

## Antioxidant and Acetylcholinesterase Inhibiting Activity of Several Aqueous Tea Infusions *in vitro*

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Received: November 29, 2007

Accepted: March 4, 2008

### Summary

A study of antioxidant activity and acetylcholinesterase (AChE) inhibitory activity of aqueous tea infusions prepared from walnut (*Juglans regia* L.), peppermint (*Mentha piperita* L.), strawberry (*Fragaria × ananassa* L.), lemon balm (*Melissa officinalis* L.), sage (*Salvia officinalis* L.), and immortelle (*Helichrysum arenarium* (L.) Moench.) is presented here. Chemical composition of selected aqueous tea infusions was determined by high-performance liquid chromatography with photodiode-array method (HPLC-PDA), and the following phenolic compounds were identified as dominant: rosmarinic acid, gallic acid (not identified in walnut and sage), caffeic acid (in sage and peppermint), neochlorogenic acid, 3-*p*-coumaroylquinic acid and quercetin 3-galactoside (in walnut) and luteolin 7-*O*-glucoside (in sage). Antioxidant activity of the selected aqueous tea infusions was measured using low-density lipoprotein (LDL) oxidation method, 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging test,  $\beta$ -carotene bleaching method, and Rancimat method (induction period of lard oxidation). Strawberry and lemon balm aqueous infusions completely inhibited LDL oxidation at the concentration of 0.005 g/L in the reacting system. Very long prolongation of the lag phase was achieved with peppermint and sage aqueous infusions. All tested infusions in the concentration range of 0.05–2.85 g/L showed very pronounced effect of DPPH scavenging activity (90–100 %) as well as the inhibition of  $\beta$ -carotene bleaching (89–100 %). In pure lipid medium, used in Rancimat method, sage and immortelle at the concentration of 0.16 % (by mass) had the highest ability to inhibit lipid peroxidation process. Screening of the AChE inhibitory activity by Ellman's method showed that the strongest inhibition was obtained with walnut and strawberry aqueous infusions at the concentration of 1.36 g/L in the reacting system. The presented results suggest that natural antioxidants could be useful and merit further investigations in the treatment and prevention of Alzheimer's disease.

**Key words:** antioxidant activity, acetylcholinesterase inhibiting activity, aqueous tea infusion, phenolic compounds

## Introduction

Alzheimer's disease (AD) is the most common type of dementia in modern societies (affects more than 20 million people worldwide). It is characterized by the loss of cholinergic innervation, reduction of choline acetyltransferase (ChAT) and enhanced acetylcholinesterase (AChE) activity (1). AChE terminates the interaction between neurotransmitter acetylcholine (ACh) and the corresponding receptor protein (acetylcholine receptor, nAChR), which is the basis of the intercellular communication in brain.

There is numerous evidence which indicates that oxidative stress may contribute to the pathogenesis of AD. Pharmacological data as well as analytical data from human tissue and body fluids have implicated oxidation products of fatty acids in the pathogenesis of AD (2). Antioxidants can act by scavenging the reactive products of lipid peroxidation and may be useful in prevention and treatment of AD. However, clinical studies carried out so far do not provide the final answer whether antioxidants are truly protective against AD or not (3).

The modulation of AChE is presently the most accepted and recognized therapeutic marker for development of cognitive enhancers (4). Clinical drug trials in patients with AD have been focusing on drugs that augment levels of AChE in brain to compensate for the loss of cholinergic function (tacrine, donepezil, rivastigmine, galanthamine). Currently used cholinesterase inhibitors, like tacrine, produce side effects such as hepatotoxicity (5). In that sense, the use of natural bioactive compounds, like antioxidants, found their possible application in the prevention and treatment of AD. Recently discovered alkaloid galanthamine, obtained from the bulbs and flowers of Caucasian snowdrop (*Galanthus woronowii*), is competitive and reversible cholinesterase inhibitor. It is believed that it enhances cholinergic function by increasing the concentration of acetylcholine in brain. In the recent years, it has been recognized as an important therapeutic option used to slow down the process of neurological degeneration in Alzheimer's disease (6). Traykova *et al.* (7) reported on radical scavenging activity of galanthamine hydrobromide and proposed that the antioxidant properties observed *in vitro* may contribute to the therapeutic effect of galanthamine hydrobromide on patients with brain degeneration.

