Acta Biologica Hungarica 61 (1), pp. 45–51 (2010)
DOI: 10.1556/ABiol.61.2010.1.5

INHIBITION OF LIPID PEROXIDATION OF HERBAL EXTRACTS (OBTAINED FROM PLANT DRUG MIXTURES OF *MYRTILLI FOLIUM, PHASEOLI FRUCTUS SINE SEMINIBUS* AND *SALVIAE FOLIUM*) USED IN TYPE 2 DIABETES MELLITUS

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(Received: January 13, 2009; accepted: March 2, 2009)

Plant drug mixtures are widely used in the adjuvant therapy of type 2 diabetes mellitus for the prevention of complications. The drug mixtures generally contribute to the efficiency of the therapy and may also reduce undesirable side effects. Two herbal extracts (lyophilized aqueous extracts of plant drug mixtures 1: *Myrtilli folium*, *Phaseoli fructus sine seminibus* and 2: *Myrtilli folium*, *Phaseoli fructus sine seminibus, Salviae folium)* were investigated in *in vitro* rat models. The content of bioactive constituents (polyphenol, flavonoid and vitamin C) in plant drug mixtures and lyophilized samples was evaluated. The antioxidant activity of lyophilized extracts was determined by measuring the ferric reducing ability of the plant, Fe^{2+} induced lipid peroxidation (LPO) in rat brain homogenates and NADPH (β-nicotinamide adenine dinucleotide phosphate reduced form) induced LPO in cerebral microsomes. The antioxidant activity of lyophilized extracts was compared to that of quercetin and rutin. Both teas of lyophilized extracts had significant reducing ability (2694 and 2771 μmol/l) and inhibited LPO (IC_{50} 28.0 and 20.6 μl in NADPH induced LPO, 17.3 and 8.7 μl in Fe^{2+} induced LPO). The high concentration of polyphenol/flavonoid (12.38–13.00 and 1.45–5.22 g/100 g, respectively) and vitamin C (0.099–0.165 g/100 g) in the herbal extracts is related to their significant antioxidant properties. The tea mixtures have significant nutritional value, since the consumption of 2 or 3 cups of tea a day covers 50% of the daily requirement of vitamin C and it is also relevant polyphenol source. The high polyphenol/flavonoid content may restore the redox imbalance and contribute to the prevention of diabetic complications.

Keywords: Medicinal plant teas – polyphenol, flavonoid – vitamin C – antioxidant property – rats – diabetes mellitus

INTRODUCTION

The formation of reactive oxigen species is involved in several disorders and diseases [1]. In diabetes the formation of reactive oxigen species leads to serious diabetic complications [8, 15, 16]. The application of medicinal plant mixtures with hypoglycemic and antioxidant effects may inhibit diabetes induced oxidative stress and contribute to a successful adjuvant therapy [12, 21, 25]. Therefore herbal teas used

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in the adjuvant therapy of type 2 diabetes mellitus may contribute to the restoration of the redox balance and may serve as a nutritional source for the supplementation of vitamins and essential elements [22].

Antioxidant properties (hydroxyl radical scavenging-, superoxide anion scavenging-, chain-breaking antioxidant activity and inhibition of lipid peroxidation (LPO)) of the plant components (*Myrtilli folium, Phaseoli fructus sine seminibus* and their mixture) of herbal erxtracts were investigated and verified earlier [24]. The antioxidant activitity of *Salviae folium* has been examined by the modified FRAP method (ferric reducing ability of plant) [23]. Since teas are generally used for therapy as extracts of plant drug mixtures for the purpose of synergism, new compositions of plant drugs have been tested in this study. The state of our health greatly depends on the food we consume, therefore herbal teas may be beneficial both as remedies for the prevention of diseases and may also serve as essential nutritional sources. Since the antioxidant effect of plants may be attributed to nutritional antioxidants, such as vitamin C and polyphenols, the total content of polyphenol and flavonoids in addition to vitamin C content and antioxidant properties (Fe^{2+} and NADPH induced LPO) have been determined in two herbal extracts.

