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Effects of Honey Addition on Antioxidative Properties of Different Herbal Teas

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Tea and herbal infusions are among the major contributors of phenolic compounds, specifically flavonoids, in our daily diet. Honey is another antioxidant-rich food that is widely used as a natural sweetener. In this work, the effects of honey addition on antioxidant properties of different herbal teas were investigated. For this purpose, 2 different types of honey (flower and pine honey) were added into 9 different herbal teas (melissa, green tea, rosehip, sage, echinacea, fennel, linden, daisy, and ginger) at 4 different temperatures (55˚C, 65˚C, 75˚C, and 85˚C), and the changes in the content of total pheolics, total flavonoids, and total antioxidant capacity were determined. The total phenolic content and the total antioxidant capacity of the honey-added-tea samples were found to be increased (up to 57% for both), especially with pine honey and at higher temperatures of honey addition. The findings of this study supported the use of honey as a natural sweetener in tea in order to be able to benefit from the health-enhancing antioxidative properties of these two promising food products.

INTRODUCTION

Tea and herbal infusions, which are popular, socially accepted, and economical, drinks [Trevisanato & Young-In Kim, 2000], can be prepared from any part of various plants, *i.e.* roots, flowers, seeds, berries, or bark, depending on the solubility of the active constituents included [Apak *et al.,* 2006]. It is well-documented that these infusions, prepared from valuable parts of herbs, are among the major contributors of phenolics in our diet [Shahidi, 2000]. Flavonoids, as the leading polyphenol group present in herbs, have been indicated to provide protection against several forms of cancer and cardiovascular diseases, as well as enhance the function of the immune system [Craig, 1999]. Brewing tea leaves in hot water has been reported to release 69–85% of the bioactive flavonoids within 3–5 minutes [Keli *et al.,* 1996] which contributes to the intake of 80 mg flavonoids per 100 mL of tea consumption [van Dokkum *et al.,* 2008].

The majority of the plant materials, that include phytochemicals possessing health-promoting antioxidant activity, are also used by the bees to collect honey nectar, leading to the transfer of these bioactive components into honey [The National Honey Board, 2002]. Honey is a natural sweetener produced by honeybees from the nectar of blossoms (floral (nectar) honey) and from secretions of living parts of plants or excretions of plant-sucking insects on the living part of plants (honeydew honey) [Persano Oddo *et al.,* 2004]. Honey is reported to be an important source of antioxidants, including flavonoids, phenolic acids, carotenoid derivatives, organic acids, Maillard reaction products, *etc.* [Gheldof & Engeseth, 2002; Aljadi & Kamaruddin, 2004]. Various literature studies pointed out the antimicrobial [Alvarez-Suarez *et al.,* 2013; Al-Waili *et al.,* 2011; Alzahrani *et al.,* 2012; Chang *et al.,* 2011; Israili, 2014], antioxidant [Alvarez-Suarez *et al.,* 2012a, b; Alzahrani *et al.,* 2012], antiinflammatory, and antitumoral properties [Alvarez-Suarez *et al.,* 2013] of honey, as well as its potential use in combination with conventional therapy as a novel antioxidant in the management of chronic diseases that are mostly related to the oxidative stress [Erejuwa *et al.,* 2012].

The use of honey can be suggested to sweeten tea as a healthier way of tea consumption with the preferred sweet taste. However, based on our current literature search, there is no data on how the antioxidant potential of herbal infusions is affected by the addition of honey. Therefore, the aim of the present work was to determine and compare the influences of flower honey (nectar honey) and pine honey (honeydew honey) addition on the total phenolic and total flavonoid contents, as well as total antioxidant capacities of different

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herbal tea samples. In addition, the effect of the infusion temperature, at which honey was added, was also investigated.

MATERIALS AND METHODS

Honey and herbal tea samples

Flower honey (from Marmaris, Mugla region of Turkey) and pine honey (from Eastern Anatolia region of Turkey) samples were collected, in duplicates in 2013, directly from beekeepers in Turkey, and tested for their total phenolic contents and total antioxidant activities before use. In order to establish the botanical origins of honey (floral, pine) samples, microscopic analysis, pollen and spore determination, conductivity, acidity, humidity, diastase and sugar profile analysis were performed along with sensory testing. Nectar honey samples were multifloral. Herbal tea samples, including melissa, green tea, rosehip, sage, echinacea, fennel, linden, daisy, and ginger teas, were supplied from a tea manufacturer in Turkey in the form of tea bags.

Sample/extract preparation

Herbal tea infusions were prepared by adding 200 mL of freshly boiled deionised water on a tea bag (2 g), and brewing for 3 min (taking the instructions on the package into consideration) without additional heating. Tea bags were removed and subsequently, flower honey or pine honey samples were added to these herbal tea infusions at 85˚C, 75˚C, 65˚C, and 55˚C of infusion temperatures, measured using a thermometer (ISOLAB Laborgerate GmbH, Germany), and at a concentration of 7.5 g honey/100 mL tea. Infusions without any honey addition were used as controls. For both the honey--added extracts and the controls, the analyses of total phenolics, flavonoids and antioxidant capacity were conducted after cooling the samples to room temperature. All honey-added and control infusions were prepared in triplicates.