There are numerous reports about inhibitory effect of antioxidants, namely polyphenols, on AChE activity. Kim *et al.* (4) showed that tea polyphenols exhibit a dramatic inhibitory effect on AChE activity and might be useful in the treatment of AD. According to Chan *et al.* (8), polyphenolic compound in apples (quercetin) may help the fight against AD. Foy *et al.* (9) studied plasma chain-breaking antioxidants  $\alpha$ -carotene,  $\beta$ -carotene, vitamins A, C and E in 79 patients suffering from AD, observing significant reductions in individual antioxidants in all patients. Their study supports the hypothesis that excessive free radical activity occurs in AD, which is manifested as a decrease in plasma chain-breaking antioxidants, especially vitamins A, C and E. Cardoso *et al.* (10) reported about high inhibitory activity of turbinatone and desoxycodifoline (glucoalkaloids isolated from *Chimarrhis turbinata*) on AChE. *Salvia lavandulaefolia* (Spanish

sage) extracts and their constituents show anticholinesterase and also, antioxidant effects (5).

The aim of this study is to examine the relationship between chemical composition and *in vitro* antioxidant and acetylcholinesterase inhibitory activity of aqueous tea infusions prepared from walnut (*Juglans regia* L.), strawberry (*Fragaria×ananassa* L.), lemon balm (*Melissa officinalis* L.), immortelle (*Helichrysum arenarium* L.), sage (*Salvia officinalis* L.) and peppermint (*Mentha×piperita* L.). These plants have been selected for this study due to their strong antioxidant capacity and high level of total phenols detected after screening of 70 medicinal plant extracts (11).

## Materials and Methods

### Chemicals

DPPH (2,2'-diphenyl-1-picrylhydrazyl) and linoleic acid were from Fluka (Switzerland). Tween 40,  $\beta$ -carotene,  $\alpha$ -tocopherol, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), AChE (from electric eel), acetylcholine iodide (ATChI) and 5,5-dithiobis(2-nitrobenzoic) acid (DTNB) were purchased from Sigma-Aldrich (Switzerland).

### Plant material

Dried flowers and leaves of walnut (*Juglans regia* L., genus *Juglans*), peppermint (*Mentha×piperita* L., genus *Mentha*), strawberry (*Fragaria×ananassa* L., genus *Fragaria*), lemon balm (*Melissa officinalis* L., genus *Melissa*), sage (*Salvia officinalis* L., genus *Salvia*) and immortelle (*Helichrysum arenarium* (L.) Moench., genus *Helichrysum*) were used for the preparation of aqueous tea infusions. Samples of immortelle were collected on the island of Vis (Croatia) during summer 2006, while all other samples were purchased from the herbal drugstore.

For the preparation of aqueous tea infusions, 15 g of air-dried plant material were infused into 200 mL of hot distilled water for 30 min, filtered through Whatman no. 4 paper and then concentrated under vacuum to dryness at a temperature between 50 and 60 °C. The obtained residue was redissolved in distilled water to achieve the concentration of 60 g/L. The yield for selected infusions in g per dried mass (gdm) was the following: 2.10 for walnut; 1.68 for strawberry; 2.40 for peppermint; 1.92 for immortelle; 2.64 for lemon balm and 2.22 for sage.

### Determination of total phenolic content in selected aqueous tea infusions

Determination of total phenolic content in aqueous tea infusions prepared from walnut, peppermint, strawberry, lemon balm, sage and immortelle was carried out according to Amerine and Ough (12) and Singleton and Rossi Jr. (13), using Folin-Ciocalteu colorimetric method, calibrated against gallic acid as the reference standard, and the results were expressed as GAE (gallic acid equivalents). Experiment was repeated three times.

### HPLC-PDA analysis of aqueous tea infusions

The analytical HPLC system employed consisted of a Varian Pro Star System (Palo Alto, CA, USA) equipped with a Pro Star Solvent Delivery Module 230, Injector Rheodyne 7125, and Pro Star 330 UV/VIS-photodiode array detector. Chromatographic separations were performed on a Pinnacle II C-18 column (250×4.6 mm i.d., 5 μm) including Pinnacle C18 guard column (10×4 mm i.d., 5 μm) (Restek, Bellefonte, USA). Gradient elution was effected using a ternary nonlinear gradient of the solvent mixture of methanol/water/acetic acid=10:88:2, by volume (solvent A), methanol/water/acetic acid=90:8:2, by volume (solvent B) and methanol (solvent C). The composition of solvent B was increased from 15 to 30 % in 15 min, then increased to 40 % in 3 min, held for 12 min and finally increased to 100 % in 5 min. Furthermore, the composition of solvent C was increased to 15 % in 2 min, then to 30 % in 11 min and returned to initial conditions in the next 2 min. Operating conditions were as follows: flow rate 0.7 mL/min, column temperature 20 °C, injection volumes 20 μL of the standards and sample extracts. The measurements were performed on a UV/VIS-photodiode array detector with the highest detection sensitivity at 278 nm. Identification of separated compounds was carried out by comparing retention times and spectral data with those of authentic standards. Identified phenolic compounds were quantified using the external standard method and quantification was based on the peak area.

