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FLAVONOIDS ARE SCAVENGERS OF SUPEROXIDE ANIONS

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Abstract—Seven flavonoids and three non-flavonoid antioxidants, i.e. butylated hydroxyanisole, chlorpromazine and BW 755 C, were studied as potential scavengers of oxygen free radicals. Superoxide anions were generated enzymatically in a xanthine–xanthine oxidase system and non-enzymatically in a phenazine methosulphate–NADH system, and assayed by reduction of nitro blue tetrazolium. The generation of malonaldehyde (MDA) by the ascorbate-stimulated air-oxidised boiled rat liver microsomes was considered as an index of the non-enzymatic formation of hydroxyl radicals. Flavonoids but not non-flavonoid antioxidants lowered the concentration of detectable superoxide anions in both enzymic and non-enzymic systems which generated these SOD-sensitive radicals. The most effective inhibitors of superoxide anions were quercetin, myricetin and rutin. Four out of seven investigated flavonoids seemed also to suppress the activity of xanthine oxidase as measured by a decrease in uric acid biosynthesis. All ten investigated compounds inhibited the MDA formation by rat liver microsomes. Non-flavonoid antioxidants were more potent MDA inhibitors than flavonoids. It is concluded that antioxidant properties of flavonoids are effected mainly via scavenging of superoxide anions whereas non-flavonoid antioxidants act on further links of free radical chain reactions, most likely by scavenging of hydroxyl radicals.

The first product of the univalent reduction of oxygen is superoxide anion which during its interaction with hydrogen peroxide generates hydroxyl radicals. The secretory function of the vascular endothelium is influenced by these oxygen-derived free radicals. Superoxide anions destroy endothelium-derived relaxing factor (EDRF) [1] while hydroxyl radicals and lipid peroxides inhibit prostacyclin generation [2]. Quercetin and other flavonoids have been shown to scavenge free radicals [3–6] and their vasoprotective action has been associated with this particular property [6].

Using an enzymic [7, 8] and non-enzymic [9] biological generators of free radicals we have compared the free radical scavenging properties of quercetin and six other flavonoids with those of three well-known antioxidants, i.e. butylated hydroxyanisole (BHA), BW 755 C and chlorpromazine [10, 11].

MATERIALS AND METHODS

Xanthine oxidase (EC 1.1.3.22) activity was evaluated by the spectrophotometric measurement of the formation of uric acid from xanthine [8]. A 100 μM solution of xanthine in 0.1 M phosphate buffer pH 7.8 with 0.04 units/ml of xanthine oxidase was incubated for 10 min at room temperature and read at 295 nm against a blank sample which did not contain the enzyme. Various concentrations of flavonoids and non-flavonoid antioxidants were added to samples before the enzyme had been instilled and their effect on the generation of uric acid was used to calculate regression lines and IC_{50} values.

Superoxide anions were estimated by the spectrophotometric measurement of the product of the reduction of nitro blue tetrazolium (NTB) [9]. Superoxide anions were generated in an enzymic (xanthine–xanthine oxidase) and a non-enzymic (phenazine methosulphate–NADH) [9] system. The former comprised a solution of 100 μM of xanthine, 600 μM of NTB in 0.1 M phosphate buffer pH 7.4 and 0.07 units/ml of xanthine oxidase. This mixture was incubated at 25° for 10 min and read at 560 nm against blank samples which did not contain the enzyme. The non-enzymic generation of superoxide anions was measured in samples which contained 10 μM phenazine methosulphate, 78 μM NADH and 25 μM NBT in 0.1 M phosphate buffer pH 7.4. After 2 min of the incubation at room temperature the colour was read at 560 nm against blank samples which contained no phenazine methosulphate. IC_{50} values for the inhibition of the generation of superoxide anions by the investigated compounds were calculated as described above. The influence of superoxide dismutase (SOD) on enzymatic and non-enzymatic reduction of NBT was checked using Specord UV–VIS spectrophotometer. In the case of the non-enzymic system only the incubation mixture differed from that used with flavonoids and it was as follows: 630 μM of NBT, 30 μM of phenazine methosulphate and 156 μM NADH in 0.1 M phosphate buffer pH 7.4. Enzymic formation of superoxide anions was also estimated by reduction of cytochrome *c*. Samples containing 0.07 units/ml of xanthine oxidase, 100 μM of xanthine and 50 μM of cytochrome *c* were incubated for 3 min at the room temperature and read at 550 nm. Malonaldehyde (MDA) formation by the boiled rat liver microsomes in which lipid peroxidation had been stimulated by 200 μM of ascorbic acid was measured by a modi-

fication of the thiobarbituric acid method [6, 10], following the incubation of samples in the presence and in the absence of the investigated flavonoids and antioxidants at 37° for 90 min.

