Hepatoprotective Activity of Xanthones and Xanthonolignoids Against *tert*-Butylhydroperoxide-Induced Toxicity in Isolated Rat Hepatocytes—Comparison with Silybin

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Purpose. Synthesize and evaluate the protective activity against *tert*-butylhydroperoxide-induced toxicity in freshly isolated rat hepatocytes of *trans*-kielcorin, *trans*-isokielcorin B, as well as their respective building blocks 3,4-dihydroxy-2-methoxyxanthone and 2,3-dihydroxy-4-methoxyxanthone.

Methods. Wistar rats, weighing 200-250g were used. Hepatocyte isolation was performed by collagenase perfusion. Incubations were performed at 37°C, using 1 million cells per milliliter in modified Krebs—Henseleit buffer. The protective activity was evaluated by measuring reduced and oxidized glutathione, lipid peroxidation and cell viability after inducing toxicity with *tert*-butylhydroperoxide (1.0 mM, 30 min), with or without the studied compounds in the concentrations of 0.025, 0.050, 0.100 and 0.200 mM. Silybin was tested in the same experimental conditions to serve as a positive control.

Results. Using these concentrations, the tested compounds prevented *tert*-butylhydroperoxide-induced lipid peroxidation and cell death in freshly isolated rat hepatocytes. All compounds were also effective in preventing perturbation of cell glutathione homeostasis in some extent. 3,4-Dihydroxy-2-methoxyxanthone and 2,3-dihydroxy-4-methoxyxanthone were more effective than *trans*-kielcorin and *trans*-isokielcorin B respectively. Silybin was less effective in protecting cells against lipid peroxidation and loss of cell viability than the four xanthonic derivatives.

Conclusions. The tested compounds protected the freshly isolated rat hepatocytes against *tert*-butylhydroperoxide-induced toxicity.

KEY WORDS: xanthonolignoids; xanthones; rat hepatocytes; hepatoprotective activity; *tert*-butylhydroperoxide; lipid peroxidation; glutathione.

INTRODUCTION

Research on therapeutic agents for use in liver ailments has been developed extensively among natural products obtained from plant sources used in popular medicine (1). The flavolignan mixture, silymarin and its most active component, silybin, obtained from the plant *Silybum marianum*, have been studied most intensely for antihepatotoxic effects (2), and as a result they are now used clinically in the treatment of many liver diseases.

Some species from the Guttiferae and Gentianaceae families have also been used in popular medicine for the treatment and prevention of liver diseases and for other medicinal applications (3,4) but, to our knowledge, the nature of the compound(s) responsible for hepatoprotection are still unknown.

Xanthones and xanthonolignoids are important compounds extracted from these Guttiferae and especially Kielmeyra species (5). In view of the postulated hepatoprotective activity of these plant extracts, and considering the structural and electronic similarity between xanthonolignoids and the known antihepatotoxic flavolignoids, it was deemed of interest to examine xanthonic derivatives with respect to this activity. To this end *trans*-kielcorin (1), *trans*-isokielcorin B (2), as well as their respective building blocks 3,4-dihydroxy-2-methoxyxanthone (3) and 2,3-dihydroxy-4-methoxyxanthone (4) (Fig. 1) were synthesized (5,6) and evaluated against *tert*-butylhydroperoxide-induced toxicity in freshly isolated rat hepatocytes.

The incubation of isolated hepatocytes with this organic hydroperoxide results in marked cell death preceded by intracellular glutathione depletion and extensive lipid peroxidation which can be prevented by antioxidants and free radical scavengers (7,8).

The prevention of *tert*-butylhydroperoxide-induced cell toxicity by (1), (2), (3) and (4) was evaluated by measuring reduced and oxidized glutathione, lipid peroxidation and cell viability. The results obtained for each xanthonolignoid were then compared with the results of the respective xanthone building block. Silybin (5) was tested in the same experimental conditions to serve as a positive control.

