Review

Potential of Phenolic Antioxidants

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Received: 08-07-2009

Abstract

In vitro studies have shown a link between the consumption of food rich in (poly)phenols, especially flavonoids, and reduced risk of coronary heart diseases. Several flavonoids have been reported to prevent low density lipoprotein cholesterol oxidation *in vitro*. The aim of our work was to determine antioxidant and radical scavenging activity of flavonoids myricetin, quercetin, rutin, luteolin, apigenin, kaempferol, catechin, epicatechin and epigallocatechin gallate and to study the influence of chemical structure and flavonoid interactions on the ability to inhibit oxidation and scavenge free radicals. Two *in vitro* methods, i.e. oxidation of β-carotene in an emulsion system and DPPH (1,1-Diphenyl-2-picrylhydrazyl) radical scavenging assay, were applied. In addition, a review of *in vivo* studies that investigate the effectiveness of food flavonoids in reducing oxidative damage in human body was done and their results were analyzed and compared to *in vitro* results obtained in our laboratory. Although all tested flavonoids except apigenin showed strong antioxidant and antiradical properties *in vitro*, these findings can not be completely confirmed from the reviewed *in vivo* human studies since those results are sometimes contradictory and inconsistent.

Keywords: Phenolics, Flavonoids, Antioxidant activity, Antiradical activity

1. Introduction

A number of epidemiological, *in vitro* and animal studies suggest that food (poly)phenols, especially flavonoids, may protect against cardiovascular diseases (CHD).[1] Flavonoids are water-soluble plant pigments that are characterized by an aromatic ring structure with one or more hydroxyl groups. They belong to a larger group of plant (poly)phenols, which can be divided into more than 10 different subclasses, including flavonols, catechins, (pro)anthocyanidins, lignans, and lignins.[2] Their bioactivity has been associated to their antioxidant properties, that is to ability to protect against damage caused by reactive oxygen species. They are free-radical scavengers and can prevent low density lipoprotein (LDL) cholesterol oxidation *in vitro*.[3,4] Since oxidation of LDL cholesterol is thought to promote atherosclerosis, it is plausible that flavonoids may delay the development of atherosclerosis and ultimately decrease CHD mortality.[5]

While vitamins and carotenoids have been widely studied for their antioxidant effects long before 1990, it is only from this date that polyphenols were taken in the consideration as potential antioxidants as they were shown to be effective in protecting LDL from oxidation *in vitro*[6]. The main factor that has delayed the research on polyphenols is the considerable diversity and complexity of their chemical structures.[6]

The truly research on flavonoid antioxidant properties begun following the results of Zutphen Elderly Study,[7] a prospective cohort study including 806 men aged 65–84 that has shown a correlation between the intake of food rich in antioxidant flavonoids (mainly quercetin, kaempferol, myricetin, apigenin and luteolin) and the reduced rate of CHD mortality in elderly men.

Numerous animal studies provide evidence that food flavonoids may have cardiovascular protective properties. Muramatsu et al.[8] have found that tea catechins decrease plasma total cholesterol and atherogenic index in cholesterol fed mice. Furthermore, the total antioxidant capacity was increased in mice receiving the infusions of green tea and aromatic plant *Pelargonium purpureum* (little robin) in comparison to mice receiving water.[9]

The review conducted by Leifert et al.[10] discusses *ex vivo* and *in vivo* animal studies and human trials which suggest the cardioprotective actions of grape polyphenols. Khan et al.[11] have reviewed the preventive effects of tea

and tea polyphenols against cancer and cardiovascular diseases reported in laboratory animals and in human epidemiological or case-control studies, while Kris-Etherton et al.[12] have investigated the epidemiological evidence for an association between the intake of bioactive compounds in food and incidence of some chronic diseases.

In this paper some common food flavonoids like myricetin, quercetin, rutin, luteolin, apigenin, kaempferol, catechin, epicatechin and epigallocatechin gallate have been studied in order to determine their antioxidant and antiradical activity with two *in vitro* tests (β-carotene bleaching test in emulsion system and DPPH free radical (DPPH•) test). Specifically, the relationship between their chemical structure (Figure 1) and the ability to inhibit oxidation and scavenge free radicals as well as their potential interactions have been examined. In addition, the obtained

