bioactivities of herbal tea compositions.

2. RESULTS and DISCUSSION

2.1. Evaluation of brewing techniques and additives on herbal infusions

There are numerous studies comparing the bioactivities and phytochemical compositions of different extracts prepared by using different methods, different infusion temperatures, multiple extraction, different brewing times [11,12,19,20]. Metal exposure is a possible undesirable result of food and beverage consumption. Boularbah et al. measured the toxicity of discharging heavy metals from the teapots [14]. According to the results, stainless steel teapot did not exhibit any toxicity from heavy metals and possibly that outcome originates for relatively small release of metals into the water. Teapots containing copper (the tin layer was missing in places) was found to be the most toxic, leading to exposure of the water to the copper alloy. Additionally, this teapot released the high amounts of Zn as well as Cu. The non-toxic stainless steel teapot released very small amount of Zn (1.64 mg/L). This study showed that chemical analysis of teapot leachates for heavy metals generally affirms the toxicity data provided by MetPAD, which is definite for heavy metal toxicity heavy metals. Boularbah et al. recommended stainless steel teapots, to bypass further daily metal intake, instead of those containing metal alloys, which may deliver metals during the infusion process [14]. Similarly, Petit et al. examined the lead intoxication from metallic teapots [21] and Ojezele et al. calculated the metal transfer capacity of cooking utensils [22]. Heavy metals, might cause harmful effects and cause serious health problems, especially for fetuses, infants and children due to the exposure concentration per body weight. Researches showed that the solubility of metals present in plants increases with extraction time [23]. Although high rate of metal concentration in plants is mainly caused by soil characteristics, place of harvesting, altitude, chemical fertilizer or other medicine additions, and genetic characteristics, tools using during collection, equipment used during transportation and shipping, storage conditions as well as extraction materials highly affect metal concentration [14,21,22]. Remarkably, accumulation of metal ions in plants leads formation of free radicals and stimulates the antioxidative defense systems in plants. The excess exposure to metals can damage to plant cells stimulating the free radical generation and ROS. Heavy metals influence the metabolic pathways interacting with organic compounds in the plant cell components when they enter the plant tissues. In addition, heavy metals can cause the formation of free radicals and replace essential metals [24]. During heavy metal exposure phenolic compounds act as metal chelators. Antioxidant activity of phenolic components is due to their huge tendency to chelate metals via various routes such as direct chelation, Fenton reaction etc., Furthermore, metal ions have the ability to decompose lipid hydroperoxide (LOOH) and result in lipid alkoxyl radicals, which trigger free radical chain oxidation. Phenolic antioxidants similarly can inhibit lipid peroxidation by trapping the lipid alkoxyl radical [25].

2.2. Assessment of polyphenolic composition of linden infusions

European Medicines Agency announced the traditional use of *Tilia cordata* and *Tilia platyphyllos* prepared in boiling water as herbal infusions in relief of the symptoms of common cold, chronic coughing and mental stress in various countries [7]. In Europe, herbal teas containing *Tiliae* flowers are recommended for common cold. Although people use *Tiliae* flowers as an infusion prepared by boiled water, there are many studies investigating the biological activities of Tiliae flowers by using different extraction techniques and materials. Karioti et al. compared the phytochemical composition of decoction, infusion, tinctures and alcoholic extracts of *Tiliae* samples [10]. Majer et al. examined the antioxidant activities of *Tiliae* flowers with different sunlight exposures [26]. Cittan et al. compared the antioxidant activity and phenolic content of *Tilia cordata* fruit samples prepared by ultrasound-assisted extraction and infusion [27]. According to the results, ultrasound-assisted extraction technique with methanol was considered to have higher effect then traditional water infusion. Moreover, protocatechuic acid was determined to be the major phenolic compound in both extracts.

In the light of previous studies, infusions of *Tiliae* samples by using different type of teapot materials: glass, ceramic, stainless steel, copper and aluminum were prepared. Antioxidant activity, phenolic contents and protocatechuic acid contents of whole extracts were measured. Antioxidant activity and phenolic content results were shown in Tables 1-3. The infusion prepared by ceramic pot showed the highest total phenolic content (672.80±1.81), total phenolic acid content (61.16±2.50), total flavonoid content (17.64±1.2), DPPH radical scavenging (EC_{50} 636.35±23.05 µg/mL), FRAP activity (1.64±0.04), CUPRAC activity (791.27±2.96) and TOAC (672.00±1.40) whereas not the highest protocatechuic acid content (0.762%).