Calibration curves of the standards were made by diluting stock solutions of standards in 80 % aqueous methanol to yield (in mg/L): luteolin 5–50, eriocitrin and luteolin 7-O-glucoside 10–150, and rosmarinic acid 10–100. The concentrations of the following phenolic acids: gallic acid, vanillic acid, *trans*-4-coumaric acid, ferulic acid, *trans*-cinnamic acid and *o*-hydroxycinnamic acid; as well as vanillin and (+)-catechin in selected aqueous infusions were determined by a Varian UV/VIS-photodiode array 330 detector, a ternary gradient liquid Pro Star 230 pump, column heater model 500 and Star chromatography workstation version 6.0. The (+)-catechin and phenolic acids were separated on ChromSep Omnisphere C18 column (250 mL×4.6 mm; purchased from Varian) and maintained at 30 °C. Infusate samples were filtered through a 0.45-μm membrane and directly injected through a 20-μL fixed loop into the guard C18 column.

Samples were prepared and analysed in triplicate. Data are presented as a mean value±standard deviation.

### Low-density lipoprotein isolation and oxidation

Low-density lipoprotein (LDL) was isolated from 3 normolipidemic donors by density gradient ultracentrifugation as described by Jürgens *et al.* (14) using the fixed angle rotor Ti70 on a Beckman preparative ultracentrifuge. To avoid the LDL oxidation during its isolation, EDTA (1 g/L) was present in all steps of the process and all the buffers were flushed with argon. The purity of LDL fraction was checked by electrophoresis using Radiophor electrophoresis system with Lipidophor agar medium. Protein concentration was measured by the method of Lowry *et al.* (15). LDL concentration refers to its protein content.

Prior to copper-induced oxidation, LDL was dialysed exhaustively overnight against 200-fold volume of 0.01 M PBS (phosphate buffer saline, 0.9 % NaCl, pH=7.4), without EDTA in the argon atmosphere. The prepared samples contained LDL in the absence or presence of 0.01 and 0.005 g/L of aqueous tea infusions. Copper-induced oxidation of these LDL samples (0.1 μM) was triggered at 37 °C by 2.5 μM CuSO<sub>4</sub> under aerated conditions. The effect of the selected aqueous tea infusions on LDL oxidation was followed using Varian Cary 50 UV/VIS spectrophotometer. The increase in the absorbance at 234 nm, indicative of the conjugated diene formation, was observed during the copper-induced oxidation. A kinetic study of the time course of LDL oxidation in terms of the lag, propagation and decomposition phases was performed according to an established protocol (15,16). In the LDL oxidation processes in control and tested samples, absorbance at 234 nm at each time point of oxidation was normalized with respect to the absorbance at the beginning of oxidation.

All experiments were done in triplicate and the results presented here refer to one chosen LDL preparation out of these three.

### Measurement of the DPPH (2,2'-diphenyl-1-picrylhydrazyl) radical scavenging activity

Procedure of the measurement of the DPPH (2,2'-diphenyl-1-picrylhydrazyl) radical scavenging activity in aqueous tea infusions prepared from walnut, lemon balm, immortelle, sage, peppermint and strawberry was described by Kulišić *et al.* (17). The percentage inhibition of the DPPH radical was calculated according to the formula (18):

$$\text{Inhibition} = ((A_{C(0)} - A_{A(t)}) / A_{C(0)}) \times 100 \quad /1/$$

where  $A_{C(0)}$  is the absorbance of the control at  $t=0$  min and  $A_{A(t)}$  is the absorbance of the antioxidant at  $t=1$  h. All measurements were done in triplicate.

### Determination of antioxidant activity using the β-carotene bleaching (BCB) method

Determination of antioxidant activity using the β-carotene bleaching method was described by Kulišić *et al.* (17). The percentage of the inhibition of β-carotene bleaching was calculated from the formula (19):

$$\text{Inhibition of } \beta\text{-carotene bleaching} = (A_{A(120)} / A_{C(0)}) \times 100 \quad /2/$$

where  $A_{A(120)}$  is the absorbance of the antioxidant at  $t=120$  min and  $A_{C(0)}$  is the absorbance of the control at  $t=0$  min. All measurements were done in triplicate.

