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Ecotoxicological effects of the antioxidant additive propyl gallate in five aquatic systems

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ABSTRACT

Propyl gallate is an antioxidant widely used in foods, cosmetics and pharmaceuticals. The occurrence and fate of additives in the aquatic environment is an emerging issue in environmental chemistry. To date, there is little available information about the adverse effects of propyl gallate on aquatic organisms. Therefore, the toxic effects were investigated, using five model systems from four trophic levels. The most sensitive system was the hepatoma fish cell line PLHC-1 according to total protein content, with an EC_{50} of 10 μM and a NOAEL of 1 μM at 72 h, followed by the immobilization of *Daphnia magna*, the inhibition of bioluminescence of *Vibrio fischeri*, the salmonid fish cell line RTG-2 and the inhibition of the growth of *Chlorella vulgaris*. Although protein content, neutral red uptake, methylthiazol metabolization and acetylcholinesterase activity were reduced in PLHC-1 cells, stimulations were observed for lysosomal function, succinate dehydrogenase, glucose-6-phosphate dehydrogenase and ethoxyresorufin-O-deethylase activities. No changes were observed in metallothionein levels. The main morphological observations were the loss of cells and the induction of cell death mainly by necrosis but also by apoptosis. The protective and toxic effects of propyl gallate were evaluated. General antioxidants and calcium chelators did not modify the toxicity of propyl gallate, but an iron-dependent lipid peroxidation inhibitor gave 22% protection. The results also suggest that propyl gallate cytotoxicity is dependent on glutathione levels, which were modulated by malic acid diethyl ester and 2-oxothiazolidine-4-carboxylic acid. According to the results, propyl gallate should be classified as toxic to aquatic organisms.

1. Introduction

Propyl gallate is an antioxidant widely used to preserve and stabilize the freshness, nutritional value, flavor and color of foods, fats and oils, and medicinal preparations (JEFCA, 1996). It is an additive authorised (INS No. 310) in the European Union and in many others countries, where it is listed as a common preservative, and is generally recognized as safe. It has been used since 1948 to stabilize cosmetic, and food-packaging materials, and foods containing fats, and as an

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Abbreviations: AChE, acetylcholinesterase; EC_{50}, mean effective concentration; EROD, ethoxyresorufin-O-deethylase; G6PDH, glucose-6-phosphate dehydrogenase; MTS, 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt; NOAEL, nonobserved adverse effect level; SDH, succinate dehydrogenase

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additive in edible fats, oils, mayonnaise, shortening, baked products, pressure-sensitive adhesives, lubricating oil additives and transforming oils (Van der Heijden et al., 1986). Propyl gallate has been evaluated by the Joint FAO/WHO Expert Committee on Food Additives on several occasions. In 1976, the Committee established an ADI in humans of 0.2 mg/kg body weight. The acute oral toxicity of propyl gallate in mice, rats, hamsters and rabbits varies from 2000 to 3800 mg/kg body weight (Van der Heijden et al., 1986).

Although there are several studies that demonstrate the benefits of propyl gallate as an antioxidant (Raghavan and Hultin, 2005) and as a chemopreventive agent (Hirose et al., 1993; Karthikeyan et al., 2005), the safety of this chemical is questioned due to its controversial effects. Kobayashi et al. (2004) and Kawanishi et al. (2005) showed that propyl gallate exerted prooxidant properties. Wargovich et al. (1996) demonstrated that propyl gallate increased the number of aberrant crypt foci after benzo(a)pyrene induction in the F344 rat colon. Moreover, in a study carried out by the National Toxicology Program Technical Report (1982), it was reported that propyl gallate induced preputial gland tumors, islet-cell tumors of the pancreas and pheochromocytomas of the adrenal glands. Therefore, in order to clarify the discrepancy between the different effects of propyl gallate, further studies on toxicity should be performed to re-evaluate its safety.

Propyl gallate is a fine white powder, odorless with a slightly bitter taste. It is soluble in ethanol but practically insoluble in water, 3.5 g/L (Budavari, 1989) and presents an estimated octanol/water partition coefficient of 0.967 (McCoy et al., 1990). Propyl gallate is mainly absorbed by the gastrointestinal tract. The available evidence indicates that the gallate esters, as propyl gallate, are hydrolyzed in the body to gallic acid and 4-O-methyl gallic acid by cellular carboxylesterase. In addition, propyl gallate is converted to a dimmer and ellagic acid via auto-oxidation (Nakagawa et al., 1995). Free gallic acid or a conjugated derivative of 4-O-methyl gallic acid with glucuronic acid, are excreted in the urine (World Health Organization, 1993).

Although propyl gallate is not currently considered an emerging or priority pollutant, due to its widespread use, it may be released into the environment by various waste streams with the possible generation of adverse ecological effects. It is known that the discharge of sewage into surface waters represents a major source of pollutants. Domestic wastes are discharged mainly into sewage systems, while industrial wastes are discharged either into the sewage system or directly into surface waters (Walker et al., 2006). This fact, together with the physical properties of propyl gallate, supports the possible presence of the additive in the aquatic environment. However, the occurrence of propyl gallate in industrial wastewaters and surface waters has not been quantified.

Nowadays, little information is available about the aquatic ecotoxicity of additives in general and of propyl gallate in particular. Therefore, the aim of the present study was to provide ecotoxicity data regarding the hazard of propyl gallate to aquatic organisms. In order to study the toxicity of propyl gallate in aquatic organisms, a representative and cost-effective test battery comprising organisms from four trophic levels of the aquatic ecosystem has been applied. Five ecological model systems with different endpoints were employed at several exposure time periods. The battery included the inhibition of bioluminescence of the bacterium *Vibrio fischeri* (decomposer), the inhibition of the growth of the alga *Chlorella vulgaris* (first producer) and the immobilization of the cladoceran *Daphnia magna* (first consumer). Cell morphology, total protein content, neutral red uptake, methylthiazol (MTS) metabolization, lysosomal function, succinate dehydrogenase (SDH) activity and glucose-6-phosphate dehydrogenase (G6PDH) leakage and activity, acetylcholinesterase (AChE) activity, metallothionein levels and ethoxyresorufin-O-deethylase (EROD) activity were studied in two fish cell lines: PLHC-1 derived from a hepatocellular carcinoma of the topminnow *Poeceliopsis lucida* and RTG-2 derived from the gonad of the rainbow trout *Oncorhynchus mykiss* (second consumer). Similar approaches have been shown useful for the ecotoxicological evaluation of other chemicals, such as bromobenzene (Zurita et al., 2007) or butylated hydroxyanisole, another antioxidant additive (Jos et al., 2005).

2. Materials and methods

2.1. Toxicant exposure

Stock solutions of propyl gallate (Sigma) were prepared in dimethyl sulfoxide. A range of different concentrations of exposure solutions of propyl gallate was prepared in the different complete culture media, according to the appropriate assay, and sterilized by filtration through a 0.22 μm Millipore® filter. High quality deionized water was used for the preparation of the different media. After replacing the medium with the exposure solutions, the systems were incubated for adequate exposure time. The concentration of dimethyl sulfoxide in all controls and exposure groups was ⩽0.2% (v/v).