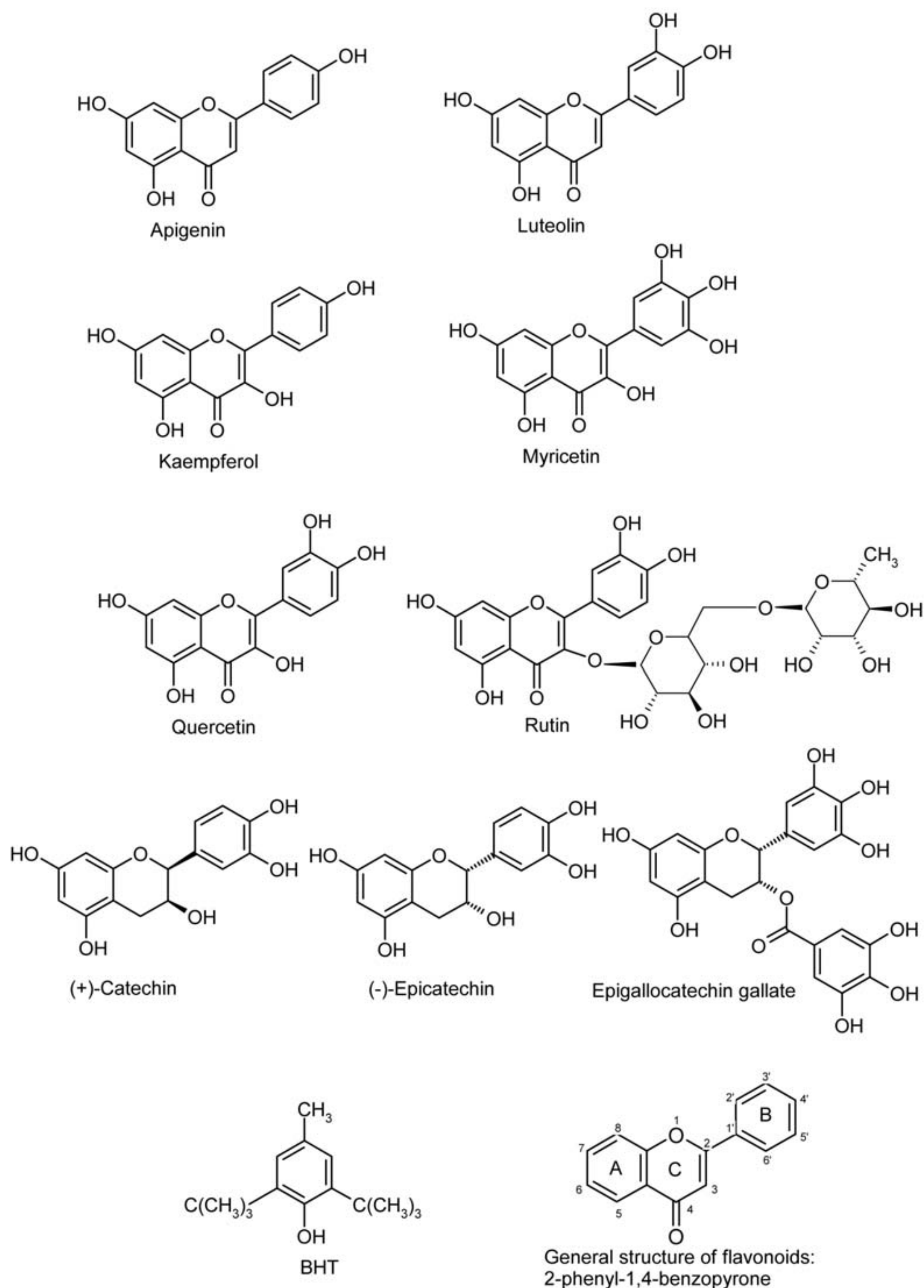

Figure 1. Chemical structures of apigenin, luteolin, kaempferol, myricetin, quercetin, rutin, (+)-catechin, (-)-epicatechin, epigallocatechin gallate (EGCg), 3,5-Di-tert-4-butylhydroxytoluene (BHT) and general structure of flavonoids (2-phenyl-1,4-benzopyrone).

results have been compared with those of *in vivo* studies that investigate the effectiveness of food flavonoids in reducing oxidative damage in human body.

2. Experimental

Antioxidant and antiradical activity of nine naturally occurring flavonoids (myricetin, quercetin, rutin, kaempferol, apigenin, luteolin, catechin, epicatechin end epigallocatechin gallate (EGCg) and one synthetic antioxidant 3,5-Di-tert-4-butylhydroxytoluene (BHT) were determined with β-carotene bleaching test in emulsion system and DPPH• scavenging test. Furthermore, antiradical activities of several combinations of the studied phenolic compounds were evaluated in order to investigate possible interactions between those compounds and their effect on antiradical activity.

2. 1. Chemicals and Standards

All solvents and chemicals (analytical grade) used for the sample preparation were purchased from Merck (Darmstadt, Germany) and from Fluka (Germany). Standards of BHT, luteolin, (+)-catechin and (-)-epicatechin were supplied by SigmaAldrich (Germany), rutin was provided by Acros Organics while (-)-epigallocatechin gallate, myricetin, kaempferol and apigenin were acquired from Fluka (Germany). For the β-carotene-linoleic acid assay β-carotene, linoleic acid and Tween 40 were provided by Fluka as well as DPPH needed for DPPH radical scavenging test.