Analytical protocols

Hydroxymethylfurfural (HMF) content of honey samples was determined using the spectrophotometric method described in Turkish Honey Standard [TS 3036, 2002]. The method was based on the colorimetric reaction among *p*-toluidine, barbituric acid and HMF forming a red colored complex. The absorbance was measured at 550 nm and the HMF was quantified using the following formula:

$$HMF(mg/kg) = A_{550} \times 192$$

where A_{550} is the absorbance measured at 550 nm and 192 is a theoretical value linked to the molar extinction coefficient of HMF.

Total phenolic (TP) content was determined according to the Folin-Ciocalteau method described previously by Velioglu *et al.* [1998]. In brief, 0.1 mL of sample was added to 0.75 mL of Folin-Ciocalteau reagent. The mixture was allowed to stand for 5 min and then 0.75 mL of 6% sodium carbonate solution was added to the mixture. After 2 h of incubation at room temperature, absorbance was read at 725 nm. The results were expressed as mg gallic acid equivalent (GAE)/L tea.

Total flavonoid (TF) content was measured using the colorimetric assay developed by Zhishen *et al.* [1999]. At time zero, 1 mL of sample was mixed with 0.3 mL of 5% $NaNO_2$ solution. After 5 min, 0.3 mL of 10% $AlCl_3$ was added. At the 6th min, 2 mL of 1 mol/L NaOH was added to the mixture. Immediately, 2.4 mL of distilled water was added and the absorbance was read at 510 nm. The results were given as mg catechin equivalent (CE)/ L tea.

Total antioxidant capacity (TAC) was estimated using two *in vitro* tests in parallel. The DPPH (1,1-diphenyl-2-picrylhydrazyl) method was performed as described by Kumaran & Karunakaran [2006]. 0.1 mL of each sample extract was mixed with 2 mL of 0.1 mmol/L DPPH in methanol. After 30 min of incubation at room temperature, the absorbance of the mixture was measured at 517 nm. The CUPRAC (Cupric Reducing Antioxidant Capacity) method was applied using the protocol reported by Apak *et al.* [2004]. 0.1 mL of extract was mixed with 1 mL of 10 mmol/L $CuCl_2$, 7.5 mmol/L neocuproine and 1 mol/L NH4Ac (pH:7). Immediately, 1 mL of distilled water was added to the mixture to make the final volume of 4.1 mL. After 60 min of incubation at room temperature, absorbance was read at 450 nm. The results were given as Trolox (6-hydroxy- 2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent (TE)/ L tea.

Statistical analysis

Statistical analysis was applied to the data obtained from the samples that were subjected to each assay in triplicates. Minitab software (version 16.1.0) was used for the one-way ANOVA and pairwise comparisons between the treatments (honey varieties and temperatures) were done using Tukey test with a 95% confidence level. The correlation coefficients (R^2) for results of the two spectrophotometric assays were calculated using Microsoft Office Excel 2011 software (Microsoft Corporation, Redmond, WA, US).

RESULTS

Hydroxymethylfurfural (HMF) content of honey samples

The HMF contents of the flower and pine honey samples were determined to check for an acceptable quality of the honey samples that were subjected to high temperatures. The maximum value for HMF content of honey after processing and/or blending, is fixed as 40 mg/kg by the Codex standard [Codex Stan 12–1981, Rev 2 2001]. The concentrations found in the current study were very low (below the Codex limit) both in flower and pine honey samples, ranging between 4.6–8.1 mg/kg.

Changes in total phenolic and total flavonoid contents of tea samples added-with-honey

The results obtained for TP and TF contents were represented in Table 1. Melissa, green tea, and rosehip were the first three teas determined to have the highest TP contents (549, 465, and 397 mg GAE/L tea, respectively) than their controls, followed by sage, echinacea, fennel, linden, daisy, and ginger, respectively (71–268 mg GAE/L tea). The flower honey led to significant increases ($p<0.05$) in TP content of sage tea at all

TABLE 1. The changes in total phenolic and total flavonoid contents of 9 different herbal teas with flower honey and pine honey addition at 4 different tea temperatures*

