Biochemical Pharmacology, Vol. 40, No. 4, pp. 793–797, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

STRUCTURE–ACTIVITY RELATIONSHIPS OF POLYMETHOXYFLAVONES AND OTHER FLAVONOIDS AS INHIBITORS OF NON-ENZYMIC LIPID PEROXIDATION

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(*Received* 24 *November* 1989; *accepted* 12 *March* 1990)

Abstract—Polymethoxylated flavones and C-glycosyl derivatives isolated from medicinal plants besides other flavonoid compounds were studied for their influence on lipid peroxidation induced by $FeSO_4$+cysteine in rat liver microsomes. A number of hydroxyflavones (e.g. luteolin); C-glycosyl-flavones (e.g. orientin); methoxyflavones (e.g. gardenin D) and flavonols (e.g. datiscetin), as well as the flavanol leucocyanidol and the biflavone amentoflavone behaved as inhibitors of non-enzymic lipid peroxidation. Structure–activity relationships were established and it was observed that the structural features for active polyhydroxylated compounds were different from those of polymethoxylated flavones, antiperoxidative flavonoids possessing a high lipophilicity.

The process of lipid peroxidation may contribute to cell ageing and pathological disorders such as some forms of liver injury, atherosclerosis and cancer. The peroxidative reaction is initiated by oxygen species which attack polyunsaturated fatty acids, starting a propagation cycle which involves lipid and peroxyl radicals as well as lipid hydroperoxides. As a result, peroxidation and radical propagation lead to destabilization and disintegration of cell membranes. The propagation cycle is broken by enzymic inactivation of oxygen species or by non-enzymic reactions due to the intervention of free radical scavengers and antioxidants [1–3].

In order to find new compounds able to control lipid peroxidation a large number of natural products, some of them belonging to the flavonoid class, have been investigated [4–6]. On the other hand, it is interesting to note that some of the pharmacological properties of this group of compounds have been related to their actions as free radical scavengers and inhibitors of peroxidation [7–10].

In the present study we have assessed the inhibitory ability of polymethoxyflavones and other flavonoids isolated from medicinal plants, using $FeSO_4$+cysteine-induced peroxidation in rat liver microsomes. We have also included a large range of synthetic flavonoids to gain more insight into the structure–activity relationships of this class of compounds as inhibitors of non-enzymic lipid peroxidation.

MATERIALS AND METHODS

Spinosin and vicenin-2 were isolated from *Cayaponia tayuya*; 5-*O*-demethylnobiletin and gardenin D from *Sideritis mugronensis*; cirsiliol, cirsimaritin, 8-methoxycirsilineol, sideritoflavone and xanthomicrol were isolated from *Sideritis javalambrensis*. Some flavonoids were generous gifts: diosmin (Faes), troxerutin (Almirall), hesperidin methylchalcone (Pierre Fabre), leucocyanidol (Rovi) and hesperidin (Seber). The rest of the flavonoids were purchased from Sigma, Aldrich and Roth. All other chemicals were from Sigma. Microsomes were prepared from the livers of male Wistar rats weighing 200–250 g and the microsomal fractions were isolated by differential centrifugation [11]. Microsomal pellets were suspended in 0.15 M KCl, 0.1 M Tris-HCl buffer, pH 7.4. Aliquots of this microsomal suspension were stored at −70° and thawed before use. Protein was determined by the method of Lowry *et al.* [12] using bovine serum albumin as standard. Final protein concentration in the incubation samples was 1.5 mg/mL. Assays were carried out in triplicate and peroxidation was induced by addition of $FeSO_4$ and cysteine (final concentrations 5 and 500 μM, respectively). Appropriate controls were performed to discard a direct interaction of flavonoids with the components of this system. Products of peroxidation were measured by the thiobarbituric acid method [13]. 1,1,3,3-Tetramethoxypropane was used as external standard and results were expressed as nmoles of malondialdehide equivalents (MDA). Half maximal inhibitory concentration (IC_{50}) was determined from the curves percentage of inhibition/flavonoid concentration and statistical analysis was performed using two-tailed Student's *t*-test for unpaired samples.

