Protective Effects of Phenolic Compounds on CCl_4-Induced Toxicity in Isolated Rat Hepatocytes

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Flavonoids, Phenolic Acids, CCl_4 Hepatocytes, Hepatoprotective Agents

The protective effects of a series of phenolic compounds, phenolic acids and flavonoids on the cytotoxicity of CCl_4 in rat hepatocytes were studied. A number of flavones, 7,8-dihydroxyflavone, luteolin and hypolaetin-8-glucoside, flavonols, morin, quercetin, robinetin and gossypin, phenolic acids, gallic, caffeic and chlorogenic acids, as well as the flavane (+)-catechin significantly inhibited alanine amine transferase (ALT) release. Catechol groups are determinant for the protective activity of flavonoids and cinnamic acid derivatives, as well as the resorcinol or pyrogallol moieties in the B ring of flavonoids. In benzoic acid derivatives a pyrogallol group is required. This feature is associated with the inhibition of ALT spontaneous release.

Introduction

Some natural phenolic compounds, such as silymarin and (+)-catechin possess hepatoprotective activity and have been reported to prevent animal poisoning by several hepatotoxic substances [1–3].

Carbon tetrachloride (CCl_4) can be considered as a model molecule for alkylating agents that are converted to radical species able to initiate lipid peroxidation. Liver necrosis induced by CCl_4 is depending on metabolic activation by the NADPH-cytochrome P-450 system of the liver endoplasmic reticulum, with formation of the trichloromethyl ($CCl_3^{\cdot}$) and trichloromethylperoxyl ($CCl_3O_2^{\cdot}$) radicals. The latter initiates lipid peroxidation while the $CCl_3^{\cdot}$ radical is involved in covalent binding to components of the membrane [4].

Studies in isolated rat hepatocytes have made it apparent that CCl_4 toxicity is not related to membrane solubilization, but to metabolic generation of free radicals [5] and have led to the development of a widely used *in vitro* model for the screening of potential hepatoprotective agents.

In this work we have studied the influence of a series of phenolic compounds, flavonoids and phenolic acids, on CCl_4-induced toxicity in isolated rat hepatocytes, in order to establish their possible hepatoprotective properties.

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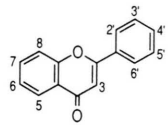
Results

Since the determination of transaminase activity in the serum is widely adopted in the assessment of liver injury, CCl_4 toxicity was evaluated by means of the alanine amino transferase (ALT) activity present in the incubation medium after 90 min. In isolated rat hepatocytes CCl_4 challenge caused a concentration-dependent cell damage as it was observed by trypan blue staining and increase in ALT leakage. In our experimental conditions we obtained a significant increase in ALT activity in cells treated with 10 mM CCl_4 (23.96 ± 1.24 UI/g wet cells, mean ± S.E., $n = 12$) compared with reference preparations (8.51 ± 0.58 UI/g wet cells, mean ± S.E., $n = 14$; $p < 0.01$). This toxicity was also confirmed by the trypan blue exclusion test as almost 100% of the hepatocytes treated with 10 mM CCl_4 appeared stained (% cell viability = 0.46 ± 0.2, $n = 8$), while in reference preparations % cell viability was 71.9 ± 1.4 ($n = 8$) after 90 min incubation time.

Compounds were added to cell suspensions at the initial screening concentration of 2 mM. Only those which afforded a protection of at least 50% were tested at a range of concentrations (0.75–2 mM) to calculate the effective concentration 50% (EC_{50}).

The degree of leakage in ALT activity, generally considered to be a reliable parameter to reflect the extent of CCl_4 toxicity was significantly decreased in cells treated with a number of flavones (7,8-dihydroxyflavone, luteolin and hypolaetin-8-glucoside), flavonols (morin, quercetin, robinetin and

Table I. Structure, percentage of protection at 2 mM and effective concentration 50% (EC_{50}) of the flavonoids tested.