MATERIALS AND METHODS

Chemicals

All reagents were of analytical grade. Water for HPLC was deionized and tridistilled. Collagenase (grade II), *tert*-butylhydroperoxide (*t*-BHP), bovine serum albumin (fraction V), HEPES, reduced glutathione (GSH), glutathione reductase (EC 1.6.4.2), β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH), β-nicotinamide adenine dinucleotide reduced form (β-NADH), pyruvic acid, tris(hydroxymethyl)aminomethane (tris), ethylene glycol-bis (β-aminoethylether) N,N,N′,N′-tetraacetic acid (EGTA) and 2-thiobarbituric acid were purchased from Sigma Chemical Company (St. Louis, MO, USA). Chloroacetic acid, methanol (gradient grade), perchloric acid and all other chemicals were obtained from Merck (D-6100 Darmstadt, Germany). Silybin was a gift from Madaus A. G. (Germany). 3,4-Dihydroxy-2-methoxyxanthone, 2,3-dihydroxy-4-methoxyxanthone, *trans*-kielcorin and *trans*-isokielcorin B were synthesized in the Organic Chemistry Department, Faculty of Pharmacy, Porto University.

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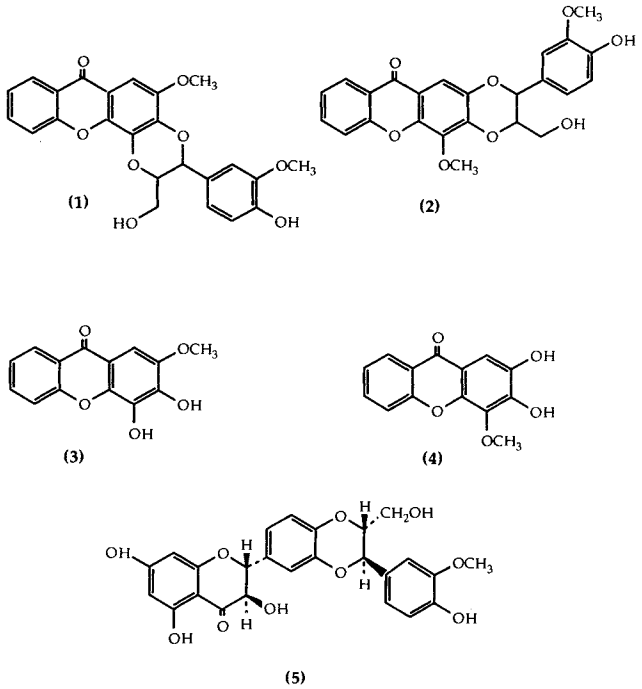

Fig. 1. The structure of the xanthonolignoids *trans*-kielcorin (1) and *trans*-isokielcorin B (2), the xanthones 3,4-dihydroxy-2-methoxyxanthone (3) and 2,3-dihydroxy-4-methoxyxanthone (4) and the flavolignan silybin (5).

Animals

This research adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985). Adult male Wistar rats, weighing 200–250g were used. The rats remained in polyethylene cages, lined with wood shavings, with wire mesh at the top, at an ambient temperature of 20 ± 1°C, humidity between 40 and 60% and 12/12 hours light/dark cycle in our animal house at least two weeks before sacrifice. Surgical procedures, which were performed under diethylether anaesthesia, occurred always between 9.00 am and 10.00 am.

Hepatocyte Isolation and Incubation

Hepatocyte isolation was performed by collagenase perfusion as previously described by Moldéus et al (9). Cell viability at the beginning of the experiments was between 85 and 95%. Incubations were performed at 37°C, using 10^6 cells per milliliter in Krebs-Henseleit buffer supplemented with 25 mM HEPES, pH 7.4, and gassed with carbogen.