Honey–temperature	Total phenolic content (mg GAE/L)								
	Melissa	Green tea	Rosehip	Sage	Echinacea	Fennel	Linden	Daisy	Ginger
Control	549±70^{a}	465±42^{b}	397±63^{b}	268±28^{b}	266±61^{c}	123±21^{b}	86±7^{b}	73±17^{b}	71±9^{cd}
Flower-55˚C	596±41^{a}	533±78^{ab}	493±43^{ab}	343±26^{a}	389±85^{ab}	163±27^{ab}	134±29^{ab}	55±13^{b}	58±2^{d}
Flower-65˚C	584±96^{a}	543±36^{ab}	521±54^{a}	327±23^{a}	449±101^{a}	152±38^{ab}	116±10^{ab}	58±17^{b}	60±2^{d}
Flower-75˚C	601±53^{a}	547±68^{ab}	525±61^{a}	343±19^{a}	361±64^{abc}	174±28^{a}	107±27^{ab}	60±25^{b}	57±4^{d}
Flower-85˚C	600±65^{a}	563±63^{a}	436±77^{ab}	333±23^{a}	378±63^{ab}	166±33^{ab}	144±16^{a}	73±25^{b}	93±10^{bc}
Pine-55˚C	659±101^{a}	544±61^{ab}	530±59^{a}	336±8^{a}	291±61^{bc}	155±18^{ab}	135±15^{a}	134±33^{a}	127±25^{a}
Pine-65˚C	680±111^{a}	544±37^{ab}	476±108^{ab}	336±17^{a}	279±51^{bc}	145±38^{ab}	140±16^{a}	132±28^{a}	128±11^{a}
Pine-75˚C	640±57^{a}	552±21^{a}	460±56^{ab}	313±23^{a}	293±65^{bc}	134±22^{ab}	136±32^{a}	141±28^{a}	111±16^{ab}
Pine-85˚C	584±72^{a}	553±32^{a}	417±68^{ab}	340±12^{a}	308±53^{bc}	160±34^{ab}	152±33^{a}	138±22^{a}	132±11^{a}
Honey–temperature	Total flavonoid content (mg CE/L)								
	Melissa	Green tea	Rosehip	Sage	Echinacea	Fennel	Linden	Daisy	Ginger
Control	3705±170^{c}	726±40^{a}	962±88^{a}	1421±121^{b}	799±87^{a}	219±35^{b}	179±29^{c}	145±7de	323±43^{a}
Flower-55˚C	4104±135^{ab}	550±40de	743±74^{cd}	1675±122^{ab}	627±75^{bcd}	157±9^{c}	181±23^{bc}	131±11^{e}	249±10^{bcd}
Flower-65˚C	4018±72^{abc}	541±63^{e}	702±51^{d}	1583±74^{ab}	644±66^{bcd}	162±9^{c}	200±31^{abc}	145±7^{cde}	258±16^{bcd}
Flower-75˚C	3876±156^{bc}	571±51^{cde}	803±97^{bcd}	1703±53^{a}	575±103^{d}	166±17^{c}	216±34^{abc}	146±8^{cde}	244±7^{cd}
Flower-85˚C	3858±447^{bc}	563±64^{cde}	686±76^{d}	1650±279^{ab}	623±99^{cd}	164±6^{c}	181±20^{bc}	154±11^{bcd}	223±17^{d}
Pine-55˚C	4128±107^{ab}	629±28^{bcd}	846±48^{abc}	1768±161^{a}	780±79^{ab}	240±25^{ab}	220±32^{abc}	175±12^{a}	293±23^{abc}
Pine-65˚C	4271±262^{a}	637±22^{bc}	900±58^{ab}	1851±203^{a}	762±84^{abc}	262±12^{a}	262±49^{a}	170±13^{ab}	290±46^{abc}
Pine-75˚C	4171±129^{ab}	682±29^{ab}	880±87^{abc}	1564±197^{ab}	759±81^{abc}	245±22^{ab}	262±44^{a}	162±9^{abc}	308±34^{ab}
Pine-85˚C	3977±211^{abc}	658±22^{ab}	786±36^{bcd}	1756±141^{a}	777±46^{abc}	235±22^{ab}	243±44^{ab}	162±8^{abc}	304±48^{abc}

* Data represent average values ± standard deviation of three independent samples. Different letters in the columns represent statistically significant differences ($p<0.05$). Control samples were tea samples with no added-honey. GAE: gallic acid equivalent; CE: catechin equivalent.

infusion temperatures of honey addition. On the other hand, flower honey-added green tea and linden tea gave significantly higher ($p<0.05$) values at 85˚C, rosehip tea at 65˚C and 75˚C, echinacea tea at 55˚C, 65˚C, and 85˚C, and fennel tea at 75˚C of honey addition temperatures. Flower honey did not result in any significant changes ($p>0.05$) in TP content of melissa, daisy, and ginger tea samples.

The pine honey addition into sage, linden, daisy, and ginger tea resulted in significantly higher ($p<0.05$) TP contents, in comparison to their controls, at all temperatures. While significant increases were obtained in TP contents of green tea at 75˚C and 85˚C and rosehip tea at 55˚C, pine honey addition did not make a significant change in TP contents of melissa, echinacea, and fennel tea samples, at any temperature.

The TF contents of melissa, sage, and rosehip tea were found to be the highest among the analysed tea samples (3705, 1421, and 962 mg CE/L tea, respectively), followed by echinacea, green tea, ginger, fennel, linden, and daisy teas, respectively (145–799 mg CE/L tea). The TF content results obtained for the flower honey addition indicated significant increases ($p<0.05$) in TF content of melissa tea at 55˚C and sage tea at 75˚C, only. On the other hand, flower honey-added green tea, rosehip, echinacea, fennel, and ginger tea samples were determined to have significantly reduced ($p<0.05$) TF contents at any temperature of honey addition. TF contents of daisy and linden tea did not change significantly with flower honey.

The TF content measurements for pine honey-added tea samples revealed that pine honey addition resulted in significant increases ($p<0.05$) in TF contents of melissa tea at 55˚C, 65˚C, and 75˚C, sage tea at 55˚C, 65˚C, and 85˚C, fennel tea at 65˚C, linden tea at 65˚C, 75˚C, and 85˚C, and daisy tea at all 4 infusion temperatures of honey addition. On the other hand, significant reductions were obtained, with pine honey addition, in green tea at 65˚C and in rosehip tea at 85˚C. TF contents of echinacea and ginger tea were not affected significantly with the addition of pine honey.

Changes in total antioxidant capacity of tea samples added-with-honey

The changes in total antioxidant capacity (TAC) values of different tea samples, with flower honey and pine honey ad-

TABLE 2. The changes in total antioxidant capacity of 9 different herbal teas with flower honey and pine honey addition at 4 different tea temperatures.

