

## Structure–Activity Relationship and Classification of Flavonoids as Inhibitors of Xanthine Oxidase and Superoxide Scavengers

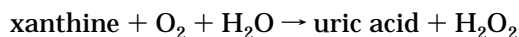
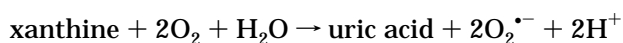
Paul Cos, Li Ying, Mario Calomme, Jia P. Hu, Kanyanga Cimanga, Bart Van Poel, Luc Pieters, Arnold J. Vlietinck, and Dirk Vanden Berghe\*

Department of Pharmaceutical Sciences, University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium

Received May 9, 1997

The structure–activity relationship of flavonoids as inhibitors of xanthine oxidase and as scavengers of the superoxide radical, produced by the action of the enzyme xanthine oxidase, was investigated. The hydroxyl groups at C-5 and C-7 and the double bond between C-2 and C-3 were essential for a high inhibitory activity on xanthine oxidase. Flavones showed slightly higher inhibitory activity than flavonols. All flavonoid derivatives except isorhamnetin (**30**) were less active than the original compounds. For a high superoxide scavenging activity on the other hand, a hydroxyl group at C-3' in ring B and at C-3 were essential. According to their effect on xanthine oxidase and as superoxide scavengers, the flavonoids could be classified into six groups: superoxide scavengers without inhibitory activity on xanthine oxidase (category A), xanthine oxidase inhibitors without any additional superoxide scavenging activity (category B), xanthine oxidase inhibitors with an additional superoxide scavenging activity (category C), xanthine oxidase inhibitors with an additional pro-oxidant effect on the production of superoxide (category D), flavonoids with a marginal effect on xanthine oxidase but with a pro-oxidant effect on the production of superoxide (category E), and finally, flavonoids with no effect on xanthine oxidase or superoxide (category F).

The enzyme xanthine oxidase catalyses the oxidation of hypoxanthine and xanthine to uric acid, which plays a crucial role in gout.<sup>1</sup> During the reoxidation of xanthine oxidase, molecular oxygen acts as electron acceptor, producing superoxide radical and hydrogen peroxide.<sup>2</sup> These reactions can be written as follows:<sup>3</sup>



Consequently, xanthine oxidase is considered to be an important biological source of superoxide radicals. These and other reactive oxygen species (ROS) contribute to the oxidative stress on the organism and are involved in many pathological processes such as inflammation, atherosclerosis, cancer, aging, etc.<sup>4</sup>

Flavonoids are a group of natural products with many biological and pharmacological activities; antibacterial, antiviral, antioxidant, and antimutagenic effects and inhibition of several enzymes have been demonstrated.<sup>5,6</sup> It has been reported that flavonoids inhibit xanthine oxidase<sup>7</sup> and have superoxide scavenging activities.<sup>8,9</sup> Therefore, flavonoids could be a promising remedy for human gout and ischemia by decreasing both uric acid and superoxide concentrations in human tissues.<sup>10</sup> In the present work, the structure–activity relationship and classification of flavonoids as inhibitors of xanthine oxidase and scavengers of superoxide were investigated.

### Results and Discussion

Both inhibition of xanthine oxidase and the scavenging effect on the superoxide anion were measured in one