2.2. Model systems

2.2.1. *V. fischeri*

Bioluminescence inhibition in the marine bacterium *V. fischeri* was evaluated at 5 and 15 min according to Cordina et al. (1993) by using freeze-dried bacteria incubated at 15 °C from Microtox® test (Microbics Corp. Carlsbad, USA).

2.2.2. *C. vulgaris*

Growth inhibition of the alga *C. vulgaris var viridis*, kindly provided by Dr. Muñoz-Reoyo (CISA, Spain), was evaluated up to 72 h in 96-well culture plates seeded with 200 μl/well of a 1,000,000 cells/ml algae culture in exponential growth phase in Bold's Basal Medium. The plates were in constant agitation at a temperature of 22 °C, under a water-saturated sterile atmosphere containing 5% CO_2 and a cold light source of 8000 lux. Absorbency at 450 nm was read on a Multiscan RC plate reader (Labsystem, Helsinki, Finland). As quality criteria, the control cultures had to grow at least tenfold in 48 h (Ramos et al., 1996).

2.2.3. *D. magna*

D. magna, kindly provided by Dr. Muñoz-Reoyo (CISA, Spain), was maintained at 20 °C and fed with *C. vulgaris*. Acute toxicity immobilization tests were performed up to 72 h in standard reference water according to Organization for Economic Co-operation and Development (2004) Guideline 202 in four replicate groups of 10 neonates per 25 ml per concentration, in 70 ml polystyrene flasks (Costar, Cambridge, MA, USA).

2.2.4. *PLHC-1 cells*

The hepatoma PLHC-1 cell line was derived from a hepatocellular carcinoma induced with 7,12-dimethylbenz(a)anthracene in an adult female *P. lucida*, a topminnow from the Sonoran desert (ATCC® ♯ CRL-2406). The cells retain some of the characteristic morphology of primary liver hepatocytes, are epithelial, present an average population doubling time of 39.4 h, express aryl hydrocarbon receptors and basal and inducible P450IA activity (Ryan and Hightower, 1994; Fent, 2001). PLHC-1 cells were grown at 30 °C in a humidified incubator containing 5% CO_2 and propagated in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% foetal bovine serum (Gibco™), L-glutamine (BioWhittaker), sodium pyruvate (BioWhitaker) and nonessential amino acids (BioWhittaker). PLHC-1 cells in exponential growth phase were plated by applying 0.2 ml of 450,000 cells/ml in each well of 96-well tissue culture plates (Costar). After 24 h at 30 °C, the cultures received 0.2 ml medium containing the test chemical and were incubated for a further 24, 48 or 72 h. For the morphological study, PLHC-1 cells were seeded in Lab-Tek® tissue culture 8 well chamber slides applying 0.5 ml of 500,000 cells/ml (Nunc, Inc., Naperville, IL) previously coated with MatrigelTM (BD Biosciences). They were then exposed to propyl gallate for 24, 48 and 72 h, fixed in 70% methanol and stained with Mayer's hematoxylin and eosin or subjected to in situ hybridization (TUNEL) to detect induction of apoptosis (Enzo, Diagnostics, Farmingdale, US).

2.2.5. *RTG-2 cells*

The RTG-2 salmonid fish cell line, derived from the gonad of rainbow trout (*O. mykiss*) was kindly provided by Dr. Castaño (ISCIII, Spain). The cells present long spindle-like and fibroblast-like morphology, an average population doubling time of 72 h and basal and inducible P450IA activity. The cells were grown in EMEM supplemented with 10% foetal bovine serum (Biochrom), L-glutamine (BioWhittaker) and nonessential amino acids (BioWhittaker). RTG-2 cells in exponential growth phase were plated in 0.2 ml of 45,000 cells/ml in 96-well tissue-culture plates (Costar). After 48 h at 20 °C, the culture medium was replaced with 0.2 ml test medium and then incubated for a further 24 or 48 h (Castaño et al., 2003). For the morphological study, RTG-2 cells were seeded in Lab-Tek® tissue culture chamber slides applying 0.5 ml of 50,000 cells/ml (Nunc, Inc., Naperville, IL). They were then exposed to propyl gallate for 24 and 48 h, fixed in 70% methanol and stained with Mayer's hematoxylin and eosin or subjected to in situ hybridization (TUNEL) to detect induction of apoptosis (Enzo, Diagnostics, Farmingdale, USA).

2.2.6. *Fish cell bioindicators*

Total cellular protein content was quantified in situ, using Coomassie brilliant blue G-250 (Repetto and Sanz, 1993) in the same 96-well tissue-culture plates in which exposure originally took place (Zurita et al., 2005). Absorbency at 620 nm was read on a Multiscan RC plate reader (Labsystem, Helsinki, Finland). Neutral red uptake was evaluated according to Babich and Borenfreund (1987) and lysosomal function relative to protein content (Repetto and Sanz, 1993). The MTS tetrazolium reduction assay was performed according to the procedure of Baltrop et al. (1991). The MTS tetrazolium compound is bioreduced by cells into a colored formazan product soluble in the culture medium. G6PDH activity, in cells and in culture medium, was determined as described by García-Alfonso et al. (1998). AChE activity on intact cells was measured by adapting the method of Repetto et al. (1994). Metallothionein induction in cells was determined by atomic absorption spectrophotometry using the cadmium/haemoglobin affinity assay (Eaton and Cherian, 1991). EROD activity, a catalytic measurement of cytochrome P4501A induction, was determined by a direct fluorometric method described by Hahn et al. (1996).

2.2.7. *Modulation studies*

Before the exposure to 1000 μM propyl gallate, PLHC-1 cell cultures were pre-treated for 30 min with 25 μM α-tocopherol succinate (Sigma), 20 mM mannitol (Sigma), 10 mM sodium benzoate (Sigma), 1 μM deferoxiamine mesylate (Sigma), 250 μM 1,4-dithiotreitol (Merck), 5 μM BAPTA-AM (Sigma) or 500 μM EGTA (Sigma), and for 24 h with 90 μM malic acid diethyl ester (Sigma) or 10 mM 2-oxothiazolidine-4-carboxylic acid (Sigma). After 24 h, the leakage of G6PDH was quantified as previously described. A similar assay was carried out with the simultaneous application of 6 mM paraquat (Aldrich) and 5 μM propyl gallate.

2.3. *Calculations and statistical analysis*

Vibrio assays were repeated three times using $n = 3$ tubes per concentration. Daphnia tests were performed three times using 4 groups of $n = 10$ individuals per concentration. Each assay in 96-well tissue cultures plates (algae and fish cells) was carried out three times using $n = 6$ wells per concentration. Values for enzyme activities, lysosomal function and metallothionein levels were corrected for cell culture total protein content to avoid misinterpretation due to the influence of the chemical tested on cell proliferation and cell detachment. Statistical analysis was carried out using analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. Mean effective concentration (EC_{50}) values were determined by probit analysis. The estimated nonobserved adverse effect levels (NOAEL) was identified as the highest concentration of a substance, which causes no detectable adverse effect on the target organism under defined conditions of exposure.