Honey–temperature	Total antioxidant capacity, DPPH Method (mg TE/L)								
	Melissa	Green tea	Rosehip	Sage	Echinacea	Fennel	Linden	Daisy	Ginger
Control	1111±179^{ab}	962±162^{b}	684±113^{a}	441±29^{b}	273±51^{abc}	78±22^{b}	64±13^{c}	43±12^{c}	100±15^{b}
Flower-55˚C	989±114^{ab}	1069±207^{ab}	613±137^{a}	522±67^{ab}	261±28^{abc}	82±8^{ab}	105±14^{ab}	23±9^{c}	133±32^{ab}
Flower-65˚C	975±123^{ab}	1106±121^{ab}	629±135^{a}	541±21^{ab}	220±13^{c}	87±14^{ab}	97±12^{ab}	33±9^{c}	125±19^{ab}
Flower-75˚C	940±126^{b}	1100±68^{ab}	637±140^{a}	545±59^{ab}	220±13^{bc}	97±13^{ab}	89±14^{abc}	44±10^{bc}	157±17^{a}
Flower-85˚C	937±94^{b}	1181±181^{a}	586±119^{a}	642±72^{a}	231±24^{bc}	77±8^{b}	76±9^{bc}	37±9^{c}	124±16^{ab}
Pine-55˚C	1058±157^{ab}	1131±43^{ab}	594±32^{a}	544±51^{ab}	316±28^{ab}	107±9^{a}	85±20^{abc}	60±5^{ab}	98±15^{b}
Pine-65˚C	1102±132^{ab}	1079±30^{ab}	584±65^{a}	575±46^{a}	348±80^{a}	98±13^{ab}	102±11^{ab}	61±4^{ab}	100±17^{b}
Pine-75˚C	1179±130^{ab}	1114±81^{ab}	604±47^{a}	527±48^{ab}	313±54^{ab}	107±11^{a}	110±34^{a}	70±6^{a}	106±19^{b}
Pine-85˚C	1221±124^{a}	1055±76^{ab}	519±56^{a}	544±58^{ab}	287±73^{abc}	104±16^{a}	89±21^{abc}	63±7^{ab}	102±17^{b}
Honey–temperature	Total antioxidant capacity, CUPRAC Method (mg TE/L)								
	Melissa	Green tea	Rosehip	Sage	Echinacea	Fennel	Linden	Daisy	Ginger
Control	2212±84^{a}	1813±277^{b}	1424±182^{ab}	911±126^{b}	610±48^{b}	238±34^{b}	215±21^{c}	219±40^{c}	256±47^{b}
Flower-55˚C	2300±105^{a}	1859±176^{ab}	1484±82^{ab}	1202±84^{a}	660±35^{ab}	299±24^{a}	283±41^{ab}	265±57^{abc}	276±26^{ab}
Flower-65˚C	2390±78^{a}	1895±281^{ab}	1428±96^{ab}	1119±100^{a}	667±57^{ab}	294±41^{ab}	281±27^{ab}	267±49^{abc}	280±29^{ab}
Flower-75˚C	2262±106^{a}	1852±104^{ab}	1611±107^{a}	1217±67^{a}	661±67^{ab}	358±23^{a}	252±24^{bc}	266±80^{abc}	276±49^{ab}
Flower-85˚C	2329±28^{a}	1810±314^{ab}	1326±100^{ab}	1155±112^{a}	674±39^{ab}	308±34^{a}	272±26^{abc}	251±53^{bc}	271±27^{ab}
Pine-55˚C	2161±285^{ab}	1929±188^{ab}	1382±124^{ab}	1167±78^{a}	730±29^{a}	339±39^{a}	322±51^{a}	339±18^{a}	314±45^{ab}
Pine-65˚C	2145±171^{ab}	1892±166^{ab}	1428±260^{ab}	1142±111^{a}	706±67^{a}	345±41^{a}	339±54^{a}	309±25^{ab}	297±42^{ab}
Pine-75˚C	2155±202^{ab}	2067±70^{ab}	1389±176^{ab}	1103±123^{a}	702±38^{a}	333±49^{a}	337±49^{a}	301±26^{ab}	339±44^{a}
Pine-85˚C	1956±224^{b}	2199±103^{a}	1214±242^{b}	1128±85^{a}	729±29^{a}	303±30^{a}	302±34^{ab}	278±21^{abc}	296±62^{ab}

* Data represent average values ± standard deviation of three independent samples. Different letters in the columns represent statistically significant differences ($p<0.05$). Control samples were tea samples with no added-honey. DPPH: 1,1-diphenyl-2-picrylhydrazyl; Cupric Reducing Antioxidant Capacity; TE: Trolox (6-hydroxy- 2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent.

dition at 4 different infusion temperatures (55˚C, 65˚C, 75˚C, and 85˚C), were determined using 2 *in vitro* tests in parallel, which were DPPH and CUPRAC methods (Table 2). The highest TAC values, determined with DPPH method, were measured for melissa, green tea, and rosehip tea samples (1111, 962, and 684 mg TE/L, respectively), followed by sage, echinacea, ginger, fennel, linden, and daisy tea samples, respectively (43–441 mg TE/L) (Table 2). The results of DPPH method indicated that flower honey led to a significant increase ($p<0.05$) in TAC of green tea and sage tea at 85˚C, linden tea at 55˚C and 65˚C, and ginger tea at 75˚C. There was no change observed in TAC of flower honey-added melissa, rosehip, echinacea, fennel, and daisy tea samples, in comparison to their control infusions.

