Superoxide Scavenging Properties of Flavonoids in a Non-Enzymic System

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The superoxide anion scavenging activity of 38 flavonoids, some of them isolated from *Sideritis mugronensis, Sideritis javalambrensis* and *Cayaponia tayuya* were investigated by measurement of their inhibition of nitroblue tetrazolium reduction. Isoorientin, orientin, amentoflavone, leucocyanidol, eriodictyol, datiscetin and robinetin behaved as potent scavengers and structure-activity relationships were established.

Introduction

Superoxide anion ($O_2^{\cdot-}$) can dismutate to H_2O_2 and O_2 and is also able to give raise to hydroxyl radical by the iron-catalyzed Fenton reaction [1]. These activated oxygen species induce polyunsaturated fatty acids peroxidation and radical propagation, which possibly determine destabilization and rupture of cell membranes. Thus, oxygen free radicals have been implicated in the development of different conditions, *e.g.* inflammatory-immune injury, reperfusion injury, cancer and atherosclerosis [1–3].

Flavonoids are natural antioxidants possessing a wide range of biological activities. A number of them have shown inhibitory effects in different experimental models of inflammation [4]. It has been suggested that the prevention of biooxidative processes by this class of compounds is due to an oxygen-free radical scavenging effect which prevents membrane lipid peroxidation [5] and the ability to remove superoxide radicals at the inflamed site is one of the possible mechanisms responsible for inhibition of the inflammatory process [6].

In the present work we have tested a series of natural flavonoids, like polymethoxyflavones isolated by us from the species *Sideritis mugronensis* and *Sideritis javalambrensis* (Lamiaceae) as well as C-glycosyl flavones from *Cayaponia tayuya* (Cucurbitaceae), as superoxide scavengers by their ability to inhibit NADH-initiated nitroblue tetrazolium reduction. In addition, 30 commercially available compounds have been included to permit the establishment of structure-activity relationships. As a whole, the possible superoxide scavenging effect of 25 flavonoids has been studied for the first time.

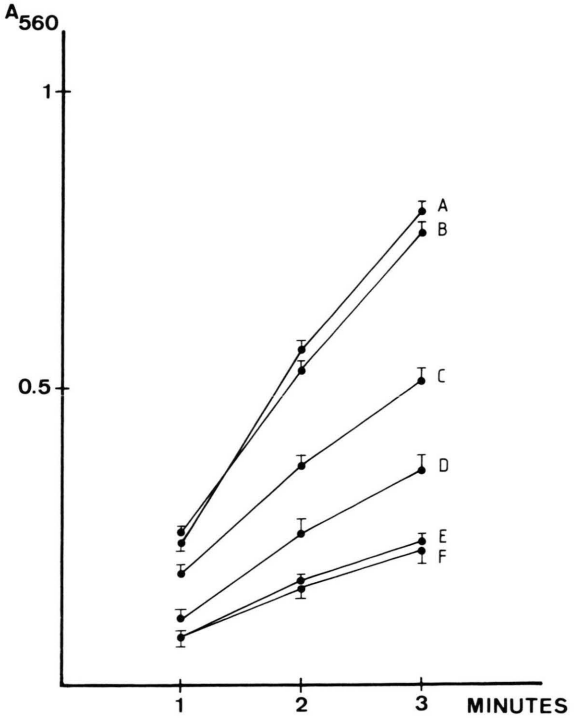

Fig. 1. Inhibition of NBT reduction by SOD. A (control); B (SOD 1 unit/ml); C (SOD 5 units/ml); D (SOD 10 units/ml); E (SOD 50 units/ml); F (SOD 100 units/ml). Data are the mean of 3 experiments.

Abbreviations: NBT, nitroblue tetrazolium; PMS, phenazine methosulfate; SOD, superoxide dismutase.

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Results

Under aerobic conditions, the addition of PMS to the reaction mixture provoked NBT reduction, a process in which $O_2^{\cdot}$ may serve as an electron donor. The reaction was inhibited by SOD, with 50% of the maximum inhibition achieved by 11.33 units/ml. Fig. 1 shows the time course of the absorbance change at 560 nm.