Table 1. Spectrophotometric determination of phenolic profile of TP infusions.

Analysis	*Aluminum*	*Ceramic*	*Copper*	*Glass*	*Stainless steel*
Total phenolic content[A]	521.33±1.30[a]	672.80±1.81[b]	566.40±1.99[c]	511.93±1.87[d]	514.46±1.66[d]
Total flavonoid content[B]	9.32±0.26[a]	17.64±1.24[b]	12.85±0.30[c]	9.29±0.97[a]	8.52±0.46[a]
Total phenolic acid content[C]	25.23±1.16[a]	61.16±2.50[b]	49.53±2.82[c]	35.26±3.81[d]	32.5±1.77[d]

[A] Results were expressed as the mean of triplicates ± standard deviation (S.D.) and as mg gallic acid equivalents (GAE) in 1 g sample.
[B] Results were expressed as the mean of triplicates ± standard deviation (S.D.) and as mg quercetin equivalents (QE) in 1 g sample.
[C] Results were expressed as the mean of triplicates ± standard deviation (S.D.) and as mg caffeic acid equivalents (CAE) in 1 g sample.
[a-e] Different letters in the same row indicate significance ($p < 0.05$).

Table 2. Quantification data for protocatechuic acid content of TP infusions.

Compound/ Infusion	Aluminum	CV %	Ceramic	CV %	Copper	CV %	Glass	CV %	Stainless steel	CV %
Protocatechuic acid (w/w%)	0.905	1.66	0.762	3.04	1.037	0.82	0.858	3.02	0.875	2.70

*CV: Coefficient of Variation

Contrary to expectations, the infusion prepared in glass pot showed lower activity in general even lowest CUPRAC (702.57±13.85) and TOAC (511.93±1.22) activities. As assumed infusions prepared in aluminum pot showed lowest TPC (521.33±1.30), TPA (25.23±1.16), DPPH radical scavenging (EC_{50} 741.09±25.81µg/mL) and FRAP (0.97±0.05) activity. From these results, it can be interpreted that, along with the phenolic, volatile composition of *T. platyphyllos* have contributed the antoxidant activity of the extracts.

Flavoring compounds comparably affect the composition and bioactivity of herbal teas as well. According to Stahl et al., milk addition to black tea lowered the antioxidant activity [28]. Sugar addition was also lowered the DPPH scavenging activity when compared with the standard black tea. Toydemir et al. examined the antioxidant activities of flower and pine honey added various herbal teas [18]. In respect to linden, TPC, total flavonoid content, TOAC and CUPRAC values were greater with addition of honey. The

antioxidant activity and the phenolic composition were greater in linden samples added with stevioside a natural diterpenoid compound which is used as sweetener.

Table 3. *In vitro* antioxidant activities of TP infusions.

Analysis	Aluminum	Ceramic	Copper	Glass	Stainless steel
DPPH scavenging activity[A]	741.09±25.81^{a}	636.35±23.05^{b}	677.79±51.61^{ab}	682.84±8.05^{ab}	717.70±32.41^{ab}
FRAP[B]	0.97±0.05^{a}	1.64±0.04^{b}	1.27±0.07^{c}	1.01±0.01^{a}	1.05±0.05^{a}
CUPRAC[C]	716.95±51.78^{ab}	791.27±2.96^{a}	733.22±17.78^{ab}	702.57±13.85^{b}	745.21±34.71^{ab}
Total antioxidant capacity[D]	521.33±1.22^{a}	672.80±1.40^{b}	566.40±1.44^{c}	511.93±1.22^{d}	514.46±1.41^{e}

P.S. 1) EC_{50} value of the reference compound "BHT" in DPPH scavenging activity is found to be 390.18 ± 4.86 µg/mL. 2) FRAP activity of the reference compound "BHT" is found to be 4.07 ± 0.52 mM FeSO4 eq. in 1 g sample.
[A] Results were expressed as the mean of triplicates ± standard deviation (S.D.) and DPPH activity was expressed as EC_{50} in µg/mL equivalents.
[B] Results were expressed as the mean of triplicates ± standard deviation (S.D.) and as Mm $FeSO_4$ equivalents in 1 g sample.
[C] Results were expressed as the mean of triplicates ± standard deviation (S.D.) and as mg ascorbic acid equivalents (AAE) in 1 g sample.
[a-e] Different letters in the same row indicate significance ($p < 0.05$).