The differences in TAC of pine-honey added tea infusions, measured using DPPH method, indicated significantly higher values ($p<0.05$) in pine honey-added sage tea at 65˚C, fennel tea at 55˚C, 75˚C, and 85˚C, linden tea at 65˚C and 75˚C, and daisy tea at all four temperatures of honey addition, in comparison to their control samples. Whereas, the TAC of melissa, green tea, rosehip, echinacea, and ginger tea did not show any significant change with the inclusion of pine honey.

The highest TAC values, determined using CUPRAC method, were again in melissa, green tea, and rosehip tea (2212, 1813, and 1424 mg TE/L), followed by sage, echinacea, ginger, fennel, daisy, and linden, respectively (215–911 mg TE/L). Flower honey increased the TAC of sage tea significantly ($p<0.05$) at all infusion temperatures. In addition, substantial increases were also obtained for flower honey-added fennel tea at 55˚C, 75˚C, and 85˚C, and linden tea at 55˚C and 65˚C. The TAC values of the remaining 6 tea samples, analysed with CUPRAC method, were not found to be affected significantly with flower honey addition at different temperatures.

Pine honey-added sage, echinacea, fennel, and linden tea samples, analysed using CUPRAC method, were found to have significantly higher ($p<0.05$) TAC values at all temperatures of honey addition, compared to their control samples. Moreover, pine honey also provided significant increases in TAC of green tea at 85˚C, daisy tea at 55˚C, 65˚C, and 75˚C, and ginger tea at 75˚C. The TAC of rosehip tea did not change significantly with the inclusion of pine honey at 4 different infusion temperatures, whereas melissa tea was

measured to have reduced TAC values when pine honey was added at 85˚C.

The correlations between spectrophotometric assay results

The linear correlation coefficients (R^2) were calculated for plots of TP *versus* TF, TP *versus* DPPH, TP *versus* CUPRAC, TF *versus* DPPH, TF *versus* CUPRAC, and DPPH *versus* CUPRAC assay results. The lowest correlation was observed between TP and TF content values (R^2=0.30118), while a highly linear relationship was determined between the results of DPPH and CUPRAC methods (R^2=0.90073) (Figure 2A). Additionally, the linear curves obtained for CUPRAC *versus* TP (R^2=0.73632) (Figure 2B) and CUPRAC *versus* TF (R^2=0.54313) (Figure 2C) results had higher correlation coefficients than those observed for DPPH *versus* TP (R^2=0.70238) and DPPH *versus* TF (R^2=0.44324) results.

DISCUSSION

The effect of different types of honey

Flower honey and pine honey addition either led to significant increases ($p<0.05$) or did not significantly change the TP contents of the honey-added tea samples compared to their controls. Flower honey provided a significantly higher ($p<0.05$) TP content value in echinacea tea at 65˚C in comparison to the value obtained with pine honey addition at the same temperature. On the other hand, pine honey-added daisy and ginger teas were measured to be significantly higher in their TP contents, at all temperatures of honey addition, compared to their flower-honey added counterparts (Table 1). It could be linked to the fact that honeys with darker color, as in the case of pine honey, have been reported to possess higher amounts of total phenolic compounds in recent research studies [Alvarez-Suarez *et al.,* 2010; Escuredo *et al.,* 2013; Kus *et al.,* 2014 Wilczynska, 2010]. Kus *et al.* [2014] determined the TP contents of lighter honeys investigated in their study (black locust, goldenrod, rapeseed, and lime) to range in between 142.8–192.5 mg GAE/kg; while this range for darker honeys (heather and buckwheat) was found to be from 306.2 to 1113.0 mg GAE/kg. In another study, the highest TP contents were again measured for darker colored honeys, including chestnut honey (1313 mg GAE/kg) and heather honey (1789 mg GAE/kg) [Escuredo *et al.,* 2013]. The TP contents of the flower and pine honeys that we used in our work were 510 and 680 mg GAE/kg, respectively.

The reason for not obtaining the same effect of pine honey for all the analysed tea samples could be related with the different phenolic profiles of the herbal tea samples or the lack of the specificity of the Folin-Ciocalteau method for phenolic compounds [Capanoglu *et al.,* 2008]. The Folin-Ciocalteau method was reported to be suffering from a number of interfering substances, including specifically sugars, aromatic amines, ascorbic acid [Box, 1983], and amino acids and proteins [Meda *et al.,* 2005] that can also react with Folin-Ciocalteau reagent. Thus, it was strongly suggested that corrections for those interfering substances should be made in order to establish a uniformly acceptable method of TP to compare the obtained results rationally [Prior *et al.,* 2005].