3. Results

3.1. Effects on V. fischeri, C. vulgaris and D. magna

The results of this study demonstrated that both the marine bacterium *V. fischeri* and the crustacean *D. magna* were very sensitive to propyl gallate, showing dose-dependent curves. The inhibition of bioluminescence in *V. fischeri* presented EC_{50} values of 226 μM at 5 min and 184 μM at 15 min of exposure (Fig. 1(a)). A similar range of effects was observed for the immobilization of *D. magna* with EC_{50} values ranging from 203 to 158 μM (Fig. 1(c)). The proliferation of the fresh-water algae *C. vulgaris* was inhibited in a concentration-dependent manner, showing EC_{50} values of 1090, 997 and 690 μM after 24, 48 and 72 h, respectively (Fig. 1(b)). This model system presented a lower sensitivity to propyl gallate than the other studied models.

3.2. Effects on the hepatoma fish cell line PLHC-1

The effects of different concentrations of propyl gallate were also investigated using PLHC-1 cells at morphological, basal cytotoxicity (total protein content, G6PDH leakage, neutral red uptake and MTS metabolization) and biochemical levels (lysosomal function, SDH activity, G6PDH activity, AChE activity, metallothionein content and EROD activity). The results obtained for the different endpoints evaluated on PLHC-1 cells are shown in Figs. 2 and 3. The uptake of neutral red was the most sensitive bioindicator at basal cytotoxicity level in the fish cell line PLHC-1, with EC_{50} values below 100 μM (Fig. 2(c)). In contrast, the leakage of G6PDH, a marker of cell death, was the least sensitive bioindicator to propyl gallate, with levels over 450 μM needed to increase the leakage by 50% (Fig. 2(b)). Cell number, evaluated by quantification of total protein content, presented similar changes as neutral red uptake (Fig. 2(a)). The metabolization of MTS was also inhibited with EC_{50} values ranging from 35 to 315 μM (Fig. 2(d)).

At the biochemical level, G6PDH activity was clearly increased from 5 μM, showing a rise up to 100% at 100 μM (Fig. 3(c)). Lysosomal function and SDH activity were also altered by propyl gallate, being stimulated from 5 to 100 μM (Figs. 3(a) and 3(b)). A slight increase was observed in AChE activity at low concentrations of propyl gallate (Fig. 3(d)). EROD activity was stimulated from 10 to 500 μM, showing a 60% induction with 100 μM propyl gallate (Fig. 3(f)). No significant increases were observed in metallothionein levels after 24 h of exposure (Fig. 3(e)). EROD activity was induced in PLHC-1 cells exposed to different concentrations of propyl gallate, showed a 60% induction at 100 μM (Fig. 3(f)).

Morphological changes induced by propyl gallate were investigated in the hepatoma fish cell line PLHC-1 (Fig. 4). As described by Ryan and Hightower (1994), the control cultures retain some of the characteristic morphology of hepatocytes. PLHC-1 cells present polygonal form, sinuous borders, with secretion vesicles around the cellular surface and are disposed in a uniform monolayer. They have abundant deposits of glycogen, tight junctions near the apical surface and basolateral interdigitations. Morphological

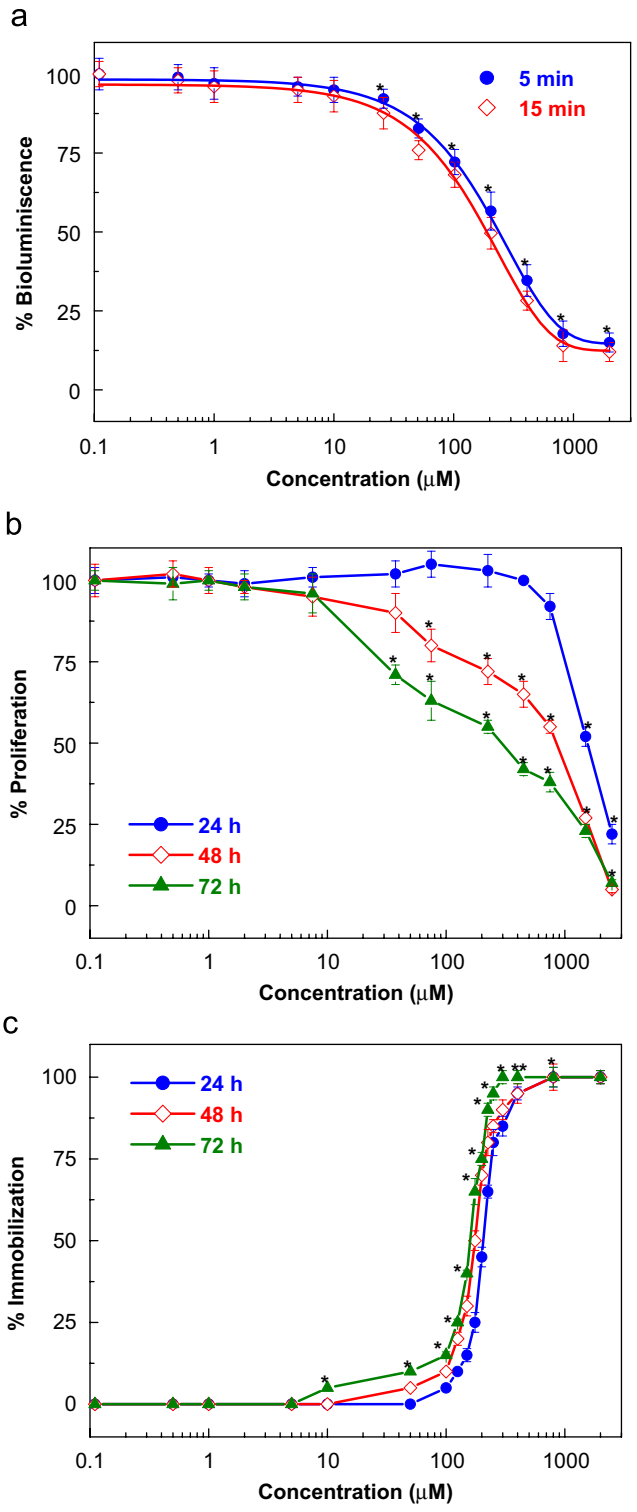

Fig. 1 – Effects of exposure to different concentrations of propyl gallate studied as (a) bioluminescence inhibition of the bacterium *V. fischeri* at 5 (●) and 15 min (◇) ($n = 3$); (b) proliferation of the alga *Chlorella vulgaris* ($n = 6$) and (c) immobilization of the cladoceran *D. magna* ($n = 40$) at 24 (●), 48 (◇) and 72 h (▲). Data expressed in % of unexposed controls (mean ± SEM) of three experiments. * indicates significant difference from control value ($p < 0.05$).