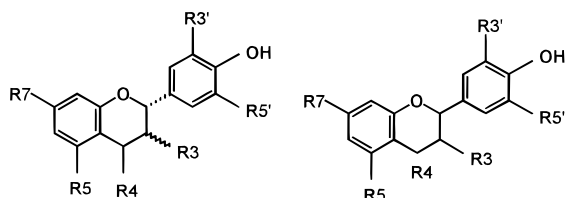
assay. Inhibition of xanthine oxidase results in a decreased production of uric acid, which can be measured spectrophotometrically, and a decreased production of superoxide, measured by the nitrite method. For each flavonoid tested two IC<sub>50</sub> values (50% inhibitory concentrations) can be calculated by linear regression analysis: 50% inhibition of xanthine oxidase (= 50% decrease of uric acid production) and 50% reduction of the superoxide level. The half-maximal inhibitory concentrations of the flavonoid aglycons are listed in Table 1. (±)-Taxifolin (**1**), (+)-catechin (**2**), (–)-epicatechin (**3**), and (–)-epigallocatechin (**4**) (Chart 1) did not inhibit xanthine oxidase up to the highest concentration tested (100 μM). 4'-Hydroxyflavone (**5**) and naringenin (**6**) were tested in concentrations up to 30 and 50 μM since their solubility was limited. No inhibition of uric acid production was observed. It was concluded that flavanones, dihydroflavonols, and flavanols were not capable of inhibiting xanthine oxidase. Therefore, the effects measured on superoxide in the presence of dihydroflavonols and flavanols were considered as superoxide-scavenging activities. Only the flavonols quercetin (**15**,  $p < 0.0001$ ), fisetin (**16**,  $p < 0.0001$ ), and myricetin (**18**,  $p < 0.0001$ ) have lower IC<sub>50</sub> values for the reduction of the superoxide level than for the inhibition of xanthine oxidase, which indicates an additional superoxide scavenging activity. The presence of a hydroxyl group at C-3' in ring B and at C-3 is associated with a high superoxide scavenging activity, which is confirmed by the lack of scavenging activity in the case of the flavonols galangin (**13**), kaempferol (**14**), and morin (**17**) (Chart 2). The highest superoxide-scavenging activities were observed for epigallocatechin

\* To whom correspondence should be addressed. Phone: (32) 3 820 25 42. Fax: (32) 3 820 25 44. E-mail: microfar@uia.ua.ac.be.

**Table 1.** IC<sub>50</sub> Values of Flavonoids and Allopurinol for Inhibition of Xanthine Oxidase and Reduction of Superoxide Level

compd		xanthine oxidase IC <sub>50</sub> (μM) ± SD	superoxide IC <sub>50</sub> (μM) ± SD	category <sup>a</sup>
(±) taxifolin	<b>1</b>	>100	1.73 ± 0.12	A
(+)-catechin	<b>2</b>	>100	1.61 ± 0.04	A
(-)-epicatechin	<b>3</b>	>100	1.59 ± 0.08	A
(-)-epigallocatechin	<b>4</b>	>100	0.48 ± 0.02	A
4'-hydroxyflavanone	<b>5</b>	>30	>18	F
naringenin	<b>6</b>	>50	>50	F
7-hydroxyflavanone	<b>7</b>	38.0 ± 7.0	>100	E
chrysin	<b>8</b>	0.84 ± 0.13	1.87 ± 0.21	D
apigenin	<b>9</b>	0.70 ± 0.23	1.33 ± 0.04	D
luteolin	<b>10</b>	0.55 ± 0.04	1.13 ± 0.16	D
baicalein	<b>11</b>	2.79 ± 0.01	2.72 ± 0.02	B
3-hydroxyflavone	<b>12</b>	>100	>100	F
galangin	<b>13</b>	1.80 ± 0.07	6.74 ± 0.32	D
kaempferol	<b>14</b>	1.06 ± 0.03	0.84 ± 0.04	B
quercetin	<b>15</b>	2.62 ± 0.13	1.63 ± 0.02	C
fisetin	<b>16</b>	4.33 ± 0.19	1.84 ± 0.07	C
morin	<b>17</b>	10.1 ± 0.70	9.1 ± 0.08	B
myricetin	<b>18</b>	2.38 ± 0.13	0.33 ± 0.03	C
allopurinol		0.24 ± 0.01	0.23 ± 0.01	B
luteolin + epigallocatechin (1:1)		0.76 ± 0.08	0.19 ± 0.02	
manganese superoxide dismutase			(4.89 ± 0.18) × 10 <sup>-4</sup>	

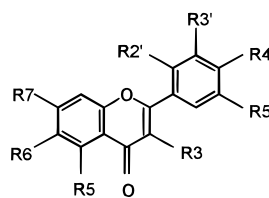
<sup>a</sup> According to their effect on xanthine oxidase and as superoxide scavenger the flavonoids were classified into specific categories (see Table 3).