The addition of pine and flower honey, at 4 infusion temperatures, was determined to affect the TF contents of different tea samples in different ways, including the effects of all significant increases/decreases or not any significant changes ($p<0.05$). It was remarkable that flower honey addition led to significant decreases ($p<0.05$) in TF contents of five (out of nine) tea samples, including green tea, rosehip, echinacea, fennel, and ginger, at all temperatures of honey addition. On the other hand, pine honey addition did not significantly change or even increased the TF contents of tea samples (except for green tea at 55˚C and 65˚C, and rosehip tea at 85˚C) (Table 1). Silici *et al.* [2013] reported catechin and epicatechin as the only compounds that were determined as the kind of flavonoids in honeydew honey samples, which were also determined to constitute the largest content (53% of detected total phenolics) of total phenolics in the analysed honeydew honey samples. On the other hand, the contribution of catechin and epicatechin components to the TP content of nectar honeys was found to be 33% of the detected phenolics [Silici *et al.,* 2013]. This could have an influence on these higher TF contents of pine honey-added-tea samples in our study, since the results for TF content analysis have been expressed as catechin equivalents (Table 1).

The results obtained by DPPH method, for the changes in TAC of different tea samples added-with-flower honey revealed significant increases ($p<0.05$) in TAC of green tea (at 85˚C), sage tea (at 85˚C), linden tea (at 55˚C and 65˚C), and ginger tea (at 75˚C). On the other hand, again with the same method pine honey was observed to lead to significant increases ($p<0.05$) in TAC of sage tea (at 65˚C), fennel tea (at 55˚C, 75˚C, and 85˚C), linden tea (at 65˚C and 75˚C) and daisy tea (at all temperatures). When the flower honey and pine honey were compared for their influences on TAC, at the same temperature of honey addition, pine honey was found to differ from flower honey with its significantly higher ($p<0.05$) contribution to the TAC of echinacea tea (at 65˚C), fennel tea (at 85˚C), and daisy tea (at all 4 temperatures). For the other tea samples, pine honey and flower honey did not significantly differ ($p>0.05$) (Table 2).

The TAC values measured with CUPRAC method indicated significantly increased ($p<0.05$) TAC by the effect of pine honey addition in five (out of nine) tea samples, including sage, echinacea, fennel, linden, and daisy, independent from the infusion temperatures tested (except for the daisy tea at 85˚C). Flower honey was found to contribute significantly to the TAC of sage tea (at all temperatures), fennel tea (at 55˚C, 75˚C, and 85˚C), and linden tea (at 55˚C and 65˚C). In addition, the comparison of the flower honey-added and pine honey-added tea samples, at the same temperature of honey addition, revealed no significant differences regarding their TAC measured by CUPRAC method (except for melissa tea at 85˚C and linden tea at 75˚C). On the other hand, pine honey had a greater contribution to the TAC values of tea samples in comparison to their respective control tea samples (Table 2). These relatively higher TAC values provided by pine honey could be explained based on the findings of other studies, which have pointed out that honey samples that are darker in their color have higher antioxidant capacities in general [Alvarez-Suarez *et al.,* 2010; Kus *et al.,* 2014; Wilczynska, 2010] since honey color depends on the potential alkalinity and ash content, as well as on the antioxidatively

active pigments, such as carotenoids and flavonoids [Frankel *et al.,* 1998]. Alvarez-Suarez *et al.* [2010] reported the TAC values of honeys tested in their study to range in between 1035 and 2945 μmol TE/kg which was linearly correlated with the color range of the honeys changing from light to amber. Accordingly, the TAC of the darker-colored pine honey (4075 mg TE/kg), used in this work, was higher in comparison to the TAC of the lighter flower honey (3545 mg TE/kg).

The effect of different infusion temperatures of honey addition

The results obtained for TP contents of honey-added-tea samples pointed out that the highest values, although not all were statistically different from control samples, were generally obtained with the addition of flower/pine honey at infusion temperatures of 75˚C and 85˚C (except for echinacea tea for flower honey addition, and melissa and rosehip teas for pine honey addition). The TF contents of flower honey-added-tea samples were again mainly higher at 75˚C and 85˚C of honey addition temperatures compared to the other infusion temperatures of honey addition. However, it should be emphasized that these relatively higher values obtained at 75˚C/85˚C, in comparison to the other infusion temperatures, of flower honey addition were mostly significantly lower or were not significantly different from the respective control tea samples (Table 1). On the other hand, when pine honey was added into tea samples at 65˚C and 75˚C of infusion temperatures, it provided relatively higher TF contents in comparison to the other infusion temperatures of honey addition. Whereas some of these TF content values, obtained for 65˚C/75˚C of pine honey addition temperatures, were still lower than the values obtained for respective control tea samples (including green tea, rosehip, and ginger tea samples) (Table 1).

The TAC of flower/pine honey added-tea samples, determined using DPPH method, were again found to be higher at 75˚C and 85˚C of honey addition temperatures compared to the other infusion temperatures of honey addition. On the other hand, the measurement of TAC values with CUPRAC method gave higher values at 65˚C and 75˚C of infusion temperatures for flower honey addition, and at 55˚C and 65˚C of infusion temperatures for pine honey addition (Table 2).