2. 2. Preparation of Sample Solutions

Flavonoid solutions were prepared in methanol in 1 mg/ml concentration and stored in refrigerator for further analysis. The exact concentrations for each standard are given in Fig. 2. Furthermore, the methanol solutions of flavonoid mixtures with total concentration of 1 mg/ml and equal mass fractions of flavonoids were prepared (Table 1) and stored in refrigerator for further measurements.

2. 3. β-carotene-linoleic Acid Assay

The antioxidant activity was elucidated on a heat-induced oxidation of an aqueous system of β-carotene and linoleic acid as described by Škerget et al.[13] A stock solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 ml of chloroform (HPLC grade). 20 µl of linoleic acid and 200 µl of Tween-40 were added as emulsifier to 1 ml of this solution since β-carotene is not water soluble. Chloroform was completely evaporated using a vacuum evaporator. Successively, 50 ml of oxygen-saturated distilled water were added and the mixture was shaken. 5 ml of this reaction mixture were dispensed into test tubes, and 200 µl volumes of sample solutions, prepared in 1 mg/ml concentrations in methanol, were added. The emulsions were incubated for 2 h at 50 °C. The absorbance of the sample was measured on the Varian UV-VIS Spectrophotometer at 470 nm at the beginning (t = 0 min) and at the end of the experiment (t = 120 min). The same procedure was repeated with a control sample which was prepared with 200 µl of pure methanol. Antioxidant activity of the sample (s) was calculated as percent inhibition of oxidation versus control sample (c) using the equation:

$$\%Antioxidant\ activity_{emulsion\ system} = \left[1 - \frac{A_s^0 - A_s^{120}}{A_c^0 - A_c^{120}}\right] \times 100 \quad (1)$$

where A_s is the absorbance of sample at t = 0 min and at t = 120 min, and A_c is the absorbance of the control sample at t = 0 min and at t = 120 min. All tests were carried out in triplicate.

2. 4. DPPH Radical Scavenging Activity

The samples were measured in terms of hydrogen-donating or radical scavenging ability, using the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) in a spectrophotometric test as described by Majhenič et al.[14] The effect of antioxidants on DPPH• scavenging is thought to be due to their hydrogen-donating ability. DPPH• is a stable free radical and accepts an electron or hydrogen radical to become a stable molecule. The reduction capability (on the DPPH•) is determined by the decrease in its absorbance at its absorption maximum at 515 nm that is induced by the antioxidant. This is visualized as a change in color from violet to yellow.[14] Briefly, 6×10^{-5} M DPPH• solution in methanol was prepared, and then 3 ml of this solution were mixed with 77 µl of the sample solution (A) prepared in 1 mg/ml concentrations in methanol. After the incubation for 15 min in the darkness, the absorbance was measured at 515 nm. Decrease in the absorbance of the DPPH• solution indicates an increase in DPPH• scavenging activity. This activity is given as the percent DPPH• scavenging which is calculated as:

$$\%Inhibiton = \left[\frac{A_B - A_A}{A_B}\right] \times 100 \quad (2)$$

The control sample (B) contained 3 ml of DPPH• solution and 77 µl of methanol. The measurements of DPPH• scavenging activity were carried out for three sample replications.

Furthermore, in order to evaluate the possible interactions among the investigated phenols, combinations of two, three and four compounds were studied using DPPH test. In particular, the combinations of catechin and epicatechin; catechin and EGCg; epicatechin and EGCg; catechin, epicatechin and EGCg; catechin, epicatechin and

quercetin; catechin, epicatechin, quercetin and myricetin were analyzed. In the mixtures each compound had the same mass fraction and the total phenol concentration was 1 mg/ml. The antiradical activity of these solutions was measured under the same experimental conditions of the individual pure compounds and expressed as the percent DPPH· scavenging.

2. 5. Statistical Analysis

Experimental results were expressed as means ± standard deviation (SD) of three parallel measurements.