**Chart 1**

compound	structure	R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>	R <sub>7</sub>	R <sub>3'</sub>	R <sub>5'</sub>
1	B	OH	= O	OH	OH	OH	H
2	A	OH (β)	H	OH	OH	OH	H
3	A	OH (α)	H	OH	OH	OH	H
4	A	OH (α)	H	OH	OH	OH	OH
5	B	H	= O	H	H	H	H
6	B	H	= O	OH	OH	H	H

(**4**) and myricetin (**18**), which are compounds with a hydroxyl group at C-3 and three hydroxyl groups in ring B.

Among the flavones and flavonols (see Table 1), only 3-hydroxyflavone (**12**) was ineffective in inhibiting xanthine oxidase. 7-Hydroxyflavone (**7**) showed inhibitory effects, but they were much weaker than those of chrysin (**8**). These observations illustrate the importance of the C-5 and the C-7 hydroxyl groups regarding the inhibition of xanthine oxidase. This conclusion was supported by a higher IC<sub>50</sub> value of fisetin (**16**) compared to quercetin (**15**). The inhibition of xanthine oxidase by baicalein (**11**) was lower than observed for chrysin (**8**), indicating that an additional hydroxyl group at C-6 reduces the inhibitory effect. Comparing the inhibition of xanthine oxidase by flavones with their corresponding flavonols, it can be concluded that the absence of a hydroxyl group at C-3 enhances slightly the inhibitory

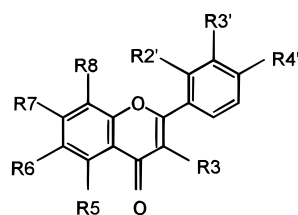
**Chart 2**

compound	R <sub>3</sub>	R <sub>5</sub>	R <sub>6</sub>	R <sub>7</sub>	R <sub>2'</sub>	R <sub>3'</sub>	R <sub>4'</sub>	R <sub>5'</sub>
7	H	H	H	OH	H	H	H	H
8	H	OH	H	OH	H	H	H	H
9	H	OH	H	OH	H	H	OH	H
10	H	OH	H	OH	H	OH	OH	H
11	H	OH	OH	OH	H	H	H	H
12	OH	H	H	H	H	H	H	H
13	OH	OH	H	OH	H	H	H	H
14	OH	OH	H	OH	H	H	OH	H
15	OH	OH	H	OH	H	OH	OH	H
16	OH	H	H	OH	H	OH	OH	H
17	OH	OH	H	OH	OH	H	OH	H
18	OH	OH	H	OH	H	OH	OH	OH

effect on xanthine oxidase. The IC<sub>50</sub> values of the flavones **8–10** were quite similar, indicating that B-ring substitution at C-3' and C-4' has little or no effect on the inhibition of xanthine oxidase. In the series of the flavonols, however, the highest inhibition was observed for kaempferol (**14**). Morin (**17**), with a 2'-hydroxyl substituent in the B-ring, showed the lowest activity.

All flavonoid derivatives (**19–32**, Chart 3), except isorhamnetin (**30**), showed a much lower inhibition of xanthine oxidase than the original compounds (see Table 2). The inhibitory effect decreased when the C-7

## Chart 3



compound	R <sub>3</sub>	R <sub>5</sub>	R <sub>6</sub>	R <sub>7</sub>	R <sub>8</sub>	R <sub>2'</sub>	R <sub>3'</sub>	R <sub>4'</sub>
19	OH	OH	glucose	OH	xylose	H	H	OH
20	OH	OH	xylose	OH	glucose	H	H	OH
21	H	OH	OCH <sub>3</sub>	OCH <sub>3</sub>	H	H	H	O-glucose
22	OH	OH	glucose	O-glucose	H	H	H	OH
23	H	OH	H	O-neohesperidose	H	H	OCH <sub>3</sub>	OH
24	H	OH	H	O-neohesperidose	H	H	OH	OH
25	<sup>a</sup>	OH	H	OH	H	H	H	OH
26	O-rutinose	OH	H	OH	H	H	H	OH
27	O-rutinose	OH	H	OH	H	H	OH	OH
28	O-rhamnose	OH	H	OH	H	H	OH	OH
29	OH	OH	H	OCH <sub>3</sub>	H	H	OH	OCH <sub>3</sub>
30	OH	OH	H	OH	H	H	OCH <sub>3</sub>	OH
31	H	OCH <sub>3</sub>	OCH <sub>3</sub>	OCH <sub>3</sub>	OCH <sub>3</sub>	OCH <sub>3</sub>	H	OCH <sub>3</sub>
32	H	OCH <sub>3</sub>	OCH <sub>3</sub>	OCH <sub>3</sub>	OCH <sub>3</sub>	H	H	OCH <sub>3</sub>

