remains unknown in which cell type within the midgut gland this synthesis takes place. So far, all attempts at a more specific localization have failed. A newly available technique of separating the various cell types of the midgut gland[13] may provide a more promising approach.

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Inhibitory effects of phenolic compounds on CCl_4-induced microsomal lipid peroxidation

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Summary. The antiperoxidative effects of 35 phenolic compounds, most of them belonging to the flavonoid class, were investigated using CCl_4-induced peroxidation of rat liver microsomes. This system was rather insensitive to gallic acid, methyl gallate and ellagic acid. Nevertheless it was inhibited by flavonoids and structure/activity relationships were established. The most potent compounds were gardenin D, luteolin, apigenin (flavones), datiscetin, morin, galangin (flavonols), eriodictyol (flavanone), amentoflavone (biflavone) and the reference compound, (+)-catechin. The natural polymethoxyflavone gardenin D has shown a potency comparable to that of (+)-catechin and higher than that of silybin. Thus, it may be considered as a new type of natural antioxidant with potential therapeutical applications.
Key words. Phenolic compounds; CCl_4-induced peroxidation; TBA-reactive substances.

The toxicity of a large number of xenobiotics depends on their conversion into free radicals, which initiate the process of lipid peroxidation in cell membranes. Carbon tetrachloride (CCl_4) is probably the best-studied liver toxicant; it stimulates endogenous peroxidative changes in rat liver microsomes during incubation at 37 °C in the presence of NADPH[1].

CCl_4 is activated by the NADPH-cytochrome P-450 system of the liver endoplasmic reticulum, with formation of the trichloromethyl radical ($CCl_3\cdot$) and in aerobic conditions, of the more reactive trichloromethyl peroxy radical ($CCl_3O_2\cdot$). The latter initiates peroxidation of the polyunsaturated fatty acids, while $CCl_3\cdot$ is more important in covalent binding to both lipid and protein components of the membrane[2,3]. The onset of fatty infiltration depends upon haloalkylation, which could be involved in the pathogenesis of liver necrosis determining an increased susceptibility of the cell to oxidative stress. Therefore, covalent binding should be implicated more in the pathogenesis of cell death during chronic CCl_4 intoxication[4], whereas acute cell death seems to be mainly dependent upon lipid peroxidation[5].

Flavonoids are a widely distributed group of natural antioxidants, some of them exerting protective effects against peroxidation-induced cell damage[6]. As the stimulatory effect of CCl_4 on lipid peroxidation provides a convenient method for studying the effectiveness of potential scavengers and antioxidants[7], we have selected it to assess the activity of a number of natural phenolic compounds, most of them belonging to the flavonoid class, in order to find new antiperoxidative agents with potential therapeutical applications.

Materials and methods

Drugs. Some compounds were isolated from plants: gardenin D and 5-O-demethylnobiletin (*Sideritis mugronensis*[8]); hypolaetin-8-O-β-D-glucoside (*Sideritis leucantha*[9]); taxifolin (*Rhamnus lycioides*[10]) and methyl gallate

(*Pistacia lentiscus*, unpublished results). Other drugs were commercially available: fisetin, 3-hydroxyflavone and ellagic acid (Aldrich); troxerutin (Almirall); diosmin (Faes); quercetin (Merck); acacetin, amentoflavone, apigenin, datiscetin, eriodictyol, galangin, orientin, isoorientin, luteolin, robinetin and silybin (Roth); leucocyanidol (Rovi); rhoifolin, naringenin, naringin, (+)-catechin, (−)-epicatechin, chrysin, morin, rutin and gallic acid (Sigma Chem.). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP, EDTA, 2-thiobarbituric acid and 1,1,3,3-tetramethoxypropane were purchased from Sigma Chem. All other chemicals were of analytical grade.