3. Results and Discussion

3. 1. *In Vitro* Results

As can be seen from the Figure 2, in the linoleic emulsion system oxidation of β-carotene was significantly inhibited (> 40%) with all flavonoid solutions (kaempferol, quercetin, myricetin, luteolin, catechin, rutin, epicatechin and EGCg) except with apigenin which resulted in 11.3% inhibition. This low inhibition might be caused by the absence of C-3 hydroxyl group which is thought to be responsible for the high inhibition of β–carotene oxidation.[15] The highest inhibitions of β–carotene oxidation were shown by kaempferol, quercetin, myricetin and luteolin (99.4%, 98.8%, 98.8% and 94.3% respectively) and are comparable to that of synthetic antioxidant BHT (98.7%). Kaempferol, quercetin and myricetin have a free hydroxyl group at the C-3 position. On the other hand, luteolin has two adjacent hydroxyl groups at positions 3' and 4' in which the hydroxyl group at C-3' enhances the reactivity of the group at the C-4' position. Furthermore, it appears that the glycosylation of the hydroxyl group at C-3 lowered antioxidant capacity of rutin (67.9% inhibition of β-carotene oxidation) in comparison to its aglycon, quercetin. In fact, Heim et al.[16] have observed that aglycones are generally stronger antioxidants than their corresponding glycosides.

In their study on antioxidative activity of flavonoids, Burda et al.[15] have concluded that flavonoid ability to inhibit β–carotene oxidation is influenced not only by the free hydroxyl group at C-3 position, but also by the presence of double bond between C-2 and C-3. It is possible that the three flavan-3-ols tested (catechin, epictechin and EGCg) show lower antioxidant activity (75.2%, 60.1% and 40.2% respectively) because of the absence of the double bond on the main ring. Furthermore, the galloylation of hydroxyl group at C-3 might be the reason of lower inhibition shown by EGCg in comparison to the other two catechins. In fact, Battestin et al.[17] demonstrated that the products of degalloylation of EGCg, epigallocatechin and gallic acid show higher antioxidant activity than the EGCg itself.

The results obtained with the antiradical test are shown in Figure 2. Kaempferol, EGCg, quercetin, epicatechin, catechin, luteolin and rutin have shown to be very effective DPPH· scavengers with inhibitions (94.5%, 94.0%, 93.4%, 93.2%, 92.7%, 92.4% and 92.3%) higher than that of synthetic antioxidant BHT (86.9%). All of these compounds, except EGCg, luteolin and rutin, have a free hydroxyl group at the C-3 position and it appears that the presence of this hydroxyl group is essential for a strong antiradical activity.[15]

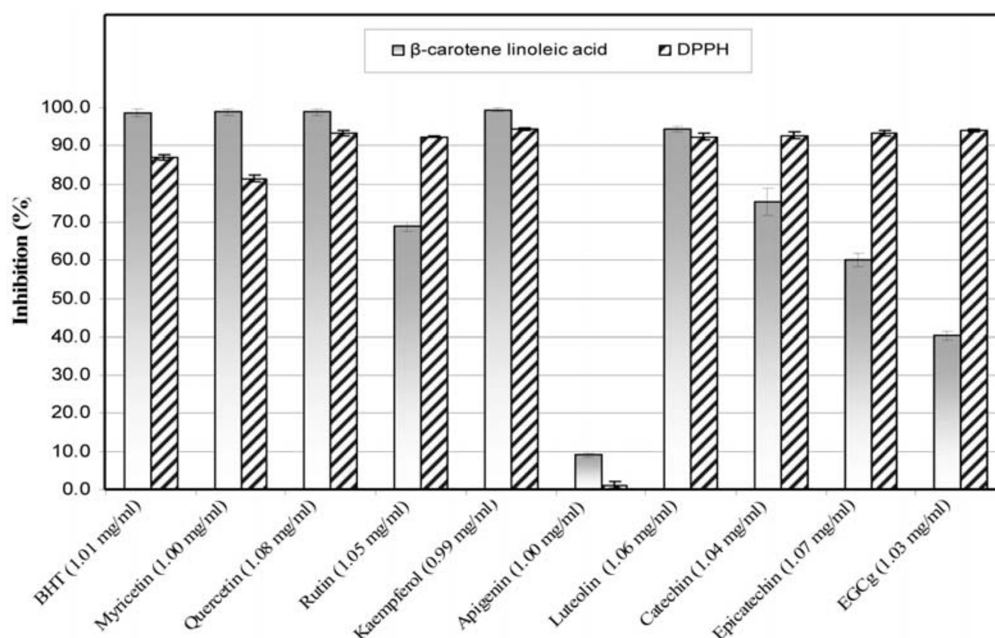

Figure 2. The antioxidant activities of phenolic compounds obtained using DPPH· assay and β-carotene–linoleic acid emulsion systems.

In the case of the DPPH· assay the glycosylation of the hydroxyl group at C-3 did not change notably the activity of rutin in comparison to its aglycone quercetin. Similarly, the galloylation of the hydroxyl group at C-3 did not decrease the antiradical activity of EGCg. It seems that the presence of a free hydroxyl groups at C-3' and C-4' is essential for the antiradical activity of rutin, EGCg and luteolin. In the case of rutin and luteolin the antiradical effectiveness of this groups is strengthened by the double bond between C-2 and C-3.[15]

Although myricetin contains hydroxyl groups at the C-3, 3', 4' and 5', the lower than expected antiradical activity (81.4%) can be explained by its high sensitivity to oxidation, which causes rapid oxidation and partial decomposition during the measurement as it was already observed by Burda et al.[15] The lowest inhibition was shown by apigenin (1.2%) which can be explained by the absence of C-3 and/or C-3' hydroxyl groups.