The following substances were tested: quercetin-(Fluka), rutin(Kock and Light Colnbrook, U.K.), 3-cyanidol (Zyma—Catergen *ex* tablets), meciadonol ZY 15029 (Zyma), troxerutin (Zyma—Venoruton *ex* ampulis), myricetin (obtained from the Department of Pharmacognosy, Academy of Medicine, Wrocław, Poland), quercetin (Schuchardt Chemische Fabrik), BW 755 C (obtained from Wellcome Research Labs, Beckenham, U.K.), BHA (butylated hydroxyanisole, Koch and Light), chlorpromazine (Polfa, Poland).

The following reagents were used: xanthine oxidase (Sigma, U.S.A.), xanthine(POCH Gliwice, Poland), NBT (Laboratorium Syntezy Organicznej, Jaworzno, Poland), *N*-methylphenazonium methosulphate (International Enzymes Limited), NADH (Sigma), superoxide dismutase (EC 1.15.1.1,3000 u/mg)(Sigma), cytochrome *c* from horse heart type III (Sigma).

RESULTS

The enzymic oxidation of xanthine to uric acid was inhibited by four flavonols out of seven investigated flavonoids (Table 1). The potency of their inhibitory action was encompassed within a narrow range of IC_{50} = 10–39 μM for aglycons (quercetin, myricetin), natural glycoside (rutin) and semisynthetic glycoside (troxerutin). Inactive as inhibitors of xanthine oxidase were the remaining three flavonoids and three investigated synthetic free radical scavengers when used at concentrations of 100–300 μM. These comprised two flavans (cyanidol and meciadonol) and a flavonol glycoside (quercitrin) as well as chlorpromazine, BW 755 C and BHA. Superoxide dismutase (SOD 1–100 units/ml) had no effect on the generation of uric acid from xanthine by xanthine oxidase.

Table 1. The influence of investigated compounds on xanthine oxidase activity

Tested compound	IC_{50} μM	*b*	*r*	N
Quercetin	10.0 ± 1.1	74.9	0.93	9
Myricetin	16.5 ± 1.3	59.5	0.88	8
Rutin	37.8 ± 0.9	59.2	0.81	7
Troxerutin	39.5 ± 1.0	50.2	0.88	7
Quercitrin	inactive at 100–300 μM			3
Cyanidol	inactive at 30–300 μM			10
Meciadonol	inactive at 30–300 μM			10
Chlorpromazine	inactive at 10–300 μM			4
BHA	inactive at 10–300 μM			3
BW 755 C	inactive at 1–100 μM			3
Superoxide dismutase	inactive at 1–100 units/ml			3

Samples containing 0.04 units/ml of xanthine oxidase and 100 μM of xanthine were incubated in phosphate buffer pH 7.8 for 10 min at room temperature. Then uric acid was estimated at 295 nm.

IC_{50} values were calculated from regression lines where: *x* was log of tested compound concentration and *y* was percent inhibition of enzyme activity.

b = slope of the regression line.

r = correlation coefficient.

N = number of experiments.

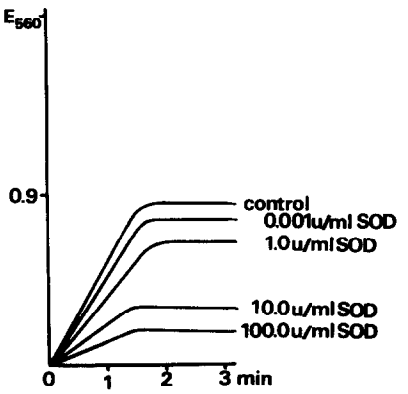

Fig. 1. The influence of superoxide dismutase on reduction of nitro blue tetrazolium by superoxide anions generated by xanthine oxidase in the presence of 100 μM of xanthine. Experimental conditions: analogical as in Table 2.