CCl_4-induced lipid peroxidation. Adult male Wistar rats (200–250 g) were killed by cervical dislocation and microsomes were prepared as described by Slater and Sawyer[1]. Microsomal pellets were resuspended in 1.15% KCl and protein was measured by the procedure of Lowry et al.[11]. Aliquots of this microsomal suspension were stored at − 70 °C and thawed before use. Incubations of microsomal suspensions (1.5 mg protein/ml) were performed at 37 °C according to Mansuy et al.[12] with 0.1 mM EDTA and an NADPH-generating system containing 1 mM NADP, 10 mM glucose-6-phosphate and 2 units of glucose-6-phosphate dehydrogenase in 100 mM potassium phosphate pH 7.4, with the addition of 10 µl of 2 M CCl_4 in ethanol. After 10 min incubation the reaction was stopped by adding the thiobarbituric acid (TBA) reagent and the absorbance was read at 535 nm. Some flavonoids needed the addition of ethanol to increase their solubility. In these cases the amount of organic solvent in the incubation mixture never exceeded 1%, a concentration that did not affect the reaction, as demonstrated using the appropriate controls. Compounds were tested at concentrations in the range of 10–330 µM and controls were performed to discard any direct interaction of test compounds with the components of the system, which could affect the determination of TBA-reactive material. 1,1,3,3-Tetramethoxypropane was used as external standard and assays were carried out in triplicate.

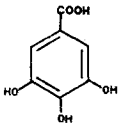
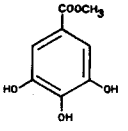
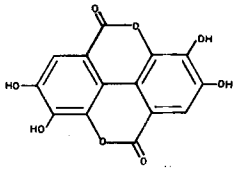
Statistical analysis. The concentrations leading to 50% inhibition (IC_{50}) of TBA-reactive material formation were determined from curves percentage of inhibition/flavonoid concentration. Statistical analysis was performed using Dunnett's t-test.

Results

As shown in table 1, CCl_4-induced lipid peroxidation was rather insensitive to gallic acid, its methyl ester and to ellagic acid, which was reported to be a potent inhibitor of iron and NADPH-dependent microsomal lipid peroxidation[13].

In the series of flavonoids, datiscetin, morin, galangin (flavonols), gardenin D, luteolin, apigenin (flavones), eriodictyol (flavanone) and amentoflavone (biflavone) were the most potent inhibitors of TBA-reactive material formation, besides the reference compound (+)-catechin (table 2), which showed an IC_{50} higher than that previously reported[14].

Table 1. Structure, percentage of inhibition at 100 µM and inhibitory concentration 50 (IC_{50}) of the phenolic acids tested.

Name	%I(100 µM)	IC_{50} (µM)
Gallic acid	9.5 ± 1.1**	> 100
		
Methyl gallate	0.7 ± 3.2	> 100
		
Ellagic acid	21.2 ± 1.6**	> 100
		

Data are the mean ± SE from two separate experiments. ** $p < 0.01$.

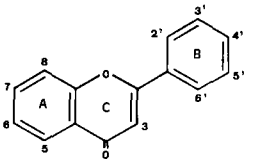
The inhibitory activity is not dependent on the number of free hydroxyl groups present in the flavone or flavane skeleton, but on the pattern of hydroxylation. Free hydroxyl groups in the rings A (C-5 and C-7) and/or C (C-3) participate in the inhibition of peroxidation, while the presence of hydroxyls in the B ring is not necessary, though it can increase the activity, with some differences according to the structure type. The hydroxyls at C-3', C-4' reported as structural determinants of antiperoxidative effects in non-enzymic systems[15] are important substitutions in flavones (where their influence is equivalent to that shown by a hydroxyl at C-4'), flavanones and flavanes, in contrast to flavonols. In this last group the structural requirements for activity are the hydroxyl at C-2' and the pyrogallol group (C-3', C-4', C-5').

