Biochemical Pharmacology, Vol. 38, No. 17, pp. 2859–2865, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

ANTIOXIDANT AND PRO-OXIDANT ACTIONS OF THE PLANT PHENOLICS QUERCETIN, GOSSYPOL AND MYRICETIN

EFFECTS ON LIPID PEROXIDATION, HYDROXYL RADICAL GENERATION AND BLEOMYCIN-DEPENDENT DAMAGE TO DNA

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(*Received* 18 *November* 1988; *accepted* 14 *March* 1989)

Abstract—The plant-derived phenolic compounds gossypol, quercetin and myricetin are powerful inhibitors of iron-induced lipid peroxidation in rat liver microsomes, under all five experimental conditions tested and at low micromolar concentrations ($IC_{50} \leq 1.5\ \mu M$). However, they greatly accelerate the generation of hydroxyl radicals (·OH) from H_2O_2 in the presence of Fe^{3+}–EDTA at pH 7.4, as measured by the deoxyribose assay. At 100 μM, the three phenolic compounds enhanced ·OH formation up to eight-fold. The hydroxyl radical generation was inhibited by catalase and superoxide dismutase, suggesting a mechanism in which the phenols oxidize to produce superoxide radical, which then assists ·OH generation from H_2O_2 in the presence of Fe^{3+}–EDTA. At concentrations up to 75 μM, quercetin and myricetin also accelerate bleomycin-dependent DNA damage in the presence of Fe^{3+}, possibly by reducing the Fe^{3+}–bleomycin–DNA complex to the Fe^{2+} form. Hence these naturally-occurring substances can have pro-oxidant effects under some reaction conditions and cannot be classified simplistically as "antioxidants".

Plant tissues are rich in a wide variety of phenolic compounds, such as chalcones, tocopherols and the hydroxylated flavonoids [1, 2]. Common foods of plant origin contain a variety of glycosylated and aglycone flavonoids in amounts ranging from trace to several grams per kilogram fresh weight [1–4]. Flavonoids have been reported to have multiple biological effects, including antioxidant ability [1–16]. Thus flavonoids and other plant phenolics have been reported to inhibit the enzyme lipoxygenase [3, 6, 14], to decrease the rate of non-enzymic lipid peroxidation of fatty acids [12], liposomes [16], mitochondria [4, 8], tissue homogenates [11] and microsomes [7, 8, 15], and to inhibit oxidant generation by the neutrophil NADPH oxidase system [6] or by the enzyme xanthine oxidase [9, 10].

However, plant phenolics have sometimes been reported to show pro-oxidant properties. Thus several flavonoids, e.g. quercetin [13], are mutagenic in bacterial test systems. They appear to oxidize, producing oxygen-derived species, such as H_2O_2, that lead to DNA damage [13]. Flavonoids also damage isolated mitochondria by a similar mechanism [17]. Gossypol, a polyphenol isolated from the cotton plant, has been clinically tested as an antifertility agent, but it has been reported to catalyse O_2-dependent DNA degradation *in vitro* [18] and to generate superoxide radical ($O_2^{\cdot-}$) in the presence of liver microsomes amd NADPH [19], although gossypol inhibits lipoxygenase activity [20] and microsomal lipid peroxidation [19].

Polyphenolic compounds also have a well-established iron chelating ability. Many of them are theoretically capable of reducing Fe^{3+} to Fe^{2+}; some can even mobilize iron from ferritin [21]. Fe^{2+} ions play important stimulatory roles in free-radical reactions by decomposing lipid peroxides to chain-propagating alkoxyl radicals and by reacting with H_2O_2 to give hydroxyl radical (·OH) and other highly-reactive species (reviewed in Ref. 22).

We have therefore undertaken a study of the effect of two flavonoids (quercetin and myricetin) and of gossypol (structures shown in Fig. 1) on iron-dependent radical-generating systems. The systems chosen were iron-dependent lipid peroxidation in rat liver microsomes, iron-dependent generation of ·OH from H_2O_2, and iron-dependent damage to DNA by the anti-tumour antibiotic bleomycin.

MATERIALS AND METHODS

Myricetin was from Aldrich Chemical Co. (Gillingham, U.K.). All other reagents were of the highest quality available from Sigma Chemical Co. (Poole, U.K.) or from BDH Ltd (Dagenham, U.K.).