Flavones	5	6	7	8	3′	4′	% Protection [2 mM]	EC_{50} [mM]
7,8-Dihydroxyflavone	H	H	OH	OH	H	H	102.6 ± 2.5**	0.64
Luteolin	OH	H	OH	H	OH	OH	38.3 ± 14.1*	n.d.
Hypolaetin-8-glucoside	OH	H	OH	OG	OH	OH	38.7 ± 7.8*	n.d.
Gardenin-D	OH	OCH_3	OCH_3	OCH_3	OH	OCH_3	13.8 ± 8.7	n.d.
Diosmin	OH	H	OGR	H	OH	OCH_3	24.3 ± 14.8	n.d.

Flavonols	3	5	7	8	2′	3′	4′	5′	% Protection [2 mM]	EC_{50} [mM]
Datiscetin	OH	OH	OH	H	OH	H	H	H	4.5 ± 4.9	n.d.
Morin	OH	OH	OH	H	OH	H	OH	H	55.3 ± 9.7**	1.73
Quercetin	OH	OH	OH	H	H	OH	OH	H	42.8 ± 6.6**	n.d.
Robinetin	OH	H	OH	H	H	OH	OH	OH	119.8 ± 4.6**	1.02
Gossypin	OH	OH	OH	OG	H	OH	OH	H	58.6 ± 2.6**	1.63

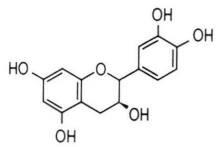
n.d., not determined.
Data are the mean ± S.E. from two separate experiments.
* $p < 0.05$ G = glucose.
** $p < 0.01$ R = rhamnose.

gossypin), phenolic acids (gallic, caffeic and chlorogenic acids) and the flavane (+)-catechin.

Table I shows percentages of protection at 2 mM, EC_{50} and structures of the flavonoids tested. The most potent compounds were the flavone 7,8-dihydroxyflavone and the flavonols robinetin, gossypin and morin, which were more effective than the standard compound (+)-catechin (Table II).

Table II. Structure, percentage of protection at 2 mM and effective concentration 50% (EC_{50}) of the flavanol tested.

Flavanol	% Protection [mM]	EC_{50} [mM]
Catechin	33.3 ± 9.4*	n.d.



n.d., not determined.
Data are the mean ± S.E. from two separate experiments.
* $p < 0.05$.

The results obtained for benzoic acid derivatives can be observed in Table III and those for cinnamic acid derivatives in Table IV. Gallic, caffeic and chlorogenic acids caused a dose-related protection of hepatic injury as judged by the decrease in ALT activity in comparison to respective controls, whereas the results in the presence of ellagic and sinapic acids were not significant.

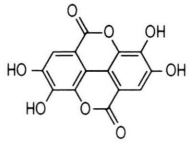
As indicated in Table V, robinetin and gallic acid at 2 mM inhibited spontaneous release of ALT activity, as observed in reference groups, thus suggesting that their high protective effect against CCl_4 toxicity was due, at least in part, to a direct action on enzyme release. Quercetin and gossypin caused a significant inhibition of spontaneous release but to a lesser extent.

Discussion

With the limitations derived from *in vitro* experiments, inhibition of CCl_4 toxicity is an assay method suitable for screening of antihepatotoxic compounds [6, 7]. From our results, some conclusions can be drawn on the structure/activity relationships for the two groups of natural phenolic com-

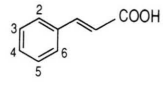
Table III. Structure, percentage of protection at 2 mM and effective concentration 50% (EC_{50}) of the benzoic acid derivatives tested.

Name	2	3	4	5	% Protection [2 mM]	EC_{50} [mM]
Gallic	H	OH	OH	OH	91.6 ± 2.7*	1.17
Gentisic	OH	H	H	OH	20.9 ± 10.9	n.d.
Protocatechuic	H	OH	OH	H	14.5 ± 11.1	n.d.
Vanillic	H	OCH_3	OH	H	−19.9 ± 13.8	n.d.
Siryngic	H	OCH_3	OH	OCH_3	12.1 ± 10.7	n.d.
Ellagic					−41.3 ± 19.1	n.d.

