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SHORT COMMUNICATION

Potentiating effects of honey on antioxidant properties of lemon-flavoured black tea

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Abstract

Health benefits including antioxidant potential of black tea (*Camellia sinensis*), lemon (*Citrus limon*) and honey bees (*Apis mellifera*) have been extensively reported. Nevertheless, nothing is reported about the effects of their concomitant use. Herein, those effects were evaluated in infusions of lemon-flavoured black tea with three different kinds of honey (light amber, amber and dark amber) from *Lavandula stoechas*, *Erica* sp. pl. and other indigenous floral species from north-east Portugal, a region with high amounts of this food product. Data obtained showed that the use of honey (dark amber > amber > light amber) potentiates the antioxidant activity of lemon-flavoured black tea, increasing the reducing power and lipid peroxidation inhibition properties, as also the antioxidant contents such as phenolics, flavonoids and organic acids including ascorbic acid.

Keywords: black tea, lemon flavoured, honey, antioxidant activity, antioxidants

Introduction

Tea (*Camellia sinensis*), known as the most popular beverage in the East, arouses great interest among scientists due to its beneficial health effects. Tea flavonoids consumption has been linked to lower incidences of chronic diseases such as cardiovascular disease and cancer (Henning et al. 2005). The health benefits associated with tea consumption have been attributed in part to the antioxidant and free radical-scavenging activity of the most abundant tea flavonols, mainly catechins or flavan-3-ols (Henning et al. 2004; Friedman et al. 2005; Seeram et al. 2006; Milasienė et al. 2007; Rusak et al. 2008).

To produce black tea, after the leaves are rolled, which disrupts cellular compartmentation and brings phenolic compounds into contact with polyphenol oxidases, the young *C. sinensis* leaves undergo oxidation (e.g. fermentation) for 90–120 min. During this period, catechins are converted to complex condensation products, the theaflavins and their polymers, thearubigins (Henning et al. 2004; Milasienė et al. 2007; Rusak et al. 2008).

The health benefits of lemon (*Citrus limon*) (Joshipura et al. 2001) and radical-scavenging activity

of its peels and juices (Guimarães et al. 2010) have also been attributed to the presence of bioactive compounds, such as phenolic compounds and vitamin C, all powerful antioxidants. Due to its special flavour, it has been used to prepare flavoured teas widely related to traditional and complementary healthcare approaches, such as reducing skin cancer (Hakim and Harris 2001) or helping during cold and flu season (Robinson et al. 2009). The same approach has been carried out with honey, the nectar collected from many plants and processed by honey bees (*Apis mellifera*) (Robinson et al. 2009). Moreover, it plays an important role in human health by combating damage caused by oxidizing agents, namely reducing the risk of heart disease, cancer, immune system decline, cataracts, different inflammatory processes, etc. (The National Honey Board 2003). The antioxidant activity of honey has been extensively reported and attributed to the antioxidants present that include both enzymatic: catalase, glucose oxidase, peroxidase and non-enzymatic substances: ascorbic acid, α -tocopherol, carotenoids, amino acids, proteins, organic acids, Maillard reaction products and more than 150

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polyphenolic compounds, including flavonoids, flavonols, phenolic acids, catechins and cinnamic acid derivatives (Ferrerres et al. 1994; Gheldof et al. 2002; Baltrušaityte et al. 2007; Bertoneclj et al. 2007; Buratti et al. 2007; Ferreira et al. 2009).

Although it has already been demonstrated that black tea, lemon and honey have antioxidant activity, nothing is reported about the effects of their concomitant use. In this study, those effects were evaluated in infusions of lemon-flavoured black tea with three different kinds of honey (light amber, amber and dark amber) from *Lavandula stoechas*, *Erica* sp. pl. and other indigenous floral species from north-east Portugal, a region with high amounts of this food product.

Materials and methods

Standards and reagents

The standard trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), gallic acid, catechin and organic acid standards were purchased from Sigma (St Louis, MO, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). All other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

Samples and samples preparation

Lemon (*Citrus limon*)-flavoured black tea (*C. sinensis*) bags were obtained in a local supermarket.