The xanthine–xanthine oxidase system also generated superoxide anions as measured by the reduction of NBT, the reaction which had been inhibited by superoxide dismutase in a concentration-dependent manner (Fig. 1). All seven investigated flavonoids inhibited the development of the colour produced during the reaction of superoxide anions with NBT, although the potency of their inhibitory action comprised a broad range of IC_{50} concentration between 20 and 225 μM (Table 2). Non-flavonoid antioxidants (NFA) were of no influence on the NBT reduction in this system (Table 2).

The specificity of the NBT method for the detection of the inhibition of superoxide anions by flavonols was additionally verified by replacement of NBT by cytochrome *c* in the xanthine–xanthine oxi-

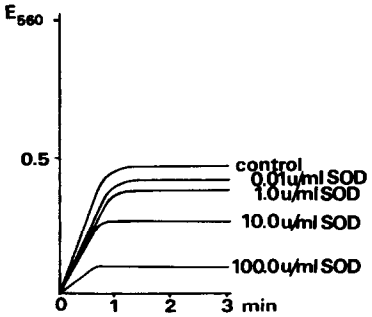

Fig. 2. The influence of superoxide dismutase on the reduction of nitro blue tetrazolium (NBT) by superoxide anions generated by phenazine methosulphate and NADH. The composition of the incubation mixture was as follows: 630 μM NBT, 30 μM phenazine methosulphate and 156 μM NADH in 0.1 M phosphate buffer pH 7.4.

Table 2. The influence of investigated compounds on nitro blue tetrazolium reduction caused by xanthine oxidase

Tested compounds	IC_{50} μM	b	r	N
Quercetin	42.3 ± 1.8	53.9	0.77	25
Myricetin	20.1 ± 1.1	73.8	0.87	8
Quercitrin	75.0 ± 0.3	195.3	0.91	6
Rutin	42.7 ± 1.4	54.8	0.88	12
Troxerutin	224.7 ± 0.7	57.7	0.93	9
Cyanidol	101.6 ± 1.2	31.0	0.85	9
Meciadonol	134.9 ± 1.2	28.2	0.95	9
Chlorpromazine	inactive at 1–300 μM			10
BHA	inactive at 0.01–100 μM			16
BW 755 C	inactive at 0.01–300 μM			19
Superoxide dismutase	72.8 ± 3.6 mU/ml	13.0	0.97	9

Samples containing 0.07 units/ml of xanthine oxidase and 100 μM of xanthine were incubated with 600 μM of nitro blue tetrazolium in 0.1 M phosphate buffer pH 7.4 in room temperature for 10 min. The extinction of the sample was read at 560 nm against a blank containing no enzyme. IC_{50} values were calculated as in Table 1.

dase systems. In this system, quercetin (50 μM) but not BW 755 C (300 μM) inhibited the reduction of cytochrome *c* to the same extent as NBT.

Our non-enzymic system (phenazine methosulphate–NADH) generated superoxide anions since superoxide dismutase in the concentration-dependent manner inhibited the reduction of NBT (Fig. 2). In that system all of the investigated flavonoids (at a range of IC_{50} from 12 to 78 μM) inhibited the reduction of NBT, whereas NFA were not active (Table 3).

All of the investigated flavonoids and NFA inhibited non-enzymic lipid oxidation in boiled liver microsomes as measured by the formation of MDA. On average NFA were by far more potent inhibitors than flavonoids. BW 755 C, for example, was ten times more potent as an inhibitor of formation of MDA than the most active flavonoid inhibitor—myricetin (Table 4).

DISCUSSION

The xanthine–xanthine oxidase system is frequently used as a generator of superoxide anions [7]. However, its primary function is to oxidize xanthine or hypoxanthine to uric acid. Therefore, looking for the free-radical scavenging properties of flavonoids in the enzymic system one has also to look for a possible inhibitory action of flavonoids on the primary function of the enzyme. We have confirmed the previous report [8] that quercetin but not quercitrin is an inhibitor of xanthine oxidase when uric acid is measured as a final product of the reaction. We have also found that three other flavonols (myricetin, rutin and troxerutin) inhibit, whereas two flavans (cyanidol and meciadonol) as well as three NFA (chlorpromazine, BHA and BW 755 C) do not inhibit the enzymatic activity (Table 1). In most cases flavonols are, while flavans and NFA are not, inhibitors of xanthine oxidase. However, the obvious exceptions are a flavonol rhamnoside—quercitrin—and dihydroquercetin [8]. Quercetin was found to inhibit superoxide generation by xanthine oxidase at a concentration of 10^{-5} M [12]. Also, the inhibition of superoxide generation by neutrophils in the presence of quercetin was described [13].