Rat liver microsomes were prepared and their peroxidation in the presence of iron ions and ascorbic acid was measured by the thiobarbituric acid method as described in [23]. In brief, reaction mixtures contained, in a final volume of 1.0 ml, 0.25 mg microsomal protein, 25 μl ethanol or phenolic compound dissolved in ethanol, and 10 mM KH_2PO_4–KOH buffer, pH 7.4. Peroxidation was started by adding, to give the final concentrations stated, Fe^{2+} (100 μM

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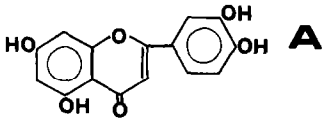

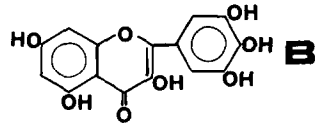

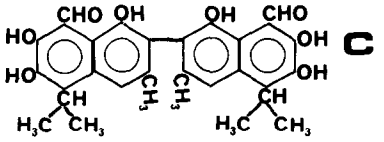

Fig. 1. Structures of quercetin (A), myricetin (B) and gossypol (C).

as ferrous ammonium sulphate), Fe^{3+} (100 μM, as ferric chloride), Fe^{3+}–ascorbate (both 100 μM), Fe^{3+}–EDTA (both 100 μM) or Fe^{3+}–ADP/NADPH (Fe^{3+}, 100 μM; ADP, 1.7 mM; NADPH, 400 μM). ADP and Fe^{3+} were pre-mixed before addition to the reaction mixture. Incubations were performed at 37°, usually for 20 min, and peroxidation was then determined by the TBA test [23]. Solutions of iron salts were made up freshly before use. The order of addition to the reaction mixtures was iron (± EDTA or ± ADP), microsomes, ethanol (± phenolic compound). Ascorbate or NADPH were added to start the peroxidation.

Hydroxyl radicals were generated by incubating the following reagents at the final concentrations stated in a volume of 1.2 ml for 20–60 min at 37°: KH_2PO_4–KOH buffer, pH 7.4 (10 mM), H_2O_2 (1.42 mM), $FeCl_3$ or ferrous ammonium sulphate (20 μM), deoxyribose (2.8 mM), and ascorbate or phenols (at the concentrations stated). Where indicated, the iron salts were pre-mixed with chelating agent (final concentration of chelator 100 μM in reaction mixture) before addition to the reaction system. Chelators used were EDTA, ADP or citrate. Solutions of iron salts, H_2O_2, ascorbate and ADP were made up fresh just before use. The extent of deoxyribose degradation by ·OH was measured by the thiobarbituric acid method [24]. 1 ml of 1% (w/v) TBA in 0.05 M NaOH plus 1 ml of 2.8% (w/v) trichloroacetic acid were added. After heating at 100° for 15 min on a heating block, and cooling, A_{532} was measured.

Units of catalase and superoxide dismutase (SOD) (bovine copper–zinc enzyme) are as defined in the Sigma catalogue.

RESULTS

Effect of quercetin, gossypol and myricetin on iron-dependent hydroxyl radical generation

Quercetin, gossypol and myricetin are poorly sol-

Table 1. Iron-dependent hydroxyl radical generation: effect of ascorbate, gossypol, quercetin and myricetin

A. Effect of ascorbate on extent of deoxyribose degradation.

Additions	No ascorbate	Plus ascorbate
Fe^{3+}	0.057 ± 0.013 (12)	0.231 ± 0.005 (12)†
Fe^{3+}–EDTA	0.138 ± 0.006 (18)*	0.576 ± 0.015 (12)*†
Fe^{3+}–citrate	0.069 ± 0.003 (14)	0.281 ± 0.004 (10)*†
Fe^{3+}–ADP	0.054 ± 0.002 (14)	0.231 ± 0.017 (10)†
Fe^{2+}	0.072 (2)	0.267 (2)
Fe^{2+}–EDTA	0.164 ± 0.030 (6)*	0.800 (2)
Fe^{2+}–citrate	0.106 ± 0.009 (6)	0.387 (2)
Fe^{2+}–ADP	0.172 ± 0.013 (6)*	0.299 (2)