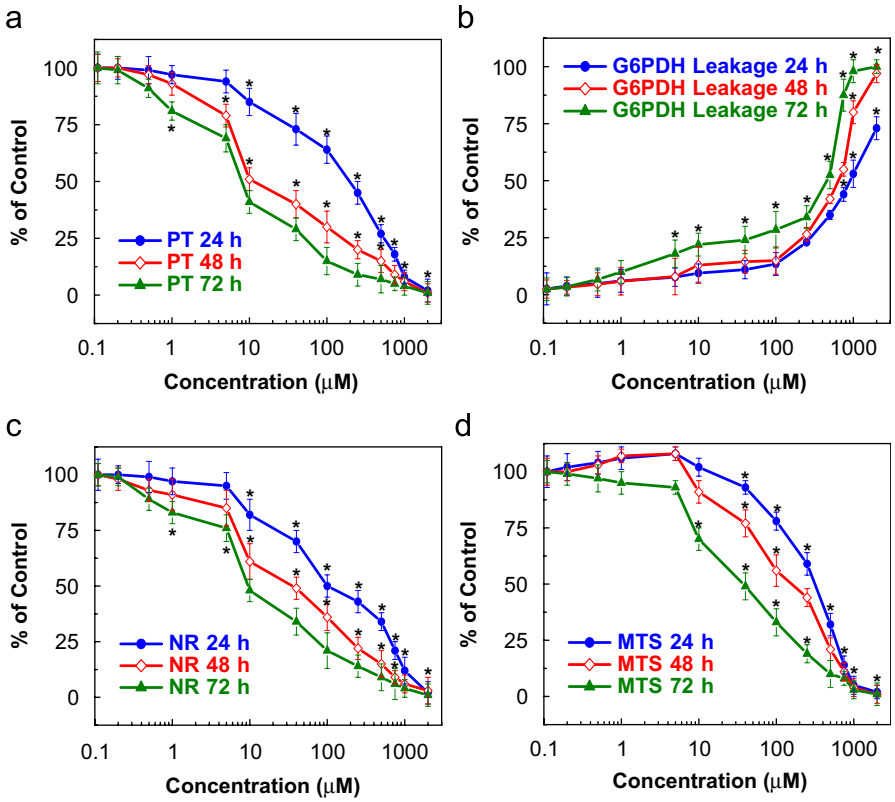

Fig. 2 – Effects of propyl gallate on PLHC-1 cells (a) total protein content, (b) G6PDH leakage, (c) neutral red uptake and (d) MTS metabolization after exposure to different concentrations for 24 (●), 48 (◇) and 72 h (▲). Data expressed in percent of unexposed controls (mean ± SEM) of three experiments using $n = 6$. ∗ indicates significant difference from control value ($p < 0.05$).

changes were detected after 24 h exposure to 10 μM and were more marked from 100 μM propyl gallate. The morphological alterations included loss of cells, decrease in secretion vesicles and cell tumefaction. Death was evident in many cells after 24 h of exposure from 100 μM, mainly by necrosis but also by apoptosis, confirmed by in situ hybridization (TUNEL). PLHC-1 cells treated with propyl gallate and stained with the cationic dye neutral red presented clear differences with regard to the corresponding untreated PLHC-1 controls. A reduction of the lysosomal function was observed, with balonization of the cells.

3.3. *Effects on the fibroblastic-like fish cell line RTG-2*

The last model system evaluated was the salmonid fish cell line RTG-2. The results for the different endpoints studied are shown in Figs. 5 and 6. Propyl gallate also presented a dose-dependent toxicity in RTG-2 cell cultures. However, this cell line was less sensitive than PLHC-1. The most sensitive endpoint in RTG-2 cells was the metabolization of MTS (Fig. 5(d)). The results obtained for the content of total protein and the uptake of neutral red were very similar, both bioindicators being inhibited with high concentrations of propyl gallate, with EC_{50} values ranging from 520 to 970 μM (Figs. 5(a) and (b)). A progressive concentration-dependent increase in G6PDH leakage, a marker of cell death, was also detected from 250 μM (Fig. 5(b)).

The activity of G6PDH was stimulated as in PLHC-1 cells, but this took place at higher concentrations, from 100 to 1000 μM, with an 80% rise (Fig. 6(c)). Nevertheless, no significant increase was detected in lysosomal function (Fig. 6(a)) and SDH activity (Fig. 6(b)). Metallothionein levels did not change after 24 h of exposure to propyl gallate (Fig. 6(d)). A slight increase was detected in EROD activity in RTG-2 cells after 24 h of exposure, with a maximum level of induction with 100 μM propyl gallate (Fig. 6(e)).

Morphological changes, induced by propyl gallate were also investigated in RTG-2 cells (Fig. 7). The control cultures show fusiform cells, arranged in plaques and disposed in parallel. They have well-defined borders, eosinophilic cytoplasm and central nuclei. Morphological alterations were detected from 100 μM at 24 h and were more evident from 500 μM, including a marked reduction in cell number and the induction of death by necrosis and apoptosis. Perinuclear vacuolization was observed as a first step in the development of hydropic degeneration. When cells were stained with neutral red, a general loss of lysosomes and of their perinuclear pattern of distribution was detected.

Table 1 includes the EC_{50} values for the different systems and biomarkers studied in the proposed ecotoxicological test battery. Considering all the data obtained, the sensitivity of the model systems to propyl gallate decreased as follows: PLHC-1 cells > *D. magna* > *V. fischeri* > RTG-2 cells > *C. vulgaris*. The estimated NOAELs were 1, 10, 15, 20, and 50 μM propyl