Table 1. Antiradical activity of pure phenolic compounds and their combinations.

Compound	Concentration (mg/ml)	Inhibition (%)
Catechin (C)	1.04	92.7 ± 0.9
Epicatechin (E)	1.07	93.2 ± 0.7
Epigallocatechin gallate(EGCg)	1.03	94.0 ± 0.2
Quercetin (Q)	1.08	93.4 ± 0.7
Myricetin (M)	1.00	81.4 ± 1.0
C + E		72.5 ± 0.1
C	0.52	
E	0.54	
C + EGCg		94.0 ± 0.5
C	0.52	
EGCg	0.52	
E + EGCg		93.5 ± 0.7
C	0.52	
EGCg	0.52	
C + E + EGCg		93.3 ± 0.8
C	0.35	
E	0.36	
EGCg	0.34	
C + E + Q		72.7 ± 0.2
C	0.35	
E	0.36	
Q	0.36	
C + E + Q + M		85.5 ± 0.7
C	0.26	
E	0.27	
Q	0.27	
M	0.25	

The results of antiradical activities of mixtures containing two, three and four flavonoids as well as the activities of pure standards are shown in Table 1. All mixtures that contained EGCg resulted in an antiradical activity comparable to that of the pure flavonoids. On the other hand, although catechin, epicatechin and quercetin had antiradical activities higher than 90%, the mixtures containing 50% of catechin and 50% of epicatechin showed lower inhibition of DPPH· (72.5%). Analogous results were obtained with the mixtures containing catechin, epicatechin and quercetin (72.7%). The combination of catechin, epicatechin, quercetin and myricetin resulted in a slightly higher inhibition (85.5%). However, it was still lower than their average inhibition (90.3%).

The obtained results show that the antioxidant and the antiradical activities of the investigated flavonoids depend on the number and location of hydroxyl groups on the aromatic ring and on the existence of the double bond between C-2 and C-3 positions. Furthermore, the results of antiradical activities of flavonoid mixtures suggest the presence of interactions between flavonoids that could affect the overall antiradical activity of mixtures.

Furthermore, it is important to notice that in our study all measurements for antiradical scavenging activity were done according to the method previously described in the literature[14] in which the uniform incubation time interval of 15 minutes was taken and that no kinetics was studied.

According to Brand-Williams et al.[18] there are three different kinetic types in which compounds can react with the DPPH radical. The first type is a fast reaction where a compound reacts quickly with the DPPH free radical reaching the steady state almost immediately. The second type is a little slower reaction when the steady state is reached within 30 minutes and, finally, there is a slow reaction which requires a couple of hours to reach a steady state[18]. The example of a slow kinetic behavior is the reaction of BHT with DPPH·[18, 19], while kaempferol exhibits fast reaction with DPPH free radical.[20, 21, 22]

A more detailed study should consider the reaction time in order to estimate the antiradical activity. For such purpose, Villaño et al.[20] have used the parameter called antiradical efficiency (AE) which takes into account both the ability of phenolic compounds to transfer labile H atoms to radicals as well as the time needed to reach the steady state.

3.2. Comparison with *In Vivo* Results

3. 2. 1. *In Vivo* Studies

The results from *in vitro* studies conducted in our laboratory suggest that eight out of nine analyzed flavonoids possess strong antioxidant and antiradical properties. In order to examine the potential of these flavonoids when introduced into the human body we have reviewed some *in vivo* studies that investigate their antioxidant activity (Table 2).

3. 2. 1. 1. Catechins

Catechins are main phenolics present in the tea (*Camellia sinensis*) leaves and constitute up to 30% of their dry weight.[23] The main tea catechins are (-)-EGCGg(-)-

Table 2. Potential of natural antioxidants: *in vivo* evidence.

