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The predictive value of the antioxidant capacity of structurally related flavonoids using the Trolox equivalent antioxidant capacity (TEAC) assay

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Abstract

The antioxidant capacity of a series of structurally related flavonoids is quantified by the amount of ABTS [2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate)] radical anions ($ABTS^{\bullet-}$) that is able to react with the flavonoid and expressed as the Trolox equivalent antioxidant capacity (TEAC). To evaluate the predictive value of the TEAC, the antioxidant activity of this series of flavonoids was also assessed in other in vitro assays, measuring the effect on hydroxyl scavenging, lipid peroxidation and doxorubicin-induced toxicity as typical scavenging or damage assays. The flavonoids tested were mono HER, di HER, tri HER, tetra HER and tri HEQ, differing in the number of aromatic hydroxyl groups. It was found that these compounds showed both a fast and slow scavenging effect in the TEAC assay and therefore the TEAC at 10 s ('fast' TEAC) and 6 min ('total' TEAC) was determined. Both this 'total' and 'fast' TEAC are negatively correlated with hydroxyl radical scavenging. The 'total' TEAC showed a better correlation than the 'fast' TEAC with the inhibition of lipid peroxidation and the protection against doxorubicin-induced toxicity. This indicates that beside the fast reaction of scavengers with the ABTS radical, also the slow reaction should be taken into consideration. It is concluded that the antioxidant capacity, assessed with the modified TEAC assay, can be useful to predict the in vivo antioxidant effect in a series of structurally related compounds.

Keywords: Antioxidants; ABTS; Antioxidant capacity; Free radicals; In vitro assay; TEAC

1. Introduction

An accumulating amount of data proves the pivotal role of free radicals in various (patho)physiological processes, like ageing, cancer and the toxicity of numerous compounds (Bast, 1994; Halliwell, 1987). This has stimulated research on the potential of intervening in these processes with antioxidants. Various in vitro tests to evaluate the efficacy of the antioxidants have been reported (for overview see Halliwell, Aeschbach, Löliger & Aruoma, 1995). These tests can roughly be divided in chemical assays, for the evaluation of the scavenging capacity, i.e. determination of the rate constant of the scavenging reaction between the antioxidant and reactive oxygen species, and more 'biological' assays, in which free radical damage is measured, e.g. quantification of the ability of antioxidants to inhibit free radical processes such as the peroxidation of biomembranes.

Recently, much attention has been focussed on determination of the total antioxidant capacity of compounds using the Trolox equivalent antioxidant capacity (TEAC) assay. This assay was first described by Miller, Rice-Evans, Davies, Gopinathan and Milner (1993), assessing the capacity of a compound to scavenge ABTS radicals. To circumvent interference in the radical generation process (Strube, Haenen, van den Berg & Bast, 1997), a modified TEAC assay using pregenerated ABTS radicals, as recommended by Re, Pellegrini, Proteggente, Pannala, Yang & Rice-Evans

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(1999) and van den Berg, Haenen, van den Berg and Bast (1999), was used.

In this report, we describe the results of a comparison study of the antioxidant capacity of a series of structurally related flavonoids using different methods. The different flavonoids have shown to give a wide variation in an antioxidative effect, i.e. the scavenging of peroxynitrite (Haenen, Paquay, Korthouwer & Bast, 1997). Next to the TEAC assay we measured the hydroxyl radical ($OH^{\bullet}$) scavenging activity, the activity to protect biomembranes against lipid oxidation (microsomal lipid peroxidation) and the protection against doxorubicin-induced toxicity in isolated left atria of mice hearts of these compounds, by comparing the antioxidant activity of the different compounds in the various assays, the predictive value of the TEAC assay is evaluated.

2. Materials and methods

2.1. Chemicals

2,2′-Azobis-(2-amidinopropane)HCl (ABAP) was purchased from Polysciences (Warrington, USA). 2,2′-Azinobis-(3-ethylbenzthiazoline-6-sulfonate) obtained as sulfonic acid ($ABTS^{2-}$) was from Sigma (St. Louis, USA). The hydroxyethylrutosides (HER) and trihydroxyethyl quercetin (HEQ) were a gift from Zyma (Nyon, Switzerland). All other chemicals used were of analytical grade.