In a series of experiments, aliquots of cell suspensions were simultaneously and separately treated with 10 µl/ml of (1), (3) and (5), dissolved in DMSO, at the concentrations of 0.025, 0.050, 0.100 and 0.200 mM, or only with DMSO, during 5 min before treatment with *t*-BHP. In another series of experiments, aliquots of cell suspensions were treated in the same manner, but with (2) and (4) instead of (1) and (3). After the 5 min of incubations, 10 µl/ml of *t*-BHP dissolved in Krebs were added to the cell suspensions at a final concentration of 1.0 mM. Only 10 µl/ml of DMSO and 10 µl/ml of Krebs were added to control cells. After 30 min of incubation with *t*-BHP, samples were taken out for measurement of trypan blue exclusion, lactate dehydrogenase (LDH) leakage, malondialdehyde, GSH and oxidised glutathione (GSSG) contents.

Analytical Procedures

Cell viability was determined during the course of the experiments by the trypan blue exclusion test and by cell LDH leakage.

For GSH measurement, cell suspension aliquots (100 µl) were precipitated with 900 µl of perchloric acid (5% final acid concentration), centrifuged 5 min at 6000 rpm, the supernatant filtered through 0.45 µm filter and 100 µl of each sample injected into an HPLC system with electrochemical detection as described before (10). For GSSG determination 100 µl of cell suspensions were diluted to 1 ml with 0.05M tris L-serine / Na-borate, pH 7.4 (final concentration 25 mM/50 mM) to prevent catabolism of glutathione by γ-glutamyltranspeptidase and frozen at −80°C. After thawing at room temperature, the cell samples were disrupted with a Politron homogenizer at maximum speed during 1 min (the efficacy of this disruption was certified by microscope). GSSG was then measured as described earlier (10).

The extent of lipid peroxidation, which can be determined as the formation of malondialdehyde after the breakdown of polyunsaturated fatty acids, was measured by the thiobarbituric acid reactive substances (TBARS) assay at 535 nm. Briefly, 250 µl of cell suspension was precipitated with 500 µl of 10% trichloroacetic acid and centrifuged 20 seconds at 6000 rpm. 500 µl of the supernatant were added with an equal volume of 1% thiobarbituric acid and the mixture was heated during 10 min in a boiling water bath, allowed to cool and the absorvance measured at 535 nm.

Statistical Evaluations

Data are expressed as means ± standard error (M±SE). Significant differences between control and t-BHP treated cells was determined by Student's t test. Significant differences between *t*-BHP and *t*-BHP + tested compound treated cells were determined by ANOVA followed by Fisher PLSD test. Differences were considered significant for $p \leq 0.05$.

RESULTS

Incubation of rat hepatocytes with 1.0 mM *t*-BHP during 30 min caused a marked loss in cell viability which was monitored as an increase in trypan blue uptake (Fig. 2) and as an increase in LHD leakage (Fig. 3). It caused also an increase of TBARS (Fig. 4), a significant depletion in GSH content (Fig. 5), a significant increase in GSSG formation (Fig. 6), a significant increase of the ratio GSSG/GSH (Fig. 7) and a significant depletion in total glutathione (GSH+GSSG) from 29.32±1.17 to 23.99±1.13 µg/ml. In all cases, p was ≤ 0.0001.

Concomitant incubation of rat hepatocytes with (1), (2), (3), (4) or (5) (0.025, 0.050, 0.100 and 0.200 mM) prevented to different extent the effects of *t*-BHP on trypan blue uptake, LDH leakage, production of TBARS, and GSH homeostasis. The protective effects of these five compounds was con-