RESULTS AND DISCUSSION

Control incubations of rat liver microsomes with $FeSO_4$ + cysteine produced 13.40 ± 0.48 nmol MDA (mean ± SD, from 22 experiments). Flavonoids were tested at the initial screening concentration of 100 μM in two separate experiments and those compounds which did not inhibit peroxidation by at least 50% of control were not tested further. Figure 1

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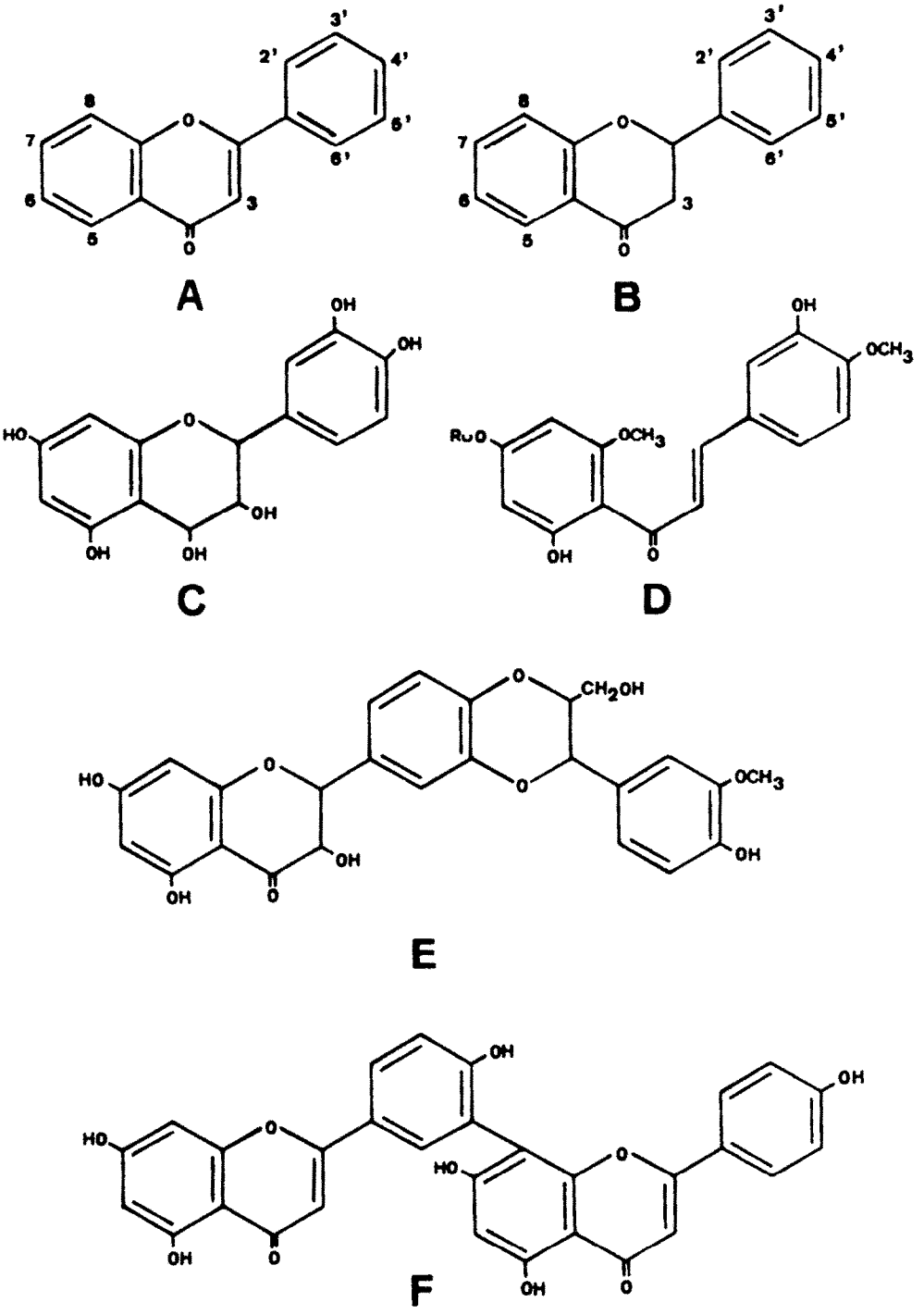

Fig. 1. Structures of flavonoids. Flavones and flavonols (A); flavanones (B); leucocyanidol (C); hesperidin-methylchalcone (D); silybin (E); and amentoflavone (F).

shows the structures of flavonoids and substituents in basic structure. Percentage of inhibition at 100 μM and IC_{50} are indicated in Table 1.

