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QUARTERLY

Short Communication

A study of the genotoxic potential of flavonoids using short-term bacterial assays*

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Genotoxic activities of flavonoids (quercetin, rhamnetin, isorhamnetin, apigenin, luteolin) were investigated using two short-term bacterial assays.

In the "repair test" in *Salmonella typhimurium* (strains TA1538 *uvrB*$^-$ and TA1978 *uvrB*$^+$) the flavonoids studied did not introduce any damage into the DNA recognized by *UvrABC* nuclease (correndonuclease II).

The results of the SOS-Chromotest in *Escherichia coli* K-12 strains PQ37 (*tag*$^+$, *alk*$^+$) and PQ243 (*tagA, alkA*) indicated that flavonoids only weakly induced the SOS system.

The addition of a liver activation system (S9 mix) did not increase the mutagenic effect of the flavonoids tested.

Two compounds: rhamnetin, isorhamnetin and their putative metabolites formed in the presence of the S9 mix did not alkylate DNA at N-3 of adenine.

Flavonoids are natural compounds which are regularly consumed by humans (about 1 g/day) in a diet containing fruit and vegetables [1 - 3]. They were found to act as antiviral, antibiotic, antifungal and antispasmogenic agents, moreover they show antineoplastic activity and a variety of other properties [4, 5]. On the other hand, some flavonoids have been shown, in tests *in vitro* to exhibit mutagenic activity, but their carcinogenicity was not confirmed by feeding studies in animals [6 - 10]. In addition, there is experimental evidence from both *in vitro* and *in vivo* tests that flavonoids act as natural antimutagens or anticancerogens [11].

The data on genotoxic properties of flavonoids are fragmentary. The only compound studied so far in detail is flavonol – quercetin [12].

In the present work the genotoxic effects of flavonoids were reevaluated using short-term bacterial assays.

For this purpose, we examined:

– I. The ability of flavonoids (quercetin, rhamnetin, isorhamnetin, apigenin, luteolin) to introduce into DNA damages recognized by correndonuclease II;

– II. Induction of the SOS repair system in *E. coli* K-12 by flavonoids in the SOS-Chromotest;

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[1]Abbreviations: DMSO, dimethylsulphoxide; MMS, methyl methanesulphonate; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; 4-NQO, 4-nitroquinoline-1-oxide; AFB_1, aflatoxin B_1; ONPG, *O*-nitrophenl-β-galactopyranoside; PNPP, *p*-nitrophenyl phosphate disodium.

– III. Alkylation of DNA at N-3 of adenine by rhamnetin and isorhamnetin. The chemical structure of these two compounds (Fig. 1) suggests that they might alkylate DNA.

MATERIALS AND METHODS

Chemicals. The following chemicals were obtained from the sources listed below:

MNNG[1] and MMS from Sigma; 4-NQO from Fluka AG; AFB_1 and ONPG from Calbiochem; PNPP from Merck; Aroclor 1254 from Analabs Inc.; sodium dodecyl sulphate, Bacto trypton and Bacto yeast extract from Difco; DMSO, mitomycin C and 2-aminofluoren from Serva.

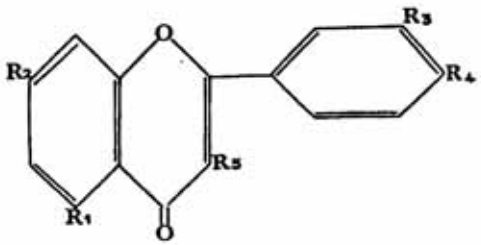
Flavonoids (quercetin, rhamnetin, isorhamnetin, apigenin, luteolin) were isolated from medicinal herbs at the Department of Pharmacognosy, Medical School in Warsaw. The chemical structures of flavonoids are shown in Fig. 1.

All control mutagens and flavonoids were dissolved in DMSO.

Fresh solutions of all chemicals were prepared immediately before use.

Bacterial strains. *E. coli* K-12 strains PQ37 *sfiA*::Mud(AP *lac*)*cts*, *lacU169*, *mal*$^+$, *uvrA*, *galE*, *galY*, *PhoC*, *F*$^-$, *rfa*, *thr*, *leu*, *his*, *pyrD*, *thi*, *trp*::*Muc*$^+$, *sr1300*::*Tn10*, *rpoB* and PQ243 (as PQ37 but *tagA*, *alkA*) used in the SOS-Chromotest were gifts from P. Quillardet and M. Hofnung U.B.M.T.G. Institut Pasteur, Paris, France.