When all the results were evaluated in general, it could be concluded that the addition of flower/pine honey into different tea samples at 75˚C and (to a lesser extent) at 85˚C gave relatively high values of TP and TF contents, as well as TAC, in comparison to the other tested temperatures. The percent changes in TP content (Figure 1A) and TAC values, determined using CUPRAC method (Figure 1B), obtained with flower/pine honey addition at 75˚C are given in Figure 1 as the representative graphs. The flower and pine honey additions into tea samples at 75˚C were determined to lead up to 41% and 57% increases in TP contents (Figure 1A), and up to 50% and 57% increases in TAC values, determined using CUPRAC method (Figure 1B), respectively. These higher values at higher temperatures may depend on the formation of Maillard reaction products, melanoidins, which have been reported to act as antioxidants [Brudzynski & Miotto, 2011a,b,c; Turkmen *et al.,* 2006]. Turkmen *et al.* [2006], who studied the effect of heating honey to 50˚C, 60˚C, and 70˚C on the antioxidant activity and brown pigment formation due to Maillard reaction, determined that both of the measured values increased with the increased temperature. The authors evaluated that the increase in brown pigment formation, due to the formation of Maillard reaction products, was accompanied with the increase in antioxidant activity, which was more remarkable in heated honey samples at 70˚C than those at 50˚C and 60˚C [Turkmen *et al.,* 2006]. In addition, these Maillard reaction products were also reported to react with Folin-Ciocalteau reagent [Verzelloni *et al.,* 2007] which could explain the higher TP content values in honey added tea samples, specifically at higher temperatures of honey addition. In another study, Brudzynski & Miotto [2011a] hypothesized that phenolics in honey may be components of melanoidin structure, and they tested the melanoidin fractions of unheated and heat-treated honey samples for their total phe-

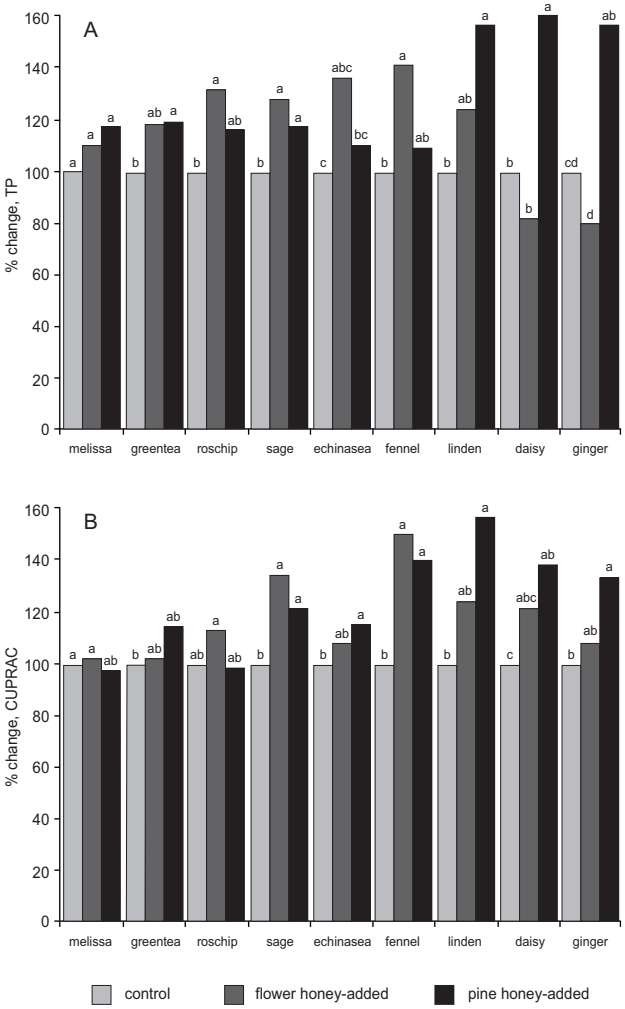

FIGURE 1. The percent changes in (A) Total phenolic (TP) contents and (B) Total antioxidant capacity (TAC) values, determined using Cupric Reducing Antioxidant Capacity (CUPRAC) assay, of the analysed tea samples with flower and pine honey addition at 75˚C. Different letters on the columns represent the statistically significant ($p<0.05$) differences observed with flower or pine honey addition into a herbal infusion at 75˚C. (See Table 1 (for TP content data) and Table 2 (for TAC data obtained *via* CUPRAC method) for the complete statistical data).