<sup>a</sup>: O-(6''-p-coumaroyl)glucose

**Table 2.** IC<sub>50</sub> Values of Flavonoid Derivatives for Inhibition of Xanthine Oxidase and Reduction of Superoxide Level

compd		xanthine oxidase IC <sub>50</sub> (μM) ± SD	superoxide IC <sub>50</sub> (μM) ± SD	category <sup>a</sup>
6-glucosyl-8-xylosylapigenin	<b>19</b>	>100	>100	F
6-xylosyl-8-glucosylapigenin	<b>20</b>	>100	>100	F
cirsimarín	<b>21</b>	>100	>100	F
saponarin	<b>22</b>	>100	>100	F
7-neohesperidosyl-3'-methylluteolin	<b>23</b>	>100	>100	F
7-neohesperidosylluteolin	<b>24</b>	11.9 ± 0.11	2.37 ± 0.14	C
tiliroside	<b>25</b>	>100	>100	F
3-rutinosylkaempferol	<b>26</b>	14.8 ± 2.0	3.4 ± 0.4	C
rutin	<b>27</b>	52.2 ± 0.6	10.6 ± 1.6	C
quercitrín	<b>28</b>	>100	8.1 ± 0.35	A
4',7-dimethylquercetin	<b>29</b>	7.7 ± 1.1	2.6 ± 0.2	C
isorhamnetin	<b>30</b>	2.51 ± 0.05	2.75 ± 0.04	B
nobiletin	<b>31</b>	>100	>100	F
tangeretin	<b>32</b>	>100	>100	F

<sup>a</sup> According to their effect on xanthine oxidase and as superoxide scavenger the flavonoids were classified into specific categories (see Table 3).

hydroxyl group, which is important for a high inhibitory activity, was derivatized as an *O*-glycoside or a methyl ether and also when the C-3 hydroxyl group was glycosylated. C-Glycosyl groups at C-6 or C-8 strongly decreased the inhibitory effect. This indicates that steric interactions reduce the inhibitory effect of flavonoids on xanthine oxidase.

It appears that for inhibition of xanthine oxidase by flavonoids the hydroxyl groups at C-5 and C-7 and the double bond between C-2 and C-3 are important. The presence of a hydroxyl group at C-3 slightly decreases the inhibitory activity. The structure-activity relation-

ship of flavonoids is different for inhibition of xanthine oxidase and scavenging of superoxide. The structure of flavanones, dihydroflavonols, and flavanols differs from flavones and flavonols by the presence of a single bond between C-2 and C-3 in the former and a double bond in the latter. Apparently, this structural difference influences the inhibitory effect on xanthine oxidase. With a double bond between C-2 and C-3, ring B will be coplanar with rings A and C due to the conjugation. Saturation of this double bond will destroy conjugation and coplanarity. This suggests that a planar flavonoid structure is important for inhibition of xanthine oxidase,

**Table 3.** Summary of the Classification of Flavonoids into Six Categories According to Their Inhibition of Xanthine Oxidase and Superoxide Scavenging Activity

category	inhibition of xanthine oxidase <sup>a</sup>	superoxide scavenging activity <sup>a</sup>	example
A	o	+	(-)-epigallocatechin
B	+	o	baicalein
C	+	+	myricetin
D	+	-	galangin
E	o	-	7-hydroxyflavone
F	o	o	naringenin

<sup>a</sup> Key: o, no effect; +, effect; -, pro-oxidant effect.

which can explain the lower inhibition of xanthine oxidase by morin (**17**), with a 2'-hydroxyl group in the B-ring, compared to the other flavonols (**13**–**16** and **18**).