S. typhimurium strains TA1538, TA1978 used in the repair test (in *S. typhimurium*) were gifts from Prof. B.N. Ames, Biochemistry Department, University of California, Berkeley, CA. U.S.A.



	R_1	R_2	R_3	R_4	R_5	
Quercetin	OH	OH	OH	OH	OH	Flavonol
Rhamnetin	OH	OCH_3	OH	OH	OH	
Isorhamnetin	OH	OH	OCH_3	OH	OH	
Apigenin	OH	OH	H	OH	H	Flavone
Luteolin	OH	OH	OH	OH	H	

Fig. 1. Chemical structures of flavonoids.

Bacterial tests. A. The repair test in *S. typhimurium* (strains TA1538 *uvrB*$^-$ and TA1978 *uvrB*$^+$) performed according to Ames *et al.* [13]: comparison of the repair-deficient and repair competent strains for zones of growth inhibition.

B. The SOS-Chromotest was conducted under standard conditions described by Quillardet *et al.* [14] and Quillardet & Hofnung [15].

It is based on induction of the *sfiA* gene (belonging to the SOS system) by a DNA damaging factor. In the PQ37 *uvrA* strain of *E. coli* K-12 the gene of β-galactosidase is linked to the promotor of the *sfiA* gene [15, 16]. Units of the enzyme activity were calculated using a simplified version of the formula used for calculation of international units (U) [17].

The mutagenic activity of a compound at the given concentration C (R(C)) may be expressed by the ratio of β-galactosidase activity to alkaline phosphatase activity. The SOS induction factor I(F) for a compound at concentration C is defined as I(F) = R(C)/R(0) in which R(0) is the mutagenic activity measured in the absence of a compound.

C. The SOS-Chromotest adapted by Quillardet & Hofnung [15, 16] also was used for detecting alkylation of DNA at N-3 of adenine by two flavonoids: rhamnetin and isorhamnetin. This method is based on the comparison of induction of the *lacZ* gene fused with the promotor region of *sfiA* gene of the *E. coli* strains PQ37 and PQ243 which are proficient and deficient in 3-methyladenine-DNA-glycosylase I and II, encoded by the *tagA* and *alkA* genes, respectively [18].

Liver homogenate fraction (S9). The liver homogenate fraction was prepared by the method of Maron & Ames [19] using Aroclor 1254 treated Wistar male rats. Fraction S9 was stored at –20°C and served as the source of soluble microsomal enzymes. The average concentration of protein in the S9 fraction was 38 mg/ml (36 - 42 mg/ml). Protein was determined according to Lowry *et al.* [20].

S9 mix. The S9 mix used in the SOS-Chromotest was prepared according to Quillardet & Hofnung [15].

RESULTS AND DISCUSSION

The ability of flavonoids to introduce into DNA lesions recognized by correndonuclease II was studied in the repair test in *S. typhimurium*. The zones of growth inhibition by flavonoids (quercetin, rhamnetin, isorhamnetin, apigenin, luteolin) at 50 - 200 µg/plate for strains TA1538 (*uvrB*$^-$) and TA1978 (*uvrB*$^+$) were formed to be practically irrespective of the presence or the absence of metabolic activation by S9 mix (Table 1). As a positive control in the above test we used mitomycin C in the absence and 2-aminofluorene in the presence of S9 fraction; with both compounds the zone of growth inhibition was for TA1538 *uvrB*$^-$ was over twice as large as for TA1978 *uvrB*$^+$. According to Ames *et al.* [13] if a tested compound is more toxic to an *uvrB*$^-$ strain than to *uvrB*$^+$, its action can be caused by covalent binding to DNA. Thus, the results obtained suggest that neither of the flavonoids tested introduced into DNA any damages recognized by correndonuclease II.

The ability of flavonoids to induce the SOS system was tested on the *E. coli* K-12 strain PQ37 with and without metabolic activation. Tables 2 and 3 appeared to be very low induction of the SOS system by flavonoids in the PQ37 *uvrA* strain.