Antioxidant	Dose (mg/d)	Antioxidant source	Participant N. and assay period	Effect	Reference
Catechins	–	Green tea extract	20 F 4 week	No enhance of antioxidant status.	Freese et al.[25]
Total catechins EGCg	254 82	Green tea extract	18 M 60 min	Increase in plasma antioxidant capacity.	Nakagawa et al.[26]
Polyphenols	–	Green and black tea	13 MF (3 × 7d) 22 MF (2 × 4w)	No influence on inhibition of lipid peroxidation.	Hodgson et al.[27]
Polyphenols	446.6 583.0	Decaffeinated black tea Decaffeinated green tea	133 MF (heavy smokers) 4 months	Green tea may protect from oxidative damages and reduce risk from diseases caused by smoking associated free radicals.	Hakim et al.[28]
Catechins	18.6	Green tea extract	16 M (smokers and non smokers) 2x3 weeks	No long-term effects on oxidation parameters within the blood or urine compartments.	Young et al.[29]
EGCg Cyanidin glycosides	236 226	Fruit juice	27 M (non smokers) 5 × 2 weeks	Enhance of antioxidant status. Decrease of oxidative DNA damage in lymphocytes.	Bub et al.[30]
Total phenolics Quercetin	3 × 31.3 3 × 9.0	Grape skin extract	15 MF 2 × 1 week	No influence on reduction of plasma protein oxidation.	Young et al.[31]
Quercetin	4.8, 6.4., 9.6	Black currant and apple juice	5 MF 2 × 1 week	Prooxidant effect on plasma proteins. Decrease in plasma lipid concentration.	Young et al.[32]
Quercetin	89.7 1.4	Onion Black tea	32 MF 14 days	No effect against lipid peroxidation.	O'Relly et al.[33]
Rutin	500	Rutin supplements	18 F 6 weeks	No influence on plasma antioxidant status.	Boyle et al.[34]
Apigenin	3.73–4.49	Parsley	5 MF 3 × 1 week	No influence on reduction of plasma protein oxidation.	Nielsen et al.[35]

M=male, F=female.

epigallocatechin, (-)-epicatechin gallate, (-)-epicatechin and (+)-catechin.[24] Numerous human intervention studies with green and black tea were conducted in the recent years.

Freese et al.[25] investigated whether the green tea polyphenolic catechins can act as antioxidants when introduced to human body and concluded that an amount of encapsulated green tea extract (3 grams per day) which corresponds to 10 cups of tea per day for 4 weeks does not have specific effects on several indicators related to risk of cardiovascular diseases in comparison with placebo treatment.

The effect of the green tea catechin consumption was also investigated by Nakagawa et al.[26] The antioxidant capacity of plasma was investigated by measuring plasma phosphatidylcholine hydroperoxide (PCOOH) levels as a marker of oxidized lipoproteins 60 minutes after the green tea extract supplementation. A decrease in plasma concentrations of PCOOH suggested that drinking green tea may contribute to prevent cardiovascular disease by increasing plasma antioxidant capacity in humans.

In contrary, two controlled intervention studies conducted in Australia[27] did not support the suggestion that polyphenolic antioxidants derived from black and green tea protect against cardiovascular diseases. The studies examined the effects of tea consumption on lipid peroxidation by measurements of urinary F2-isoprostane excretion (an index of lipid peroxdation and oxidative DNA damage in white blood cells). In the first study, 13 subjects with elevated blood pressure consumed green tea, black tea and hot water containing caffeine for 7 days each. In the second study, the effects of the consumption of 1250 ml of the black tea per day were compared with the hot water consumption in 22 subjects with mildly raised serum concentrations of total cholesterol. In both studies, F2-isoprostane excretion was not altered after regular tea ingestion in comparison with hot water and it was concluded that polyphenolic antioxidants derived from tea do not inhibit *in vivo* lipid peroxidation.

Hakim et al.[28] have also studied antioxidant effects of tea polyphenols. The influence of high consumption (4 cups per day) of decaffeinated green or black tea on oxidative DNA damage was measured by urinary 8-hydroxydeoxyguanosine (8-OHdG) among smokers over a 4 month period. One cup of decaffeinated green tea contained 145.75 mg of total poyphenols (from which 73.49 mg of total catechins) while one cup of decaffeinated black tea contained 111.65 mg of total polyphenols (from which

8.11 mg of total catechins). A decrease in urinary 8-OHdG (–31%) was measured only in the case of green tea consumption suggesting that a regular green tea drinking might protect smokers from the oxidative damages and could reduce the risk of cancer or other diseases caused by the free radicals associated with smoking.

Young et al.[29] investigated the effect of the green tea extract used as a food antioxidant on markers of oxidative status in a diet low in flavonoids. Eight smokers and eight non-smokers participated in a double blind randomized 2 × 3 week cross-over study with 2 weeks wash-out before each intervention with green tea extract (GTE) corresponding to a daily intake of 18.6 mg of catechins. Subjects were divided into groups A and B, with four smokers and four non-smokers in each group and no long-term effects on oxidation parameters were observed within the blood or urine compartments.

The effect of the tea catechins was also investigated by Bub et al.[30] in a randomized crossover study with 27 healthy non smoking men consuming 2 polyphenol-rich juices (one providing 236 mg of cyanidin glycosides and other containing green tea and 226 mg of EGCg) for 2 weeks. The juice intervention reduced oxidative DNA damage in lymphocytes and decreased plasma malondialdehyde (marker of lipid oxidation).