B. Effect of gossypol, quercetin and myricetin on extent of Fe^{3+}–chelate-induced deoxyribose generation.

Iron complex	No inhibitor	Gossypol	Quercetin	Myricetin
Fe^{3+}	0.057 ± 0.013 (12)	0.017 ± 0.009 (4)‡	0.053 ± 0.006 (8)	n.t.
Fe^{3+}–EDTA	0.138 ± 0.006 (18)	0.801 ± 0.045 (12)‡	0.776 ± 0.061 (12)‡	1.082 ± 0.066 (6)‡
Fe^{3+}–citrate	0.069 ± 0.003 (14)	0.062 ± 0.004 (4)	0.073 ± 0.005 (4)	0.075 (2)
Fe^{3+}–ADP	0.054 ± 0.002 (14)	0.010 ± 0.006 (4)‡	0.052 ± 0.006 (4)	0.058 (2)

Values show extent of deoxyribose generation as A_{532} value after 1 hr in (N) tests with SE.

(A) Iron salts were 20 μM with 100 μM EDTA, citrate, ADP and ascorbate. The symbols * and † denote significant differences $P < 0.05$ by Student's unpaired t-test in comparison with iron ions alone (*) or in corresponding sample lacking ascorbate (†).

(B) Gossypol, quercetin and myricetin were added at 100 μM and their absorbance values at 532 nm of 0.092 ± 0.012, 0.017 ± 0.002 and 0.018 ± 0.002 have been subtracted where relevant. ‡ indicates significant difference with respect to phenol-free control, $P < 0.05$. n.t. = not tested.

uble in aqueous solution at pH 7.4. Solutions for the experiments described below were prepared either by sonicating the solids with buffer at pH 7.4 for 10 min, or by dissolving in alkaline solutions and then readjusting the pH to 7.4 immediately before use. Ethanol or other organic solvents could not be used to solubilize the compounds, as the solvents are themselves powerful scavengers of ·OH.

Hydroxyl radicals were measured by their ability to degrade the sugar deoxyribose into a thiobarbituric acid (TBA)–reactive material, measured at 532 nm after heating with TBA [24]. As shown in Table 1A, addition of Fe^{3+} ions to H_2O_2 at pH 7.4 produced very little ·OH generation. As reported previously [25], addition of Fe^{3+}–EDTA to H_2O_2 gave more ·OH generation. Chelating Fe^{3+} to ADP or to citrate produced slight but not significant ($P > 0.05$) stimulations over the ·OH generation observed with Fe^{3+} alone. Addition of Fe^{2+}, Fe^{2+}–EDTA, Fe^{2+}–citrate or Fe^{2+}–ADP to H_2O_2 gave greater rates of ·OH generation than was the case for Fe^{3+} (Table 1). Addition of ascorbic acid to any of these reaction mixtures greatly increased the generation of ·OH, as expected [26] (Table 1). Deoxyribose degradation in ascorbate-containing systems could be inhibited by catalase or by scavengers of ·OH, but not by superoxide dismutase (see below).

Quercetin and myricetin, at the concentrations used, did not interfere with the deoxyribose assay, whereas gossypol produced a slight absorbance at 532 nm after heating with TBA: this was corrected for, in all experiments, as described in the legend to Table 1B. Inclusion of gossypol, myricetin or quercetin in reaction mixtures containing H_2O_2 and Fe^{3+}–citrate or Fe^{3+}–ADP did not cause any reproducible increase in ·OH generation. In fact, gossypol decreased the amount of ·OH generated. However, addition of any one of these phenols to reaction mixtures containing Fe^{3+}–EDTA and H_2O_2 caused a striking six- to eight-fold stimulation of deoxyribose degradation (Tables 1B and 2, see also Tables 3 and 4). This deoxyribose degradation was markedly inhibited by omitting H_2O_2 or iron–EDTA from the reaction mixture, or, as shown in Table 2, by adding catalase or scavengers of ·OH (mannitol, formate, dimethylsulphoxide). In the presence of ascorbic acid, however, the stimulatory effects of these compounds were much less pronounced (Table 3 shows a typical experimental result), except under conditions where the reaction was allowed to run for so long that ascorbic acid concentrations in the reaction mixture became depleted (data not shown).

