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HEPATOPROTECTIVE ACTIVITY OF POLYPHENOLIC COMPOUNDS FROM *CYNARA SCOLYMUS* AGAINST CCL_4 TOXICITY IN ISOLATED RAT HEPATOCYTES

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ABSTRACT.—The hepatoprotective activity against CCl_4 toxicity in isolated rat hepatocytes of some polyphenolic compounds, such as cynarin, isochlorogenic acid, chlorogenic acid, luteolin-7-glucoside, and two organic acids, caffeic and quinic, from *Cynara scolymus*, is tested. Only cynarine and, to a lesser extent, caffeic acid showed cytoprotective action. The possible relationship between the molecular structure and the protective effect found is discussed.

Among all the plants used in folk medicine against liver complaints, *Silybum marianum* Gaertner and *Cynara scolymus* L. (Compositae) can be considered the most important. Problems arise, however, upon attempting to demonstrate the hepatoprotective action of *C. scolymus* by means of an experimental model of acute hepatitis. The experiments carried out have not been conclusive in this field (1). Due to the great complexity of hepatic function and the inherent variability of the toxic process studied, it is very difficult to prove a hepatoprotective effect in tests performed on whole animals.

CCl_4 intoxication in the rat is an experimental model widely used to study necrosis and steatosis of the liver (2). The toxic manifestations of CCl_4 in isolated rat hepatocytes have been described by several researchers (3-5) who find a suitable correlation with the induced cell injury in vivo. However, the large doses of CCl_4 applied (10-100 mM) make the prevention of cellular lesions by means of hepatoprotective agents very problematic. Thanks to the work by Perrissoud *et al.* (6) in which they were able to obtain toxic effects with low doses the above problem can be solved.

In the present study we have used isolated rat hepatocytes for the determination of the hepatoprotective activity of certain polyphenolic constituents of *C. scolymus* against CCl_4 intoxication. Given that CCl_4 needs metabolic activation by the cytochrome P-450 system (7), we have rejected the use of cultured rat hepatocytes to perform this study due to the rapid loss of the above system produced during the development of cultures (8).

EXPERIMENTAL

ANIMALS.—Male Sprague-Dawley rats (wt. 200-240 g) were fasted 18 h before the isolation of the hepatocytes. H_2O was available ad libitum.

MATERIALS.—The following compounds were purchased: glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) uv test (Boehringer Mannheim), quinic acid (Fluka), DMSO (Merck), CCl_4 (Proebus), caffeic acid, isochlorogenic acid, and luteolin-7-glucoside (Sarsyntex), chlorogenic acid and bovine serum albumin fr. V (Sigma), and collagenase type I from *Clostridium histolyticum* (Worthington). (+)-Cyanidan-3-ol was a gift from Lab. Zyma (Switzerland) and cynarin from Lab. Ferrer (Spain). All chemicals used were of analytical grade.

ISOLATION OF HEPATOCYTES.—Isolated hepatocytes were obtained by the collagenase perfusion method (9) with some modifications (10). After cannulation of the portal vein with a polyvinyl catheter in an anaesthesized animal (sodium pentobarbital 200 mg/kg ip), perfusion was started with Ca^{++} free Krebs Ringer-bicarbonate buffer, gassed with 95% O_2/5% CO_2 (pH 7.4), and warmed at 37°. After 10 min of perfusion (flow rate 20 ml/min), the liver was excised from the abdominal cavity, arranged upon a porous holder, and a recirculating circuit was established with 100 ml buffer solution supplemented with bovine serum albumin (15 mg/ml), collagenase (0.4 mg/ml, 148 U/mg), and $CaCl_2$ (2.5 mM) for 12-20 min (flow rate 30-35 ml/min). The liver was then transferred into a beaker containing buffer solution (50 ml) plus bovine serum albumin and $CaCl_2$ at the same concentrations as above and gently dispersed with two for-

ceps. The crude cell suspension was then filtered through nylon gauze (150 μm), preincubated under 95% O_2/5% CO_2 at 37° for 15 min, and gently shaken (40 cycles/min). The cell suspension was then filtered through nylon gauze (50 μm) into centrifuge tubes and centrifuged at 50 g for 90 sec. The supernatant was aspirated off, and the loosely packed pellet of cells was carefully resuspended in buffer. This washing procedure was repeated twice. Finally, the pellet was resuspended in an adequate volume of buffer to give $1.1 \pm 0.1 \times 10^7$ cells/ml and poured into 10-ml flasks (1.5 ml cell suspension/flask). The incubation was carried out under 95% O_2/5% CO_2 at 37° with shaking (65-70 cycles/min).