Any compound that did not inhibit by at least 50% of control at the initial screening concentration of 100 μM was not tested further. By this criterion, the active flavonoids were tested over a range of concentrations of 10 to 100 μM to determine their half-maximal concentration for inhibition of NBT reduction. This was calculated from the percent inhibition-log concentration regression line.

We have shown that 17 out of the 39 investigated flavonoids are potent scavengers of $O_2^{\cdot}$ anions generated by a non-enzymic system comprising PMS and NADH. Some compounds, such as (+)-catechin and quercetin, have been mentioned as strong scavengers in both enzymic and non-enzymic systems [7–9], although they have behaved in our experiments as inhibitors less potent than expected.

The structure of flavonoids tested and their inhibition of NBT reduction are presented in Table I. Our results provide new information on the structure-activity relationship underlying the $O_2^{\cdot}$ radical scavenging effect of flavonoids. The differences in activity are mainly accounted for by the number and position of free hydroxyls, as the most active compounds possess from 3 to 6 OH and their inhibitory potency increases with the number of free OH functions in the molecule. Flavones and flavanones exhibited a similar behaviour, with a slight increase in potency resulting from the resonance between B and C rings.

The results presented in this paper confirm the importance of an *o*-dihydroxy function observed by other authors [7, 8] in the flavone and flavanone groups, followed by 4′-OH substitution. Thus, eriodictyol and apigenin are more potent than naringenin and chrysin, respectively. Further hydroxylation in the 3 position seems to be detrimental, while features such as a resorcinol moiety, in either A or B rings, or a pyrogallol group in B ring, as well as a free 2′-OH increase the inhibitory potency.

Blockade of active OH by glycosylation or introduction of hydroxyethyl groups is detrimental, while if it occurs on a OH having a negative influence, the activity of the compound increases. This can be observed by comparing the aglycone-glycoside pairs apigenin–rhoifolin and quercetin–rutin, as well as rutin with troxerutin.

The introduction of one methoxy group decreases the potency of the resulting compound, as seen for acacetin and tamarixetin, which are monomethoxy derivatives of apigenin and quercetin, while polymethoxylated flavones are weak inhibitors of NBT reduction or inactive, which suggests that the presence of methoxy groups can counteract the influence of an *o*-dihydroxy function.

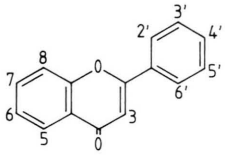
The presence of a sugar moiety directly attached to the flavonoid nucleus can affect the activity in different ways, depending on the pattern of hydroxylation present in the compound. Thus, 8-C-glycosides possessing 5,7,4′-OH are less potent than the corresponding aglycones (as seen in the C-glycosides of apigenin), nevertheless in 5,7,3′,4′-OH derivatives the glycosilation at C-8 does not have any noticeable effect while at C-6 it increases the potency (*cf.* luteolin with isoorientin and orientin).

Discussion

We have established the superoxide scavenging activity of a series of flavonoids belonging to different structural groups and possessing from 3 to 6 free hydroxyls. In our experiments it has been demonstrated for the first time that isoorientin, orientin, amentoflavone, leucocyanidol, eriodictyol, datiscetin and robinetin possess a potency comparable to that of previously known scavengers of superoxide anions, such as morin, rutin or luteolin [7]. In the series of flavonoids isolated by us from medicinal plants, vicenin-2, sideritoflavone, 5-O-demethylnobiletin, spinosin, gardenin D, cirsimaritin and 8-methoxycirsilineol have shown inhibitory effects to a lesser extent, while cirsiliol and xanthomicrol are inactive. This scavenging effect can depend on hydrogen atom donation [9] and may provide a basis for the pharmacological activity and therapeutical applications of flavonoids. Vasoprotective properties have been related to protection of endothelium-derived re-

Table I. Structure, percentage of inhibition (% I) at 100 μM and inhibitory concentration 50 (IC_{50}) of the flavonoids tested.