A reduction in uric acid production results automatically in an equivalent reduction in superoxide.<sup>2,11</sup> This means that the rate of uric acid reduction equals the rate of superoxide reduction in the case of a xanthine oxidase inhibitor without any additional superoxide-scavenging activity (see Table 3, category B). In the case of an additional superoxide-scavenging activity (see Table 3, category C) the superoxide concentration reduction is higher so that the corresponding IC values of the flavonoid for superoxide are lower than those for uric acid. The IC<sub>100</sub> values must be equivalent. In the case of a xanthine oxidase inhibitor with a pro-oxidant effect, i.e., generation of superoxide radicals during uric acid production, the corresponding IC values of superoxide must be higher than those for uric acid (see Table 3, category D). Those theoretical examples were confirmed by IC experiments with selected flavonoids.

The relative concentration of uric acid (as a measure of residual xanthine oxidase activity) and superoxide, compared to appropriate control experiments, as a function of the concentration of the flavonoid tested, is displayed in Figure 1 for compounds **4**, **11**, **18**, and **13**. It appears that all flavonoids tested can be classified into different categories according to these concentration plots and the calculated IC<sub>50</sub> values. In Figure 1, panel A shows the results for compound **4** (epigallocatechin) as an example of category A. The uric acid production is not affected by an increasing concentration of **4**, which means that xanthine oxidase is not inhibited, but nevertheless the superoxide level decreases. The corresponding IC<sub>50</sub> can be considered as a measure of the superoxide scavenging activity. Compound **1** and the flavanols with a hydroxyl group at C-3' in ring B and a hydroxyl group at C-3 (**2**–**4**) showed this kind of activity and were classified into category A (superoxide scavengers without xanthine oxidase inhibitory activity). The flavonoid derivative quercitrin (**28**) also belongs to this category.

The results obtained for compound **11** (baicalein), an example of category B, are shown in Figure 1, panel B. The two curves are overlapping, and the IC<sub>50</sub> values for uric acid and superoxide are almost identical (see Table 1). Compounds **11** (baicalein), **14** (kaempferol), **17** (morin), and **30** (isorhamnetin) are the only flavonoids tested showing this pattern, as well as allopurinol, a known xanthine oxidase inhibitor used against gout.<sup>12</sup> These compounds, classified into category B, can be considered as pure inhibitors of xanthine oxidase, without any additional superoxide-scavenging activity.

The results obtained for compound **18** (myricetin) are characteristic for category C (Figure 1, panel C). Both the uric acid and the superoxide level decrease with an increasing concentration of test compound, and the superoxide curve lies below the uric acid curve. This means that the IC<sub>50</sub> value for superoxide is lower than that for uric acid, indicating that this class of compounds shows inhibition of xanthine oxidase and possesses an additional superoxide-scavenging activity. The IC<sub>100</sub> value for superoxide and uric acid are equivalent (Figure 1, panel C). Some flavonoid derivatives (**24**, **26**, **27** and **29**) and all flavonols measured with hydroxyl groups at C-5 and C-7, except compounds **14** and **17**, are classified into category C.

Compound **13** (galangin), as the only flavonol, and several flavones, show a different behavior, illustrated for **13** in panel D of Figure 1. The relative percentage of superoxide detected is higher than the relative uric acid level, and the superoxide curve is situated above the uric acid curve. The IC<sub>50</sub> value for superoxide is higher than that for uric acid. This means that the superoxide level is higher than expected, based on the inhibition of xanthine oxidase; i.e., in these test conditions this class of flavonoids shows some pro-oxidant effect on the superoxide level. Except for compound **7** (7-hydroxyflavone) and **11** (baicalein), all flavones tested show the same behavior as **13** and are classified into category D.

Compound **7** shows a marginal effect on xanthine oxidase (IC<sub>50</sub> higher than 30 μM) but has a pro-oxidant effect on the production of superoxide and is classified into category E.