In this system a series of flavonoids not previously reported as inhibitors of lipid peroxidation demonstrated a significant activity. Luteolin and orientin were the most potent compounds, comparable to known synthetic antioxidants such as propyl gallate [13]. We confirmed the antiperoxidative activity of chrysin, 3-hydroxyflavone, fisetin, morin, quercetin, rutin and silybin, which had been studied in other systems [6–8], with some differences in potency. Apart from these known inhibitors, a number of flavonoids effectively inhibited $FeSO_4$+cysteine-induced peroxidation: isoorientin, acacetin, gardenin D, cirsimaritin, 8-methoxycirsilineol, 5-*O*-

Table 1. Substituents, percentage of inhibition (%I) at 100 μM and inhibitory concentration 50 (IC_{50}) of the flavonoids tested. Flavones (A)

Name	5	6	7	8	3′	4′	%I (100 μM)	IC_{50} (μM)
Flavone	H	H	H	H	H	H	10.8†	>100‡
Chrysin	OH	H	OH	H	H	H	96.4†	26.4
Apigenin	OH	H	OH	H	H	OH	74.5†	55.2
Rhoifolin	OH	H	ONh	H	H	OH	70.1†	66.1
Acacetin	OH	H	OH	H	H	OCH_3	89.0†	37.6
Spinosin	OH	So	OCH_3	H	H	OH	15.6	>100‡
Vitexin	OH	H	OH	Gl	H	OH	8.1	>100‡
Vicenin-2	OH	Gl	OH	Gl	H	OH	11.6	>100‡
Luteolin	OH	H	OH	H	OH	OH	97.1†	5.8
Diosmin	OH	H	ORu	H	OH	OCH_3	1.5	>100‡
Isoorientin	OH	Gl	OH	H	OH	OH	99.1†	15.4
Orientin	OH	H	OH	Gl	OH	OH	97.7†	9.5
Cirsimaritin	OH	OCH_3	OCH_3	H	H	OH	71.5†	70.2
Cirsiliol	OH	OCH_3	OCH_3	H	OH	OH	1.6	>100‡
Xanthomicrol	OH	OCH_3	OCH_3	OCH_3	H	OH	4.0	>100‡
Sideritoflavone	OH	OCH_3	OCH_3	OCH_3	OH	OH	5.4	>100‡
Gardenin D	OH	OCH_3	OCH_3	OCH_3	OH	OCH_3	62.5†	47.7
8-Methoxycirsilineol	OH	OCH_3	OCH_3	OCH_3	OCH_3	OH	74.2†	71.7
5-*O*-Demethylnobiletin	OH	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	82.0†	73.8

* $P < 0.05$, † $P < 0.01$.
Gl, glucose; Nh, neohesperidose; Ru, rutinose; So, sophorose.
‡ 50% of inhibition was not reached.

Table 1. Continued. Flavonols (A)

Name	3	5	7	2′	3′	4′	5′	%I (100 μM)	IC_{50} (μM)
3-Hydroxyflavone	OH	H	H	H	H	H	H	98.3†	17.4
Galangin	OH	OH	OH	H	H	H	H	98.5†	24.6
Datiscetin	OH	OH	OH	OH	H	H	H	97.3†	12.1
Kaempferol	OH	OH	OH	H	H	OH	H	98.7†	23.8
Fisetin	OH	H	OH	H	OH	OH	H	96.2†	29.2
Morin	OH	OH	OH	OH	H	OH	H	98.2†	11.1
Quercetin	OH	OH	OH	H	OH	OH	H	98.0†	17.6
Robinetin	OH	H	OH	H	OH	OH	OH	97.5†	35.7
Rutin	ORu	OH	OH	H	OH	OH	H	98.9†	19.5
Troxerutin	ORu	OH	OHE	H	OHE	OHE	H	78.6†	44.1
Tamarixetin	OH	OH	OH	H	OH	OCH_3	H	−2.6	>100‡

* $P < 0.05$, † $P < 0.01$.
Ru, rutinose; OHE, O-hydroxyethyl.
‡ 50% of inhibition was not reached.