Three different coloured honeys, light amber, amber and dark amber, were obtained in north-east Portugal (Parque Natural de Montesinho) from experienced producers. This is a region of honey production with large areas of spontaneous vegetation. *L. stoechas* and *Erica* sp. pl are the two main nectar sources for honeybees, allowing the production of unifloral honeys, to the extent of natural limitations, with light amber and dark amber, respectively. Medium coloured honeys, amber colour, are also found in this region and usually named as forest honeys. Those are a mixture of several floral sources which includes the nectar of *Castanea sativa* as well as honeydews from *Quercus* sp.

For the preparation of infusions, the bag (1 g) was poured in 100 ml of boiling water and stood for 5 min; afterwards honey spoon of one (5 g) was dissolved in the infusion. The stock solution was concentrated at 50 mg of honey/ml of infusion or 10 mg of tea/ml of infusion. Four samples were used as controls: infusion without honey (stock solution concentrated at 10 mg of tea/ml of infusion); light amber, amber and dark amber honeys prepared in distilled water (stock solutions concentrated at 500 mg of honey/ml of

infusion). In the case of honey controls, the concentration had to be increased to achieve antioxidant potential.

Several dilutions were carried out and the obtained infusion or control solutions were submitted to further analyses.

Honey colour analysis

Honey samples were first heated to 50°C to assure the dissolution of any sugar crystals, and the colour was determined with the photometer C221 (Hanna Instruments, Woonsocket, RI, USA). The honeys were classified according to the Pfund scale obtained directly from the instrument reading.

Evaluation of antioxidant activity

The antioxidant activity of the samples was evaluated by DPPH radical-scavenging activity, reducing power (ELX800 Microplate Reader, Bio-Tek Instruments, Bedfordshire, UK), inhibition of β -carotene bleaching in the presence of linoleic acid radicals and inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS) assay in porcine brain homogenates (Specord 200 spectrophotometer, Analytikjena, Jena, Germany) (Queiróz et al. 2009; Barros et al. 2010). The sample concentrations providing 50% of antioxidant activity or 0.5 of absorbance (EC_{50}) were calculated from the graphs of antioxidant activity percentages (DPPH, β -carotene bleaching and TBARS assays) or absorbance at 690 nm (reducing power assay) against sample concentrations. Trolox was used as standard.

Evaluation of antioxidants

Phenolics. The solution (0.5 ml) was added to a Folin–Ciocalteu solution (1:10 v/v; 2.5 ml) and sodium carbonate (75 g/l, 2 ml). The tubes were vortexed and incubated at 40°C for 30 min. Absorbance was then measured at 765 nm. Gallic acid was used to calculate the standard curve (0.05 – 0.8 mM; $y = 1.529x + 0.0104$; $R^2 = 0.9996$), and the results were expressed as mg of gallic acid equivalents per ml of infusion.

Flavonoids. The solution (0.5 ml) was mixed with distilled water (2 ml) and $NaNO_2$ solution (5%, 0.15 ml). After 6 min, $AlCl_3$ solution (10%, 0.15 ml) was added and allowed to stand further 6 min. $NaOH$ solution (4%, 2 ml) was added to the mixture, followed by distilled water until a final volume of 5 ml. The mixture was properly mixed and allowed to stand for 15 min. The intensity of pink colour was measured at 510 nm. Catechin was used to calculate the standard curve (0.03 – 1 mM; $y = 0.9148x - 0.0036$; $R^2 = 0.9995$) and the results were expressed as mg of catechin equivalents (CE) per ml of infusion.

Organic acids. Analysis was carried out by ultra-fast liquid chromatograph (UFLC) coupled to photodiode

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Table I. Antioxidant activity (EC₅₀ values) and antioxidants of the infusions of lemon-flavoured black tea with three different kinds of honey (mean ± SD).