Table 3. The influence of investigated compounds on superoxide generation by phenazine methosulphate and NADH

Tested compound	IC_{50} μM	b	r	N
Quercetin	12.5 ± 0.9	58.5	0.88	8
Myricetin	14.3 ± 0.8	83.7	0.87	8
Quercitrin	45.9 ± 0.7	41.1	0.86	6
Rutin	15.4 ± 0.6	37.6	0.84	6
Troxerutin	77.6 ± 0.8	29.0	0.95	5
Cyanidol	67.9 ± 0.7	47.6	0.91	8
Meciadonol	51.9 ± 0.7	49.5	0.91	7
Chlorpromazine	inactive at 1–100 μM			7
BHA	inactive at 1–300 μM			9
BW 755 C	inactive at 1–100 μM			6
Superoxide dismutase	2185 ± 4.2 mU/ml	21.8	0.94	10

Samples containing 10 μM of phenazine methosulfate, 78 μM NADH and 25 μM nitro blue tetrazolium in 0.1 M phosphate buffer pH 7.4 were incubated for 2 min at the room temperature and read at 560 nm against a blank containing no phenazine methosulphate. IC_{50} values were calculated as in Table 1.

Table 4. The influence of tested compounds on malonaldehyde generation in boiled rat liver microsomes stimulated with ascorbate

Compound	IC_{50} μM	*b*	*r*	N
*Quercetin	9.4 ± 0.6	98.7	0.85	6
Myricetin	1.4 ± 1.0	89.5	0.91	10
Quercitrin	7.6 ± 1.7	56.4	0.80	12
*Rutin	71.9 ± 0.5	128.7	0.91	5
Troxerutin	123.2 ± 0.9	55.0	0.91	6
*Cyanidol	64.3 ± 1.4	34.4	0.89	6
*Meciadonol	61.0 ± 1.4	33.0	0.89	6
†Chlorpromazine	47.6 ± 0.2	414.0	0.85	10
BHA	0.47 ± 1.7	54.2	0.80	17
†BW 755 C	0.16 ± 0.4	240.0	0.83	12
Superoxide dismutase inactive at 1–1000 units/ml				4

Inactive rat liver microsomes were incubated with 200 μM ascorbate in 0.1 M phosphate buffer pH in 37° for 90 min. Then trichloracetic acid was added and samples were centrifuged. Supernatant was heated with thiobarbituric acid and estimated at 532 nm.

* Published previously [6].

† Published previously [10].

trophils in the presence of quercetin was described [13].

We have shown that all seven investigated flavonoids are scavengers of the SOD-sensitive free radicals which are generated during the activity of xanthine oxidase. NFA are deprived of this property (Table 2). In other words, they are flavonoids which are not inhibitors of xanthine oxidase but they are superoxide anion scavengers (quercitrin, cyanidol, meciadonol). In the next series of experiments it has been shown that all seven of the investigated flavonoids are scavengers of superoxide anions when these SOD-sensitive free radicals are generated by a non-enzymic system comprising phenazine methosulphate and NADH. Again NFA show no activity in this system (Table 3). Therefore, it is obvious that all of the investigated flavonoids are superoxide anion scavengers whereas some of them are also xanthine oxidase inhibitors. A net effect of flavonoids with the dual action of the xanthine oxidase functioning is very complicated, therefore a comparison between the results presented in Tables 1 and 2 has not been made. Unlike flavonoids NFA are not scavengers of superoxide anions.