MATERIALS AND METHODS

Quercetin, rutin, sucrose, phenazine methosulphate, KH_2PO_4, 2-deoxy-D-ribose, nitro blue tetrazolium chloride monohydrate, HEPES (2-[4-(hydroxyethyl)-1-piperazine]-ethanesulfonic acid), TRIS (tris-hydroxymethyl-aminomethane), TBA (2-thiobarbituric acid), TCA (trichloroacetic acid) and ADP (adenosine diphosphate) were purchased from Sigma-Aldrich Co., NADPH (β-nicotinamide adenine dinucleotide phosphate, reduced form) was obtained from Boehringer Co., other chemical compounds were purchased from Merck LtD.

The samples (leaves of *Vaccinum myrtillus* L., *Ericaceae*; pericarp of *Phaseolus vulgaris* L., *Fabaceae*; leaves of *Salvia officinalis* L., *Lamiaceae*) were collected from the Model Farm of Corvinus University and Botanical Garden of Vácrátót in 2005. The plant samples were dried at room temperature. The constituents of plant drug mixtures (PDM1: 10 g of *Myrtilli folium*, 10 g of *Phaseoli fructus sine seminibus,* PDM2: 10 g of *Myrtilli folium*, 10 g of *Phaseoli fructus sine seminibus,* 10 g of *Salviae folium*) are listed in the Hungarian Pharmacopoeia [13] and almost all European Pharmacopoeias, e.g. German Pharmacopoeia [6].

The preparation of tea was performed as follows: a known amount of PDM sample (50 g) was infused in 2000 ml deionized water for 10 minutes and filtered while hot. After being cooled down the tea was lyophilized. The yields of lyophilized herbal extracts (HE1 from PDM1 and HE2 from PDM2) were 9.22 g for HE1 and 16.40 g for HE2.

The tea was prepared from lyophilized extracts (5 g) with 100 ml of water.

The *total polyphenol content* of the herbs was determined by the Hungarian Pharmacopoeia (Ph.Hg. VIII., 2004) by spectrometry. The absorbance was read at 765 nm and the results were expressed in gallic acid equivalent.

The flavonoid content was determined by the modified Glasl method [7]. The absorbance was measured at 420 nm against a blank solution prepared without Al-reagent. The results were expressed in hyperoside equivalent.

The vitamin C content of the samples was determined by spectrometry according to Pharmacopoeia Hungarica [13]. The absorbance was measured at 525 nm. The reference solution was prepared likewise, the only difference was that the dipyridyl solution was replaced by ethanol.

For measurement of the ferric reducing ability of the plant or plasma (FRAP) a method described by Benzie and Strain and modified by Ladó et al. was used [2, 9]. FRAP results were compared with those of quercetin and rutin.

Animal experiment: Ten male Hannover-Wistar rats (breeding colony, Gedeon Richter), 180–220 g in weight were used. The rats were fed with normal diet (ssniff® R/M-Z+H, ssniff Spezieldiäten GmbH, D-59494 Soest). The constituents of the diet were as follows: 19.0% crude protein, 3.5% crude fat, 3.6% crude fiber, 6.5% crude ash, 55.9% N free extracts, 4.8% sugar. The energy content of the diet was 13.4 MJ/kg. The rats were anaesthetized (35 mg/bwkg) and in deep narcosis they were exanquinated via abdominal vein. All experiments were conducted according to the guidelines of animal experimentation as determined by Act 243/1998 (XII. 31). XXVIII of the Hungarian Parliament and approved by the Gedeon Richter Animal Experimentation Ethics Committee.

In all cases the results of quercetin and rutin were considered as controls, since they proved to be good antioxidants.

Fe^{2+} induced lipid peroxidation (LPO) in brain homogenates: Whole rat brains were homogenized in 9 vol. buffer (pH = 7.4; HEPES, 15 mM, NaCl /140 mM/; KCl /3.6 mM/; $CaCl_2$ /1.5 mM/; $MgCl_2$ /0.7 mM/; KH_2PO_4 /1.4 mM/ and glucose /10 mM/). The 200 µl homogenate (protein content: 10 mg/ml) and $Fe(NH_4)_2(SO_4)_2$ solution (5 µl, 8 mM) were incubated at 37 °C for 20 min. LPO was terminated by the addition of a TCA solution (12.5%) in 0.8 M HCl. The sample was centrifuged (2000 G) at 4 °C for 10 min. The supernatant (0.5 ml) was mixed with a TBA solution (1 ml, 1%). The sample was placed in boiling water for color development for a period of 20 min, then optical density was determined spectrophotometrically at 535 nm [3]. For the determination of protein content the method of Lowry et al. was applied and bovine serum albumin was used for standard [10].