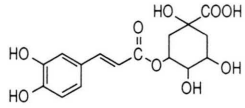


n.d., not determined.
Data are the mean ± S.E. from two separate experiments.
* $p < 0.01$.

Table IV. Structure, percentage of protection at 2 mM and effective concentration 50% (EC_{50}) of the cinnamic acid derivatives tested.



Name	2	3	4	5	% Protection [2 mM]	EC_{50} [mM]
o-Coumaric	OH	H	H	H	−1.0 ± 9.8	n.d.
m-Coumaric	H	OH	H	H	15.9 ± 6.1	n.d.
p-Coumaric	H	H	OH	H	26.5 ± 7.1	n.d.
Caffeic	H	OH	OH	H	89.2 ± 9.4*	1.37
Ferulic	H	OCH_3	OH	H	−23.8 ± 13.0	n.d.
Sinapic	H	OCH_3	OH	OCH_3	−43.4 ± 17.8	n.d.
Chlorogenic					58.5 ± 12.2	1.61



n.d., not determined.
Data are the mean ± S.E. from two separate experiments.
* $p < 0.01$.

pounds studied, flavonoids and phenolic acids. Catechol groups are determinant for the protective activity of flavonoids, especially at C-7, C-8, as well as the resorcinol or pyrogallol groups in the B ring. We have also observed that flavonols are more active than their corresponding flavones, as deduced from the pairs quercetin-luteolin and gossypin-hypolaetin-8-glucoside, indicating the contribution of the free hydroxyl at C-3 in compounds possessing a free catechol group.

Table V. Effects of polyphenolic compounds (2 mM) on spontaneous release of ALT activity (IU/g wet cells).

Name	ALT activity
7,8-Dihydroxyflavone	10.98 ± 0.56 (a)
Morin	10.17 ± 1.49 (a)
Robinetin	2.16 ± 0.41** (a)
Gossypin	9.43 ± 0.59* (a)
Gallic acid	2.57 ± 0.32** (a)
Chlorogenic acid	11.87 ± 1.34 (a)
Caffeic acid	8.32 ± 1.14 (b)
Quercetin	7.92 ± 0.65* (b)

Spontaneous release of ALT activity in reference group: (a) 13.23 ± 0.98; (b) 10.77 ± 0.70 IU/g wet cells.
Data are the mean ± S.E. from two separate experiments.
* $p < 0.05$.
** $p < 0.01$.

In phenolic acids the presence of a catechol group is determinant for activity in the case of cinnamic acid derivatives (caffeic and chlorogenic acids), whereas in benzoic acid derivatives a pyrogallol group is required (gallic acid). The formation of a lactone (ellagic acid) or the blockade of active catechol (ferulic acid) or pyrogallol (syringic acid) groups by methylation abolish the protective activity. It is noteworthy that inhibition of ALT spontaneous release occurs in compounds containing a pyrogallol moiety (robinetin and gallic acid).

For comparative purposes, we have included in our experiments the flavonoids luteolin and quercetin, previously reported as protective agents against CCl_4-induced cytotoxicity [5, 8, 9], which have shown a significant activity.

In the group of phenolic acids, our results confirm the activity of caffeic acid [10, 11], nevertheless in our study chlorogenic acid has exerted protective effects, while it has been reported as inactive [11]. This discrepancy can be due to differences in the experimental conditions. In this respect, it is interesting to note that chlorogenic acid significantly decreases transaminase levels in rats fed peroxidized oil [12].

The present work demonstrates for the first time the protective effects of phenolic compounds like 7,8-dihydroxyflavone, hypolaetin-8-glucoside, morin, robinetin, gossypin and gallic acid. The precise mechanisms underlying their activity remain to be established.