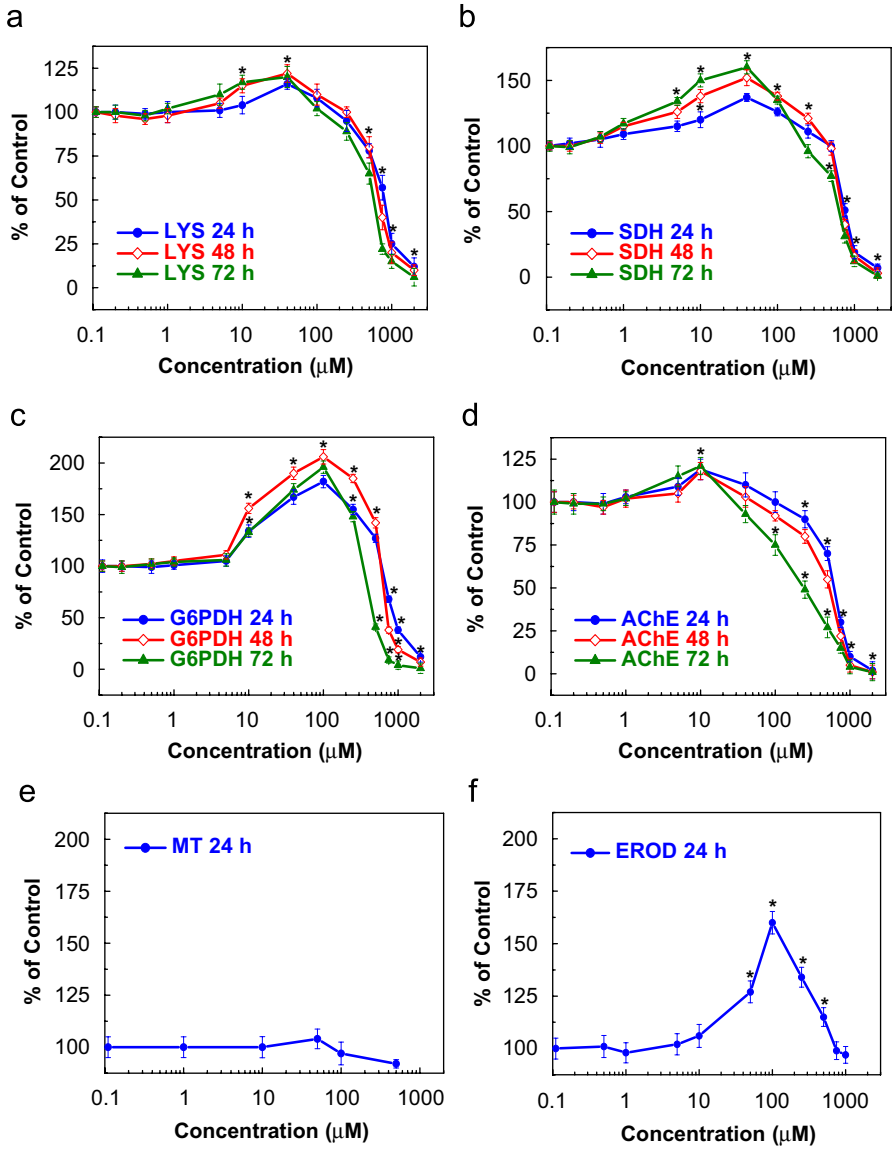

Fig. 3 – Effects of propyl gallate on PLHC-1 fish cell cultures (a) lysosomal function, (b) SDH activity, (c) G6PDH activity and (d) AChE activity at 24 (●), 48 (◇) and 72 h (▲); (e) metallothionein levels and (f) EROD activity after 24 h (●). Data expressed in % of unexposed controls (mean ± SEM) of three experiments using $n = 6$. * indicates significant difference from control value ($p < 0.05$).

gallate for PLHC-1 cells, *D. magna*, *V. fischeri*, *C. vulgaris*, and RTG-2 cells, respectively.

3.4. *Modulation of cytotoxicity*

The protective and toxic effects of propyl gallate were studied under different conditions in the fish cell line PLHC-1 measuring the leakage of G6PDH after 24 h of exposure (Fig. 8). Firstly, the possible protective effect of propyl gallate was evaluated using paraquat. The cytotoxic effects of this herbicide produced 48% cell death at 6 mM. However, it was reduced up to 36% with the simultaneous application of 5 μM propyl gallate.

Furthermore, the toxicity of 1000 μM propyl gallate was modulated by the application of nine compounds. Six of them (α-tocopherol succinate, mannitol, sodium benzoate, 1,4-dithiotreitol, BAPTA-AM and EGTA) did not modify the toxic effects of propyl gallate, since the leakage of G6PDH was kept around 50%, as was observed in the culture treated with 1000 μM propyl gallate. Nevertheless, the application of deferoxiamine mesylate and 2-oxothiazolidine-4-carboxylic acid prevented the toxicity of propyl gallate by 22% and 25%, respectively. Malic acid diethyl ester was the only chemical used that amplified the cytotoxicity induced by propyl gallate, increasing cell death by 40%.

4. Discussion

Propyl gallate is an antioxidant additive widely employed around the world to retard the spoilage of fats and oils. It is often used in combination with butylated hydroxyanisole and

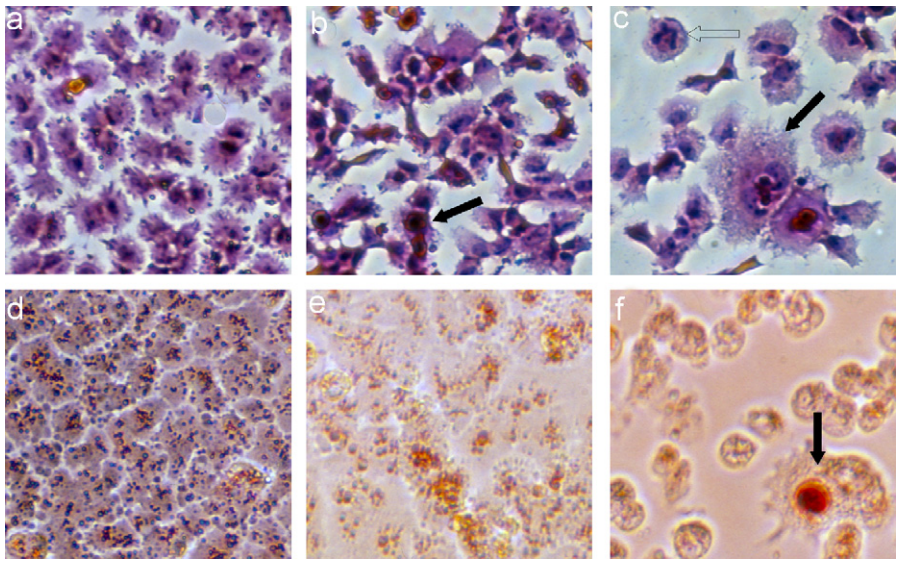

Fig. 4 – Morphology of PLHC-1 cell cultures (×1600) stained with Mayer's Hematoxylin and Eosin (a)–(c) or neutral red (d)–(f): (a) Control culture of PLHC-1 cells presents polygonal form, sinuous borders, with secretion vesicles around the cellular surface, (b) culture treated with 10 μM propyl gallate for 24 h showing a decrease in the number of cells and some death cells (→), (c) multiples changes were observed in the cultures treated with 100 μM after 24 h, including decrease in the number of cells, cell tumefaction (→) and death by apoptosis (⇒), (d) control culture of PLHC1 cells stained with neutral red, (e) culture of cells exposed for 24 h to 10 μM propyl gallate showing a general decrease in the uptake of neutral red, and (f) culture exposed for 24 h to 100 μM propyl gallate with more evident damage, reduction in cell number, balonization and reduced uptake, except for some accumulations (→).

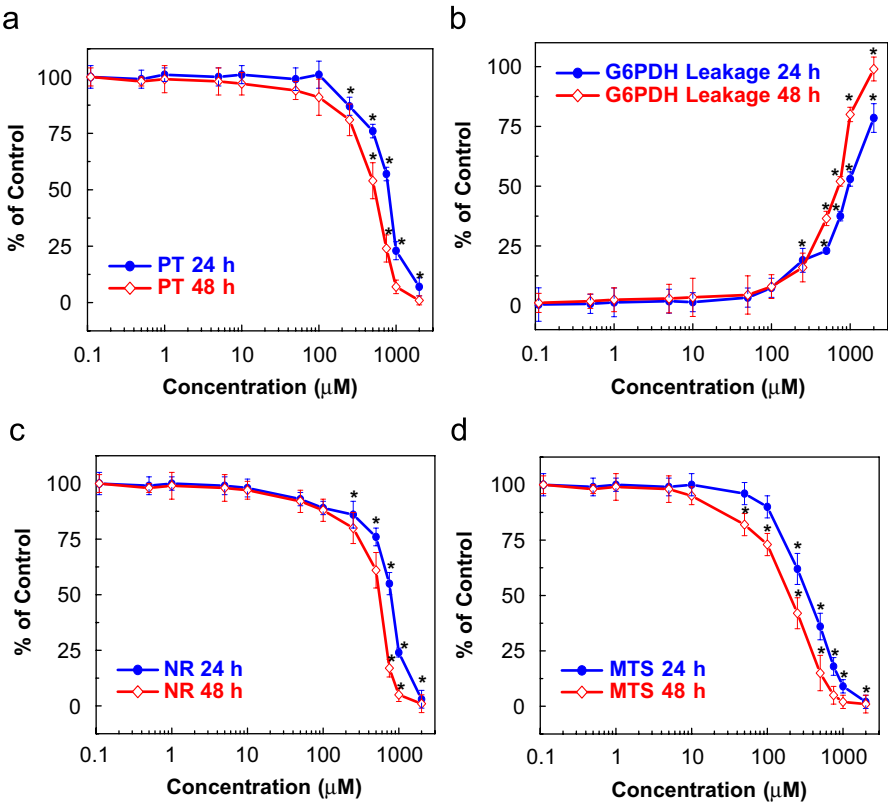

Fig. 5 – Effects of propyl gallate on RTG-2 cells (a) total protein content, (b) G6PDH leakage, (c) neutral red uptake and (d) MTS metabolization after exposure to different concentrations for 24 (●), 48 (◇) and 72 h (▲). Data expressed in % of unexposed controls (mean ± SEM) of three experiments using $n = 6$. ∗ indicates significant difference from control value ($p < 0.05$).