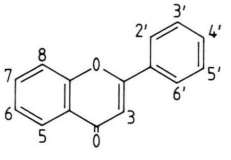
Flavones



Name	5	6	7	8	3′	4′	% I (100 μM)	IC_{50} μM
Flavone	H	H	H	H	H	H	−9.0**	
Chrysin	OH	H	OH	H	H	H	37.0**	
Apigenin	OH	H	OH	H	H	OH	82.4**	62.0
Vitexin	OH	H	OH	Gl	H	OH	30.0**	
Vicenin-2	OH	Gl	OH	Gl	H	OH	25.1**	
Rhoifolin	OH	H	ONh	H	H	OH	28.4**	
Acacetin	OH	H	OH	H	H	OCH_3	34.3**	
Spinosin	OH	So	OCH_3	H	H	OH	21.1**	
Cirsimaritin	OH	OCH_3	OCH_3	H	H	OH	11.2**	
Luteolin	OH	H	OH	H	OH	OH	97.7**	35.2
Isoorientin	OH	Gl	OH	H	OH	OH	79.5**	16.3
Orientin	OH	H	OH	Gl	OH	OH	79.9**	31.5
Diosmin	OH	H	ORu	H	OH	OCH_3	5.0	
Xanthomicrol	OH	OCH_3	OCH_3	OCH_3	H	OH	3.0	
Cirsiliol	OH	OCH_3	OCH_3	H	OH	OH	−0.8	
Sideritoflavone	OH	OCH_3	OCH_3	OCH_3	OH	OH	24.8**	
Gardenin-D	OH	OCH_3	OCH_3	OCH_3	OH	OCH_3	19.7**	
8-Methoxycirsilineol	OH	OCH_3	OCH_3	OCH_3	OCH_3	OH	9.4*	
5-Demethylnobiletin	OH	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	22.3**	

* $P < 0.05$.
** $P < 0.01$.
Gl = glucose; Nh = neohesperidose; Ru = rutinose; So = sophorose.

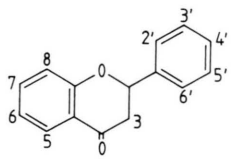
Flavonols



Name	3	5	7	2′	3′	4′	5′	% I (100 μM)	IC_{50} μM
3-Hydroxyflavone	OH	H	H	H	H	H	H	−5.3*	
Galangin	OH	OH	OH	H	H	H	H	71.3**	68.3
Datiscetin	OH	OH	OH	OH	H	H	H	91.8**	40.6
Kaempferol	OH	OH	OH	H	H	OH	H	50.2**	99.1
Fisetin	OH	H	OH	H	OH	OH	H	39.5**	
Morin	OH	OH	OH	OH	H	OH	H	98.3**	31.4
Quercetin	OH	OH	OH	H	OH	OH	H	51.9**	100.0
Rutin	ORu	OH	OH	H	OH	OH	H	75.7**	19.7
Troxerutin	ORu	OH	OHE	H	OHE	OHE	H	53.3**	73.5
Tamarixetin	OH	OH	OH	H	OH	OCH_3	H	30.0**	
Robinetin	OH	H	OH	H	OH	OH	OH	97.2**	41.2

* $P < 0.05$.
** $P < 0.01$.
Ru = rutinose; OHE = O-hydroxyethyl.

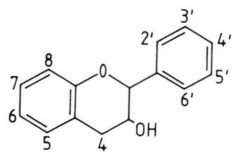
Flavanones



Name	5	7	3′	4′	% I (100 μM)	IC_{50} μM
Naringenin	OH	OH	H	OH	57.0**	91.3
Naringin	OH	ORu	H	OH	41.1**	
Eriodictyol	OH	OH	OH	OH	92.4**	47.1
Hesperidin	OH	ORu	OH	OCH_3	20.1**	

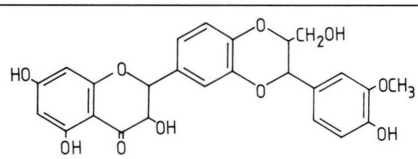
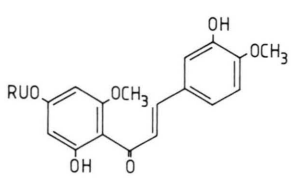
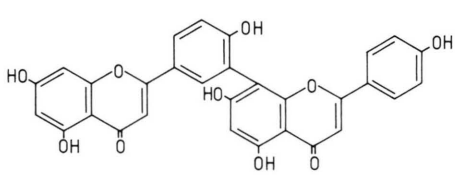
* $P < 0.05$.
** $P < 0.01$.
Ru = rutinose.