### Induction period of lard oxidation (Rancimat assay)

The induction period of lard with and without the addition of selected aqueous tea infusions was described by Kulišić *et al.* (17). The antioxidant activity index (AI) is calculated from the measured induction times, according to the following formula (20):

$$\text{AI} = \frac{\text{Induction time of lard oxidation with antioxidant}}{\text{Induction time of lard oxidation without antioxidant}} \quad /3/$$

All measurements were done in triplicate.

### Acetylcholinesterase activity

Inhibition of AChE was assessed by slightly modified colorimetric method of Ellman *et al.* (21). A typical run consisted of 25  $\mu$ L of the enzyme suspension at a final concentration of 0.008 U/mL, 1 mL of 0.1 M phosphate buffer, pH=8.0, 35  $\mu$ L of DTNB at a final concentration of 0.3 mM prepared in 0.1 M phosphate buffer, pH=7, with 0.12 M sodium bicarbonate, and 25  $\mu$ L of the test solution in ethanol. The sample was preincubated for 10 min (without substrate but with buffer and DTNB) at room temperature. The reaction was initiated by adding 25  $\mu$ L of AChE to give a final concentration of 0.5 mM. The concentration of all samples was 1.36 g/L in the reacting system. Absorbance was measured on UV/VIS (double-beam) Perkin Elmer Lambda EZ 201 spectrophotometer at 412 nm. Each sample was assayed in triplicate.

### Statistical analysis

Data were statistically analyzed by one-way analysis of variance (ANOVA), and *p*-values lower than 0.05 were considered significant.

## Results and Discussion

### Polyphenolic content of selected aqueous tea infusions

Table 1 shows the content of total phenolics in the selected aqueous tea infusions determined by Folin-Ci-

Table 1. Concentration of total phenols in aqueous tea infusions ( $\gamma$ =60 g/L) from immortelle (*Helichrysum arenarium* (L.) Moench.), walnut (*Juglans regia* L.), strawberry (*Fragaria×ananassa* L.), lemon balm (*Melissa officinalis* L.), peppermint (*Mentha×piperita* L.) and sage (*Salvia officinalis* L.)

Herb	Total phenols/(mg per L of GAE)
Immortelle	4501±195.00
Strawberry	3217±27.72
Walnut	3541±82.89
Lemon balm	5776±81.78
Sage	3676±204.00
Peppermint	2512±122.00

Values represent the average of triplicates±standard deviation; GAE=gallic acid equivalent

calteu method. Among the tested samples, the highest amounts of total phenolics were detected in lemon balm and immortelle infusions. Strawberry, walnut and sage tea infusions had lower and very similar phenolic content, while the smallest amount of total phenolics was detected in peppermint infusion.

Table 2 shows phenolic compounds identified in the selected aqueous tea infusions by HPLC-PDA analysis. Among phenolic acids, the presence of rosmarinic acid was detected as dominant in aqueous infusions, particu-

Table 2. Phenolic compounds identified in aqueous tea infusions from walnut (*Juglans regia* L.), peppermint (*Mentha×piperita* L.), immortelle (*Helichrysum arenarium* (L.) Moench.), lemon balm (*Melissa officinalis* L.) and strawberry (*Fragaria×ananassa* L.) (expressed as mg/g of dry plant material)