Table 1. Continued. Flavanones (B)

Name	5	7	3′	4′	%I (100 μM)	IC_{50} (μM)
Naringenin	OH	OH	H	OH	4.4	>100‡
Naringin	OH	ONh	H	OH	3.6	>100‡
Eriodictyol	OH	OH	OH	OH	94.2†	21.5
Hesperidin	OH	ORu	OH	OCH_3	6.0	>100‡

* $P < 0.05$, † $P < 0.01$.
Nh, neohesperidoside; Ru, rutinose.
‡ 50% of inhibition was not reached.

Table 1. Continued

Name	%I (100 μM)	IC_{50} (μM)
Flavanols (C)		
Leucocyanidol	97.7†	17.3
Chalcones (D)		
Hesperidin-methylchalcone	5.4	>100‡
Dihydroflavonols (E)		
Silybin	57.9†	96.7
Biflavones (F)		
Amentoflavone	99.6†	16.5

* $P < 0.05$, †$P < 0.01$.
‡ 50% of inhibition was not reached.

demethylnobiletin, datiscetin, galangin, robinetin, leucocyanidol and amentoflavone.

Lipid peroxidation is a complex process which can be influenced by flavonoids through different mechanisms, such as divalent metal chelation and free radical scavenging, possibly by donation of the phenolic hydrogen and formation of a flavonoid radical which in turn reacts with free radicals, thus breaking the propagating chain [14, 15]. The presence of an *ortho*-dihydroxyl group, a feature compatible with the formation of rather stable *ortho*-semiquinone anions, has been reported as necessary for inhibition of non-enzymic peroxidation [6]. In fact, we have observed that the most potent compounds possess a free catechol group at 3′, 4′ and the potency increases with the introduction of this group in polyhydroxylated flavonoids (apigenin-luteolin; vitexin-orientin). Nevertheless, the presence of a free hydroxyl group at 4′ has no influence (galangin-kaempferol), while at 2′ besides at 3 (datiscetin, morin), a configuration vinylogous of *ortho*-dihydroxyphenol, leads to an increase in the inhibitory activity. This structural feature can be important for the free radical scavenging activity shown by such compounds [16].

The introduction of a hydroxyl group at C-3 of the flavone skeleton gives raise to a significant activity (flavone-3 hydroxyflavone), supporting the contention derived from results obtained in other systems that the 3-OH is a group determinant for antiperoxidative activity [6]. It is obvious that 3-OH or 5-OH besides the carbonyl group at 4, as well as *ortho*-dihydroxyl groups may form chelates with divalent cations, one of the possible mechanisms by which flavonoids act [15].

O-Glycosylation has a slight negative influence at C-7, suggesting a partial role for the free hydroxyl at this position, while at C-3 it has no effect. C-glycosylation at 6 or 8 position decreases the activity.

The structure–activity relationship for methoxylated compounds deserves especial attention. In polyhydroxylated products methoxylation at C-4′ has a slight influence when that is the only substituent in the B ring (apigenin-acacetin), or can be detrimental if the result is the blocking of the active 3′,4′ catechol group (quercetin-tamarixetin). Interestingly, we have demonstrated the inhibitory activity of some polymethoxylated flavones which have shown a potency comparable to that reported for vitamin E in the same system [13]. The features related to the antiperoxidative activity are different from those displaced by polyhydroxylated flavonoids, since the 3′,4′ catechol group in flavones possessing two or three methoxyl groups (C-6, C-7 or C-6, C-7 and C-8) does not cause an increase in the antiperoxidative effects (cirsiliol, sideritoflavone). In addition, polymethoxylated flavones with catechol groups partially or totally blocked by methoxylation display inhibitory activity (gardenin D, 8-methoxycirsilineol, 5-*O*-demethylnobiletin).

On the other hand we have observed that hydrogenation of the 2,3 double bond decreases the activity (apigenin-naringenin, luteolin-eriodictyol).

The lack of effect of sideritoflavone and cirsiliol, which are potent lipoxygenase inhibitors [17] indicates that the inhibition of arachidonic acid metabolism by these compounds is dependent on flavonoid–enzyme interactions and not related to possible antioxidant properties.

Flavonoids active on lipid peroxidation may be an alternative to the more toxic synthetic antioxidants for the treatment of pathological conditions derived from such process, since this class of natural products may effectively inhibit peroxidation. Nevertheless, it is necessary to confirm *in vitro* results performing studies which must include the administration of the active compounds to animals.