Flavanols



Name	4	5	7	3′	4′	% I (100 μM)	IC_{50} μM
Catechin	H, H	OH	OH	OH	OH	45.8**	
Leucocyanidol	H, OH	OH	OH	OH	OH	82.9**	39.1

* $P < 0.05$.
** $P < 0.01$.

Name		% I (100 μM)	IC_{50} μM
Dihydroflavonols Silybin		70.4**	67.5
Chalcones Hesperidin-methylchalcone		34.0**	
Biflavones Amentoflavone		98.4**	31.8

* $P < 0.05$.
** $P < 0.01$.
Ru = rutinose.

laxing factor and prostacyclin synthetase from inactivation by oxygen-derived radicals and lipid peroxides [10, 11]. Furthermore, free radical scavengers of the flavonoid class, such as anthocyanosides, protect collagen fibrils from degradation by superoxide anions [12] and it is also known than flavonoids can activate collagen biosynthesis and reticulation of collagen fibrils [13].

Finally, many flavonoids are endowed with anti-inflammatory activity [4] and a superoxide scavenging effect has been demonstrated for some of them, such as fisetin, quercetin, rutin and (+)-catechin. It should be noted that sideritoflavone and cirsiliol, possessing anti-inflammatory properties [14, 15] lack the sufficient superoxide scavenging ability to justify their pharmacological effects. Nevertheless possible interactions with other free radicals should not be excluded, on account of which there would be necessary further experiments to know the role of different free radicals in the anti-inflammatory activity of this class of compounds.

Materials and Methods

The following flavonoids were tested: Spinosin and vicenin-2 were isolated from *Cayaponia tayuya* [16]; 5-O-demethylnobiletin and gardenin D from *Sideritis mugronensis* [17]; cirsiliol, cirsimaritin, 8-methoxycirsilineol, sideritoflavone and xanthomicrol were isolated from *Sideritis javalambrensis* following known procedures [18].

Other flavonoids were commercially available: fisetin, flavone, 3-hydroxyflavone (Aldrich); troxerutin (Almirall); diosmin (Faes); quercetin (Merck); hesperidin methylchalcone (Pierre Fabre SAE); acacetin, amentoflavone, apigenin, datiscetin, eriodictyol, galangin, isoorientin, luteolin, orientin, robinetin, silybin, tamarixetin and vitexin (Roth); leucocyanidol (Rovi); kaempferol (Sarsyntex); hesperidin (Seber); rhoifolin, (+)-catechin, chrysin, morin, naringenin, naringin and rutin (Sigma).

The following reagents were used: β-NADH, PMS, NBT and SOD were purchased from Sigma. All other reagents were of analytical grade.

The $O_2^{\cdot}$ scavenging effect of flavonoids was tested following the procedure of Slater and Eakins [9] with some modifications. The incubation mixture contained NADH (166 μM), NBT (43 μM), test compound and phosphate buffer 19 mM, pH 7.4, with a final volume of 3.1 ml. The addition of PMS (2.7 μM) provoked the reduction of NBT, which was followed by measuring the absorbance (560 nm, Lambda 1 Perkin-Elmer spectrophotometer) at 20 °C during 3 min, a period in which the absorbance increased linearly. Flavonoids were dissolved in 0.5% Na_2CO_3 or mixtures of 0.5% Na_2CO_3 with ethanol or dimethylsulfoxide 1/3 (v/v), according to the solubility of the compound. The amount of solvent never exceeded 1% of the final volume in the reaction and control incubations contained the same volume of the vehicle used. After substracting the appropriate blanks, reaction rate was used to calculate the percentage of inhibition due to the presence of flavonoid. Statistical analysis was performed using two-tailed Student's *t*-test for unpaired samples.

Acknowledgements

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