2.2. Antioxidant capacity assay

The ABAP modified TEAC assay was used as reported elsewhere (Van den Berg et al., 1999). Briefly, an $ABTS^{\bullet-}$ solution was prepared by mixing 2.5 mM ABAP with 20 mM $ABTS^{2-}$ stock solution in 100 mM phosphate buffer (pH 7.4), containing 150 mM NaCl (PBS). The solution was heated for 12 min at 60°C, protected from light and stored at room temperature. To check $ABTS^{\bullet-}$ formation the absorbance at 734 nm was determined (absorption had to be between 0.35 and 0.40).

For measuring antioxidant capacity 40 μl of the sample was mixed with 1960 μl of the radical solution. Absorbance was monitored at 734 nm for 6 min. The decrease in absorption at 734 nm 10 s ('fast' reaction) and 6 min ('total' reaction) after addition of a compound was used for calculating the TEAC. The antioxidant capacity of the compounds was expressed relative to that of Trolox. All compounds were dissolved in PBS.

2.3. Hydroxyl radical scavenging

Hydroxyl radical scavenging was determined according to the method described by Halliwell, Gutteridge and Aruoma (1987). Hydroxyl radicals were generated by hydrogen peroxide, ascorbate and $FeCl_3$, in the presence or the absence of 100–500 μM of the test compound. The ability of the compound to compete with deoxyribose for scavenging hydroxyl radicals gives the rate constant of the reaction between hydroxyl radicals and the scavenger (ks).

2.4. Microsomal lipid peroxidation

Untreated male Wistar rats (Harlan Olec C.P.B., Zeist, The Netherlands), 220–250 g, were killed by decapitation. Liver microsomes were prepared as previously described Haenen and Bast (1983), and stored at −80°C. Before use, the microsomes were thawed, diluted five-fold with ice-cold Tris buffer (50 mM Tris–HCl, 150 mM NaCl, pH 7.4) and, in order to remove potentially protective cytosolic contamination (e.g. endogenous GSH), subsequently washed twice with the Tris buffer by centrifugation (40 min, $115\,000 \times g$ at 4°C). Finally, the pellet was resuspended in the Tris buffer and the microsomes (final concentration 1–1.5 mg microsomal protein/ml) were incubated at 37°C. Lipid peroxidation was induced by a combination of vitamin C (0.2 mM) and Fe^{2+} (10 μM). The reactions were started with the addition of iron. Lipid peroxidation was measured with the thiobarbituric acid assay, as previously described (Haenen & Bast), and determined as the absorption at 535 vs 600 nm (ΔA 535–600).

2.5. Doxorubicin-induced cardiotoxicity in the isolated left atrium

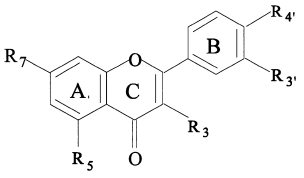
Untreated male Balb/c mice (Harlan Olec C.P.B., Zeist, The Netherlands), 18–22 g, were killed by decapitation. The hearts were rapidly excised. The isolated left atria were mounted in water jacketed organ baths, thermostated at 37°C, containing a Krebs buffer gassed with a mixture of 95% O_2 and 5% CO_2, resulting in a pH of the solution of 7.4. The composition of the Krebs buffer was (mM): NaCl (117.5), KCl (5.6), $MgSO_4$ (1.18), $CaCl_2$ (2.5), NaH_2PO_4 (1.28), $NaHCO_3$ (25) and glucose (5.5). The tension was adjusted to 0.40 g. Sixty min after mounting the atria, they were stimulated at a frequency of 4 Hz at a voltage of 1.5, the threshold voltage. Doxorubicin, at a concentration of 35 μM, was added in the absence or presence of 0.5 mM of the test compound. For tri HEQ, a concentration of 0.1 mM was used, due to the poor water solubility of this compound. After 1 h of incubation, the inotropy was measured. The reduction in the contractile force relative to the contractile force at the beginning of the incubation with doxorubicin was determined. Doxorubicin at a concentration of 35 μM induced a 50% reduction in contractile force (0% protection). The mean percentual protection provided by 0.5 mM of the test compound is given ($n = 2$).