Table 4 shows the effect of bovine erythrocyte copper–zinc superoxide dismutase. As reported previously [25], SOD inhibited the generation of ·OH from Fe^{3+}–EDTA and H_2O_2, but did not inhibit the much greater ·OH generation when ascorbate was added to the reaction mixtures [26]. SOD also substantially inhibited the enhanced ·OH generation observed in the presence of gossypol, quercetin or myricetin (Table 4).

Effect of quercetin, gossypol and myricetin on iron-dependent damage to DNA by bleomycin

Bleomycin is an anti-tumour antibiotic that binds

Table 2. Effect of gossypol, quercetin and myricetin on Fe^{3+}–EDTA-dependent hydroxyl radical generation and modification by removal of H_2O_2 or by hydroxyl radical scavengers

	Deoxyribose degradation (A_{532}) after 1 hr incubation in the presence of				
Source of hydroxyl radical	Reaction mixture (RM)	RM plus 100 units catalase	RM plus 50 mM mannitol	RM plus 50 mM formate	RM plus 20 mM dimethylsulphoxide
Fe^{3+}–EDTA	0.138 ± 0.006 (18)	0.021 ± 0.001 (6)	0.043 ± 0.003 (6)	0.050 ± 0.003 (8)	0.031 ± 0.001 (6)
Fe^{3+}–EDTA + gossypol	0.801 ± 0.045 (12)	0 (4)	0.014 ± 0.004 (4)	0.033 ± 0.006 (6)	0 (4)
Fe^{3+}–EDTA + quercetin	0.776 ± 0.060 (12)	0.012 ± 0.004 (4)	0.081 ± 0.012 (4)	0.062 ± 0.003 (6)	0.031 ± 0.002 (4)
Fe^{3+}–EDTA + myricetin	1.082 ± 0.066 (6)	0.050 ± 0.009 (4)	0.122 ± 0.015 (4)	0.093 ± 0.006 (4)	0.045 ± 0.006 (4)

Results show mean values ± SE for (N) tests. The reaction mixture (1.2 ml) contained 1.42 mM H_2O_2, 2.8 mM deoxyribose, 20 μM $FeCl_3$–100 μM EDTA, 100 μM gossypol, quercetin or myricetin and catalase or scavengers as indicated. Absorbance of the plant phenolics was corrected for as indicated in Table 1B. The phenolics all enhanced ·OH production significantly ($P < 0.001$), and catalase/scavenger treatments all inhibited ·OH generation significantly ($P < 0.001$).

Table 3. Effect of gossypol, quercetin, myricetin and ascorbic acid on hydroxyl radical generation in the presence of Fe^{3+}–EDTA

Addition to reaction mixture containing Fe^{3+}–EDTA	Extent of deoxyribose degradation A_{532}
—	0.054
Gossypol	0.140
Quercetin	0.074
Myricetin	0.203
Ascorbic acid	0.992
Ascorbic acid + gossypol	1.075
Ascorbic acid + quercetin	0.998
Ascorbic acid + myricetin	1.045

Results show mean values of duplicate determinations for each condition. All tubes contained Fe^{3+}–EDTA, deoxyribose, buffer and H_2O_2 at the concentrations given in Materials and Methods. The incubations were performed for 5 min only and the ascorbate concentration was raised to 500 μM to avoid depletion. Where indicated, ascorbic acid (500 μM), quercetin (100 μM), gossypol (100 μM) or myricetin (100 μM) were added to give the final concentrations stated. Values for gossypol, quercetin and myricetin have been corrected for the colours produced by the phenols themselves in the TBA test (see Table 1).