NADPH induced LPO in cerebral brain microsomes: Whole brains were homogenized in ice-cold solution (10 vol., 0.25 M sucrose). The homogenate was centrifuged (15,000 G) at 4 °C for 10 min. The supernatant was centrifuged (78,000 G) at 4 °C for 60 min. The pellet was suspended in a KCl solution (0.15 M). The microsomes (0.2 mg protein) were incubated in a mixture of TRIS (50 mM, pH = 6.8), $FeCl_3$ (0.2 mM), KH_2PO_4 (1 mM), ADP (0.5 mM) at 37 °C for 20 min. The incubation volume obtained was 1 ml. LPO was initiated by the addition of NADPH (0.4 mM) and the reaction was terminated by the addition of a stopping solution (40%

TCA: 5 HCl = 2 : 1). The acidified sample was mixed with a TBA solution (1 ml, 1%). The sample was placed in boiling water for color development for a period of 20 min then centrifuged (2000 G) at 4 °C for 10 min. Optical density was determined spectrophotometrically at 535 nm [14].

Mean values and standard deviation were calculated from parallel measurements. In rat experiments IC_{50} values (concentration for 50% inhibition of LPO induced by Fe^{2+}, or NADPH) were calculated from concentration-effect curves by sigmoidal fitting, using Origin 6.0 software (Microcal). For comparison of the means, a *t*-test was used with GraphPAD software version 1.14 (1990). Significance was assessed at $P < 0.05$.

RESULTS

The pharmacological effect of medicinal plants and extracts is attributed mainly to organic constituents. Therefore the content of bioactive agents in drug mixtures and herbal extracts were determined (Table 1). Higher amounts of polyphenol and flavonoid were observed in the HE2 sample, while higher vitamin C content was measured in the HE1 sample.

The antioxidant properties of the teas of HE samples were examined in three different test systems (Table 2). Since flavonoids are known to be good antioxidants, the results were compared with the antioxidant activities of quercetin (flavonoid aglycon) and rutin (flavonoid glycoside). Quercetin proved to be a signifcant antioxidant, while rutin showed less antioxidant properties. Ferric reducing ability was measured by the FRAP method. Both lyophilized extracts had good reducing ability, although their results showed somewhat lower values than those of standard compounds.

The inhibition of LPO was measured in brain microsomes by NADPH induction and in brain homogenates by Fe^{2+} induction. In the presence of NADPH, as well as upon the effect of the free radical induction of Fe^{2+}, thiobarbituric acid derivatives (TBARs) are formed from the lipid membranes. The IC_{50} of the teas of HEs was determined by the inhibition of TBAR formation. The teas were better antioxidants

Table 1
Phytochemical characteristics of plant drug mixtures (PDM1 and PDM2) and lyophilized samples (HE1 and HE2) determined by spectrometry

	PDM1	PDM2	HE1	HE2
Polyphenol (g/100 g)	12.53 ± 0.31	15.12 ± 0.04*	13.00 ± 0.05	12.38 ± 0.18**
Flavonoid (g/100 g)	0.374 ± 0.011	0.529 ± 0.010*	1.45 ± 0.06	5.22 ± 0.10**
Vitamin C (g/100 g)	0.045 ± 0.001	0.056 ± 0.002*	0.165 ± 0.003	0.099 ± 0.004**

Values are means ± SD, n = 3. Values of PDM1 (10 g of *Myrtilli folium*, 10 g of *Phaseoli fructus sine seminibus*) was compared with that of PDM2 (10 g of *Myrtilli folium*, 10 g of *Phaseoli fructus sine seminibus,* 10 g of *Salviae folium*) and values of HE1 (lyophilized herbal extract of PDM1) was compared with that of HE2 (lyophilized herbal extract of PDM2). Results show significant difference between values of PDM1 and PDM2 (*), as well as HE1 and HE2 (**) at $P < 0.05$ level.