225	Samples	Antioxidant activity (EC ₅₀ , mg/ml)			Antioxidants			280
		DPPH scavenging activity	Reducing power	β-Carotene bleaching inhibition	TBARS inhibition	Phenolics (mg GAE/ml)	Flavonoids (mg CE/ml)	
	Control light amber honey*	533.15 ± 10.03 ^a	45.05 ± 0.06 ^a	36.17 ± 2.47 ^a	58.90 ± 0.02 ^a	0.98 ± 0.05 ^f	0.05 ± 0.00 ^c	
	Control amber honey*	224.73 ± 11.47 ^b	24.90 ± 0.25 ^b	32.26 ± 1.03 ^b	20.03 ± 0.79 ^b	1.59 ± 0.03 ^c	0.30 ± 0.02 ^c	
	Control dark amber honey*	41.64 ± 2.18 ^c	10.78 ± 0.07 ^c	17.42 ± 2.14 ^c	2.76 ± 0.17 ^c	2.56 ± 0.04 ^d	0.18 ± 0.01 ^d	
230	Infusion with light amber honey*	4.56 ± 0.21 ^d	1.87 ± 0.01 ^d	3.40 ± 0.11 ^d	0.26 ± 0.01 ^d	16.91 ± 0.16 ^c	2.13 ± 0.03 ^b	285
	Infusion with amber honey*	3.59 ± 0.25 ^d	1.83 ± 0.02 ^{ed}	1.34 ± 0.10 ^c	0.05 ± 0.00 ^d	17.78 ± 0.49 ^b	2.34 ± 0.02 ^a	
	Infusion with dark amber honey*	3.59 ± 0.42 ^d	1.70 ± 0.02 ^e	1.12 ± 0.04 ^e	0.01 ± 0.00 ^d	19.65 ± 0.22 ^a	2.42 ± 0.13 ^a	
	Control infusion [†]	0.25 ± 0.01 ^c	0.20 ± 0.00 ^a	0.17 ± 0.02 ^b	0.06 ± 0.00 ^b	141.79 ± 1.67 ^d	21.76 ± 0.54 ^b	
	Infusion with light amber honey [†]	0.46 ± 0.02 ^a	0.19 ± 0.00 ^b	0.34 ± 0.01 ^a	0.10 ± 0.00 ^a	169.15 ± 1.63 ^c	21.26 ± 0.29 ^b	
	Infusion with amber honey [†]	0.35 ± 0.03 ^b	0.18 ± 0.00 ^c	0.13 ± 0.01 ^c	0.01 ± 0.00 ^c	177.76 ± 4.94 ^b	23.38 ± 0.22 ^a	
235	Infusion with dark amber honey [†]	0.36 ± 0.04 ^b	0.17 ± 0.00 ^d	0.11 ± 0.01 ^d	0.01 ± 0.00 ^c	196.45 ± 2.17 ^a	24.16 ± 1.30 ^a	290

Q9 Note: nd, not detected; * Concentrations expressed in mg of honey by ml of infusion. In each column, different letters (a, b, c, d, e, f) mean significant differences (*p* < 0.05); [†] Concentrations expressed in mg of lemon-flavoured black tea powder by ml of infusion. In each column, different letters (a, b, c, d) mean significant differences (*p* < 0.05).

240 array detector (PDA), using a Shimadzu 20A series
Q4 UFLC (Shimadzu Corporation), following extraction
procedure and analysis conditions described by Barros
et al. (2012). Detection was carried out in a PDA,
using 215 and 245 nm as preferred wavelengths. The
245 organic acids were quantified by comparison of the
area of their peaks recorded at 215 nm with calibration
curves obtained from commercial standards of each
compound. The results were expressed as µg per ml
of infusion.

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Statistical analysis

The results are expressed as mean values ± standard
deviation (SD) or standard error (SE). The results
255 were analysed using one-way analysis of variance
(ANOVA) followed by Tukey's HSD test with
α = 0.05. This treatment was carried out using the
SPSS Version 16.0 software.

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Results and discussion

Table I shows the data regarding the antioxidant
properties of lemon-flavoured black tea infusions with
three different kinds of honey (light amber, amber and
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dark amber) evaluated by four different assays (DPPH
295 radical-scavenging capacity, reducing power and
inhibition of lipid peroxidation using β-carotene-
linoleate model system in liposomes and TBARS assay
in brain homogenates). In order to identify the
individual effects of honey or tea, the results were
300 compared with control samples and expressed
accordingly: mg of honey per ml of infusion to
compare with controls of light amber, amber or dark
amber honey, or mg of lemon-flavoured black tea per
ml of infusion to compare with the infusion control
305 (without honey).

The order observed for the honey antioxidant
properties was: dark amber > amber > light amber
(infusions or controls), which is in agreement with
previous results reported by our research group in
310 other honey samples (Ferreira et al. 2009). Moreover,
the order of antioxidants (phenolics, flavonoids and
organic acids including ascorbic acid) content in the
samples followed the same tendency (Tables I and II).
Black tea has a significant higher antioxidant potential
than light amber, amber or dark amber honeys
315 controls, because the EC₅₀ values of the infusion
with honey (≤4.56 mg honey/ml) were much lower
(higher antioxidant activity) than control honey
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Table II. Organic acids of the infusions of lemon-flavoured black tea with three different kinds of honey (mean ± SD).