In polyhydroxylated flavonoids, the blocking of active hydroxyl functions by glycosylation or methoxylation causes a decrease in their inhibitory effects, which is also seen in C-glycosides (luteolin versus isoorientin or orientin); this indicates that the linkage of bulky sugar substituents to carbons next to hydroxyl groups leads to a reduction in the inhibitory efficiency of such groups, due to steric effects.

The data obtained for the two natural polymethoxyflavones, gardenin D and 5-O-demethylnobiletin, indicate that in this group of flavonoids, the hydroxyl at C-3' favours the inhibition, and the presence of a free hydroxyl at C-5 results in a certain degree of activity.

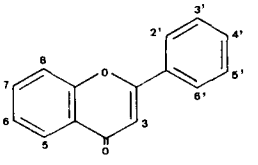
Table 2. Structure, percentage of inhibition at 100 μM and inhibitory concentration 50 (IC_{50}) of the flavonoids tested.

Flavones



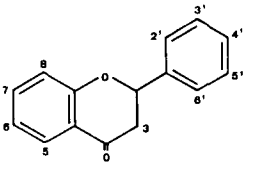
Name	5	6	7	8	3'	4'	%I(100 μM)	IC_{50} (μM)
Chrysin	OH	H	OH	H	H	H	54.7 ± 1.3**	> 100
Apigenin	OH	H	OH	H	H	OH	75.7 ± 1.7**	79.1 ± 0.8
Rhoifolin	OH	H	ONh	H	H	OH	37.9 ± 7.9*	> 100
Acacetin	OH	H	OH	H	H	OCH_3	56.3 ± 1.7**	100 ± 1.4
Luteolin	OH	H	OH	H	OH	OH	72.2 ± 2.2**	70.4 ± 1.7
Isoorientin	OH	Gl	OH	H	OH	OH	37.3 ± 8.7**	> 100
Orientin	OH	H	OH	Gl	OH	OH	43.7 ± 8.7**	> 100
Hypolaetin-8 glucoside	OH	H	OH	OGl	OH	OH	12.5 ± 4.9	> 100
Gardenin-D	OH	OCH_3	OCH_3	OCH_3	OH	OCH_3	65.1 ± 1.6**	84.6 ± 1.7
5-Demethylnobiletin	OH	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	50.5 ± 1.3**	> 100

Flavonols



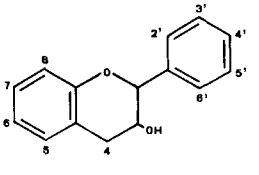
Name	3	5	7	2'	3'	4'	5'	%I(100 μM)	IC_{50} (μM)
3-Hydroxyflavone	OH	H	H	H	H	H	H	39.6 ± 4.3**	> 100
Galangin	OH	OH	OH	H	H	H	H	82.6 ± 3.8**	68.9 ± 1.3
Datiscetin	OH	OH	OH	OH	H	H	H	79.2 ± 1.6**	39.5 ± 0.8
Fisetin	OH	H	OH	H	OH	OH	H	33.8 ± 0.8**	> 100
Morin	OH	OH	OH	OH	H	OH	H	82.3 ± 1.0**	48.5 ± 0.9
Quercetin	OH	OH	OH	H	OH	OH	H	51.6 ± 3.1**	> 100
Rutin	ORu	OH	OH	H	OH	OH	H	22.2 ± 4.3	> 100
Troxerrutin	ORu	OH	OHE	H	OHE	OHE	H	46.5 ± 4.1**	> 100
Robinetin	OH	H	OH	H	OH	OH	OH	55.7 ± 1.8**	96.8 ± 1.6

Flavanones



Name	5	7	3'	4'	%I(100 μM)	IC_{50} (μM)
Naringenin	OH	OH	H	OH	29.9 ± 6.2**	> 100
Naringin	OH	ONh	H	OH	5.6 ± 1.8	> 100
Eriodictyol	OH	OH	OH	OH	64.7 ± 4.2**	78.9 ± 1.3