	Strawberry	Immortelle	Walnut	Sage	Peppermint	Lemon balm
<i>trans</i> -Resveratrol	0.01±0.0056	0.06±0.0030	0.06±0.0030	0.10±0.0050	0.03±0.0010	n.d.
(+)-Catechin	1.71±0.0082	7.35±0.2570	2.45±0.1240	1.12±0.0039	2.76±0.1340	n.d.
Gallic acid	2.19±0.0081	0.36±0.0018	n.d.	n.d.	5.37±0.2150	3.82±0.1610
Vanillic acid	0.37±0.0019	n.d.	n.d.	0.26±0.0011	n.d.	n.d.
Vanillin	0.69±0.0028	0.49±0.2330	n.d.	0.09±0.0040	n.d.	0.89±0.0026
<i>trans</i> -4-Coumaric acid	0.21±0.0010	1.17±0.0051	0.27±0.0011	0.09±0.0041	0.57±0.0290	0.29±0.0130
Ferulic acid	0.09±0.0050	1.51±0.0060	0.31±0.0014	1.48±0.0073	1.30±0.0051	2.29±0.1070
<i>trans</i> -Cinnamic acid	0.02±0.0010	0.03±0.0010	0.14±0.0060	0.01±0.0006	0.03±0.0010	0.37±0.0180
<i>o</i> -Hydroxycinnamic acid	0.61±0.0031	1.86±0.0091	0.14±0.0070	1.05±0.0048	0.46±0.0020	0.14±0.0060
Neochlorogenic acid			14.26±1.1600	n.d.	n.d.	n.d.
3- <i>p</i> -Coumaroylquinic acid			4.07±1.3173	n.d.	n.d.	n.d.
Chlorogenic acid			1.75±0.5911	n.d.	n.d.	3.44±0.7923
Rosmarinic acid			n.d.	64.15±34.7461	13.28±1.0573	15.90±1.2577
Caffeic acid			n.d.	1.49±0.1558	1.02±0.1598	n.d.
Luteolin			n.d.	1.95±0.7203	n.d.	n.d.
Luteolin 7- <i>O</i> -glucoside			n.d.	37.46±3.1093	4.07±0.2394	5.51±0.7048
Eriocitrin			n.d.	n.d.	6.25±0.7710	n.d.
Quercetin 3-galactoside			27.08±2.5730	n.d.	n.d.	2.37±0.4429
Quercetin 3-arabinoside			2.77±0.4480	n.d.	n.d.	n.d.
Quercetin 3-rutinoside			n.d.	n.d.	n.d.	4.57±1.1092
Quercetin 3-rhamnoside			3.59±3.0070	n.d.	n.d.	n.d.

Values represent the average of triplicates

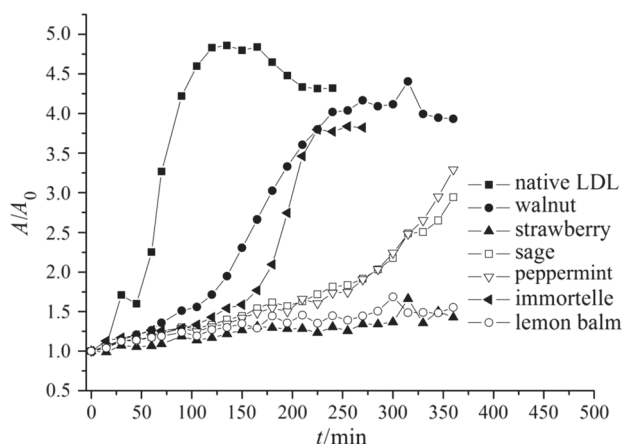
larly in sage aqueous infusion. Gallic acid was identified in strawberry, immortelle, peppermint and lemon balm infusions. Neochlorogenic and 3-*p*-coumaroylquinic acids were detected in walnut aqueous infusion. Chlorogenic acid was identified in lemon balm and walnut aqueous infusions. Ferulic acid was detected in small concentrations in all infusions. Other phenolic compounds detected in the selected infusions were: luteolin 7-*O*-glucoside, quercetin 3-galactoside, and eriocitrin.

Similar results are presented by other authors who found that water extracts from herbs of the Lamiaceae family (lemon balm, sage and peppermint) are very rich in bound forms of phenolic compounds such as hydroxycinnamic acids and flavonoids (22).

### Antioxidant activity of aqueous tea infusions

Determination of antioxidant activity of aqueous tea infusions from immortelle, strawberry, walnut, peppermint, sage and lemon balm was based on four antioxidative methods (LDL oxidation test, DPPH radical scavenging method,  $\beta$ -carotene bleaching method and Rancimat method). Each of these methods evaluates the antioxidant activity of the prepared infusions under the different conditions.

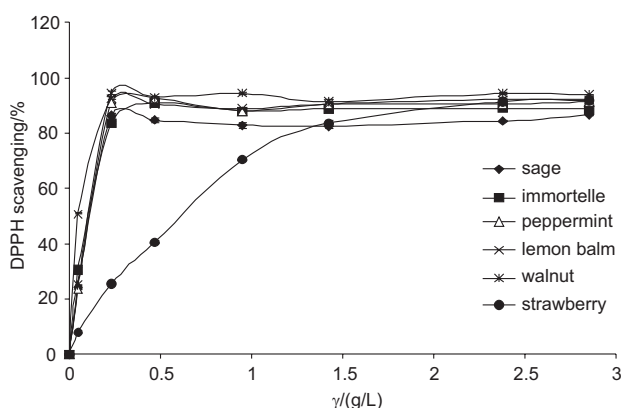
The oxidation of LDL was used as a model for investigating the activity of the polyphenols as chain-breaking antioxidants (23). Results presented in Fig. 1 show inhibition of copper-catalyzed oxidation of human LDL by selected aqueous tea infusions at the concentration of 0.005 g/L in the reacting system. All the tested infusions provoked the prolongation of the lag phase. The duration of the lag phase was (39 $\pm$ 1) min for the LDL sample in the absence of aqueous infusions. At the concentration of 0.005 g/L, strawberry and lemon balm aqueous infusions completely inhibited oxidation process of LDL. Very long prolongation of the lag phase was achieved with peppermint (243 $\pm$ 6) min and sage aqueous infusions (208 $\pm$ 10) min. Immortelle aqueous infusion prolonged the duration of the lag phase for (155 $\pm$ 2)