The ability of quercetin, rhamnetin, isorhamnetin, apigenin, luteolin to induce the SOS sys-

Table 1

The zones of growth inhibition produced by flavonoids in TA1538 (uvrB$^-$) and TA1978 (uvrB$^+$) strains of S. typhimurium in the presence or absence of a metabolic activation

Compound tested	Dose µg/plate	Diameter of growth inhibition zone (mm ± S.D.[a])			
		TA1538 (*uvrB*$^-$)	TA1978 (*uvrB*$^+$)	TA1538 (*uvrB*$^-$)	TA1978 (*uvrB*$^+$)
		–S9		+S9[b]	
Mitomycin C	1	36 ± 0	23 ± 3	NT	NT
2-Aminofluorene	50	NT	NT	16 ± 2	10 ± 1
Quercetin	50	8 ± 1	11 ± 3	10 ± 2	11 ± 3
	100	9 ± 3	9 ± 3	9 ± 1	10 ± 1
	200	9 ± 1	9 ± 3	10 ± 1	10 ±4
Rhamnetin	50	6 ± 1	6 ± 0	7 ± 0	6 ± 0
	100	6 ± 1	6 ± 0	6 ± 1	6 ± 1
	150	6 ± 1	6 ± 1	6 ± 1	6 ± 0
	200	6 ± 0	6 ± 0.5	7 ± 0	6 ± 0
Isorhamnetin	50	8 ± 3	8 ± 2	10 ± 3	7 ± 2
	100	9 ± 4	10 ± 2	8 ± 2	7 ± 1
	150	9 ± 3	10 ± 1	8 ± 0	8 ± 1
	200	10 ± 3	10 ± 2	8 ± 2	8 ± 2
Apigenin	50	9 ± 3	11 ± 2	11 ± 2	11 ± 1
	100	9 ± 2	11 ± 2	11 ± 3	11 ± 2
	150	9 ± 0.5	10 ± 1	10 ± 2	11 ± 1
	200	6 ± 0	6 ± 0.5	8 ± 2	11 ± 0
Luteolin	50	8 ± 0.5	9 ± 1	9 ± 1	6 ± 1
	100	10 ± 0.5	9 ± 1	9 ± 1	6 ± 1
	150	8 ± 1	9 ± 1	9 ± 1	10 ± 1
	200	7 ± 1	10 ± 2	8 ± 1	8 ± 1

[a]Average from 9 plates; [b]100 µl S9 per plate; NT, not tested

tem was assayed in a concentration range not toxic for strain tested (0 - 20 mg per sample). 4-NQO and AFB_1 (20 ng per sample) were used as positive controls. The response of the SOS system to flavonoids was much lower than to 4-NQO or AFB_1, irrespective of the presence or absence of the S9 mix.

In the SOS-Chromotest used to study the possible alkylation of DNA at N-3 of adenine by rhamnetin and isorhamnetin, the degree of β-galactosidase activity was low both in the double mutant PQ243 *tagA, alkA* and in the wild type PQ37, irrespective of the presence or absence of fraction S9 (Table 3).

On the other hand, MMS known to introduce approximately 10% of all methylations into N-3 of adenine [21] induced a 3.5 times higher level of β-galactosidase in PQ243 than in PQ37

Table 2

Effect of flavonoids on the SOS system induction in PQ37 strain of E. coli K-12 in the presence or absence of metabolic activation