Table 4. Action of superoxide dismutase on Fe^{3+}-dependent hydroxyl radical generation

Source of hydroxyl radical	Deoxyribose degradation (A_{532})	
	no SOD added	SOD present
Fe^{3+}–EDTA/H_2O_2 (control)	0.151 ± 0.005	0.046 ± 0.002*
+ myricetin	0.983 ± 0.027†	0.274 ± 0.016*
+ gossypol	0.890 ± 0.099†	0.093 ± 0.031*
+ quercetin	0.916 ± 0.071†	0.101 ± 0.013*
+ ascorbic acid	0.598 ± 0.023†	0.533 ± 0.040

Results show mean ± SE for four tests. All tubes contained buffer, deoxyribose, Fe^{3+}–EDTA and H_2O_2 as described in Materials and Methods, and where indicated myricetin, gossypol, quercetin or ascorbic acid were added at 100 μM. SOD was added at 100 units per 1.2 ml reaction mixture. The values for myricetin, gossypol and quercetin have been corrected as in Table 1. † indicates significant difference with respect to control, $P < 0.001$; * indicates significant difference compared to corresponding incubation lacking SOD, $P < 0.001$.

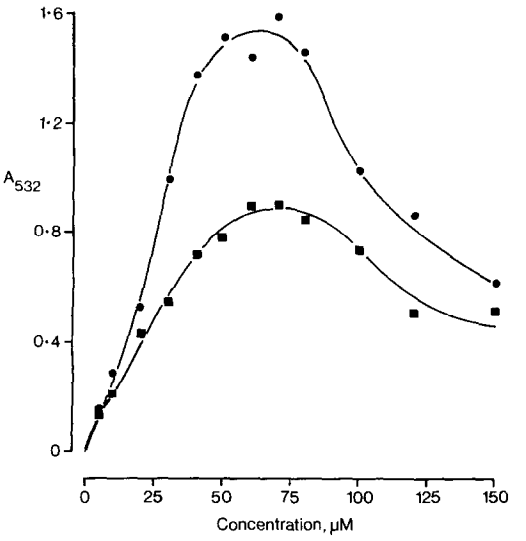

Fig. 2. Action of plant phenolics on DNA degradation in a bleomycin–Fe^{3+}–DNA system. 0.5 ml DNA (1 mg/ml) was mixed together with 0.05 ml bleomycin sulphate (1.5 units/ml, approximately 0.6 mM), 0.1 ml $MgCl_2$ (0.05 M), 0.05 ml Tris buffer (1.0 M, pH 7.4) 0.1 ml $FeCl_3$ (0.5 mM) or 0.1 ml buffer (for the reaction blank tubes), and the reaction was started by addition of 0.1 ml of either quercetin, myricetin or gossypol dissolved in ethanol. Tubes were incubated at 37° for 30 min, followed by the addition of 1 ml of 25% (v/v) HCl and 1 ml TBA reagent (1% (w/v) TBA in 0.05 M NaOH). The tubes were then heated at 100° for 10 min to develop the MDA–TBA chromogen which was read at 532 mm after cooling. Samples containing gossypol often became turbid during incubation, and developed a purple precipitate on heating at 100°. Reaction mixtures were extracted into butanol before reading at 532 nm. Results are for single determinations at each concentration tested, and show the values obtained when the absorbance due to the reaction blanks (lacking $FeCl_3$) was subtracted from the test values. (●) Myricetin; (■) quercetin.

both to iron ions and to DNA. The bleomycin–Fe^{3+}–DNA complex is inert, but addition of a reducing agent, such as ascorbic acid, produces rapid DNA damage under aerobic conditions [27, 28]. Addition of H_2O_2 to a bleomycin–Fe^{3+}–DNA complex can also result in DNA damage [28].

Figure 2 shows that addition of quercetin or myricetin led to DNA degradation in the presence of bleomycin and Fe^{3+} at pH 7.4. The stimulatory effects increased with concentration up to a maximum, but then became smaller (Fig. 2). Gossypol had no observable stimulatory effect, but its tendency to precipitate in the reaction mixture (see legend to Fig. 2) could have obscured one. The compounds were added dissolved in ethanol, but control experiments showed that ethanol had no effect on DNA degradation. The DNA degradation promoted by quercetin and myricetin was not inhibited by addition of catalase (tested up to 10^3 units) or of superoxide dismutase (tested up to 100 units) to the reaction mixture.