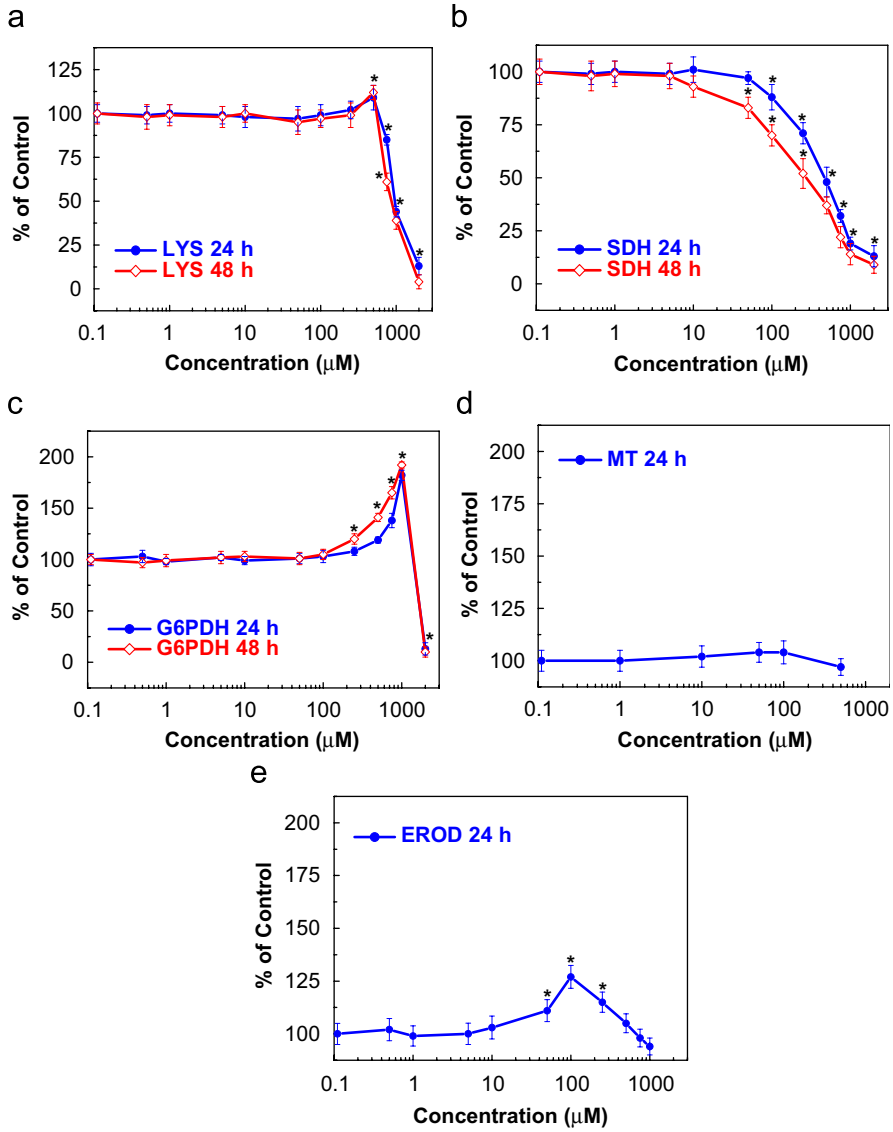

Fig. 6 – Effects of propyl gallate on RTG-2 fish cell cultures (a) lysosomal function, (b) SDH activity and (c) G6PDH activity at 24 (●), 48 (◇) and 72 h (▲); (d) metallothionein levels and (e) EROD activity after 24 h (●). Data expressed in % of unexposed controls (mean ± SEM) of three experiments using $n = 6$. ∗ indicates significant difference from control value ($p < 0.05$).

butylated hydroxytoluene because of their synergistic action. In addition, this antioxidant is a major component of many medicinal plants (Lin et al., 2000). However, despite its widespread use, very few reports are available about the aquatic effects of propyl gallate. Therefore, in order to provide ecotoxicity data regarding the hazard of propyl gallate in aquatic organisms, a test battery comprising organisms from four trophic levels of the aquatic ecosystem was used. Ecotoxicological models are applied for the detection, control and monitoring of the presence of pollutants in water (Repetto et al., 2003).

The decomposer *V. fischeri* is a basic organism in the breakdown and transformation of organic matter, contributing to the fertility and health of ecosystem. The inhibition of bioluminescence in *V. fischeri* was a very sensitive bioindicator in the proposed test battery. The toxicity of propyl gallate to this bacterium might be due to its interaction with mitochondrial oxidative phosphorylation, as was described for butylated hydroxyanisole and butylated hydroxytoluene (Fusi et al., 1991). These antioxidants uncouple oxidative phosphorylation by increasing the permeability of the mitochondrial inner membrane to protons. They also inhibit respiration by direct interaction with the electron transport chain. These cellular respiration pathways are closely linked to those implicated in bioluminescence. Consequently, the interference produced by a toxic compound will affect light production. Moreover, propyl gallate has also been reported to elicit antimicrobial activity to *Salmonella choleraesuis* (Kubo et al., 2002) and *Bacillus subtilis* (Kubo et al., 2004), contributing to its food preservative action. The cladoceran *D. magna* was also very sensitive to propyl gallate. Nevertheless, the proliferation of the alga *C. vulgaris* presented the lowest sensitivity. This