Furthermore, catechins were main polyphenols in a two weeks cross-over study of the influence of grape-skin extract on the markers of oxidative status in the blood.[31] Fifteen subjects (nine women, six men) were randomly separated in two groups and consumed 200 ml grape-skin extract in water (1 mg of extract per ml) during each of the three daily meals. The study brought to conclusion that the grape-skin extract in an amount corresponding to a total phenolics daily intake of 93.9 mg (from which 27 mg catechin, 4.8 mg caffeic acid, 4.5 mg epicatechin, 4.5 mg rutin, 3.6 mg myricetin, 2.1 mg resveratrol and 42 mg of unidentified phenolics) did not influence markers of antioxidative status.

3. 2. 1. 2. Quercetin

The effect of the intake of flavonoid-containing black currant and apple juice on markers of oxidative status was investigated in a crossover study with 3 doses of juice (750, 1000 and 1500 ml) consumed for 1 week by 4 women and 1 man corresponding to an intake of 4.8, 6.4, and 9.6 mg quercetin per day.[32] A high juice intake showed a prooxidant effect on plasma proteins, whereas malondialdehyde concentration in plasma decreased. The results suggested that there are several subcompartments within the plasma that may respond differently to the dietary components.

Forty-two (20 male and 22 female) healthy, nonsmoking subjects participated in a randomized crossover study[33] where have been investigated the effects of consuming food rich in flavonoids on indexes of oxidative damage (F2-isoprostanes and malondialdehyde (MDA) – LDL autoantibody titer). Every subject consumed daily one 150 grams onion cake (containing 89.7 mg quercetin) and one 300 milliliters cup of black tea (containing 1.4 mg quercetin). As there were no significant effects on indexes of oxidative damage it was concluded that flavonoid consumption in onion and tea does not inhibit lipid peroxidation in humans[33]

3. 2. 1. 3. Rutin

Eighteen healthy non-obese normocholesterolaemic female participated in a study of the potential antioxidant effect of rutin (quercetin-3-O-beta-rutinoside) supplementation. These 6-week randomized single-blinded placebo controlled trial conducted by Rowett Research Institute[34] resulted in no significant change in plasma antioxidant status.

3. 2. 1. 4. Apigenin

Nielsen et al.[35] studied the effect of the intake of parsley, containing high levels of the apigenin, on the biomarkers for oxidative stress. 14 subjects received a diet supplemented with parsley providing 3.73–4.49 mg apigenin and low in other naturally occurring antioxidants during the 2 weeks of intervention. No significant changes were observed in plasma protein 2-adipic semialdehyde residues, a biomarker of plasma protein oxidation.

3. 2. 2. Discussion – *In Vivo* and *In Vitro* Results

The majority of the reviewed trials studied the potential antioxidant effects of tea catechins. Different research conditions (number of subjects, their sex, age, oxidative status, markers of oxidative stress etc.) brought to contradictory results. Hakim et al.[28] conducted the study with the highest number of subjects and took in the consideration their oxidative status (heavy smokers). The positive antioxidant effects were obtained only with the green tea. Actually, the green tea is rich in catechins which are oxidized and dimerized during the production of the black tea. In the trial, one cup of green tea contained 73.49 mg of total catechins while one cup of black tea contained only 8.11 mg. In both types of the tea the most abundant catechin was EGCg (35.95 mg in green and 3.54 mg in black tea).

In our study, all three catechins showed excellent scavenging activities towards DPPH radical. In addition, their mixtures resulted in being good antiradicals and the highest inhibitions were observed with the combinations that included EGCg. The outcome of the study conducted by Hakim et al.[28] and the results of the experiments conducted in our laboratory suggest that the catechins could protect against free radicals caused by smoking.

In the case of quercetin, although we have demonstrated a good antioxidant potential with two *in vitro* tests, we can not make the comparison with the data from literature since the outcomes of the reviewed *in vivo* studies are

contradictory and the studies have numerous limitations (small number of participants, presence of other food constituent etc.).

Furthermore, contradictory results were obtained also for rutin. While our tests suggest that rutin may be a good antioxidant and antiradical agent, the study conducted by Boyle et al.[34] showed no influence of rutin supplement on the plasma antioxidant status. It has to be taken into consideration that only healthy non-obese normocholesterolaemic female volunteers in the age range 18–48 years participated in the trial. Future studies are needed to investigate the potential of rutin to increase the resistance to plasma oxidative stress. Specifically, the studies should include subjects with elevated levels of cholesterol in the blood and subjects exposed to the smoke-related free radicals.