Table 2
Antioxidant activities of 5% aqueous extracts of lyophilized samples (HE1 and HE2)

	Tea of HE1	Tea of HE2	Quercetin	Rutin
FRAP (μmol/l)	2694±14	2771±8*	3833±2	5295±3
NADPH induced LPO in microsome (IC_{50}, μl)	28.0[a]	20.6[a]	9.0±1.1	120.5±20.6
Fe^{2+} induced LPO in brain homogenates (IC_{50}, μl)	17.3[a]	8.7[a]	12.8±1.9	181.7±39.6

Values are means ± SD, n = 3, [a] n = 1. FRAP= ferric reducing ability of plant. Values of HE1 (lyophilized herbal extract of PDM1: 10 g of *Myrtilli folium*, 10 g of *Phaseoli fructus sine seminibus*) tea were compared with those of HE2 (lyophilized herbal extract of PDM2: 10 g of *Myrtilli folium*, 10 g of *Phaseoli fructus sine seminibus,* 10 g of *Salviae folium*) tea. Values of HE1 and HE2 teas were compared with those of quercetin and rutin. * Significant difference between values of tea of HEs at $P < 0.05$ level. Concentration causing 50% inhibition (IC_{50} values) was calculated.

in the Fe^{2+} induced test system than in the NADPH induced system and the tea of HE2 was a better antioxidant than the tea of HE1. Fe^{2+} induced LPO in the brain homogenate of HE2 proved to be more efficient than that of quercetin. In this system HE2 showed higher antioxidant activity than aglycon quercetin. Although Fe^{2+} induced LPO in the brain homogenate of HE1 was lower than that of quercetin, nevertheless the value indicated significant antioxidant activity.

DISCUSSION

Although several investigations were carried out on the bioactive organic ingredients (tannins, vitamins, flavonoids etc.) of plant drugs and extracts and the antioxidant properties, there are hardly any data on the measurement of extracts of drug mixtures [12]. The plant components of HEs have hypoglycemic activity as described earlier [17]. *In vivo* experiments proved that some of the plant extracts with hypoglycemic activity provided antioxidant activity in *in vitro* tests [19]. Therefore, *in vitro* measurements were made in order to determine the antioxidant effect of two extracts of medicinal plant mixtures recommended in folk medicine for the treatment of diabetes mellitus and they are also used in our days in the adjuvant therapy of type 2 diabetes mellitus. In this work antioxidant activity was found to be better for both HEs than for quercetin glycoside or rutin. The aqueous extracts of HEs inhibited LPO in brain microsomes and brain homogenates, induced enzymatically by adding NADPH and non-enzymatically by adding Fe^{2+}. These results also show the importance of applying herbal mixture extracts in the adjuvant therapy of diabetes and other metabolic diseases.

The antioxidant property of HE2 was found to be better than HE1. This fact could be explained by the higher flavoinod (Table 1) and lower heavy metal content of HE2 [22].

The measured organic agents have nutritional value as well. From nutritional aspects, the consumption of vitamin C related to the daily requirement is relevant. The vitamin C content of the tea (2 dl) prepared as described in part Material and

methods (5 g lyophilized sample/100 ml water) was 16.5 mg for HE1 and 9.9 mg for HE2. Considering the fact that the daily vitamin C requirement of a male is 60 mg (RDA and DRI data) and each extract covers more than 10% of the requirement, these tea extracts may be regarded as good sources of vitamin C. In adjuvant therapy, the consumption of one liter of tea per day is recommended, therefore the daily intake of vitamin C may be even more favorable [5, 18].

Since there are no RDA or DRI data for polyphenols and flavonoids in the literature, only the intake of these compounds in different countries could be compared. In Hungary people consume 19 mg of flavonoid a day while in the USA this value may amount to 1 g. [11]. Several publications recommend 200–300 mg polyphenol a day for the prevention of diseases, at the same time, according to a report, in the case of Irish women, the intake of polyphenol ranges between 500 and 700 mg per day [4, 20]. Our calculations show that one cup of tea from the lyophilized extract contains 1000–1300 mg polyphenol and 145–522 mg flavonoid which shows very high polyphenol/flavonoid intake. Although there was no calculated correlation between polyphenol/flavonoid content and antioxidant properties of the extracts because of the few data, the bioactive agents in HEs in high concentration presumable contribute to the beneficial effect of the tea.

ACKNOWLEDGEMENTS

The work was supported by ETT 012/2006. The authors express their thanks to Ms. E. Bíró for the excellent technical assistance.

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