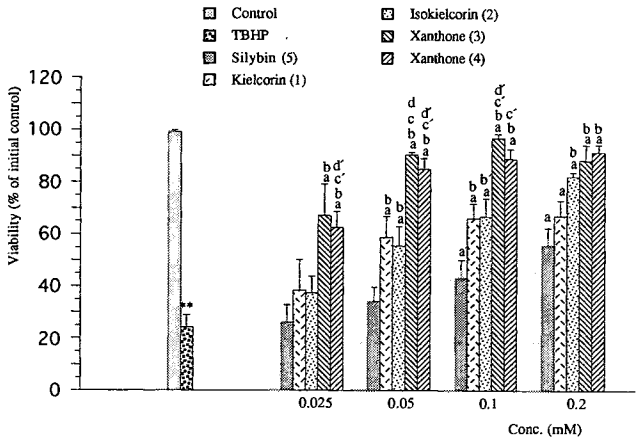

Fig. 2. Effect of silybin (5), *trans*-kielcorin (1), *trans*-isokielcorin B (2), 3,4-dihydroxy-2-methoxyxanthone (3) and 2,3-dihydroxy-4-methoxyxanthone (4) in viability expressed by trypan blue uptake by freshly isolated rat hepatocytes after 30 min of *t*-BHP exposure (means±SEM). n=4 or 8; **p≤0.0001 (between control and *t*-BHP treated cells); [a] p<0.01 and [a'] p<0.05 (when compared with *t*-BHP treated cells); [b] p<0.01, [b'] p<0.05 [when compared with (*t*-BHP + silybin) treated cells]; [c] p<0.01, [c'] p<0.05 [when compared with (*t*-BHP + *trans*-kielcorin) treated cells]; [d] p<0.01, [d'] p<0.05 [when compared with (*t*-BHP + *trans*-isokielcorin B) treated cells].

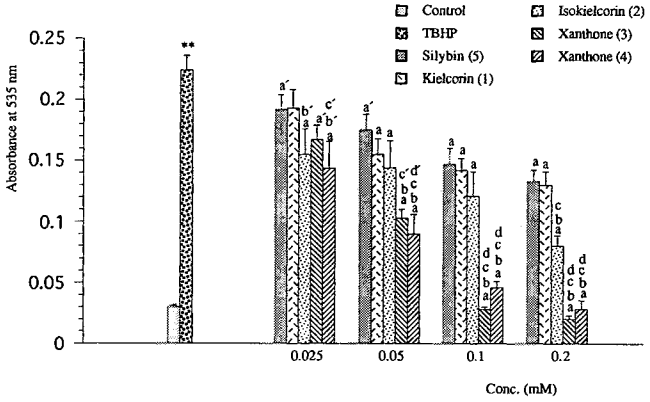

Fig. 4. Effect of silybin (5), *trans*-kielcorin (1), *trans*-isokielcorin B (2), 3,4-dihydroxy-2-methoxyxanthone (3) and 2,3-dihydroxy-4-methoxyxanthone (4) in preventing the formation of thiobarbituric acid reactive substances (TBARS) by freshly isolated rat hepatocytes after 30 min of *t*-BHP exposure (means±SEM). n=4 or 8; **p≤0.0001 (between control and *t*-BHP treated cells); [a] p<0.01 and [a'] p<0.05 (when compared with *t*-BHP treated cells); [b] p<0.01, [b'] p<0.05 (when compared with *t*-BHP + silybin treated cells); [c] p<0.01, [c'] p<0.05 [when compared with (*t*-BHP + *trans*-kielcorin) treated cells]; [d] p<0.01, [d'] p<0.05 [when compared with (*t*-BHP + *trans*-isokielcorin B) treated cells].

centration-dependent for all parameters measured, except for total glutathione where no effect was found.

3,4-Dihydroxy-2-methoxyxanthone (3) and (4) were the most effective in protecting cells against *t*-BHP-induced cell death, while (5) was the less effective (Figs. 2 and 3). Furthermore, prevention of cell death for (3) and (4) at the three higher concentrations reached a steady-state near control values. Interestingly, the prevention of cell death by (3) was not statistically different from (4), although the effect for (2) was more pronounced than for (1) at 0.200 mM ($p \leq 0.05$) (Fig. 3).

The prevention of TBARS production by (3) and (4) which was similar, was higher than by (5) at all concentrations, and higher than by (1) and (2) at the three higher concentrations (Fig. 4). Like the effect on cell viability, at 0.100 and 0.200 mM of (3) and (4) the amount of TBARS found in the cell suspensions was also similar to control levels and prevention by (2) was more effective than by (5) at 0.025 and 0.200 mM. The *t*-BHP-induced depletion of cellular GSH (Fig. 5), increase in GSSG formation (Fig. 6) and increase in the ratio GSSG/GSH (Fig. 7) were prevented by all compounds. Despite the different effects of these compounds in viability and TBARS, the effects on GSH levels were similar.