**Fig. 1.** Inhibition of LDL oxidation of aqueous tea infusions of strawberry (*Fragaria×ananassa* L.), lemon balm (*Melissa officinalis* L.), immortelle (*Helichrysum arenarium* (L.) Moench.), peppermint (*Mentha×piperita* L.), sage (*Salvia officinalis* L.) and walnut (*Juglans regia* L.) at the concentration of 0.005 g/L in the reacting system. Values represent mean value of three independent experiments

min, while walnut aqueous infusion prolonged the duration of the lag phase for (92 $\pm$ 3) min. At the concentration of 0.2 g/L in the reacting system, aqueous tea infusions inhibited the oxidation of LDL, *i.e.* it did not enter the propagation phase during the measured time (data not shown). Inhibition of *in vitro* LDL oxidation by phenolic compounds from some tested plants (walnut, strawberry and peppermint) has also been demonstrated in several studies (23–26).

DPPH radical scavenging method was used to evaluate free radical scavenging ability by the selected aqueous tea infusions. Fig. 2 shows very high rate of DPPH inhibition of all the tested samples (90–100 %), even at their low concentrations in the medium (<1 g/L). In the case of strawberry aqueous infusion, high rate of DPPH inhibition was achieved at higher concentrations of samples (>1 g/L). All samples show dose-dependent radical scavenging activity. In comparison with the tested aqueous infusions, commercial antioxidants (BHT, BHA and  $\alpha$ -tocopherol) reach the maximum of their radical scavenging effect at much lower range of concentrations. Aqueous tea infusions represent complex mixtures of different compounds and because of that their activity can hardly be compared with those of pure compounds.

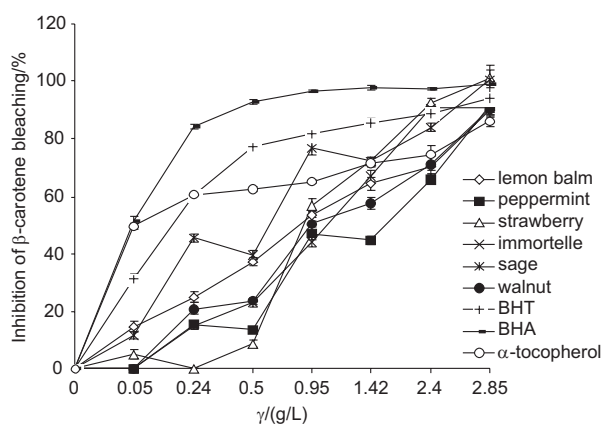


**Fig. 2.** Radical scavenging effect of aqueous tea infusions of strawberry (*Fragaria×ananassa* L.), lemon balm (*Melissa officinalis* L.), immortelle (*Helichrysum arenarium* (L.) Moench.), peppermint (*Mentha×piperita* L.), sage (*Salvia officinalis* L.) and walnut (*Juglans regia* L.). Values represent mean value of three independent experiments

Phenolic compounds from the tested aqueous infusions like rosmarinic acid, gallic acid, caffeic acid, eriocitrin, quercetin, and neochlorogenic acid are already known as strong scavengers of free radicals and thus can explain very high DPPH inhibition rate of the selected infusions in this study (27–30).

The  $\beta$ -carotene bleaching method (coupled oxidation of  $\beta$ -carotene and linoleic acid) estimates the relative ability of antioxidant compounds in the plant extracts to scavenge the radical of linoleic acid peroxide that oxidizes  $\beta$ -carotene in the emulsion phase. Behaviour of the antioxidant in emulsion is still unresolved (31). In several studies (32–34), polar compounds did not show any antioxidant effect when using this method (in case of vi-

tamin C). This is explained by polar paradox, based on the assumption that polar antioxidants, remaining in the aqueous phase of the emulsion, are more diluted in lipid phase and are thus less effective in protecting the linoleic acid. However, the inhibition of  $\beta$ -carotene bleaching by aqueous tea infusions from walnut, strawberry, sage, immortelle, mint and lemon balm was very high (80–100 %) (Fig. 3). This can be explained by structural features of compounds present in the selected aqueous infusions, which in some cases, according to Pratt and Birac (35), are probably more important than the polarity itself. Koleva *et al.* (33) noticed that a complex composition of the extracts could provoke certain interactions (synergistic, additive or antagonistic effects) between their components and/or the medium. Antioxidant activities of all the tested aqueous tea infusions were dose-dependent and, according to the results presented in Fig. 3, are comparable with commercial antioxidants (BHT, BHA and  $\alpha$ -tocopherol).