Viability of the cells was estimated by the trypan blue exclusion test (11). Preparations with a viability of less than 85% were rejected.

DETERMINATION OF CYTOTOXICITY INDUCED BY CCl_4.—After 15 min of incubation, CCl_4 was added in 10 μl of DMSO to hepatocyte suspensions at final concentrations of 0.5, 1, 1.5, and 2 mM, the control flasks receiving the same volume of DMSO alone. At each incubation time (5, 10, 15, 20, and 30 min) samples were taken for the quantification of GOT and GPT activity in a Cobas-Bio serum autoanalyzer (Roche).

For the determination of the cytoprotective activity, each one of the compounds was tested at 4 different concentrations: 0.01, 0.1, 1, and 3 mg/ml of incubation medium. The cell suspensions were preincubated for 15 min. After this period, the compounds, dissolved in 10 μl of DMSO, were poured into the medium, and the same amount of DMSO was added to the control flasks and to the flasks kept for receiving the toxin alone. CCl_4 was added 10 min later in 10 μl of DMSO to the flasks, save to the control ones, which only received DMSO. After 30 min of incubation, the enzymatic activity was assessed.

At the end of the incubation time, two samples were obtained from each flask. One of them was centrifuged, and the enzymatic activity was measured in the supernatant. The remaining sample was homogenized, measuring its total enzymatic activity. The percentage of enzymatic activity released into the incubation medium was calculated by dividing the activity present in the supernatant by the total activity measured in the homogenized sample and then multiplying by 100.

DETERMINATION OF THE INFLUENCE OF THE COMPOUNDS STUDIED ON GOT AND GPT ACTIVITY.—The compounds, at the above concentrations, were added to a standard serum (Precipath, Boehringer Mannheim), redissolved in the incubation medium, and GOT and GPT activity was determined.

Statistical analysis was carried out by Student's *t*-test for unpaired groups (12).

RESULTS AND DISCUSSION

As shown in Figure 1, the addition of CCl_4 to the incubation medium induces an increase in the leakage of GOT and GPT. Of the four CCl_4 concentrations used (0.5, 1, 1.5, and 2 mM), 2 mM gave the maximal increase in transaminase leakage and was selected for the remaining experiments. For each dose, the release of GOT was higher than GPT which suggests that GOT is more sensitive than GPT as a marker of the cytotoxicity of CCl_4. This pattern could be due to the inherent mechanism of CCl_4 injury in which a strong mitochondrial disturbance is produced (13).

Table 1 shows the percentage of enzymatic activity released after the addition of CCl_4 (2 mM) to hepatocyte suspensions prepared from fasted animals. The highest GOT and GPT release was obtained after 30 min CCl_4 incubation (5, 10, 15, 20, and 30 min incubation time tested); this time was selected for the remaining experiments. During this period, viability of control hepatocytes was preserved, given that the release of transaminase activity remained unchanged.

Tables 2 and 3 give the values of the percentage of GOT and GPT activity, respectively, for each one of the tested compounds. Generally, when the values for control and intoxicated groups are compared with respect to those obtained for the same groups in the experiment described above (Table 1), a rise in the enzymatic release is observed. DMSO used for the dissolving of compounds can disturb per se the permeability of cell membranes. In the studies of the evolution of the toxic action of CCl_4, 10 μl of DMSO were added to each test tube which means 0.6% of overall incubation volume. For the testing of hepatoprotective activity, the amount of DMSO added to each tube was 20 μl, 1.3% of the volume of cell suspension. This proportional increase in the amount of

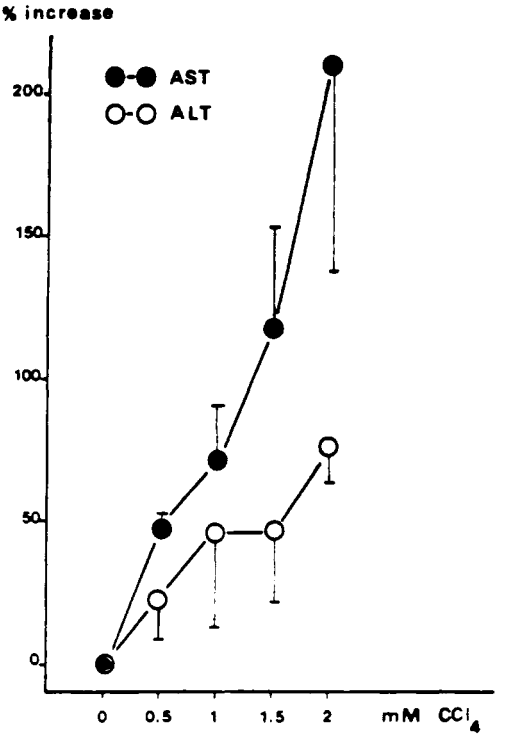

FIGURE 1. Percentage of increase in transaminase leakage with respect to the control values (0% of increase), after addition of different doses of CCl_4 to the medium. The time of incubation was 20 min. Results are means from six different flasks obtained from at least two different cell preparations. All values are significant ($p<0.01$) against control.