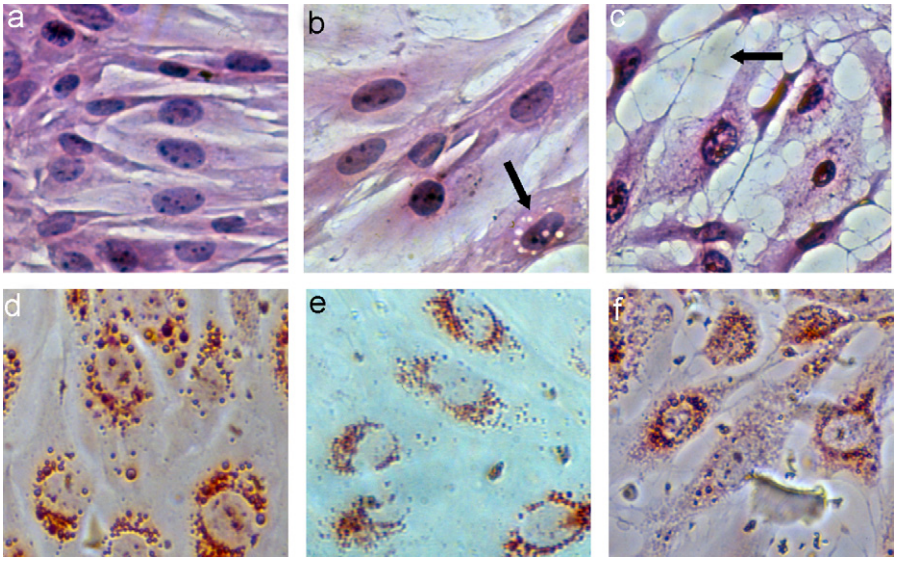

Fig. 7 – Morphology of RTG-2 cell cultures (× 1600) stained with Mayer's Hematoxylin and Eosin (a)–(c) or neutral red (d)–(f): (a) control culture of RTG-2 cells showing fusiform cells arranged in plaques in parallel, (b) cell culture exposed to 50 μM propyl gallate for 24 h showing loss of cells and perinuclear vacuolization (→) as a first step in the development of hydropic degeneration of the cytoplasm, (c) culture of cells exposed for 24 h to 1000 μM of propyl gallate, with a general vacuolization (→) and a marked hydropic degeneration, (d) control culture stained with neutral red, (e) the exposure to 50 μM propyl gallate for 24 h induced a small loss of lysosomes, and (f) after exposure to 500 μM propyl gallate for 24 h, the cells present only a small reduction in lysosomal function.

Table 1 – Toxic effects of propyl gallate on the selected models and biomarkers of the proposed ecotoxicological battery

Model system	Origin	Indicator	24 h	48 h	72 h
Vibrio fischeri	Bacteria (decomposer)	Bioluminescence	226[a]	184[b]	—
Chlorella vulgaris	Unicel. alga (producer)	Growth	1090	997	690
Daphnia magna	Cladoceran (First consumer)	Immobilization	203	178	158
PLHC-1 cell line	Topminnow (Second consumer)	Protein content	195	25	10
		G6PDH leakage	900	660	445
		Neutral red uptake	100	35	13
		MTS metabolization	315	160	35
		Lysosomal function	790	660	575
		SDH activity	750	710	610
		G6PDH activity	20	12	17
		AChE activity	605	540	235
		Metallothionein	—[c]	—	—
		EROD activity	80	—	—
RTG-2 cell line	Rainbow trout (Second consumer)	Protein content	935	532	—
		G6PDH leakage	970	725	—
		Neutral red uptake	876	519	—
		MTS metabolization	414	240	—
		Lysosomal function	950	840	—
		SDH activity	480	275	—
		G6PDH activity	820	595	—
		Metallothionein	—[c]	—	—
		EROD activity	—[c]	—	—

EC_{50} values (μM).Ec_{50} (μM), concentration of test chemical that modified each biomarker by 50% (positive or negative) in comparison with appropriate untreated controls.

[a,b] Values referred to 5 and 15 min exposure times, respectively.

[c] Not modified at the highest concentration tested.

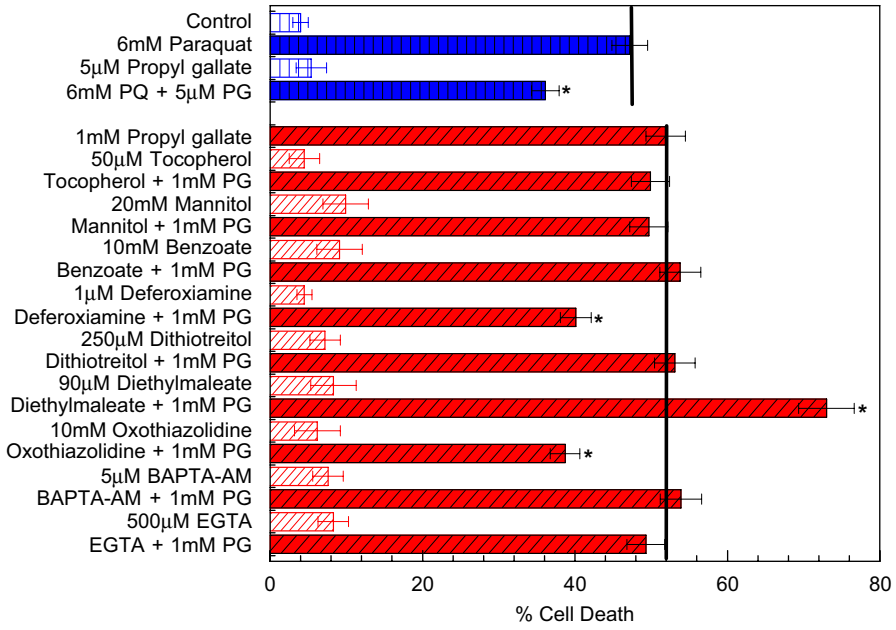

Fig. 8 – Protective effects of 5 μM propyl gallate on cell death induced by paraquat; and modulation of the toxic effects induced by 1 mM propyl gallate with the application of α-tocopherol succinate, mannitol, sodium benzoate, deferoxiamine mesylate, 1,4-dithiotreitol, malic acid diethyl ester, 2-oxothiazolidine-4-carboxylic acid, BAPTA-AM or EGTA. The effects were evaluated on PLHC-1 cells after 24 h of exposure by the quantification of the leakage of G6PDH. Data expressed in percent of unexposed controls (mean ± SEM) of three experiments using $n = 6$. * indicates significant difference from the effects induced by 6 mM paraquat (PQ) or 1 mM propyl gallate (PG), respectively ($p < 0.05$).

large difference of sensitivity to propyl gallate among the model systems may possibly be due to the characteristic metabolic activities of the different species.

From the basal cytotoxicity study carried out in two fish cell lines it is deduced that the content of total protein and the uptake of neutral red were the most sensitive biomarkers in the hepatoma fish cell line PLHC-1. However, in RTG-2 cells, the most sensitive endpoint was the metabolization of MTS. Total protein content and neutral red uptake were inhibited in both fish cell lines with a concentration-dependent toxicity. Nakagawa et al. (1995) demonstrated that addition of propyl gallate (from 50 to 2000 μM) to cultured rat hepatocytes elicited concentration-dependent cell death, accompanied by decreases in intracellular ATP. Cell death was induced in both cells from distinct concentrations of propyl gallate, as the leakage of G6PDH was significantly induced from 5 μM in PLHC-1 cells and from 250 μM in RTG-2 cells.

At the biochemical level, the G6PDH and EROD activities were increased in both fish cell lines. However, lysosomal function and SDH activity were increased only in PLHC-1 cells. Metallothionein levels did not show significant modifications in the range of concentrations tested after 24 h of exposure in the cultured cells. The main biomarker modified in both fish cell lines exposed to propyl gallate was the increase in G6PDH activity, although in a different range of concentrations. This observed stimulation may reflect the increase of the pentose phosphate pathway, an alternative metabolic route for supplying energy to the cell. It is in agreement with the reduction in metabolic activity detected in the bacterium *Vibrio fisheri*. We also found a more marked induction of EROD activity in PLHC-1 than in RTG-2 cells. This result can be correlated with the recent report by Zurita et al. (2007) in which PLHC-1 cells present 2.5-fold more basal EROD activity than RTG-2 cells.