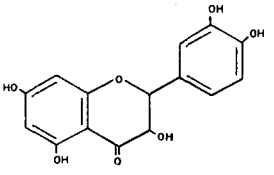
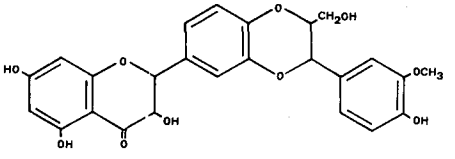
Flavanols



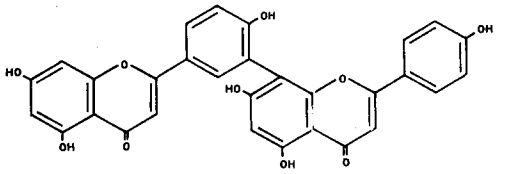
Name	4	5	7	3'	4'	%I(100 μM)	IC_{50} (μM)
Catechin	H	OH	OH	OH	OH	69.6 ± 1.9**	87.1 ± 1.7
Epicatechin	H	OH	OH	OH	OH	58.2 ± 1.8**	> 100
Leucocyanidol	OH	OH	OH	OH	OH	51.7 ± 5.5**	> 100

Table 2. Continued

Dihydroflavonols

Name	%I(100 μM)	IC_{50} (μM)
Taxifolin	17.6 ± 7.2	100
		
Silybin	34.0 ± 5.7**	100
		

Biflavones

Name	%I(100 μM)	IC_{50} (μM)
Amentoflavone	61.1 ± 1.2**	74.1 ± 0.8
		

Data are the mean ± SE from two separate experiments.
*$p < 0.05$, Gl = Glucose, **$p < 0.01$, Nh = Neohesperidose, Ru = Rutinose, OHE = O-hydroxyethyl.

On the other hand, the hydrogenation of the double bond in the C ring decreases the antiperoxidative effects of flavonoids. Finally, it can be deduced that the keto group at position 4 is not essential for the inhibitory activity, and the dimerization of an active compound does not modify its influence on peroxidation (apigenin - amentoflavone).

Discussion

Flavonoids can act at the initiation stage of peroxidation, interfering with the metabolism of CCl_4 either by scavenging CCl_4-derived radicals or by the impairment of the microsomal enzyme system necessary for CCl_4 metabolism. Another possibility is the scavenging of lipoperoxy and other radicals, thus breaking the chain reaction. Fe^{2+} ions are probably implicated in the catalysis of chain propagation reactions in CCl_4-induced hepatotoxicity, and thereby the administration of metal chelators to rats inhibits CCl_4-induced lipid peroxidation and hepatotoxicity[16]. The presence of *ortho*-dihydroxyl groups as well as C-5 or C-3 free hydroxyl groups, besides the carbonyl function at position 4, are structural determinants for the formation of chelates with divalent ions, which may participate in the mechanism of action of antiperoxidative flavonoids, as reported for rutin and quercetin[17].

The reference compound (+)-catechin and some alkyl derivatives have been reported as inhibitors of CCl_4-induced peroxidation in rat liver microsomes, acting as free radical scavengers mainly owing to the 3', 4' diphenolic group in the B ring. In this respect it has been suggested that this flavane derivative scavenges the $CCl_3\cdot$[18] or $CCl_3O_2\cdot$ radicals[19], as well as certain propagating radicals[3]. In a similar way it is likely that our flavonoids possessing active hydroxyl groups act by scavenging CCl_4-derived radicals.

Some of the flavonoids studied may inhibit lipid peroxidation by reacting with peroxy radicals of polyunsaturated fatty acids, breaking the chain reaction[20]. On the other hand, interactions of these natural products with the cytochrome P-450 system have been reported[21].

The potency of flavonoids in this system is lower than that of synthetic antioxidants, but comparable to that of vitamin E[12]. These natural antioxidants may have the advantage of having only a low toxicity in animals[22].