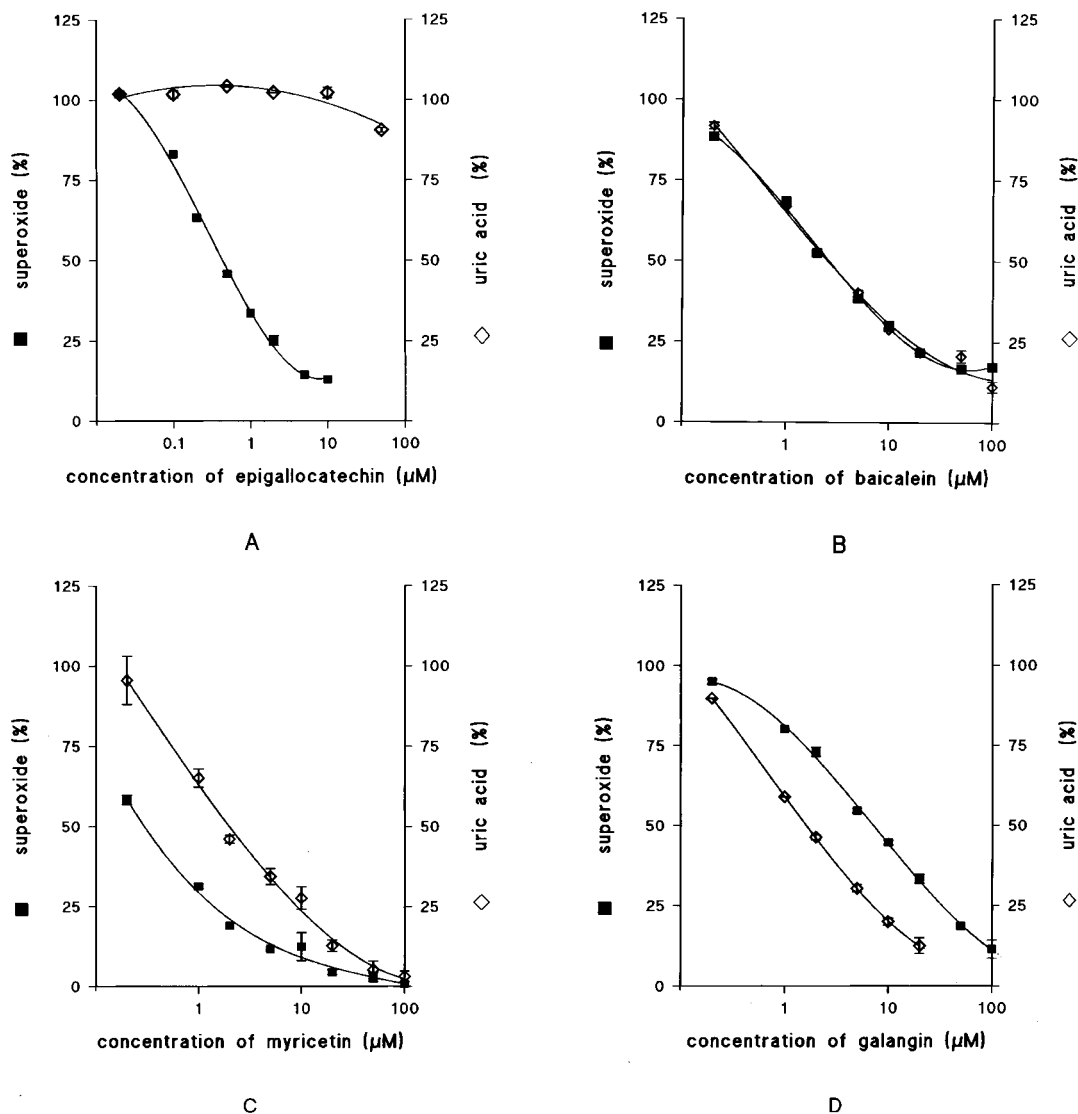
Finally, category F is composed of flavonoids that, at the concentrations tested, do not exhibit any effect on xanthine oxidase or the superoxide level, since the concentration of both uric acid and superoxide is not affected by increasing flavonoid concentrations. Compounds **5**, **6**, **12**, and many flavonoid derivatives (**19**–**23**, **25**, **31**, and **32**) belong to category F.

On the basis of these results, a mixture of two flavonoids, luteolin (**10**), a strong xanthine oxidase inhibitor, and epigallocatechin (**4**), a strong superoxide scavenger, was evaluated in the same assay. The result can be explained as the sum of the separate products (see Table 1) and confirm thereby the proposed structure–activity relationship.