		Oxalic acid	Quinic acid	Malic acid	Shikimic acid	Ascorbic acid	Citric acid	Fumaric acid	
270	Control light amber honey (µg/ml)	14.36 ± 0.31 ^d	6.08 ± 0.27 ^c	nd	nd	nd	nd	nd	325
	Control amber honey (µg/ml)	13.89 ± 0.63 ^d	0.90 ± 0.08 ^c	0.37 ± 0.02 ^c	0.18 ± 0.02 ^c	0.30 ± 0.00 ^c	25.22 ± 0.11 ^b	0.02 ± 0.00 ^d	
	Control dark amber honey (µg/ml)	17.89 ± 0.89 ^d	1.90 ± 0.18 ^c	3.58 ± 0.71 ^c	1.47 ± 0.04 ^c	0.36 ± 0.00 ^c	29.37 ± 0.18 ^b	0.13 ± 0.02 ^c	
	Control infusion (µg/ml)	59.31 ± 1.85 ^c	46.03 ± 2.80 ^d	29.36 ± 2.99 ^d	3.24 ± 0.07 ^d	2.15 ± 0.37 ^d	30.58 ± 0.13 ^b	0.10 ± 0.01 ^c	
	Infusion with light amber honey (µg/ml)	103.45 ± 11.16 ^b	97.76 ± 5.75 ^c	51.98 ± 6.79 ^c	4.58 ± 0.38 ^c	4.92 ± 0.02 ^c	30.80 ± 2.21 ^b	0.12 ± 0.01 ^c	
275	Infusion with amber honey (µg/ml)	111.16 ± 1.82 ^b	131.41 ± 0.11 ^b	63.06 ± 1.88 ^b	5.24 ± 0.27 ^b	8.92 ± 0.15 ^b	85.69 ± 9.54 ^a	0.49 ± 0.00 ^b	330
	Infusion with dark amber honey (µg/ml)	146.91 ± 2.54 ^a	183.28 ± 19.09 ^a	74.43 ± 0.09 ^a	10.80 ± 0.30 ^a	10.79 ± 0.07 ^a	95.84 ± 12.54 ^a	0.76 ± 0.01 ^a	

Notes: nd, not detected; in each column different letters (a, b, c, d) mean significant differences (*p* < 0.05).