3. Results

The time-dependent $ABTS^{\bullet-}$ scavenging capacity for the structurally related flavonoids (Fig. 1) tested is shown in Fig. 2. Trolox reacts instantaneously with the $ABTS^{\bullet-}$, and within a few seconds the reaction is completed. In contrast, tri HER reacts relative slowly with the $ABTS^{\bullet-}$, and the reaction takes several minutes. The other compounds (mono HER, di HER and tri HEQ) show combined effects and tetra HER displayed no antioxidant capacity.

In Fig. 3, the concentration-dependent effect of the compounds on the 'fast' reduction of the absorption measured at 10 s ('fast' TEAC), as well as the reduction of the absorption at 6 min ('total' TEAC) is depicted. It shows that at both time-points scavenging capacity was lineary related to the concentrations examined. The capacity factors relative to Trolox are given in Table 1.

Results obtained with the hydroxyl radical scavenging assay, the inhibition of lipid peroxidation and the ability to protect against doxorubicin-induced cardiotoxicity in the isolated left atrium are summarized in Table 2. In the hydroxyl radical scavenging assay tetra HER shows the highest scavenging activity followed by tri HER, di HER and mono HER respectively. Scavenging activity could not be determined for tri HEQ because its poor water solubility. With the lipid peroxidation assay tri HEQ showed the highest protection followed by mono HER, di HER, tri HER and tetra HER respectively. In the assay assessing protection against doxorubicin-induced cardiotoxicitity mono HER shows the highest protection followed by di HER, tri HER and tetra HER. Tri HEQ could only be tested at a lower concentration (0.1 mM) due to its poor water solubility. When mono HER was tested at the same concentration as tri HEQ a similar protection was found.



Compound	substituent R3	R5	R7	R3'	R4'
Mono HER	ORu	OH	OEtOH	OH	OH
Di HER	ORu	OH	OEtOH	OH	OEtOH
Tri HER	ORu	OH	OEtOH	OEtOH	OEtOH
Tetra HER	ORu	OEtOH	OEtOH	OEtOH	OEtOH
Tri HEQ	OH	OH	OEtOH	OEtOH	OEtOH

Ru = rutinose; Et = CH_2CH_2

Fig. 1. Chemical structures of the series of compounds tested.

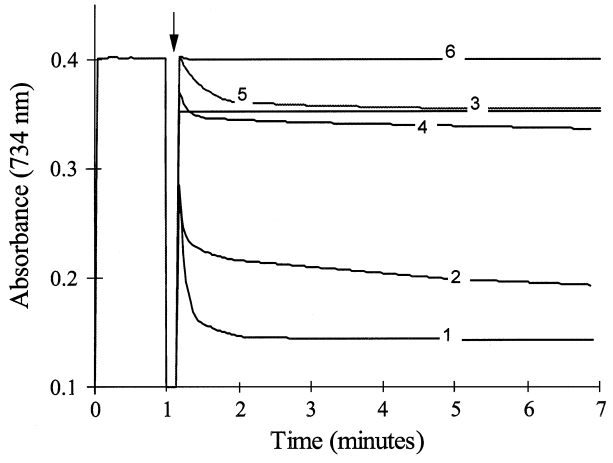


Fig. 2. Reduction of the absorption at 734 nm by the addition of Trolox and flavonoids. $ABTS^{\bullet-}$ were prepared using ABAP as radical source. The compounds were: (1) mono HER, (2) tri HEQ, (3) Trolox, (4) di HER, (5) tri HER and (6) tetra HER.