The classification of flavonoids into six groups according to their effect on xanthine oxidase and as superoxide scavengers can be useful in the search for better compounds against gout than the widely used drug allopurinol (category B), which acts as a xanthine oxidase inhibitor without an additional superoxide-scavenging activity. Further study is also needed to investigate the type of inhibition of flavonoids on xanthine oxidase.

## Experimental Section

**General Experimental Procedures.** Superoxide levels were measured by the nitrite method.<sup>13</sup> The uric acid production was calculated according to the increasing absorbance at 290 nm.<sup>14</sup> Flavonoids were dissolved in demineralized water (Milli Q), with phosphate buffer (20 mM, pH 8.3) or a small amount of NaOH (0.05 M) and immediately diluted with water to 1 mM. Fla-



**Figure 1.** The concentration of flavonoid vs the relative superoxide amount detected and the uric acid production. The results are expressed in % =  $(A_{\text{in the presence of flavonoid}}/A_{\text{in the absence of flavonoid}}) \times 100$ .

Flavonoid solutions were prepared just before the assay. Test solutions were prepared by adding xanthine (final concentration  $50 \mu\text{M}$ ), hydroxylamine (final concentration  $0.2 \text{ mM}$ ), EDTA (final concentration  $0.1 \text{ mM}$ ), and flavonoid in various concentrations. The reaction was started by adding  $0.2 \text{ mL}$  of xanthine oxidase ( $6.25 \text{ mU/mL}$ ) in a phosphate buffer solution ( $\text{pH} = 7.50$ ,  $200 \text{ mM}$ ). The mixture (total  $1 \text{ mL}$ ) was incubated for  $30 \text{ min}$  at  $37^\circ\text{C}$ . Prior to the measurement of uric acid production by measuring the UV absorbance at  $290 \text{ nm}$ , the reaction was stopped by adding  $0.1 \text{ mL}$  of  $\text{HCl}$  ( $0.58 \text{ M}$ ). The uric acid production was calculated from the differential absorbance with a blank solution in which the xanthine oxidase was replaced by buffer solution. A test mixture containing no flavonoids was prepared to measure the total uric acid production. To detect superoxide, the coloring reagent (final concentration of  $300 \mu\text{g/mL}$  sulfanilic acid,  $5 \mu\text{g/mL}$  of  $N$ -(1-naphthyl)-ethylenediamine dihydrochloride, and  $16.7\%$  (v/v) acetic acid) was added after the incubation. The mixture was allowed to stand for  $30 \text{ min}$  at room temperature, and the absorbance at  $550 \text{ nm}$  was measured.

Different concentrations of flavonoids were analyzed, and then the half-maximal inhibitory concentration

( $\text{IC}_{50}$ ) was calculated by linear regression analysis. The sensitivity of the nitrite method was evaluated with manganese superoxide dismutase (see Table 1). Different concentrations of quercetin were measured in triplicate on separate days to validate the nitrite method and the method to detect uric acid. The variability was  $10\%$  and  $8\%$  for the nitrite method and the detection of uric acid, respectively. The statistical significance between  $\text{IC}_{50}$  values of test products was evaluated with an unpaired two-tailed student's  $t$ -test. A  $p$  level of  $0.05$  was considered to be statistically significant.

**Materials.** Quercetin, myricetin, kaempferol, (+)-catechin, (-)-epicatechin, morin, naringenin, taxifolin, allopurinol, xanthine, xanthine oxidase and manganese-containing superoxide dismutase (from *Escherichia coli*) were purchased from Sigma (USA). Luteolin, 3-hydroxyflavone, fisetin, 7-hydroxyflavone, chrysin, baicalein, apigenin and galangin were purchased from Aldrich (Belgium). Isorhamnetin and 4'-hydroxyflavone were purchased from Roth (Germany). Rutin was purchased from Merck (Germany) and tilirosidine from Extrasynthese (France). Nobiletin and tangeretin were obtained from the Department of Citrus, Lakeland, FL

33802. The other flavonoids were isolated from different plant sources in the Laboratory of Pharmacognosy, Department of Pharmaceutical Sciences, University of Antwerp, Belgium.

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