A number of flavonoids tested exhibited inhibitory effects higher than those of silybin, a flavonoid with therapeutical applications which has been reported as a protective agent in other models of lipid peroxidation and hepatotoxicity[23]. In our experiments the polymethoxylated flavone gardenin D has shown an inhibitory potency comparable to that of the well-known scavenger (+)-catechin. This finding is especially interesting because natural flavonoids of this type would be more appropriate for in vivo administration than highly polar hydroxylated compounds, such as (+)-catechin. This molecule is extensively metabolized and, as a result, it is more active in vitro than in vivo, since it probably does not reach the site of free radical damage in effective concentrations[14].

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Insect anti-juvenile hormone and juvenile hormone activity from plants in the genus *Nama*

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Summary. The insect anti-juvenile hormones precocene I and II (7-methoxy-2,2-dimethyl-2H-1-benzopyran and 6,7-dimethoxy-2,2-dimethyl-2H-1-benzopyran) were identified in three of nine *Nama* (Hydrophyllaceae) species. Precocene I occurred in *N. lobbii* while precocene II occurred in *N. hispidum, N. lobbii* and *N. sandwicense. N. hispidum* contained the highest concentration (ca 0.5% dry weight) of precocene II, which was found in the leaves, stems, seed capsules, corolla, glandular trichomes, and seeds. In addition to the anti-juvenile hormone, insect juvenile hormone activity was detected in the organosoluble extracts of *N. rothrockii* and *N. sandwicense. N. sandwicense* is the first plant discovered to contain compounds with both anti- and juvenile hormone activity.
Key words. Nama; Hydrophyllaceae; precocenes; anti-juvenile hormones; juvenile hormone activity.

Certain phytochemicals are recognized to adversely impact the endocrine system of insects by mimicking the insect's natural juvenile hormone (JH) or acting as antagonists to the production or action of JH[1,2]. Since JH regulates many important physiological functions including metamorphosis[3], an insect's life cycle can be disrupted by the presence of these phytochemicals. As a consequence of JH mimic activity, some insects may undergo abnormal growth and delayed metamorphosis[4]. When JH is eliminated, due to JH antagonists or anti-juvenile hormone (AJH) activity, some insects may prematurely molt to adults, enter diapause, or become sterilized[5]. Even minute amounts of these plant compounds are often sufficient to disrupt the insect's physiology and development[6].

Compounds with juvenile hormone activity have been isolated from a variety of plants[6,7], while AJHs have been previously isolated only from the Asteraceae[5,8]. These types of compounds have never been reported to occur simultaneously in the same plant. We have now discovered in plants in the genus *Nama* (Hydrophyllaceae) compounds which disrupt the endocrine system of the large milkweed bug, *Oncopeltus fasciatus. Nama hispidum* contained the AJH, precocene II (6,7-dimethoxy-2,2-dimethyl-2H-1-benzopyran) while *N. rothrockii* had at least two compounds with JH activity. *N. sandwicense* contained precocene II (PII) as well as JH activity. The distribution and concentration of P II and the related compound P I (7-methoxy-2,2-dimethyl-2H-1-benzopyran) are reported for the nine *Nama* species collected and for the different anatomical parts of *N. hispidum*.

Experimental

Plant material. Specimens in the genus *Nama* were collected during the spring and summer of 1986 at the following localities: *N. demissum* Gray: Clark Co., Nevada, USA; *N. densum* Lemmon: Mono Co., California, USA; *N. hispidum* Gray: Pima Co., Arizona, USA; *N. jamaicense* Linn: Dominican Republic; *N. lobbii* Gray: Eldorado Co., California, USA; *N. rothrockii*: Inyo Co., California, USA; *N. sandwicense* Gray: Maui Co., Hawaii, USA; *N. stevensii* Hitchcock: Eddy Co., New