The only system in which NFA show similar properties to the investigated flavonoids is the non-enzymic oxidation of lipids as evidenced by the formation of MDA [5, 14–16]. Both NFA and flavonoids are inhibitors of the MDA formation. It is generally accepted that lipid oxidation is initiated by hydroxyl radicals [5, 12], although recently, this direct relationship has been questioned [13, 14]. However, even in the process of non-enzymic lipid oxidation there is a profound difference between NFA and flavonoids in their inhibitory mechanism in relation to the formation of MDA. We have shown here that NFA are not scavengers of superoxide anions and thus their inhibitory action on the MDA formation might be related to a direct scavenging of hydroxyl radicals. Other mechanisms like interaction with hydrogen peroxide [13], with iron or lipoxyl radicals cannot be excluded. On the other hand, the most plausible explanation for the inhibitory action of flavonoids on the formation of MDA is their scavenging action towards the precursors of hydroxyl radicals, i.e. superoxide anions. IC_{50} values for each flavonoid (except for myricetin and quercetin) in Tables 2, 3 and 4 are close enough to each other for a speculation that flavonoids prevent lipid oxidation by scavenging superoxide anions which initiate the chain of free radical reactions leading finally to lipid oxidation [5]. Myricetin and quercetin might have an additional mechanism of antioxidant action.

In summary, we believe that the antioxidant properties of flavonoids [3–6] are common to aglycons and glycosides of flavonols and flavans and that these properties depend on breaking the chain of formation of free radicals at a "high level", actually at its very beginning, i.e. at the level of superoxide anions [5]. This mechanism of the antioxidant action of flavonoids is different from that of synthetic antioxidants such as butylated hydroxyanisole, BW 755 C or chlorpromazine, which seem to be "low level" scavengers of hydroxyl radicals. This unique type of antioxidant action of flavonoids may explain their hepatoprotective [5] and vasoprotective [6] properties which are organ-directed by the selective binding of flavans (e.g. cyanidol) to hepatocytes [5] or to flavonols (e.g quercetin) to thrombocytes [6]. In this last case flavonols, by removing superoxide anions from the vicinity of mural thrombi adhering to vascular walls, may directly prolong half-life of the endothelium-derived relaxing factor (EDRF) [1] and indirectly protect prostacyclin synthetase from being destroyed by hydroxyl radicals and lipid peroxides [2]. Some of the flavonoids may have an additional mechanism of antioxidative action, e.g. quercitrin (as shown here).

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REFERENCES

1. R. J. Gryglewski, R. M. J. Palmer and S. Moncada, *Nature* **320**, 454 (1986).
2. R. J. Gryglewski, S. Bunting, S. Moncada, R. Flower and J. R. Vane, *Prostaglandins* **12**, 685 (1976).
3. L. Cavallini, A. Bindoli and N. Siliprandi, *Pharmac. Res. Commun.* **10**, 133 (1978).
4. J. Bauman, F. v. Bruchhausen and G. Wurm, *Prostaglandins* **20**, 627 (1980).
5. T. F. Slater and R. Scott, in *(+)Cyanidol in the Diseases of Liver* (Ed. H. O. Conn) p. 33. Academic Press London and Royal Society of Medicine, London (1981).
6. R. J. Gryglewski, R. Korbut, J. Robak and J. Swies, *Biochem. Pharmac.* **36**, 317 (1986).
7. J. M. McCord and I. Fridovich, *J. biol. Chem.* **243**, 5753 (1968).
8. A. Bindoli, M. Valente and L. Cavallini, *Pharmac. Res. Commun.* **17**, 831 (1985).
9. M. Nikishimi, N. A. Rao and K. Yagi, *Biochem. biophys. Res. Commun.* **46**, 849 (1972).
10. Z. Duniec, J. Robak and R. J. Gryglewski, *Biochem. Pharmac.* **32**, 2283 (1983).
11. G. Cohen and R. E. Hekkila, *J. biol. Chem.* **249**, 2447 (1974).

12. W. W. Busse, D. E. Kopp and E. Middleton, *J. Allergy clin Immun.* **73**, 801 (1984).
13. C. Pagonis, A. J. Tauber, N. Pavlotsky and E. R. Simons, *Biochem. Pharmac.* **35**, 237 (1986).
14. K. L. Fong, P. B. Cay and J. L. Poyer, *J. biol. Chem.* **248**, 7792 (1978).
15. A. Bast and M. H. M. Steeghs, *Experientia* **42**, 555 (1986).
16. O. Beloqui and A. J. Cederbaum, *Biochem. Pharmac.* **35**, 2663 (1986).