Korir et al. studied alterations on antioxidant activity of tea samples after addition of some additives such as stevia, honey and sugar [29]. Results indicated that stevia addition produced no significant modification on antioxidant activity. In contrary sugar and honey inclusion expressively decreased the antioxidant activity. As a result, stevia was determined as preferable sweetener for obtaining severe antioxidant activity. In another study, honey addition to several fruit tea infusions was reduced total phenolic content and antioxidant activity by 28% and 22% respectively. It may be hypothesized that glycosylation causes reduction on hydrogen donation of polyphenols therefor sugar and honey addition impairs antioxidant activity [30]. Lemon is rich for sugar and ascorbic acid. Even though sugar has negative effect for antioxidant activity, ascorbic acid is known efficient antioxidant substance. Our results revealed that stevia and synthetic sweetener added samples have significantly higher antioxidant activities when compared to sugar and honey added samples (Table 4). These results are expectable in the light of previous studies. Lemon added samples have mild antioxidant activity however significantly higher than sugar and honey added samples. It may be claimed that ascorbic acid compensated negative effects of sugars of lemon and resulted with nothing but mild decrease when compared to honey and sugar.

Table 4. *In vitro* antioxidant activities of TP infusions with various flavors.

Analysis	*Stevioside*	*Synthetic sweetener*	*Brown Sugar*	*White Sugar*	*Flower Honey*	*Pine Honey*	*Lemon*
DPPH scavenging activity[A]	2781.76 ±44.38^{a}	3412.50 ±98.66^{b}	6793.99 ±81.80^{a}	8873.36 ±410.81^{c}	7425.16 ±308.07^{a}	11675 ±299.27^{d}	4487.41 ±112.44^{e}
CUPRAC[C]	192.78 ±9.39^{a}	157.03 ±8.91^{b}	19.11 ±0.82^{c}	15.15 ±0.59^{c}	17.03 ±0.76^{c}	11.30 ±0.61^{c}	55.19 ±0.59^{d}
Total antioxidant capacity[D]	111.22 ±0.78^{a}	95.71 ±1.25^{b}	55.23 ±3.79^{c}	41.33 ±0.93^{d}	49.20 ±1.36^{e}	45.97 ±2.43^{de}	55.84 ±1.99^{c}

P.S. 1) EC_{50} value of the reference compound "BHT" in DPPH scavenging activity is found to be 390.18 ± 4.86 µg/mL. 2) FRAP activity of the reference compound "BHT" is found to be 4.07 ± 0.52 mM FeSO4 eq. in 1 g sample.
[A] Results were expressed as the mean of triplicates ± standard deviation (S.D.) and DPPH activity was expressed as EC50 in µg/mL equivalents.
[B] Results were expressed as the mean of triplicates ± standard deviation (S.D.) and as Mm FeSO4 equivalents in 1 g sample.
[C] Results were expressed as the mean of triplicates ± standard deviation (S.D.) and as mg ascorbic acid equivalents (AAE) in 1 g sample.
[a-e] Different letters in the same row indicate significance ($p < 0.05$).

3. CONCLUSION

In the light of the information obtained from this study, it is designated that, the brewing materials during the preparation of the herbal teas and additives are also very important to their effects and therapeutically usages. Consequently, the preparation methods and additives of the herbal teas have to be chosen according to the aim of disease treatment.

4. MATERIALS AND METHODS

4.1. Chemicals and reagents

All of the chemicals, and references used during the experiments were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Spectrophotometric calculations were conducted using Thermo Multiskan Sky Microplate Spectrophotometer. Protocatechuic acid contents were measured by Camag-HPTLC system.