Effect of quercetin, gossypol and myricetin on iron-dependent lipid peroxidation

Incubation of rat-liver microsomes with Fe^{2+}, Fe^{3+}/ascorbate, or Fe^{3+}–ADP/NADPH at pH 7.4 causes rapid peroxidation, detectable by the TBA method. Fe^{3+} ions alone or Fe^{3+}–EDTA complexes caused only very slight stimulations of peroxidation (Table 5) in freshly-prepared microsomal fractions. (These stimulations increased if microsomes had been stored at −20° for more than 3 weeks: hence microsomes no older than this were used in all our experiments.) Quercetin, gossypol and myricetin (added at micromolar concentrations, dissolved in

Table 5. Effect of quercetin, gossypol and myricetin on iron-dependent lipid peroxidation in rat liver microsomes

Addition to reaction mixture	Extent of peroxidation (A_{532})	Inhibition of peroxidation %
None (control)	0.018	—
100 μM Fe^{3+}	0.084	—
Fe^{3+} + 10 μM quercetin	0.048	55
Fe^{3+} + 10 μM gossypol	0.042	74
Fe^{3+} + 10 μM myricetin	0.046	58
100 μM Fe^{2+}	1.048	—
Fe^{2+} + 10 μM quercetin	0.352	68
Fe^{2+} + 10 μM gossypol	0.142	88
Fe^{2+} + 10 μM myricetin	0.246	78
100 μM Fe^{3+}/ascorbate	1.216	—
Fe^{3+}/ascorbate + 10 μM quercetin	0.101	93
Fe^{3+}/ascorbate + 10 μM gossypol	0.072	95
Fe^{3+}/ascorbate + 10 μM myricetin	0.077	95
100 μM Fe^{3+}–ADP/NADPH	1.071	—
Fe^{3+}–ADP/NADPH + 10 μM quercetin	0.384	65
Fe^{3+}–ADP/NADPH + 10 μM gossypol	0.134	89
Fe^{3+}–ADP/NADPH + 10 μM myricetin	0.316	72
100 μM Fe^{3+}–EDTA	0.068	—
Fe^{3+}–EDTA + 10 μM quercetin	0.051	34
Fe^{3+}–EDTA + 10 μM gossypol	0.061	14
Fe^{3+}–EDTA + 10 μM myricetin	0.047	42

Results are the means of duplicate determinations taken from a representative experiment. Percentage inhibition due to the phenolic compounds is calculated after deducting the "control" level of peroxidation from the relevant values. The assays were performed as described in Materials and Methods.

ethanol) exerted powerful inhibitory effects on peroxidation in all five systems tested. Low concentrations introduced a lag period, after which peroxidation proceeded at the previous rate (Fig. 3 shows an example using 3 μM quercetin). The exact length of these lag periods varied slightly in different microsomal preparations. Concentrations of flavonoids greater than or equal to 20 μM inhibited peroxidation almost completely in all microsomal preparations. Figure 4 shows some representative experimental results. It may be seen from both Table 5 and Fig. 4 that gossypol is more effective as an inhibitor of peroxidation ($IC_{50} \leq 0.1$ μM) than are myricetin or quercetin (IC_{50} values approximately 1.5 μM). Also, the inhibitions by the phenols in the Fe^{3+}/ascorbate system were greater than in the other peroxidation systems (Table 5). Mixtures of gossypol, quercetin or myricetin (concentration range 0–50 μM) with Fe^{3+} or Fe^{3+}–EDTA gave no stimulation of peroxidation; Table 5 shows a typical result, using 10 μM phenols, and illustrates that the phenols were again inhibitory. Ethanol itself, at the concentrations used, had no significant effect on the peroxidation process, nor did the phenols interfere with the TBA test.

DISCUSSION

When Fe^{3+}–EDTA is mixed with H_2O_2, a slow rate of ·OH generation results, which is inhibitable by SOD and catalase (Ref. 25; Tables 2 and 4). This ·OH may arise by reaction of Fe^{3+}–EDTA with H_2O_2 to form a ferryl or Fe(V) complex, which then reacts with more H_2O_2 to give perferryl EDTA and hence Fe^{2+}–EDTA. This can generate ·OH in a Fenton reaction [25, 29].

$$Fe^{2+}\text{–EDTA} + H_2O_2 \rightarrow Fe^{3+}\text{–EDTA} + \cdot OH + OH^- \quad (1)$$

Fe^{3+} alone reacts poorly, if at all, with H_2O_2. Complexation of Fe^{3+} with citrate or ADP often gives slight but not significant ($P > 0.05$) stimulation of ·OH generation. Addition of ascorbic acid to the reaction mixtures greatly increases the rate of ·OH generation (Tables 1, 3, 4), presumably by redox-cycling iron and maintaining a supply of Fe^{2+} for reaction (1).

$$Fe^{3+}\text{–complex} + \text{ascorbate} \rightarrow Fe^{2+}\text{–complex} + \text{semidehydroascorbate} \quad (2)$$

The greatly-accelerated ·OH generation in the presence of ascorbate is inhibited by catalase, but not by SOD (Ref. 26; Table 4).