Flavonoids are considered to be water-soluble chain-breaking antioxidants [18], although it should be taken into account that the introduction of several methoxyl groups leads to a change in their antiperoxidative structural features and to a decrease in hydrophilicity, which may facilitate the access to lipid structures. Thus, polymethoxyflavones may be considered as inhibitors of non-enzymic lipid peroxidation possessing features different from those of previously known active flavonoids.

Acknowledgement—This work was supported by grant PA86-0295 from C.I.C.Y.T. Spanish Ministerio de Educación y Ciencia.

REFERENCES

1. Niki E, Antioxidants in relation to lipid peroxidation. *Chem Phys Lipids* **44**: 227–253, 1987.
2. Slater TF, Free radical mechanisms in tissue injury. *Biochem J* **222**: 1–15, 1984.
3. Southorn PA, Free radicals in medicine. II. Involvement in human disease. *Mayo Clin Proc* **63**: 390–408, 1988.
4. Robak J, Duniec Z, Rzadkowska-Bodalska H, Olechnowicz-Stepien W and Cisowski W, The effect of some flavonoids on non-enzymatic lipid oxidation and enzymatic oxidation of arachidonic acid. *Pol J Pharmacol Pharm* **38**: 483–491, 1986.
5. Affany A, Salvayre R and Douste-Blazy L, Comparison of the protective effect of various flavonoids against lipid peroxidation of erythrocyte membranes (induced by cumene hydroperoxide). *Fundam Clin Pharmacol* **1**: 451–457, 1987.
6. Ratty AK and Das NP, Effects of flavonoids on non-enzymatic lipid peroxidation: structure–activity relationship. *Biochem Med Metab Biol* **39**: 69–79, 1988.
7. Valenzuela A, Guerra R and Videla LA, Antioxidant properties of the flavonoids silybin and (+)cyanidanol-3: comparison with butylated hydroxyanisole and butylated hydroxytoluene. *Planta Med* **52**: 438–440, 1986.
8. Gryglewski RJ, Korbut R, Robak J and Swies J, On the mechanism of antithrombotic action of flavonoids. *Biochem Pharmacol* **36**: 317–322, 1987.
9. Alcaraz MJ and Hoult JRS, Actions of flavonoids and the novel antiinflammatory flavone, hypolaetin-8-glucoside, on prostaglandin biosynthesis and inactivation. *Biochem Pharmacol* **34**: 2477–2482, 1985.
10. Alcaraz MJ and Villar A, Antiinflammatory effects of natural phenolic compounds. In: *Handbook of Free Radicals and Antioxidants in Biomedicine* (Eds. Miquel J, Quintanilha AT and Weber H), Vol. II, 77–85. CRC Press, Boca Raton, FL, 1989.
11. Slater TF and Sawyer BC, The stimulatory effects of carbon tetrachloride and other halogenoalkanes on peroxidative reaction in rat liver fractions *in vitro*. *Biochem J* **123**: 805–814, 1971.

12. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
13. Mansuy D, Sassi A, Dansette PM and Plat M, A new potent inhibitor of lipid peroxidation *in vitro* and *in vivo*, the hepatoprotective drug anisyldithiolthione. *Biochem Biophys Res Commun* **135**: 1015–1021, 1986.
14. Robak J and Gryglewski RJ, Flavonoids are scavengers of superoxide anions. *Biochem Pharmacol* **37**: 837–841, 1988.
15. Afanas'ev IB, Dorozhko AI, Brodskii AV, Kostyuk VA and Potapovitch AI, Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochem Pharmacol* **38**: 1763–1769, 1989.
16. Bäumann J, Wurm G and Bruchhausen Fv, Hemmung der prostaglandinsynthetase durch Flavonoide und Phenolderivate im Vergleich mit deren O_2^- Radikalfängereigenschaften. *Arch Pharm* **313**: 330–337, 1980.
17. Alcaraz MJ and Ferrándiz ML, Modification of arachidonic metabolism by flavonoids. *J Ethnopharmacol* **21**: 209–229, 1987.
18. Fraga CG, Martino VS, Ferraro GE, Coussio JD and Boveris A, Flavonoids as antioxidants evaluated by *in vitro* and *in situ* liver chemiluminescence. *Biochem Pharmacol* **36**: 717–720, 1987.