Morphological changes were also a sensitive indicator of the effects of propyl gallate. PLHC-1 cells were more sensitive than RTG-2 cells, showing alterations from 10 μM in PLHC-1 cells and from 100 μM in RTG-2 cells. The most out-standing changes were the loss of cells and the induction of cell death mainly by necrosis but also by apoptosis. It is interesting to note that PLHC-1 cells presented less marked hydropic degeneration than RTG-2 cells, probably because RTG-2 cells are much more resistant to propyl gallate cytotoxicity, and are able to express more marked damage before the death.

The extent of variation of each cytotoxic and biochemical biomarker in both fish cell lines exposed to 100 μM of propyl gallate for 24 h was compared in Fig. 9. Considering the global results obtained, the hepatoma fish cell line PLHC-1 was more sensitive to propyl gallate than the fibroblastic-like fish cell line RTG-2. The higher metabolic activity of PLHC-1 cells over fibroblastic-like fish cell lines (Fent, 2001), may facilitate the metabolic activation of propyl gallate to gallic acid. The different metabolic profile of both fish cell lines should be considered to explain the large difference between them.

Propyl gallate is hydrolyzed enzymatically to gallic acid by cellular carboxylesterase. It has been reported that gallic acid generates hydrogen peroxide in HL-60 human leukaemia cells, increasing the amount of 8-oxo-7,8-dihydro-2′-deoxyguanosine, a characteristic biomarker of oxidative stress (Kobayashi et al., 2004). The DNA base damage is well

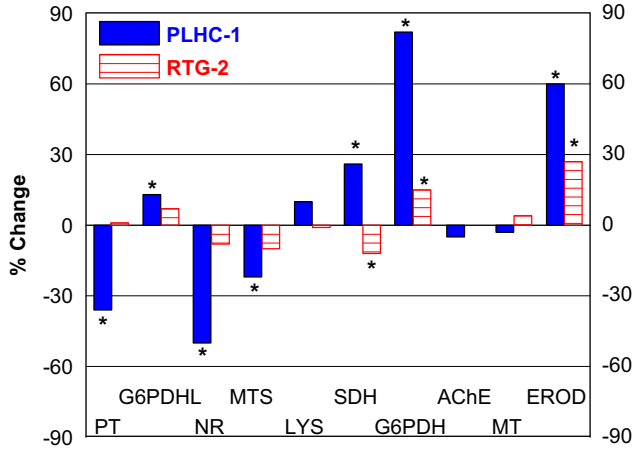

Fig. 9 – Comparison of the extent of variation of each cytotoxicity and biochemical biomarker studied after 24 h exposure to 100 μM propyl gallate in PLHC-1 and RTG-2 cells. Toxicity indicators assessed in the in vitro test systems were: cell protein content (PT), glucose-6P dehydrogenase leakage (G6PDHL), neutral red uptake (NR), methylthiazol metabolization (MTS), lysosomal function (LYS), succinate dehydrogenase activity (SDH), glucose-6P dehydrogenase activity (G6PDH), acetylcholinesterase activity (AChE), metallothionein levels (MT) and ethoxyresorufin-O-deethylase activity (EROD). Data expressed in percent of unexposed controls (mean ± SEM) of three experiments using $n = 6$. * indicates significant difference from control value ($p < 0.05$).

characterized as a premutagenic lesion in mammalian cells, because it causes misreplication of DNA that may lead to mutation or cancer (Shibutani et al., 1991).

Although there are many studies that demonstrate the antioxidant capacity of propyl gallate (Karthikeyan et al., 2005), other authors have reported prooxidant properties (Kobayashi et al., 2004; Kawanishi et al., 2005). Therefore, in order to clarify this discrepancy, the protective and toxic effects of propyl gallate were studied under different conditions in the fish cell line PLHC-1. Firstly, the possible protective effect of propyl gallate on paraquat toxicity was explored. The cytotoxic effects due to the herbicide, a oxidative stress inducer chemical, was reduced by 23% with the application of 5 μM propyl gallate, showing its capacity to act as free radical scavenger.

Secondly, the toxicity of 1000 μM propyl gallate was modulated by the application of nine compounds. As expected, general antioxidants (tocopherol succinate, mannitol, and sodium benzoate) did not modify the toxic effects of propyl gallate. The intracellular and extracellular calcium chelators, BAPTA-AM and EGTA, respectively, did not produce changes in the leakage of G6PDH, suggesting that alterations in calcium homeostasis are not the main mediators in propyl gallate induced cell death. The application of deferoxiamine mesylate, an inhibitor of iron-dependent lipid peroxidation, gave 22% protection from the deleterious effects of propyl gallate. This result shows the possible implication of iron in the mechanism of toxic action of propyl gallate, as was already reported by Kobayashi et al. (2004).

In addition, we have not found influence of the membrane permeable sulfydryl-protecting agent 1,4-dithiotreitol, on propyl gallate toxicity. However, Nakagawa et al. (1995) reported decreases in protein thiols and glutathione levels in cultured rat hepatocytes treated with propyl gallate. Therefore, the influence of the modulation of the levels of glutathione was evaluated by pre-treating the cells for 24 h with malic acid diethyl ester, an inhibitor of the synthesis of glutathione that depletes reduced glutathione without forming oxidized glutathione, or with 2-oxothiazolidine-4-carboxylic acid, a substrate for the synthesis of glutathione that increases its cellular levels. Cell death induced by propyl gallate was increased by 40% by the use of malic acid diethyl ester. Nevertheless, the pre-treatment of PLHC-1 cells with 2-oxothiazolidine-4-carboxylic acid reduced the toxicity of propyl gallate by 25%. Consequently, these results suggest that glutathione levels modulate propyl gallate cytotoxicity.

5. Conclusions

The complexity of the results obtained, showing EC_{50} values ranging from 10 to 1090 μM, confirm the different sensitivity of the model systems used. Considering all the data obtained, the sensitivity to propyl gallate decreased as follows: PLHC-1 cells > *D. magna* > *V. fischeri* > RTG-2 cells > *C. vulgaris*. The most sensitive endpoint was the content of total protein in PLHC-1 cells, with an EC_{50} of 10 μM and a NOAEL of 1 μM propyl gallate at 72 h, while the least sensitive was the inhibition of the growth of the green alga *C. vulgaris*, with an EC_{50} of 1090 μM and a NOAEL of 800 μM propyl gallate at 24 h. The large differences found in the evaluated models might be related to a variety of factors. Therefore, a single bioassay will never provide adequate information for a suitable ecotoxicological evaluation, showing that bioassay batteries are valuable tools in the ecotoxicological assessment of chemicals.

Following the EU guideline for classification, packaging and labelling of dangerous substances (Commission Directive 2001/59/EC, 2001), according to the results obtained and due to its low octanol/water partition coefficient ($\log K_{ow} < 3$), propyl gallate should be classified as "R51 Toxic to aquatic organisms". However, as there are no available reports quantifying the presence of propyl gallate in the aquatic environment, the ratio between the predicted environmental concentration and the predicted no-effect environmental concentration could not be calculated to estimate its potential risk.

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