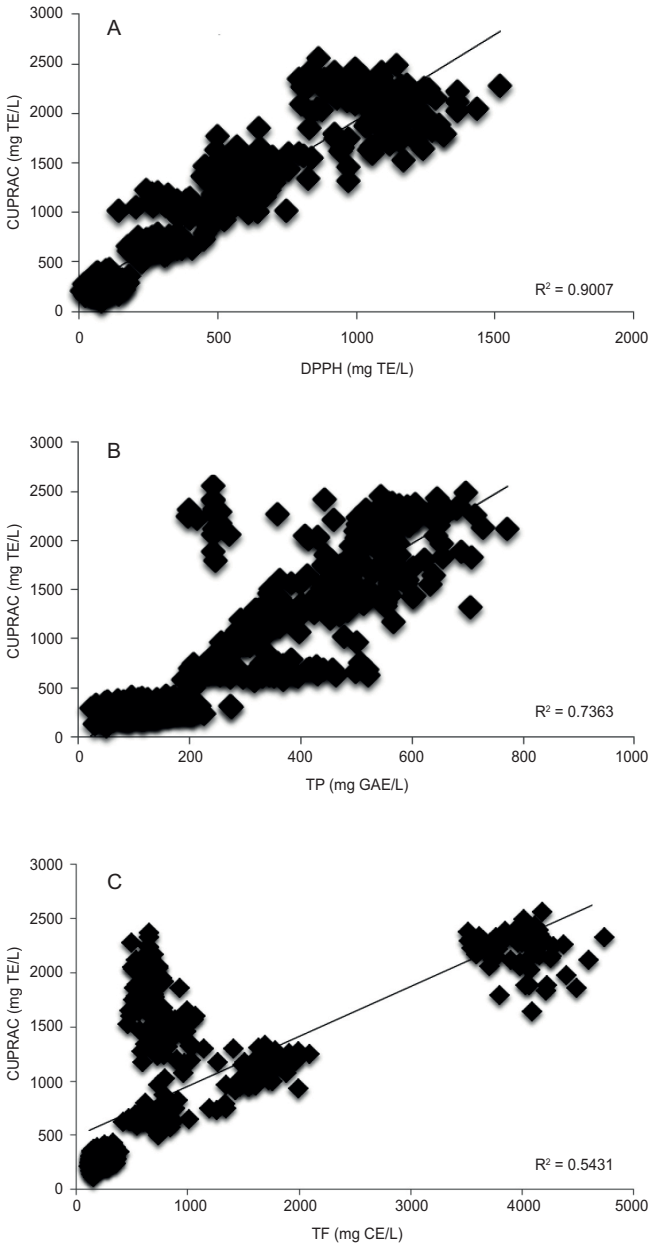

FIGURE 2. The linear correlation coefficients (R^2) calculated for plots of (A) 1,1-diphenyl-2-picrylhydrazyl (DPPH) *versus* Cupric Reducing Antioxidant Capacity (CUPRAC), (B) Total phenolics (TP) *versus* CUPRAC, and (C) Total flavonoids (TF) *versus* CUPRAC assay results.

nolic contents. Their results indicated a significant increase in the TP content in melanoidin fractions of the heat-treated honeys as compared to the TP content in melanoidin fractions of their unheated counterparts. This could also explain the reaction between Maillard reaction products and the Folin-Ciocalteau reagent.

Because of the fact that heating of honey leads to the formation of HMF (5-hydroxymethylfurfural), as a result of the hexose dehydration in acid media [Belitz & Grosch, 1999], we also checked the HMF contents of the honey samples that were subjected to high temperatures in our study, and confirmed that the HMF contents were all below the limit value (40 mg/kg). HMF is considered as an important quality parameter for honey by means of evaluating the freshness and the heating and storage history [Karabournioti & Zervalaki, 2001; Fallico *et al.,* 2004]. HMF formation in honey could be influenced by the chemical properties of honey, including pH, total acidity, mineral content, *etc*., which are dependent on the floral source from which the honey sample has been extracted [Anam & Dart, 1995; Bath & Singh, 1999]. So, the inclusion of honey samples obtained from different floral sources can provide different levels of HMF contents.

The relationships between the results of the applied spectrophotometric assays

The correlation coefficients (R^2) calculated between the applied spectrophotometric methods showed that the results of the CUPRAC assay correlated better with the TP and TF contents of different herbal tea samples, compared to the DPPH assay results. Besides, good correlations were also observed between the results of DPPH and CUPRAC assays (Figure 2). In accordance with our results, CUPRAC method was proved to correlate well with ABTS and Folin-Ciocalteau assays in herbal plant infusions [Apak *et al.,* 2006], apricot [Guclu *et al.,* 2006], and kiwifruit [Park *et al.,* 2006] extracts. Apak *et al.* [2006] reported the CUPRAC assay as the most consistent method of total antioxidant measurement in relation to Folin reagent-responsive TP content, since this method is suitable for and reacts with a variety of antioxidant compounds regardless of chemical type or hydrophilicity. Additionally, the linear correlation determined between CUPRAC and ABTS assays (R^2=0.8) has been linked to the fact that these methods are similar electron transfer-based antioxidant assays [Apak *et al.,* 2007], which can also be evaluated for the high correlation found out between DPPH and CUPRAC assays (R^2=0.90073) in this present work. However, it is worth to remark that although there are a number of methods that have been developed to assess the antioxidant capacity of either pure antioxidant compounds or products containing complex mixture of antioxidants, there is still lack of correlation between the results obtained for the same compound/product by different assays, as well as by the same assay in different laboratories [Niki, 2011].

On the other hand, lower correlation coefficients were obtained between TF assay results and the results of the other three assays. Similarly, Park *et al.* [2006] and Meda *et al.* [2005] reported low correlations between ABTS, CUPRAC or TP content results and TF contents, which was linked to the nature of the measurement technique used for total flavonoids. The aluminum chloride ($AlCl_3$) colorimetric test used for flavonoid analysis has been pointed out to be sensitive only for flavonoid groups that possess the characteristic chelating functional groups for Al binding (*i.e.* flavones and flavonols), while this method does not measure the flavonoids that do not include these functional groups (*i.e.* flavanones). This leads to the underestimation of the TF content by using this aluminum chloride method [Chang *et al.,* 2002].

CONCLUSION

The comparison on the effect of flower and pine honey addition into 9 different herbal tea samples at 4 different temperatures revealed that the TP content and TAC values

of the honey-added-tea samples were generally higher than those of the control tea samples, specifically with pine honey addition and at higher temperatures. These findings support the use of honey as a natural sweetener in tea drink in order to be able to benefit from the health-enhancing antioxidative properties of these two promising food products.

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