**Fig. 3.** Inhibition of  $\beta$ -carotene bleaching of aqueous tea infusions of strawberry (*Fragaria×ananassa* L.), lemon balm (*Melissa officinalis* L.), immortelle (*Helichrysum arenarium* (L.) Moench.), peppermint (*Mentha×piperita* L.), sage (*Salvia officinalis* L.) and walnut (*Juglans regia* L.) in comparison with commercial antioxidants (BHT, BHA and  $\alpha$ -tocopherol). Values represent mean value of three independent experiments

Different antioxidants added to lipids inhibit or retard their autoxidation. They function either by scavenging chain-carrying peroxy radicals or by diminishing the formation of initiating lipid radicals (36). Rancimat method is an accelerated oxidation test running at elevated temperatures with the sample exposed to air. This results in autoxidation within few hours. Table 3 shows induction times and antioxidant activity index of aqueous tea infusions from lemon balm, walnut, immortelle, sage, peppermint and strawberry at the concentration of 0.16 % (by mass) in the reacting system. Aqueous infusion from sage showed the largest increase of induction time in comparison with pure lard (10.83 h). Smaller increase of the induction time was present in aqueous infusions from immortelle (7.67 h), while the increase of induction time of walnut, peppermint, strawberry and lemon balm was the smallest. The induction time of pure lard was (8.41±2.1) h in the test with aqueous tea infusion from walnut and sage, and (6.30±1.6) h in the test with aqueous tea infusion from immortelle, strawberry

**Table 3.** Induction times and antioxidant activity index (AI) of aqueous tea infusions from lemon balm (*Melissa officinalis* L.), peppermint (*Mentha×piperita* L.), strawberry (*Fragaria×ananassa* L.), immortelle (*Helichrysum arenarium* (L.) Moench.), sage (*Salvia officinalis* L.) and walnut (*Juglans regia* L.) in comparison with commercial antioxidants (BHT, BHA and  $\alpha$ -tocopherol), determined by the Rancimat method

Antioxidant*	Induction time/h	AI
Walnut	14.98±2.3	1.78
Peppermint	12.62±1.8	2.00
Strawberry	11.30±1.4	1.34
Sage	19.24±2.1	2.28
Immortelle	13.97±3.0	1.66
Lemon balm	11.80±2.7	1.56
BHT	19.80±1.2	3.60
BHA	37.80±2.3	7.20
$\alpha$ -Tocopherol	35.40±1.9	6.30

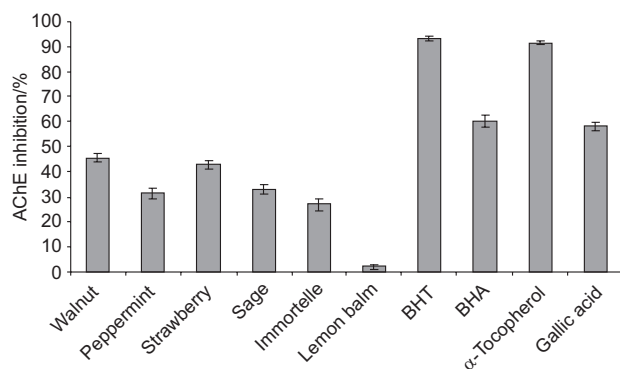
\*Concentration of the tested antioxidant added to the lard was 0.16 % (by mass) in the reacting system  
Values represent average of triplicates±standard deviation

and peppermint, while in the test with synthetic antioxidants (BHT, BHA,  $\alpha$ -tocopherol) it was (5.2±1.7) h. ANOVA analysis showed that the tested samples are significantly different. Presented results indicate that antioxidant activity in pure lard medium, detected by Rancimat method, does not correlate with total phenolic content (see Table 1). This can be explained by differences in the stability of phenolic compounds in the lipid medium under 100 °C, differences in their solubility in the lipid medium, and also by their different inhibitory activity toward lipid peroxidation process. Von Gadow *et al.* (30) reported different antioxidant activity of specific phenolic acids and flavonoids using Rancimat method. The exact mechanism of antioxidant action of phenolic compounds in lipids has not been completely explained so far (37).