The GSSG formation induced by *t*-BHP was prevented by all the compounds (Fig. 6). *trans*-Isokielcorin B (2) exhibited higher potency when compared to (1) at 0.100 and 0.200 mM and when compared to (5) at 0.200 mM. It

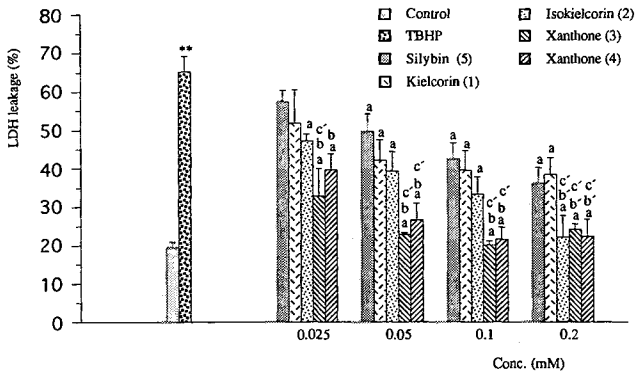

Fig. 3. Effect of silybin (5), *trans*-kielcorin (1), *trans*-isokielcorin B (2), 3,4-dihydroxy-2-methoxyxanthone (3) and 2,3-dihydroxy-4-methoxyxanthone (4) in cell viability expressed by LDH leakage by freshly isolated rat hepatocytes after 30 min of *t*-BHP exposure (means±SEM). n=4 or 8; **p≤0.0001 (between control and *t*-BHP treated cells); [a] p<0.01 (when compared with *t*-BHP treated cells); [b] p<0.01, [b'] p<0.05 [when compared with (*t*-BHP + silybin) treated cells]; [c] p<0.01, [c'] p<0.05 [when compared with (*t*-BHP + *trans*-kielcorin) treated cells].

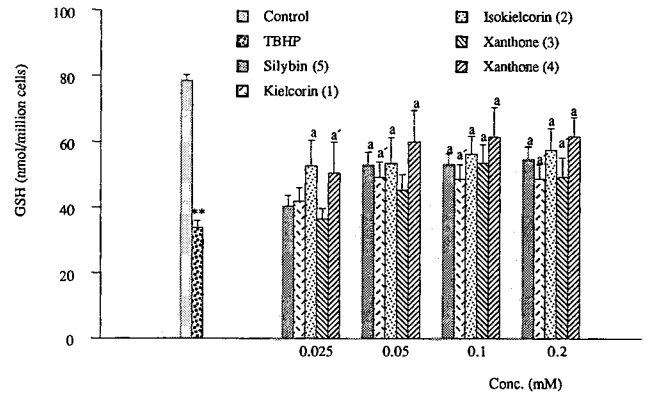

Fig. 5. Effect of silybin (5), *trans*-kielcorin (1), *trans*-isokielcorin B (2), 3,4-dihydroxy-2-methoxyxanthone (3) and 2,3-dihydroxy-4-methoxyxanthone (4) in freshly isolated rat hepatocytes GSH content after 30 min of *t*-BHP exposure (means±SEM). n=4 or 8; **p≤0.0001 (between control and *t*-BHP treated cells); [a] p<0.01 and [a'] p<0.05 (when compared with *t*-BHP treated cells).