4.2. Plant materials

The commercially available *Tilia platyphyllos* (TP) samples were purchased from the local market in Istanbul/Turkey. The classification of the commercial plant material was performed using "Flora of Turkey and the East Aegean Islands" [31].

4.3. Preparation of brews

The infusions were prepared from 2 g plant materials which contains equal amounts of bracteols and flowers, with 100 mL of freshly boiled water representing the ordinary quantity consumed by tea drinkers, in pots which are made of glass, ceramic, stainless steel, copper and aluminum, for 5 minutes with a closed cap; filtered by using filter paper. 0.5 g natural sweetener (stevioside), and synthetic sweetener (aspartame), 5 g brown and white sugar, 5 g flower and pine honey, and 10 mL of lemon juice were added on freshly prepared ceramic pot infusions, separately. The amounts of the additives were calculated by observing the packages sold for one cup of tea.

4.4. Quantitative assessment of polyphenolic content

4.4.1. Total phenolic content

The evaluation of total phenolic contents of the infusions was executed according to the method described by Singleton and Rossi [32]. Diluted infusion samples were introduced into the blend of Na_2CO_3 (20%) and FCR (Folin-Ciocalteu reagent which diluted with H_2O (1:9)). Thereafter the incubation duration at 45°C for 30 min., the absorbance of the mixtures was calculated at 765 nm. The results were expressed as gallic acid equivalents (GAE) per g dry extract (DE).

4.4.2. Total flavonoid content

Total flavonoid contents of the infusions were determined via the method described by Celep et al. [33] . Diluted specimens were blended with the admixture of Aluminum chloride (10%) and sodium acetate (1M), and the incubation process lasted for 30 min at room temperature. The absorbance was read at 415 nm. Total flavonoid content was given as mg quercetin equivalents (QE) per g dry extract.

4.4.3. Total phenolic acid content

The total phenolic acid content of the infusions was spectrophotometrically measured at 490 nm [34]. The method is based on forming a complex due to the chemical reaction of sodium molybdate-sodium nitrite with phenolic acids. The results were assessed as caffeic acid equivalents (CAE) in per g dry extract.

4.5. HPTLC quantification of protocatechuic acid

Protocatechuic acid contents of the Tiliae infusions were calculated by a method published recently by Niranjan et al. [35]. The concentration of standard solution of protocatechuic acid (250 µg/mL) was adjusted in methanol and 5 mg of total extract and each fraction were dissolved in 1 mL methanol. 0.45 µm syringe filter was used for filtration of extracts. 15 µL of Tiliae samples were applied in triplicate. Application of 2 µL to 5.5 µL of standard protocatechuic acid solution was in triplicate. Band length of the standard solutions and samples was 8 mm on silica gel glass HPTLC plates 60 F_{254} with Camag Automatic TLC Sampler IV. The mobile phase was toluene:ethyl acetate:formic acid (14:10:1) (v/v/v). Developments were executed in

Camag Automatic Developing Chamber (ADC-2). Saturation process of the chamber lasted 20 min before the development. The humidity control managed by ADC-2 via using $MgCl_2$ (33% RH) for 10 minutes. Densitometric screening was implemented by using Camag TLC Scanner IV at 282 nm and VisionCATS software in fluorescence mode after development of 70 mm. The slit dimension was arranged as 5×0.2 mm, micro and the scanning speed was 20 mm/s. Amounts of standards in the extracts were obtained by comparison of AUCs with the standards' calibration curve ($y=1.742*10^{-8}x-4.159*10^{-3}$). The coefficient variation coefficient (CV%) was 1.62% and the correlation coefficient (R) of the calibration curve was more than 0.998. The presence of standard compounds in extracts was ensured by comparison of the retention factors (R*f* 0.550) and overlapping UV spectra of each extract and standards (Figures 1-2).