When gossypol, quercetin or myricetin were added to reaction mixtures containing Fe^{3+}–EDTA and H_2O_2 at pH 7, they produced a striking stimulation of deoxyribose degradation. This may be attributed to increased ·OH generation, since the deoxyribose degradation was almost completely prevented by catalase as well as by established scavengers of ·OH (mannitol, formate, dimethylsulphoxide), as shown in Table 2. The stimulation was not additive to that produced by ascorbate (Table 3), suggesting that the phenols are somehow acting by redox-cycling the iron. The ability of phenols to reduce Fe^{3+} to Fe^{2+} is well established [21]. However, their mechanism of action must be more complex than this, since SOD almost completely prevented ·OH generation in the presence of gossypol, quercetin or myricetin. A logical explanation of our results is that Fe^{3+}–EDTA induces an oxidation of the phenols to produce superoxide (O_2^-), which is responsible for the reduction of Fe^{3+}–EDTA to Fe^{2+}–EDTA, i.e.

$$\text{Phenol} \xrightarrow{Fe^{3+}\text{–EDTA, O2}} \text{oxidized phenol} + O_2^-. \quad (3)$$

$$Fe^{3+}\text{–EDTA} + O_2^- \rightarrow \text{intermediate complexes} \rightarrow Fe^{2+}\text{–EDTA} + O_2. \quad (4)$$

Two aspects of our data on ·OH generation deserve comment. Firstly, gossypol, quercetin and myricetin are very poorly soluble at pH 7.4. They could not be dissolved in organic solvents, since such

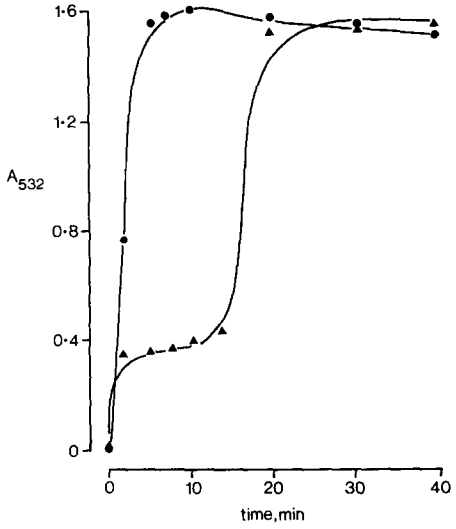

Fig. 3. Action of quercetin on Fe^{3+}–ascorbate-stimulated microsomal lipid peroxidation: production of a lag period at low quercetin concentrations. Results show mean values derived from duplicate determinations at each time point. Reaction mixtures contained, in a final volume of 1 ml, 0.25 mg microsomal protein, 40 μl ethanol, or quercetin dissolved in ethanol to give a final concentration of 3 μM, 10 mM KH_2PO_4–KOH buffer pH 7.4, 100 μM $FeCl_3$ and 100 μM ascorbate. Tubes were incubated at 37° for various times and the reaction stopped by adding the TBA reagents. (●) Control (40 μl EtOH); (▲) quercetin (3 μM).