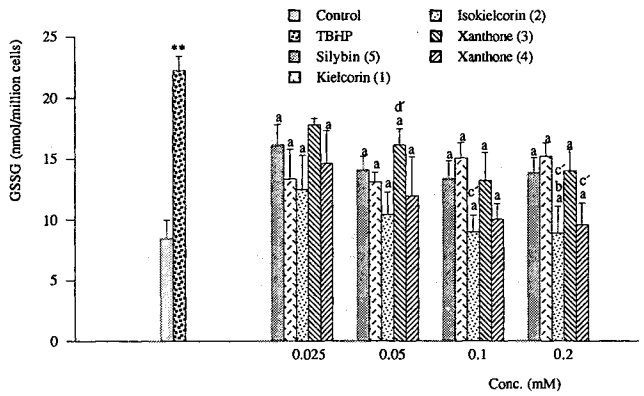

Fig. 6. Effect of silybin (5), *trans*-kielcorin (1), *trans*-isokielcorin B (2), 3,4-dihydroxy-2-methoxyxanthone (3) and 2,3-dihydroxy-4-methoxyxanthone (4) in freshly isolated rat hepatocytes GSSG formation after 30 min of *t*-BHP exposure (means±SEM). n=4 or 8; **p≤0.0001 (between control and *t*-BHP treated cells); [a] p<0.01 (when compared with *t*-BHP treated cells); [b'] p<0.05 [when compared with (*t*-BHP + silybin) treated cells]; [c'] p<0.05 [when compared with (*t*-BHP + *trans*-kielcorin) treated cells]; [d'] p<0.05 [when compared with (*t*-BHP + *trans*-isokielcorin B) treated cells].

seems also interesting that (2) and (4) prevented *t*-BHP-induced GSSG formation almost completely at the two higher concentrations.

The prevention of *t*-BHP-induced GSH depletion and GSSG formation by the studied hepatoprotective compounds changed extensively the GSSG/GSH ratio (Fig. 7). This effect was significant for all the tested compounds at all concentrations. *trans*-Isokielcorin B (2) and (4) showed a tendency to be more efficient compounds in lowering the GSSG/GSH ratio than (1) and (3). Silybin (5) effects were similar to the other compounds at all concentrations, except at 0.025 mM, where it was less effective than (2). 3,4-Dihydroxy-2-methoxyxanthone (3) and (4) were significantly different from each other at 0.025 and 0.050 mM.

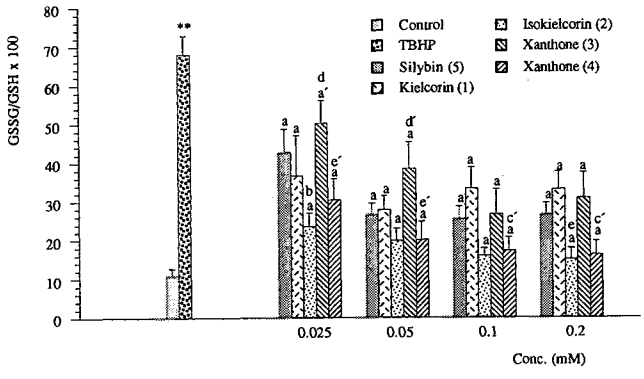

Fig. 7. Effect of silybin (5), *trans*-kielcorin (1), *trans*-isokielcorin B (2), 3,4-dihydroxy-2-methoxyxanthone (3) and 2,3-dihydroxy-4-methoxyxanthone (4) in freshly isolated rat hepatocytes GSSG/GSHx100 ratio after 30 min of *t*-BHP exposure (means±SEM). n=4 or 8; **p≤0.0001 (between control and *t*-BHP treated cells); [a] p<0.01 and [a'] p<0.05 (when compared with *t*-BHP treated cells); [b] p<0.01, [when compared with (*t*-BHP + silybin) treated cells]; [c'] p<0.05 [when compared with (*t*-BHP + *trans*-kielcorin) treated cells]; [d] p<0.01, [d'] p<0.05 [when compared with (*t*-BHP + *trans*-isokielcorin B) treated cells]; [e'] p<0.05 [when compared with (*t*-BHP + 3,4-dihydroxy-2-methoxyxanthone) treated cells].

Although total glutathione (GSH+GSSG) was significantly depleted by *t*-BHP (about 18%), all the tested compounds were ineffective in preventing this depletion in any of the tested concentrations.