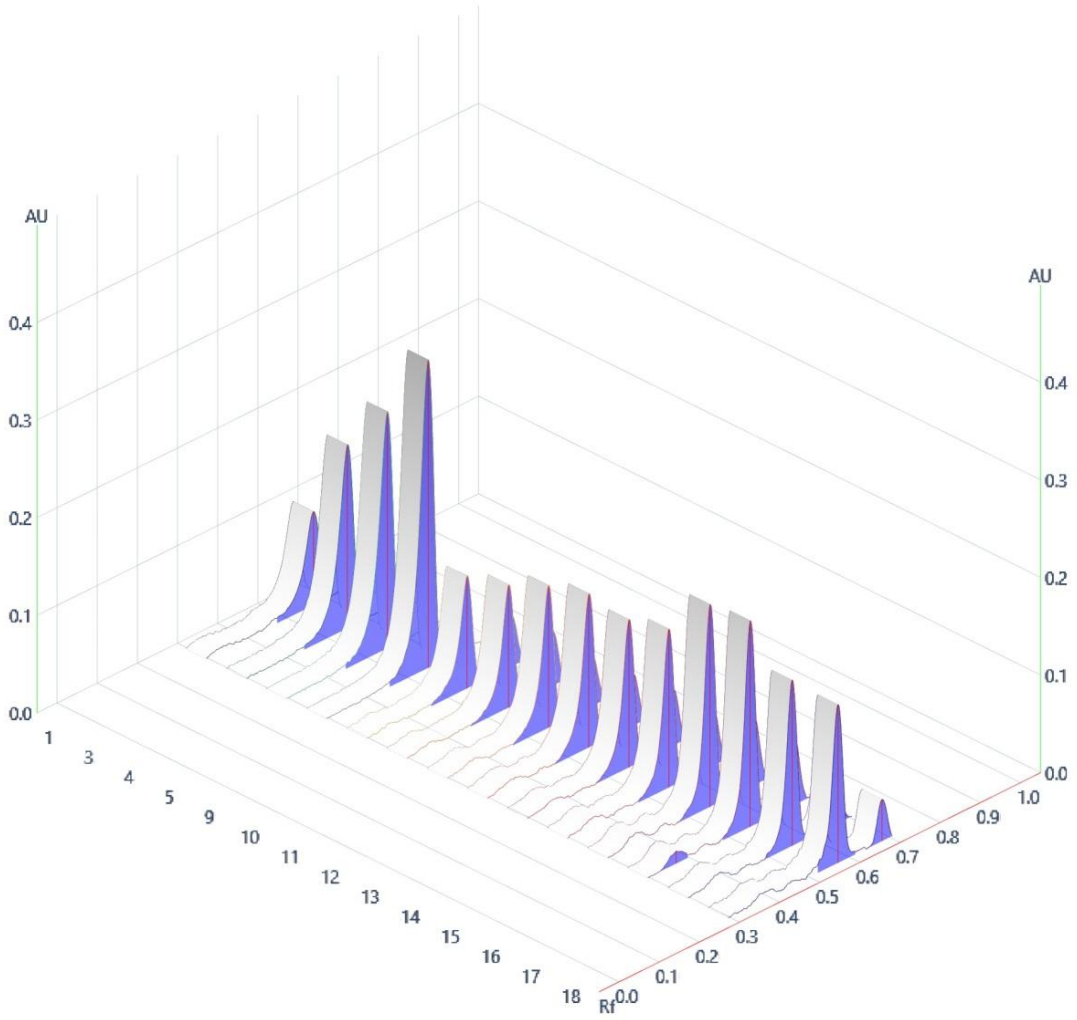

Figure 1. Separation and R*f* value of protocatechuic acid standard and Tiliae infusions prepared in different brewing materials.

4.6. Assessment of antioxidant activity based on DPPH radical-scavenging activity

Scavenging activity of DPPH radical was determined using the method stated earlier by Celep et al. [36]. Dilution of specimens were performed freshly and separately mixed with 100 µM methanolic DPPH solution. The mixtures were maintained at room temperature and at the dark for 50 min., and the calculation of the absorbance conducted at 517 nm. EC_{50} values of the infusions were calculated by using five different concentratons. Butylated hydroxytoluene (BHT) was selected as reference compound.