Inhibition of CCl_4-induced cytotoxicity may result from drug inhibition of metabolic activation of the toxicant. Thus, the interaction of some flavonoids with the cytochrome P-450 has been reported [13]. On the other hand, protective effects could be a consequence of the ability to remove reactive free radicals and possibly to prevent lipoperoxidative events, since lipid peroxidation is the key obligatory consequence in CCl_4-induced toxicity, a conclusion which is supported by the observation that antioxidants reduce CCl_4 liver injury [14].

It is worth noting that in the flavonoid group, the structure/activity relationship we have found for cell protection is quite similar to that reported for inhibition of lipid peroxidation and thus we have previously demonstrated the antioxidant properties of several compounds which have shown a significant inhibition of CCl_4-induced toxicity [15, 16]. For phenolic acids there is no clear correlation with their reported antioxidant properties [17, 18].

Previous work [5] focused attention on the hydrophilicity of protective flavonoids. Thus active compounds may stabilize the hepatocyte membrane by binding to it at the water/membrane interface. The question as to whether polarity can determine hepatoprotective activity remains controversial. In our experiments a lack of activity for methoxylated compounds, flavonoids and phenolic acids has been observed. However, cell protection is not dependent on the number of free hydroxyl groups present in the molecule, but on their position. In this respect, the activity of 7,8-dihydroxyflavone can be an example.

The relevance of the hepatoprotective effects shown by flavonoids and phenolic acids, in relation to *in vivo* situations, as well as their possible mechanisms of action will be established by further studies now in progress.

Materials and Methods

Some flavonoids were isolated from plants: hypolaetin-8-glucoside from *Sideritis leucantha* and gardenin D from *Sideritis mugronensis* Borja (Lamiaceae) following known procedures [16]; gossypin from *Hibiscus vitifolius* L. (Malvaceae) was a kind gift (Prof. A. G. R. Nair, Pondicherry University, India). Other compounds were purchased from Sigma Chemical and Roth. All chemicals used were of analytical grade.

Isolation of hepatocytes

Male Wistar rats (250–300 g), starved overnight, were used. Liver cells were isolated by a modification of the collagenase perfusion method of Seglen [19]. After cannulation of the superior *Vena cava* in an anaesthesized animal (sodium pentobarbital 60 mg/kg i.p.), perfusion was started with Ca^{2+}-free Krebs medium containing 1 mM EGTA (pH 7.4), aerated with 95% O_2/5% CO_2 and maintained at 37 °C. After 10 min perfusion was carried out with a Ca^{2+}-containing Krebs solution supplemented with collagenase (0.4 mg/ml), which recirculated for 10–12 min till complete dispersion of the liver cells. The liver was then transferred into a beaker containing Ca^{2+}-free Krebs solution and dispersed with two forceps. The cell suspension was filtered and centrifuged at 50 g for 90 sg and the pellet was gently resuspended in an adequate volume of medium to give 8×10^6 cells/ml. Viability of the cells was determined by trypan blue exclusion (85% or higher).

Cytotoxicity induced by CCl_4

Incubations were performed under 95% O_2/5% CO_2 with shaking (80 cycles/min) in the presence of 10 mM CCl_4 (dissolved in ethanol, final concentration 2%) for 90 min at 37 °C. Compounds were dissolved in DMSO (final concentration 2.4%) and added 10 min prior to toxic challenge. Solvent was also included in control and reference groups (without CCl_4). Samples in each group were tested in quadruplicate.

Cell toxicity was evaluated by measuring the alanine aminotransferase (ALT) activity released into the medium using the method of Karmen [20]. In this standard and reproducible assay, pyruvate formed from alanine and 2-oxoglutarate by ALT is immediately converted to lactate by lactate dehydrogenase with NADH oxidation, which is followed spectrophotometrically by the lost of absorbance at 340 nm. Statistical analysis was performed using Dunnett t-test. Percentage of protection was calculated as follows [21]:

$$\% \text{ Protection} = \frac{\text{ALT control} - \text{ALT sample}}{\text{ALT control} - \text{ALT reference}} \times 100$$

Effective concentration 50% (EC_{50}) was determined from % protection/log concentration regression lines.

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