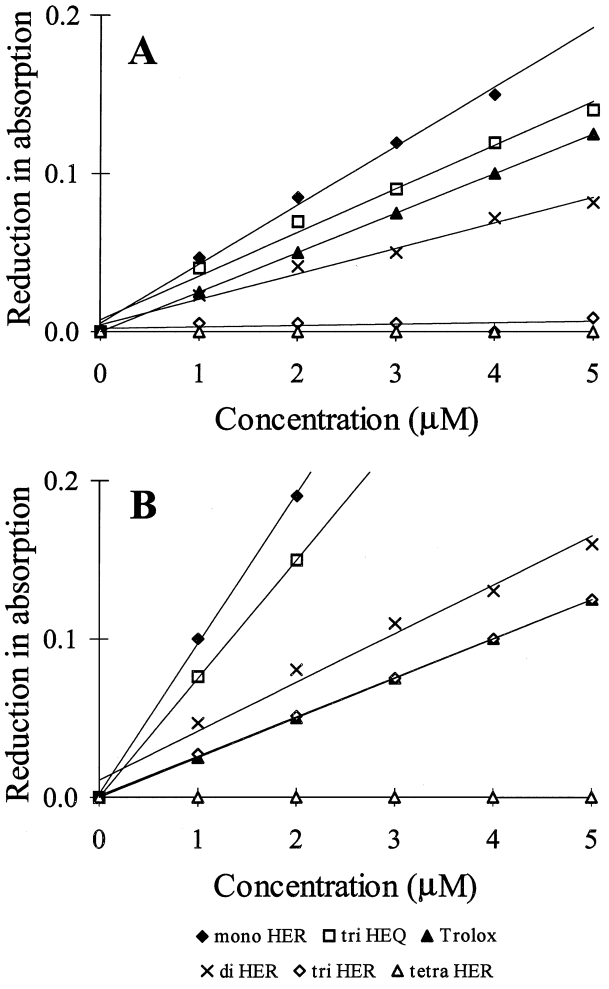


Fig. 3. Concentration dependent reduction of the absorption determined at (A) 10 s ('fast' TEAC) or (B) 6 min ('total' TEAC) after the addition of the test compounds. $ABTS^{\bullet-}$ were generated using ABAP. The compounds were: (◆) mono HER, (□) tri HEQ, (▲) Trolox, (×) di HER, (◇) tri HER and (△) tetra HER.

Table 1
Antioxidant capacity (TEAC) of a series of hydroxyethylrutosides[a]

Compound	TEAC at 10 s	TEAC at 6 min
Tetra HER	0.00 ± 0.00	0.00 ± 0.00
Tri HER	0.02 ± 0.01	1.0 ± 0.1
Di HER	0.7 ± 0.1	1.3 ± 0.2
Mono HER	1.3 ± 0.2	3.0 ± 0.2
Tri HEQ	1.1 ± 0.2	2.5 ± 0.2

[a] The TEAC at 10 s ('fast' TEAC) or at 6 min ('total' TEAC) after addition of the test compound to the solution containing the $ABTS^{\bullet-}$.

Table 2
Antioxidant parameters of a series of hydroxyethylrutosides

Compound	Hydroxyl radical scavenging (k_s, $M^{-1}s^{-1}$)	Inhibition of lipid peroxidation (EC_{50}, μM)	Protection against doxorubicin cardiotoxicity (%)[a]
Tetra HER	11.1 ± 2.2	6200 ± 200	25
Tri HER	10.2 ± 2.9	290 ± 24	28
Di HER	8.2 ± 3.5	210 ± 12	66
Mono HER	3.9 ± 2.2	15 ± 1.8	93
Tri HEQ	n.d.[b]	13 ± 1.4	41[c]

[a] The mean percentual protection provided by 0.5 mM of the test compound is given ($n = 2$).

[b] The hydroxyl radical scavenging activity could not be determined due to the poor water solubility.

[c] Due to the poor water solubility, a concentration of 0.1 mM was used. Mono Her gave a similar protection as tri HEQ when used at a concentration of 0.1 mM.

4. Discussion

The 'total' TEAC of the series of flavonoids tested correlated nicely with the number of aromatic hydroxyl groups. A similar relationship was found for another group of flavonoids (Rice-Evans, Miller & Paganga, 1997). The higher TEAC of tri HEQ compared to di HER — compounds with an equal amount of aromatic hydroxyl groups — points at a higher contribution of the hydroxyl group at the 3-position compared to that of the hydroxyl group at the 3′-position. The same structure-activity relationship has also been found for other activities (Haenen et al., 1997).

Free radicals formed in biological systems are usually very reactive and scavengers have to compete with vulnerable biological targets in the protection against free radical damage. Therefore, the fast scavenging reaction, as observed in the TEAC assay, seems more relevant than the slow scavenging reactivity. However, as the $ABTS^{\bullet-}$ has a relatively slow reactivity as compared to physiological important radicals, the slow scavenging reaction might also be relevant.