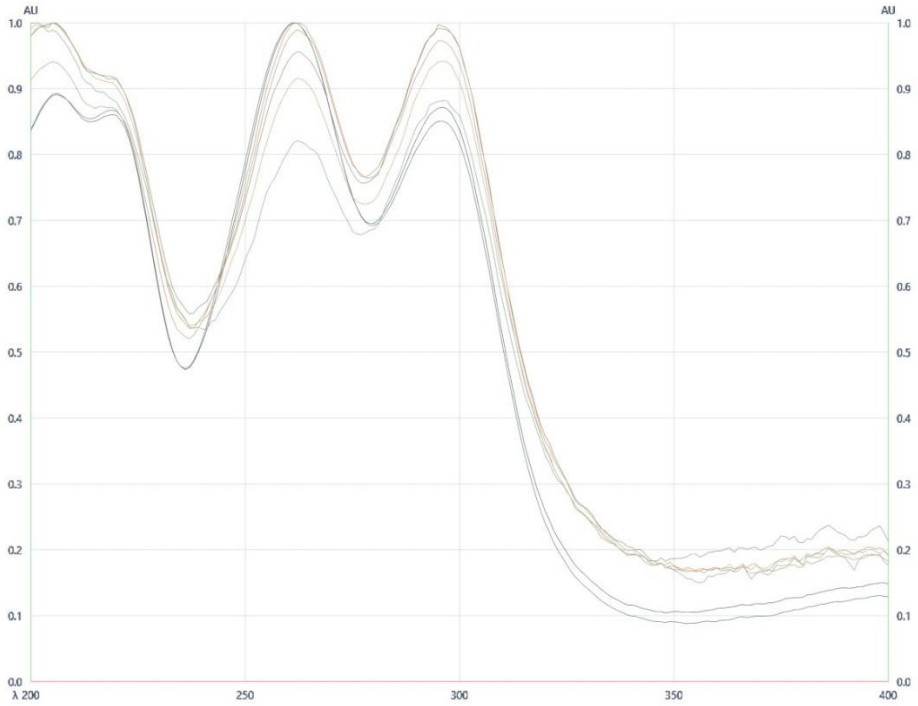

Figure 2. Overlapped UV spectra of protocatechuic acid standard and Tiliae infusions prepared in different brewing materials at 282 nm.

4.7. Assessment of antioxidant activity based on metal-reduction potential

4.7.1. Cupric reducing antioxidant capacity (CUPRAC)

CUPRAC activities of the studied samples were determined using the modified method of Apak et al. [37]. Exact same volumes of Copper sulfate (10 mM), ammonium acetate buffer (1 M, pH 7.0) and neocuproine (7.5 mM) were mixed, independently. Afterwards, infusions were added to the mixture and then incubation process lasted for 1h, and the absorbance was recorded at 450 nm. The results were expressed as mg ascorbic acid equivalent (AAE) per g dry extracts.

4.7.2. Ferric reducing antioxidant power (FRAP)

Benzie and Strain's previously described spectrophotometric method was preferred [38]. Samples were legitimately and then blended with FRAP reagent, which is composed of acetate buffer (0.3 M), 2,4,6-tripyridyl-S-triazine (10 mM) and $FeCl_3$ (20 mM). Subsequent to 30 min. incubation, the absorbance was measured at 593 nm. BHT was chosen as reference compound. The results were measured as mM $FeSO_4$ per g dry extract.

4.7.3. Estimation of total antioxidant capacity by phosphomolybdenum method

The spectrophotometric method of Prieto et al. was carried out for the estimation of total antioxidant capacity [39]. Solutions of samples were transfused into the mixture, which consists of sodium phosphate monobasic (28 mM), sulfuric acid (0.6 M) and ammonium molybdate (4 mM), for reaction. Afterwards incubation occurred for 90 min. at 95 °C, and subsequent to cooling to room temperature absorbance was read 695 nm. Total antioxidant capacity was expressed as mg AAE per g dry extract.

4.8. Statistics

All of the assays and analyses were performed in triplicate. Mean ± standard deviations were calculated in each assay. ANOVA test was used for statistical comparison of the results. The multiple comparisons were accomplished by Tukey–Kramer post hoc test. Statistically significant difference was defined as $p < 0.05$.

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