#### *Inhibitory effect of aqueous tea infusions on acetylcholinesterase activity in vitro*

Inhibitory effect of the selected aqueous tea infusions on acetylcholinesterase (AChE) activity was determined by using Ellman's colorimetric method. Acetylcholine is hydrolyzed by acetylcholinesterase producing acetic acid and thiocholine. Thiocholine reacts with the Ellman reagent DNTB (5,5-dithiobis-2-nitrobenzoic acid) to produce the anion of 5-thio-2-nitrobenzoic acid (TNB) and the increase of its absorption indicates enzyme activity (38).

To date, the use of acetylcholinesterase inhibitors is the only therapy that has shown consistent positive results in the treatment of Alzheimer's disease (39). Fig. 4 shows the rate of AChE inhibition by the tested aqueous tea infusions (at the concentration of 1.36 g/L in the reacting system) in comparison with commercial antioxidants at the same concentration (BHT, BHA, gallic acid and  $\alpha$ -tocopherol). Aqueous tea infusions from walnut and strawberry show 45 and 42.5 % inhibition of AChE,



**Fig. 4.** Inhibitory activity of aqueous tea infusions from lemon balm (*Melissa officinalis* L.), peppermint (*Mentha piperita* L.), strawberry (*Fragaria x ananassa* L.), immortelle (*Helichrysum arenarium* (L.) Moench.), sage (*Salvia officinalis* L.) and walnut (*Juglans regia* L.) on acetylcholinesterase (AChE) in comparison with pure antioxidant compounds (BHT, BHA,  $\alpha$ -tocopherol, gallic acid and catechin). Values represent mean value of three independent experiments

respectively. Chauhan *et al.* (40) reported high inhibition of amyloid beta ( $A\beta$ ) protein, the principal component of amyloid plaques in the brains of patients with AD, by the walnut extract. They proposed that polyphenolic compounds (such as flavonoids) present in walnut may be responsible for its anti-amyloidogenic activity. Sage, immortelle and peppermint aqueous infusions show weaker inhibition of AChE (27–33 %), although Perry *et al.* (41) observed its high inhibition by sage (68 %, at the concentration of 2.5 mg/mL). Similarly, the inhibition of AChE by lemon balm aqueous infusion is very weak (2 %), but in some cases it has been found rather high (>50 %) (42). This can be explained by differences in sample preparation or by different chemical profile of the samples. According to the presented results, the inhibition of AChE activity does not correlate with the total phenolic content determined in aqueous tea infusions. For example, total phenolic content is the highest in lemon balm aqueous infusion, while its inhibitory activity of AChE was very faint according to Ellman's method. It is highly probable that a specific phenolic component (like quercetin 3-galactoside) is more important than the total content of phenolic compounds for the rate of AChE inhibition. The rate of AChE inhibition by pure compounds BHT, BHA, gallic acid and  $\alpha$ -tocopherol is very high.

## Conclusions

Presented results prove that selected infusions represent a mixture of compounds with very pronounced biological activity. In that sense, aqueous tea infusions from walnut (*Juglans regia* L.), strawberry (*Fragaria x ananassa* L.), peppermint (*Mentha piperita* L.), lemon balm (*Melissa officinalis* L.), immortelle (*Helichrysum arenarium* (L.) Moench.) and sage (*Salvia officinalis* L.) showed high rate of antioxidant activity by all used methods (LDL oxidation method, DPPH radical scavenging assay,  $\beta$ -carotene bleaching method and Rancimat method). Inhibition of LDL oxidation is the highest for aqueous infusions of

strawberry and lemon balm. Furthermore, all tested infusions have very high and very similar rate of DPPH scavenging as well as the inhibition of  $\beta$ -carotene bleaching. In pure lipid medium, used in Rancimat method, the ability of inhibition of peroxidation process is the strongest in sage and immortelle infusions. The antioxidant activity of other tested infusions in pure lipid medium was also pronounced. Screening of the AChE inhibitory activity by Ellman's method showed high inhibitory activity of walnut and strawberry aqueous infusions. Such effect makes them interesting for more detailed investigation for the treatment of Alzheimer's disease.

## Acknowledgements

This work was supported by the Ministry of Science, Education and Sports of the Republic of Croatia, projects 011-2160547-1330, 011-2160547-2226 and 011-2160547-2226.

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