Flavonoid	PQ37 −S9			PQ37 +S9		
µg/sample	Uβ	UP	I(F)	Uβ	UP	I(F)
Quercetin						
0.00	1.4 ± 0.3	19.5 ± 4.9	1.0	1.1 ± 0.2	11.0 ± 2.0	1.0
0.10	1.1 ± 0.2	18.7 ± 2.7	0.8 ± 0.2	1.1 ± 0.3	11.0 ± 0.5	0.9 ± 0.3
1.00	1.4 ± 0.1	18.9 ± 3.1	0.9 ± 0.2	1.5 ± 0.8	11.3 ± 1.3	0.9 ± 0.3
5.00	2.7 ± 0.9	23.9 ± 2.3	1.3 ± 0.3	1.5 ± 0.5	10.4 ± 3.1	1.6 ± 0.8
10.00	2.9 ± 0.3	23.0 ± 5.5	1.8 ± 0.5	1.8 ± 0.7	11.7 ± 2.9	1.6 ± 0.7
20.00	2.8 ± 0.6	21.3 ± 3.1	1.5 ± 0.7	1.9 ± 0.6	15.0 ± 2.0	1.5 ± 0.5
Apigenin						
0.00	2.7 ± 0.5	12.0 ± 1.9	1.0	7.8 ± 0.4	61.3 ± 3.3	1.0
0.10	1.9 ± 0.6	12.7 ± 1.2	0.7 ± 0.3	6.3 ± 0.4	62.9 ± 2.7	0.9 ± 0.1
1.00	1.6 ± 0.3	12.6 ± 1.5	0.6 ± 0.1	7.1 ± 0.8	56.5 ± 4.7	1.2 ± 0.2
5.00	1.9 ± 0.6	10..6 ± 1.4	0.8 ± 0.2	6.1 ± 0.3	50.7 ± 2.8	1.1 ± 0.2
10.00	4.3 ± 0.5	11.4 ± 0.8	1.7 ± 0.6	5.2 ± 0.9	54.2 ± 4.1	0.9 ± 0.3
20.00	4.7 ± 0.2	13.0 ± 2.2	1.7 ± 0.2	6.6 ± 2.9	41.3 ± 9.5	1.2 ± 0.5
Luteolin						
0.00	2.3 ± 0.1	16.2 ± 1.1	1.0	2.3 ± 0.1	9.9 ± 1.5	1.0
0.10	2.0 ± 0.5	12.0 ± 1.5	1.2 ± 0.4	2.0 ± 0.1	9.7 ± 2.0	0.9 ± 0.1
1.00	2.4 ± 0.1	14.5 ± 2.1	1.2 ± 0.2	1.8 ± 0.2	8.5 ± 0.8	1.0 ± 0.2
5.00	1.8 ± 0.1	21.0 ± 1.3	0.9 ± 0.2	2.4 ± 0.5	10.8 ± 2.1	1.0 ± 0.2
10.00	2.3 ± 0.2	15.0 ± 2.8	1.2 ± 0.1	2.5 ± 0.3	11.9 ± 1.9	0.9 ± 0.1
20.00	3.4 ± 0.3	14.0 ± 2.7	1.7 ± 0.3	2.5 ± 0.2	8.8 ± 1.6	1.1 ± 0.1
Positive controls						
4-NQO*						
20 ng	17.8 ± 5.7	13.7 ± 3.3	10.4 ± 3.2	NT	NT	NT
AFB_1**						
20 ng	NT	NT	NT	5.8 ± 0.8	43.7 ± 1.6	12.1 ± 0.5

Uβ, international units of β–galactosidase activity;
UP, international units of alkaline phosphatase activity; I(F), the SOS induction factor;
Positive controls : *without S9; **with S9
Each value is an average from 5 independent experiments ± S.D.

strain of *E. coli* K-12 (Table 3). The level of this induction in both strains was similar.

On the basis of these results it is possible to suppose that rhamnetin and isorhamnetin do not alkylate DNA at N-3 of adenine.

Table 3

Effect of rhamnetin and isorhamnetin on the SOS system induction in PQ37 and PQ243 strains of E. coli K-12 in the presence or absence of metabolic activation