Apigenin is the main flavone in parsley which contains low concentration of other flavonoids.[35] This dominance of apigenin makes parsley suitable for *in vivo* study of apigenin antioxidant properties. In fact, Nielsen et al.[35] conducted an intervention study with parsley and concluded that it has no influence on reduction of plasma protein oxidation. The results are in accordance with our findings that apigenin exhibits small antioxidant and negligible antiradical activities.

4. Conclusions

Although the study conducted in our laboratory suggests that flavonoids kaempferol, quercetin, rutin, EGCg, catechin, luteolin, epicatechin and myricetin possess strong antioxidant and antiradical properties, it is difficult to draw an overall conclusion from the reviewed *in vivo* human studies since the obtained results are sometimes contradictory and inconsistent. One of the biggest shortcomings is a small number and a not representative group of the examined subjects. Cited studies rarely take into consideration a number of factors (such as sex, age, overall health, living and working environment etc.) which could have strong influence on final results. Furthermore, in these *in vivo* studies the effects of dietary antioxidants have been analyzed using the biomarkers for oxidative damage. Even the more reliable biomarkers of oxidative activity in plasma (F2-isoprostanes) and urine (F2-isoprostanes, isoprostane metabolites, 8-hydroxy-20-deoxyguanosine [8OHdG], malondialdehyde) have numerous limitations. For example, the concentrations of malondialdehyde in the urine can be affected by changes in diet.[36]

Another limitation of the reviewed trials is that, due to the complexity of food composition, it is not possible to separate a specific antioxidant or a group of them from other food constituents and to study it individually. Very often various food components react in parallel or in subsequent order leading to the final result which can not be simply associated to a particular substance. Moreover, the active compound may not be the original (poly)phenol found in food, but rather one or more of its metabolites. Our analyses of antiradical activity of phenolic combinations also suggested that there might be interactions between flavonoids. These interactions are important for the understanding of biological activity of natural antioxidants. Future studies are needed to examine the synergism of phenolic compounds and the synergism with other compounds. Furthermore, it has to be taken in consideration that the (poly)phenols may also react as pro-oxidants although these pro-oxidant effects can also be beneficial as they raise the levels of antioxidant defense by imposing a mild degree of oxidative stress.[37]

The reviewed studies were focused on the dietary antioxidants that are absorbed through the gastrointestinal tract (GI) into the rest of the body. Halliwell et al.[38] develop the argument that the high levels of antioxidants present in certain foods and beverages play an important role in protecting the gastrointestinal tract itself from oxidative damage, and in delaying the development of the stomach, colon and rectal cancer. A strong support to their theory is the fact that the carotenoids and flavonoids do not seem to be absorbed as good as vitamins C and E are. Since their concentrations can be much higher in the lumen of the GI tract than in the plasma or in other body tissues, it is more likely that flavonoids and other polyphenols exhibit their antioxidant effects before absorption, within the GI tract itself.

In conclusion, more studies are required to determine antioxidant properties of natural phenolic compounds, their metabolites and their interactions in the GI tract. *In vitro* studies in environments simulating the GI tract conditions could be helpful for a better understanding of the mechanism of the phenolic compounds' reaction against free radicals. More detailed *in vivo* studies should be conducted on individuals with defined oxidative status since the activity of an antioxidant compound is strongly dependent on the initial conditions of the environment where they act as protectors against the reactive oxygen species.

5. Acknowledgement

The authors wish to acknowledge the financial support from the Slovenian Research Agency (Project P2-0046).

6. References

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Povzetek

Epidemiološke študije kažejo, da ima konzumiranje živil bogatih s polifenoli, predvsem s flavonoidi, preventivni vpliv na koronarno srčno bolezen. Flavonoidi naj bi vplivali na zmanjšanje oksidacije LDL holesterola *in vitro*. Namen naše študije je bil določiti antioksidativno aktivnost in radikalsko reaktivnost flavonoidov kot so miricetin, kvercetin, rutin, luteolin, apigenin, kempferol, katehin, epikatehin in epikatehin galat ter preučiti vpliv kemijske strukture in interakcij na sposobnost inhibicije oksidacije in vezavo prostih radikalov. Uporabili smo dve *in vitro* metodi: oksidacijo β-karotena v emulziji in DPPH (1,1-Diphenyl-2-picrylhydrazyl) metodo. V zaključku smo podali pregled ter analizo *in vivo* študij antioksidativne učinkovitosti flavonoidov v človeškem telesu ter jih primerjali z rezultati naših *in vitro* raziskav. Čeprav so vsi testirani flavonoidi razen apigenina pokazali močne antioksidativne in antiradikalske lastnosti *in vitro*, se ti rezultati ne ujemajo popolnoma z *in vivo* študijami, ki so včasih protislovne in nedosledne.