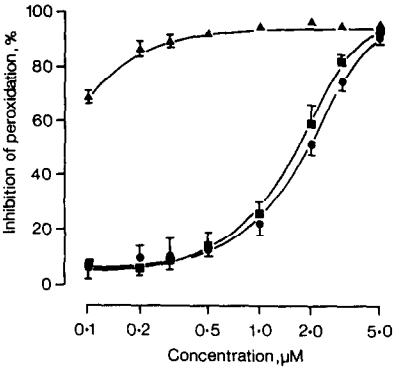

Fig. 4. Concentration-dependent inhibition by gossypol, quercetin and myricetin of microsomal lipid peroxidation in the presence of Fe^{3+}–ascorbate. Reaction mixtures contained, in a final volume of 1 ml, the following reagents added in the order stated: 100 μM $FeCl_3$, 0.25 mg microsomal protein, 0–100 μl ethanol (or phenolic compound dissolved in ethanol to give the final concentration stated), 10 mM KH_2PO_4–KOH buffer pH 7.4, and 100 μM ascorbate to start the reaction. Tubes were incubated at 37° for 20 min and peroxidation measured by the TBA test. Values are expressed as % inhibition of peroxidation, values of 0% being the extent of peroxidation in the presence of ethanol only. Results show mean values ± SE for 4–6 tests at each concentration. (●) Myricetin; (■) quercetin; (▲) gossypol.

solvents are themselves powerful scavengers of ·OH. Thus, it is possible that the concentrations of the phenols in our reaction systems were rather less than those expected, especially as solutions made up at alkaline pH values tend to precipitate on pH adjustment and incubation at pH 7.4. Thus the phenols may be even more effective in stimulating ·OH generation than is shown by our data.

Secondly, the stimulatory effect of the phenols was shown with Fe^{3+}–EDTA, but not with Fe^{3+} alone or with the more physiologically-relevant [22] Fe^{3+}–ADP or Fe^{3+}–citrate complexes. Presumably, complexation of Fe^{3+} with EDTA alters the redox potential and iron solubility [30] so as to facilitate oxidation of the phenols by iron and reduction of the iron by O_2^-. It will be necessary to identify a physiological Fe^{3+}–chelate that does interact with the phenols before our results on ·OH generation can be said to have relevance *in vivo*. Recent studies (S. M. Rankin and D. S. Leake, personal communication), have shown that some flavonoids (including myricetin) accelerate oxidative modification of human low density lipoproteins, including that mediated by macrophages. This suggests that our observations may be relevant to a physiologically-important process. Further, our experiments show that quercetin and myricetin accelerate DNA damage by a bleomycin–Fe^{3+}–DNA complex. They probably do this by reducing the Fe^{3+}–complex to an Fe^{2+}–complex. It is unlikely in this reaction system that quercetin and myricetin oxidize to produce H_2O_2, which can accelerate DNA degradation by a Fe^{3+}–bleomycin complex [28], since catalase had no effect on the rate of DNA degradation. The reason why higher concentrations of quercetin and myricetin give lower rates of DNA degradation in the presence of Fe^{3+}–bleomycin (Fig. 2) is unknown: one possibility is that their iron-binding capacity allows them to withdraw iron from the bleomycin–iron–DNA complex at higher phenol concentrations. Gossypol did not stimulate bleomycin-dependent DNA degradation, but we cannot rule out that this was due to solubility problems, since it had a tendency to precipitate out in the reaction mixtures.

Despite their ability to stimulate ·OH generation and (in two cases) bleomycin-dependent DNA degradation, none of these phenolic compounds produced any acceleration of microsomal lipid peroxidation in the presence of Fe^{3+} or Fe^{3+}–EDTA. Indeed, they are powerful inhibitors of microsomal peroxidation in the presence of a variety of iron complexes (Table 5; Fig. 4). This inhibitory effect is especially marked when peroxidation is stimulated by adding Fe^{3+}/ascorbate. Gossypol is more effective, on a molar basis, than quercetin or myricetin, perhaps because of its increased hydrophobicity (4 aromatic rings, see Fig. 1), which would allow it to enter the membranes more readily. The fact that, at low concentration, the phenols introduce a "lag period" into the peroxidation process (Fig. 3) suggests that they act as lipid-soluble chain-breaking inhibitors of the peroxidation process, scavenging intermediate peroxyl and alkoxyl radicals. This chain-breaking activity seems to outweigh any iron-reducing activity, at least in the microsomal system.

Thus flavonoids and other phenolics cannot simply be classified as antioxidants on the basis of experiments performed with lipid systems. They must also

be carefully examined for pro-oxidant properties before being proposed for use *in vivo*. A similar point about synthetic phenolic antioxidants has been made previously by Gutteridge [27].

Acknowledgements—We are very grateful to the ONO Pharmaceutical Company for research support.

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