To evaluate the predictive value of the antioxidant capacity, measured as the TEAC, we compared both the TEAC at 10 s (fast reaction) as well as the TEAC at 6 min (total reaction) of a series of structurally related compounds, with variable antioxidant effect, to other antioxidant activities obtained in another screening assay or in a more biological system. Hydroxyl radicals are likely to be produced in vivo and are therefore physiological more relevant than $ABTS^{\bullet-}$. In the present study, it was found that the TEAC at 10 s and 6 min was negatively correlated with the hydroxyl radical scavenging activity, indicating that substituting the aromatic hydroxyl groups with hydroxyethyl groups increases the hydroxyl radical scavenging activity. This is not unexpected because the hydroxyl radical is a much stronger oxidant than $ABTS^{\bullet-}$ and is capable of H-abstraction from the hydroxyethyl group. The $ABTS^{\bullet-}$ does not react with the hydroxyethyl group. This is in line with the finding that $ABTS^{\bullet-}$ does not react with ethanol.

The negative correlation between hydroxyl radical scavenging and the TEAC could indicate that the $ABTS^{\bullet-}$ scavenging does not reflect physiological relevant scavenging. However, also a negative correlation between hydroxyl radical scavenging and protection against doxorubicin-induced toxicity in the atrium model was found. This might suggest that hydroxyl scavenging plays a minor role in the protection of flavonoids against doxorubicin toxicity. There is also a negative correlation found between hydroxyl scavenging and the protection against lipid peroxidation, suggesting that hydroxyl radical scavenging also plays a minor role in this protection. Previously, it has been shown that hydroxyl radical scavengers do not affect lipid peroxidation (Bast & Steegs, 1986). Therefore, screening with the hydroxyl radical scavenging assay appears not to be an useful tool to predict the antioxidant effect of the flavonoids for these activities.

Free radical mediated damage to biological systems is often studied using lipid peroxidation. With this assay tri HEQ showed the highest protection followed by mono HER, di HER, tri HER and tetra HER respectively. This shows that, with exception of tri HEQ, the compound with the highest number of aromatic hydroxyl groups shows the best protecting against lipid peroxidation and substituting these hydroxyl groups decreases the EC_{50} of the flavonoids. Tri HEQ that has no rutinose group, had a higher activity then expected on the basis of the number of aromatic hydroxyl groups. The lipophilicity of HEQ is expected to be higher than that of the other compounds, since tri HEQ is the only one that does not contain the hydrophilic rutinose moiety. A higher lipophilicity will result in a higher partition of tri HEQ into lipid membranes which may explain the higher activity of tri HEQ.

By using an isolated organ, the effect on a more integrated system can also be assessed. The results with this assay show that the compound with the highest number of aromatic hydroxyl groups shows best protection against doxorubicin-induced cardiotoxicity and substituting these hydroxyl groups decreases the protection of the compounds.

We have chosen the atrium model because there is a good correlation between the clinically observed cardiotoxicity and the protection against the doxorubicin-induced negative inotropic effect in the atrium (De Jong, Schoofs, Snabilie, Bast & van der Vijgh, 1993). The isolated left atrium model has been validated using a range of compounds with known cardiotoxicity (Van den Acker, submitted). The TEAC at 10 s as well as the TEAC at 6 min correlated with the protection against lipid peroxidation and doxorubicin-induced cardiotoxicity.

Here again, tri HEQ appeared to be an exception. For tri HER, there was a remarkable difference between the fast TEAC and the total TEAC. Tri HER only reacts slowly with the $ABTS^{\bullet-}$. Therefore, its fast TEAC is almost zero, whereas it has a substantial total TEAC. Tri HER displays a considerable antioxidant effect on lipid peroxidation and the cardiotoxicity induced by doxorubicin. These antioxidant effects are similar to those of di HER, a compound that has a substantial 'fast' TEAC. When only the fast TEAC is considered, tri HER is — erroneously — classified as having no antioxidant activity, comparable to tetra HER. The total TEAC predicts the right antioxidant potential of tri HER, i.e. an antioxidant activity comparable to that of di HER. From these data it can be seen that beside the fast reaction of scavengers with the $ABTS^{\bullet-}$, also the slow reaction should be taken into consideration.

The results of our study indicate that the improved TEAC can be used to screen structurally related compounds to predict their antioxidant capacity, provided that the total TEAC is used.

Abbreviations: ABAP, 2,2′-azobis-(2-amidinopropane) HCl; ABTS, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid); HER, hydroxyethyl rutoside; HEQ, hydroxyethyl quercetin; TEAC, Trolox equivalent antioxidant capacity.

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