DMSO added to the control and intoxicated groups of the two different experiments could be responsible for the rise in enzyme leakage.

(+)-Cyanidan-3-ol, used as a reference substance whose activity has been tested in similar studies (14), shows a hepatoprotective effect against CCl_4 at the maximum dose

TABLE 1. Percentage of Enzymatic Activity Released into the Medium After Different Times of Incubation.[a]

Time (min)	GOT[b]		GPT[b]	
	Control	CCl_4	Control	CCl_4
5	7.0±1.0(3)	13.2±0.9(6)	9.3-12.4[c]	13.2±1.1(4)
10	—	12.2±2.1(6)	—	12.3±0.2(4)
15	6.7±1.1(3)	14.3±1.1(6)	8.2-10.6[c]	13.7±0.8(4)
20	—	15.5±1.2(4)	—	13.2±1.5(4)
30	6.2±0.2(3)	18.7±2.9(4)	9.6± 0.7(3)	14.3±2.0(4)

[a]The concentration of CCl_4 was 2 mM. Values are means±SD of the number of determinations in brackets from at least two different cell preparations. All values are significant ($p<0.01$) against control. Hepatocytes were obtained from fasted animals.

[b]GOT=glutamic oxaloacetic transaminase, GPT=glutamic pyruvic transaminase.

[c]Only two determinations were done.

TABLE 2. Percentage of Glutamic Oxaloacetic Transaminase (GOT) Activity Released into the Medium After Pretreatment with Several Polyphenolic Substances[a]

Substance	Control	CCl_4	0.01 mg/ml	0.1 mg/ml	1 mg/ml	3 mg/ml
(+)-Cyanidan-3-ol	12.9±2.3	21.2±1.9	16.8±2.1[b]	18.3±2.1[b]	21.0±1.3	22.4±2.5
Caffeic acid	13.2±2.1	24.0±2.8	21.0±2.0	19.9±4.8	18.6±3.6[b]	22.1±3.2
Quinic acid	12.0±2.1	23.7±4.6	18.4±3.0	21.9±2.5	21.2±2.4	21.2±2.8
Chlorogenic acid	11.8±2.3	21.6±4.3	19.5±3.5	19.4±3.7	20.6±3.9	20.6±2.7
Isochlorogenic acid	12.7±2.7	23.2±3.4	23.8±3.7	24.0±3.8	26.0±1.7[b]	
Cynarin	9.0±1.3	20.3±2.3	17.6±3.0[b]	17.9±3.8[b]	17.6±3.2[b]	16.5±3.6[b]
Luteolin-7-glucoside	13.9±1.7	24.2±2.6	21.9±3.8	20.4±4.5[b]	21.1±3.7[b]	22.8±2.9

[a]CCl_4 dose was 2 mM and incubation time 30 min. Values are means±SD for 6-9 determinations from at least two different cell preparations.

[b]$p < 0.01$ against CCl_4 values (all CCl_4 values are significant—$p < 0.01$—against control values and are not pointed out in the table).

of 0.01 mg/ml (21% GOT/22% GPT less than the values obtained for the intoxicated group). This pattern validates the experimental design used.

From the three caffeoylquinic derivatives tested, only cynarin reveals hepatoprotective properties, showing a maximum effect at the dose of 3 mg/ml (19% GOT/28% GPT less than the values obtained for the intoxicated group). At the two lower doses (0.01 and 0.1 mg/ml) it only modifies the release of GOT, but GPT is not affected. Chlorogenic acid does not alter the toxicity of CCl_4, while isochlorogenic acid induces a slight rise in GOT release at the dose of 1 mg/ml (12% of increase with respect to the values of the intoxicated group).