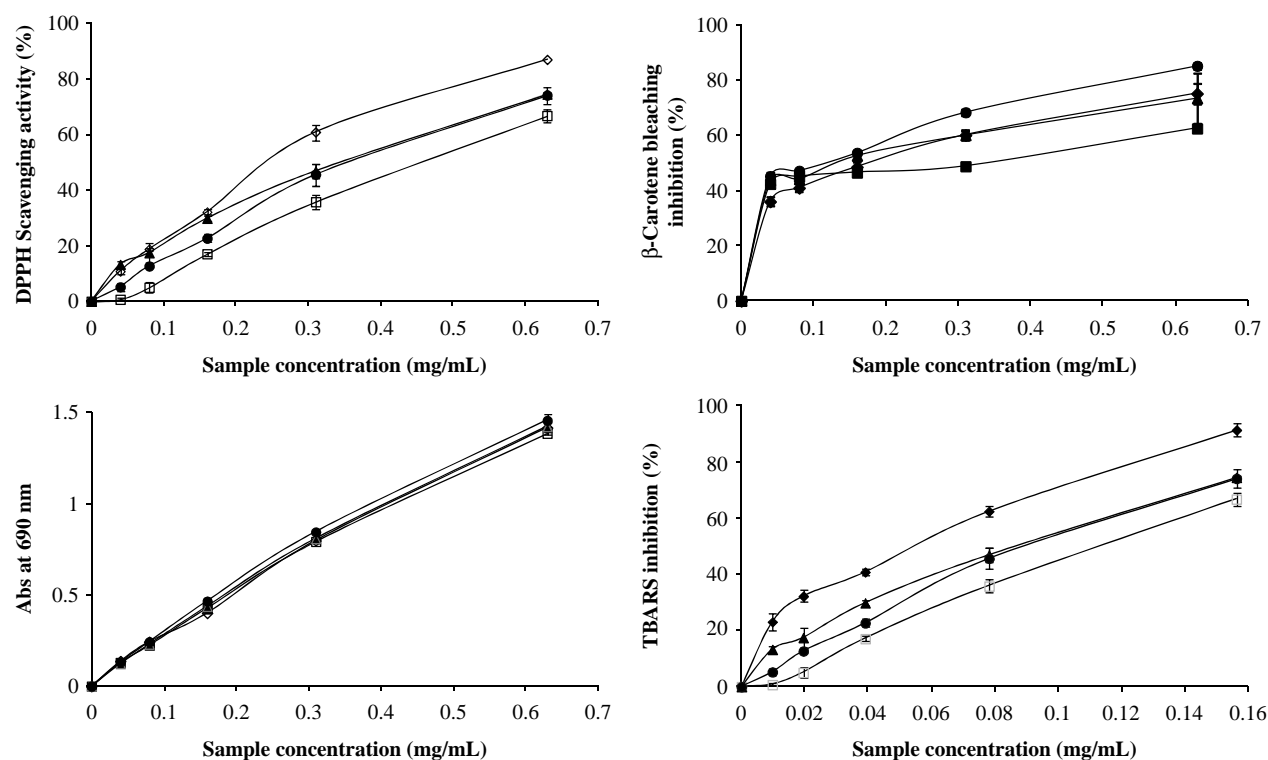


Figure 1. Antioxidant activity (DPPH scavenging activity, reducing power, β -carotene bleaching inhibition and TBARS inhibition) of the infusions of lemon-flavoured black tea with three different kinds of honey. The concentrations are expressed in mg of tea/ml of infusion: control infusion (\circ); infusion with light amber honey (\square); infusion with amber honey (\blacktriangle); infusion with dark amber honey (\bullet). Each value is expressed as mean \pm SE.

samples (≤ 533 mg honey/ml). The same was observed for antioxidants content in which infusion with dark amber honey gave the highest phenolics (19.65 mg GAE/ml), flavonoids (2.42 mg CE/ml), quinic acid (183.28 μ g/ml), oxalic acid (146.91 μ g/ml), citric acid (95.84 μ g/ml), malic acid (74.43 μ g/ml), shikimic acid (10.80 μ g/ml), ascorbic acid (10.79 μ g/ml) and fumaric acid (0.76 μ g/ml) levels.

The presence of honey (light amber, amber or dark amber) slightly increased the reducing power of lemon-flavoured black tea (Table I; Figure 1), decreasing EC_{50} values from 0.20 mg tea/ml to 0.19, 0.18 and 0.17 mg tea/ml, respectively. Furthermore, amber and dark amber honeys increased the lipid peroxidation inhibition of lemon-flavoured black tea (measured by either β -carotene-linoleate or TBARS assays), as it can be observed by the decrease in EC_{50} values (0.17–0.13 or 0.11 mg tea/ml; 0.06–0.01 mg tea/ml; Table I) or the increase in antioxidant activity percentages (Figure 1). The mentioned potentiating honey effects were not observed in the DPPH radical-scavenging activity. As expectable, the presence of light amber, amber or dark amber honey increased phenolics, flavonoids and organic acids (including ascorbic acid) content relatively to control infusion, and the highest levels were found in the infusion with dark amber honey (196.45 mg/ml, 24.16 mg/ml and 10.79 μ g/ml, respectively).

The phenolic profile of honey samples from north-east Portugal, obtained by HPLC-DAD analysis, was previously reported by other authors (Estevinho et al. 2008). These authors described that *p*-hydroxybenzoic acid, cinnamic acid, naringenin, pinocembrin and chrysin were the phenolic compounds present in most of the samples, and that phenolic compounds extract obtained from the dark honey sample had stronger antioxidant than the clear (light amber) honey sample. Phenolic compounds might be responsible for the observed antioxidant potential as they can act as free radical scavengers and redox-active metal chelators (Galleano et al. 2010). Organic acids such as ascorbic, citric and oxalic acids could also have an important contribution since they have been also described as potent antioxidants (Barros et al. 2012).

Conclusion

The use of honey (in the order dark amber > amber > light amber) potentiates the antioxidant activity of lemon-flavoured black tea, which is already a mixture of two powerful antioxidant matrixes (lemon and tea), increasing the reducing power and lipid peroxidation inhibition properties, as also the antioxidants content such as phenolics, flavonoids and ascorbic acid. Moreover, the mixture of honey and black tea

is much more favourable when antioxidants are considered compared to the use of honey alone.

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