Flavonols	PQ37 (wild type)			PQ243 (*tagA, alkA*)		
µg/sample	Uβ	UP	I(F)	Uβ	UP	I(F)
Rhamnetin						
0.00	1.1 ± 0.1	19.2 ± 1.2	1.0	1.4 ± 0.3	24.8 ± 1.4	1.0
0.10	1.6 ± 0.2	17.0 ± 5.3	1.0 ± 0.1	1.6 ± 0.2	31.6 ± 6.4	0.9 ± 0.1
1.00	2.1 ± 0.3	15.6 ± 3.2	1.1 ± 0.4	1.9 ± 0.2	34.7 ± 4.3	1.0 ± 0.2
5.00	2.4 ± 0.5	16.0 ± 4.7	1.6 ± 0.6	1.7 ± 0.3	26.7 ± 4.3	1.5 ± 0.4
10.00	2.3 ± 0.8	15.7 ± 6.2	1.8 ± 0.7	2.4 ± 0.8	26.3 ± 4.5	1.9 ± 0.5
20.00	1.6 ± 0.7	15.6 ± 5.3	1.8 ± 0.7	2.8 ± 0.2	26.6 ± 6.5	1.8 ± 0.9
Rhamnetin + S9						
0.00	2.2 ± 0.4	15.0 ± 0.9	1.0	1.8 ± 0.4	22.7 ± 2.9	1.0
0.10	1.8 ± 0.3	18.9 ± 3.0	0.6 ± 0.4	1.5 ± 0.2	25.3 ± 4.1	0.6 ± 0.2
1.00	1.6 ± 0.4	20.0 ± 1.8	0.8 ± 0.1	1.8 ± 0.4	23.8 ± 6.3	0.7 ± 0.1
5.00	1.8 ± 0.6	17.3 ± 5.1	1.0 ± 0.2	1.9 ± 0.2	18.5 ± 5.3	0.8 ± 0.4
10.00	1.9 ± 0.5	18.5 ± 5.0	1.2 ± 0.4	2.4 ± 0.4	22.0 ± 4.4	0.9 ± 0.4
20.00	2.0 ± 0.6	17.3 ± 6.0	1.3 ± 0.2	1.9 ± 0.8	22.0 ± 3.1	0.9 ± 0.2
Isorhamnetin						
0.00	1.1 ± 0.1	19.5 ± 2.7	1.0	1.5 ± 0.2	21.4 ± 3.2	1.0
0.10	1.4 ± 0.4	20.9 ± 3.0	0.8 ± 0.1	1.2 ± 0.1	32.3 ± 4.5	0.8 ± 0.3
1.00	1.4 ± 0.2	19.4 ± 6.1	0.9 ± 0.2	1.3 ± 0.6	32.8 ± 8.6	0.8 ± 0.3
5.00	1.9 ± 0.8	16.3 ± 3.1	1.7 ± 0.1	1.8 ± 0.7	27.1 ± 2.4	1.1 ± 0.2
10.00	2.1 ± 0.5	16.0 ± 1.8	1.5 ± 0.2	1.6 ± 0.3	25.8 ± 5.8	1.1 ± 0.2
20.00	1.7 ± 0.4	16.3 ± 1.2	1.8 ± 0.3	1.6 ± 0.4	27.3 ± 3.8	1.1 ± 0.4
Isorhamnetin + S9						
0.00	1.1 ± 0.1	11.2 ± 0.6	1.0	2.0 ± 0.3	24.1 ± 2.1	1.0
0.10	2.4 ± 0.3	15.0 ± 2.0	1.0 ± 0.2	1.8 ± 0.3	18.1 ± 5.5	0.9 ± 0.5
1.00	2.6 ± 0.2	14.7 ± 3.0	1.2 ± 0.1	1.8 ± 0.2	17.7 ± 4.1	0.9 ± 0.2
5.00	2.2 ± 0.4	17.5 ± 1.8	1.6 ± 0.2	2.6 ± 0.6	19.0 ± 6.1	0.8 ± 0.2
10.00	2.3 ± 0.4	16.0 ± 2.1	1.4 ± 0.3	3.1 ± 0.7	20.8 ± 1.2	0.9 ± 0.4
20.00	1.9 ± 0.3	15.8 ± 1.4	1.4 ± 0.4	3.1 ± 0.5	20.8 ± 3.2	1.1 ± 0.1
MMS						
20 nM			2.0 ± 0.5			7.0 ± 1.1
4-NQO*						
20 ng	16.0 ± 5.70	23.6 ± 6.6	10.9 ± 5.3	18.7 ± 5.3	21.0 ± 4.5	10.5 ± 2.2
AFB_1**						
20 ng	13.6 ± 6.1	10.9 ± 3.2	15.3 ± 6.6	12.5 ± 2.6	19.9 ± 3.3	8.5 ± 0.8

Abbreviations as in Table 2.

The flavonoids studied are able to induce the SOS system both in the presence and absence of metabolic activation. However, in the SOS-Chromotest, we have observed a weak response to both flavonoids either with or without metabolic activation in *E. coli* K-12 strain PQ37. Moreover, metabolic activation by S9 mix decreased the SOS response with all flavonoids tested (Tables 2, 3). In our experiments the metabolic activation increased also the toxicity of the compounds tested.

The maximum values obtained for the SOS induction factor with metabolic activation were 1.8 and 1.3 for quercetin and rhamnetin, respectively and 1.8 without activation for either compounds in the *E. coli* strain PQ37 (Tables 2, 3).

The decrease of quercetin genotoxicity by S9 activation in the induction of SOS system is well known [22]. Liver enzymes are to enhance the mutagenicity of quercetin in the Ames test [23, 24] and lower its genotoxicity in other assays [25].

Reuft *et al.* [26] suggested that quercetin might cause DNA damage *via* more than one mechanism and not only by metabolic activation. Metabolic activation of flavonoids by mammalian enzymatic systems can occur *via* pathways which are not well understood.

Our results, i.e. the lack of DNA alkylation at N-3 of adenine and lack of damages recognised by correndonuclease II, suggest that flavonoids and/or their putative metabolites do not bind covalently to DNA.

It is possible, that the mutagenic activity of flavonoids could be caused by other interactions with replicative apparatus as suggested by Reuff [26].

Investigations on genotoxicity of flavonoids could help to gain knowledge on the precise nature of lesions in DNA induced by mutagens/carcinogens such as flavonoids and get more insight into the underlying mutational mechanism.

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