Likewise, the activity of caffeic and quinic acids that form part of the molecular structure of the above mentioned compounds were tested. Quinic acid does not modify the CCl_4 toxicity. On the contrary, caffeic acid shows evident hepatoprotective activity at the dose of 1 mg/ml (22% GOT/35% GPT less than the values obtained for the intoxicated group). Finally, luteolin-7-glucoside, that presents an *o*-diphenolic group in its molecule like caffeic acid, acts exclusively upon GOT activity, inducing the maximum drop at the 0.01 mg/ml dose (16% less than the value obtained for the intoxicated group).

TABLE 3. Percentage of Glutamic Pyruvic Transaminase (GPT) Activity Released into the Medium After Pretreatment with Several Polyphenolic Substances[a]

Substance	Control	CCl_4	0.01 mg/ml	0.1 mg/ml	1 mg/ml	3 mg/ml
(+)-Cyanidan-3-ol	12.5±2.2	17.2±2.7	13.5±2.3[b]	14.3±0.9[b]	17.3±2.3	20.1±4.9
Caffeic acid	13.6±2.2	20.5±2.4	19.0±2.7	17.5±6.3	13.4±2.7[b]	16.7±4.8
Quinic acid	12.3±2.8	19.3±3.7	15.5±2.7	18.1±1.6	17.4±2.0	17.0±1.9
Chlorogenic acid	11.5±3.2	18.1±3.0	16.7±3.6	15.3±3.9	17.7±4.0	15.9±3.0
Isochlorogenic acid	12.1±0.6	19.8±2.6	18.5±1.7	18.5±1.8	19.1±1.4	
Cynarin	11.4±1.7	18.5±2.5	16.3±3.3	16.8±3.8	13.9±2.9[b]	13.3±3.3[b]
Luteolin-7-glucoside	13.2±1.1	18.0±1.8	15.9±1.8	14.9±2.5	15.9±2.9	20.9±1.7

[a]CCl_4 dose was 2 mM and incubation time 30 min. Values are means±SD for 6-9 determinations from at least two different cell preparations.

[b]$p < 0.01$ Against CCl_4 values (all CCl_4 values are significant—$p < 0.01$—against control values and are not pointed out in the table).

None of these compounds interfered in any significant way with the transaminase activity, as deduced from the results shown in Table 4. For this reason, the obtained values are probably due to a protective effect of these polyphenolic substances against CCl_4 cytotoxicity.

TABLE 4. Percentage of Variation in Transaminase Activity After Addition to a Standard Serum of Different Doses of Several Polyphenolic Substances[a]

Substance	Dose (mg/ml)	GOT[b] U/liter	GPT[b] U/liter
Control	—	127	105
(+)-Cyanidan-3-ol .	0.1	127	103
Cynarin	1.0	126	103
Cynarin	3.0	123	110
Caffeic acid	1.0	123	97
Luteolin-7-glucoside .	1.0	139	111

[a]No significant differences (Wilcoxon-test, $z=0.3651$ for GOT values and $z=0.6741$ for GPT values) were observed (values are means of 3 different determinations).

[b]GOT=glutamic oxaloacetic transaminase, GPT=glutamic pyruvic transaminase.

In general, polyphenolic compounds and, specifically, caffeic acid and derivatives, have antioxidant properties (15). Younes and Siegers (16) demonstrated a protective activity in vitro against glutathione-depletion induced lipoperoxidation for quercetin, rutin, and others. It would be possible to assign the observed effects to the antioxidant activity of these compounds, which prevents the CCl_4-induced oxidation of the phospholipids that are constituents of the hepatocyte membranes. Similarly, it would be possible to attribute the hepatoprotective action of cynarin to the product of its hydrolysis, caffeic acid. This hydrolysis can take place at pH 7.4 in the incubation medium. Nevertheless, the fact that chlorogenic acid and isochlorogenic acid, related to cynarin and similarly containing the caffeic acid molecule in their structures, are devoid of activity does not support this possibility.

At lower doses, cynarin and luteolin-7-glucoside only affect GOT leakage. This fact would mean a certain selectivity of these compounds for mitochondria, as has been reported for other polyphenolic derivatives (17,18). Given that CCl_4 causes great mitochondrial injury, this characteristic of cynarin and luteolin-7-glucoside would reflect a specific interaction with the toxin not ascribed exclusively to a redox process. In this way, it is interesting to note that cynarin, among the caffeoylquinic derivatives tested, is the only one possessing two caffeic substituents in an axial position (19,20). This could reflect a certain relationship between the hepatoprotective activity of this compound and the spatial orientation of the two molecules of caffeic acid. Nevertheless, further studies in this field, through the use of other toxin such as galactosamine, phalloidin, etc, that act in different ways than CCl_4 are necessary to confirm the above hypothesis.

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