DISCUSSION AND CONCLUSIONS

In this study, compounds (1), (2), (3), (4) and (5) showed protective effects against *t*-BHP-induced toxicity in freshly isolated rat hepatocytes. Both xanthones were more potent than (1) and (2) in preventing loss of cell viability and lipid peroxidation, although (2) and (4) seemed to be most effective in preventing loss of glutathione homeostasis. All the four studied compounds had a better profile than (5) in protecting the hepatocytes against *t*-BHP-induced toxicity. Total glutathione (GSH+GSSG) depletion was not prevented by any of the studied compounds.

An important aspect of *t*-BHP-induced hepatotoxicity is its reduction by glutathione peroxidase to the corresponding alcohol at the expense of GSH which is converted to GSSG (11). Under severe exposure to *t*-BHP, the reduction of GSSG by glutathione reductase or the regeneration of NADPH may be insufficient, resulting in the accumulation of GSSG. A deep depletion of intracellular GSH induced by *t*-BHP can increase susceptibility to irreversible injury by oxidative intoxication and by free radicals which can result in lipid peroxidation, protein oxidation, protein inactivation, disturbances in calcium homeostasis and consequent loss of cell viability (12-13).

Another aspect of *t*-BHP-induced hepatotoxicity is its metabolization mediated by cytocrome P-450 system which catalyzes its cleavage to the correspondent peroxy and alkoxy radicals (14) which are implicated in the initiation and propagation of lipid peroxidation. This is an additional factor for the depletion of GSH since it can scavenge free radicals by being oxidised to GSSG (15).

The maintenance of GSH homeostasis is by no means very important for the hepatoprotective activity of natural compounds as in the example of (5) (16). However, the fact that the pattern of protection of cellular GSH homeostasis by the studied compounds was not the same as their prevention of cell death means that this protection is not enough to explain the antihepatotoxic activity. Nevertheless, it was observed that (2) and (4) were more effective than (1) and (3) respectively in preventing GSSG accumulation. This may help to explain the higher potency of (2) in protecting cell viability at 0.200 mM when compared to (1), but in the case of (3) and (4), there was no difference at all between them with respect to this parameter. The complete prevention of TBARS formation by the xanthones at the two higher concentrations possibly plays a more important role in protecting cell viability. In fact, the effects found for all the tested compounds in the protection against lipid peroxidation are probably the most important contribution for the differences found in the cell viability, since they are very well correlated. The best results found were for (3) and (4) which prevented any increase in lipid peroxidation for the two higher concentrations and nearly prevented any loss of cell viability for the three higher concentrations.

Although all compounds were effective in preventing GSH depletion and GSSG increase, they were ineffective in

preventing total glutathione (GSH + GSSG) depletion. This depletion can be due to covalent binding of glutathione to some electrophilic species which can appear during *t*-BHP metabolism, or to glutathione oxidation with protein thiols induced by the oxidative status of the cells.

The different pattern found for the five compounds against the increase in lipid peroxidation and loss of glutathione homeostasis does probably mean that we are in the presence of multiple ways of hepatoprotection, probably by a different capacity of reacting with lipid carbon, lipid peroxy, or alkoxyl radicals. Also, a different solubility of the five compounds inside the biomembranes could explain their different effects in counteracting lipid peroxidation.

Another hypothesis is a possible inhibition of cytochrome P-450, resulting in a lower metabolism of *t*-BHP by this enzymatic system. However, for the concentrations used in this study at least the protection shown for (5) could hardly be attributed to cytochrome P-450 inhibition (17).

To our knowledge, this is the first report about hepatoprotective activity of (1), (2), (3) and (4). Also, this is the first time that an *in vitro* hepatoprotective activity is found for any xanthone or any xanthonolignoid, although antioxidant properties have already been found for other xanthones (18).

The presence of (1) and (3) in plants used in folk medicine for treatment of liver ailments can be important for this particular clinical action. Also, the availability of synthetic xanthonolignoids and xanthones will help to develop structure-activity relationships necessary to establish and increase our knowledge about their mechanism(s) of action.

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