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Phloroglucinol: Antioxidant properties and effects on cellular oxidative markers in human HepG2 cell line

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ABSTRACT

Phloroglucinol is an ubiquitous secondary metabolite encountered in a free state or polymerised as phlorotannins in brown macroalgae, and present in higher plants. FRAP and TEAC assays measured the antioxidant properties of phloroglucinol in non-biological conditions. Additionally, the biological effects of phloroglucinol (4–400 μM) were scrutinised using cellular oxidative stress markers, such as the generation of ROS, antioxidant defences (concentration of GSH and activities of GPx, GR and GST), and levels of MDA as a marker for lipid peroxidation. The direct effect was assessed immediately after an incubation period, whereas for the protective effect, the incubation period was followed by 3-h treatment with the pro-oxidant *t*-BOOH. The results indicated that despite having a higher radical scavenging capacity than Trolox after 30 min, phloroglucinol was not a suitable antioxidant standard for phlorotannins. Regarding the biological effects, phloroglucinol had no impact on cell viability, reduced levels of ROS and increased antioxidant defences in the direct treatment for most concentrations. The results of the protective effect were mitigated as phloroglucinol failed to protect from ROS generation but evoked a significant recovery of the stress-altered cellular antioxidant defences to restful conditions. Additionally, MDA levels were greatly reduced, preventing a radical chain oxidation.

1. Introduction

Phloroglucinol (1,3,5-trihydroxybenzene) is the monomeric building unit of phlorotannins, phenolic compounds known only from brown algae (Phaeophyceae). The free form is found in some Fucales at concentrations over 0.5% dry weight (DW) for *Cystoseira discors* and *Cystoseira tamariscifolia* (*Sargassaceae*; Ragan and Glombitza, 1986), and in *Ecklonia cava* (*Laminariaceae*) at 0.016% DW (Kang et al., 2006). It is also present in *Himanthalia elongata* (*Himanthaliaceae*; Ragan and Glombitza, 1986), consumed as "Sea spaghetti" in Brittany, France (Guiry and Blunden, 1991) and also used as a fertilizer on artichokes and onions in Brittany (Guiry, pers. comm.) When polymerised as phlorotannins, their concentration in Fucales is frequently around 10% and may reach as much as 20% DW. Beside the free form occurring in macroalgae, alkali degradative studies of phlorotannins have shown that phloroglucinol can be released from the polymeric chain, despite a poor yield (Ragan and Glombitza, 1986). In higher plants (Tracheophyta), it is also the back bone of over 700 secondary metabolites encountered in Angiosperm species such as *Hypericum* sp., *Melicope sessiliflora*, *Helichrysum caespititium*, *Kaempferia pandurata* and *Acacia meamsii* and many others (Singh et al., 2010).

The ability of monomeric phenolic compounds as antioxidants depends on the degree of hydroxylation and extent of conjugation (Hodnick et al., 1988), and phloroglucinol displays an elevated degree of hydroxylation with an aromatic nucleus surrounded with three hydroxyl groups. Phloroglucinol and its derivatives exhibit a wide range of activities, such as anti-inflammatory, cytotoxicity, DNA strand-scission, enzyme inhibition (Barwell et al., 1989), antithrombotic and profibrinolytic activities (Bae, 2011) and are included in patented compositions as anticancer, anti-depressant, anti-microbial, anti-protozoal, anti-spasmodic, anti-viral, and in dermatology products (see review from Singh et al. (2009)). Polymers of phloroglucinol have been shown to exhibit antioxidant activities (Zubia et al., 2009), and so has the free form of phloroglucinol (Kang et al., 2006). Kang et al. (2010) have recently investigated the protective effect of phloroglucinol, isolated from *Ecklonia cava*, against γ-ray radiation-induced oxidative damage.

Abbreviations: ABTS, 2,2′-azinobis-(3-ethylbenzothiazolin)-6-sulfonate; DCFH, dichlorofluorescin; DW, dry weight; DNPH, 2,4-dinitrophenylhydrazone; FBS, foetal bovine serum; FRAP, ferric reducing/antioxidant power; GR, glutathione reductase; GSH, reduced glutathione; GST, glutathione-S-transferase; MCB, monochlorobimane; MDA, malondialdehyde; NADH, nicotine adenine dinucleotide (reduced); NADPH, nicotine adenine dinucleotide phosphate reduced; OPT, *o*-phthalaldehyde; ROS, reactive oxygen species; *t*-BOOH, *tert*-butyl hydroperoxide; TCA, trichloroacetic acid; TEAC, Trolox equivalent antioxidant capacity; TPTZ, 2,4,6-tripyridy-s-triazine.

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Phloroglucinol significantly decreased the level of radiation-induced intracellular ROS and damage to cellular components such as lipids, DNA and proteins. In the same line, Kim and Kim (2010) recently suggested a possible mechanism for the reactivity of phloroglucinol on chronic inflammation via NF-κB and AP-1 signaling pathway, notably implicating the reduction of endogenous and exogenous ROS levels, and a decrease in protein and DNA oxidation. However, to the best of our knowledge, there is no mention of antioxidant activities from phloroglucinol in the context of hepatoprotection in the literature.

The aims of the present study were therefore to investigate the antioxidant properties of phloroglucinol, both in routine spectrophotometric assays to examine its potential role as a standard for natural phloroglucinol derivatives, and in cellular assays to assess their direct and protective effects on biomarkers of human HepG2 cells.

2. Materials and methods

2.1. Reagents

Chemicals and solvents used were analytical grade available commercially unless otherwise specified. The following chemicals were used in the assays: 2,2′-azinobis-(3-ethylbenzothiazoline 6-sulfonate) (ABTS), dichlorofluorescin (DCFH), 2,4-dinitrophenylhydrazone (DNPH), EDTA, gentamycin, glutathione reductase (GR), reduced glutathione (GSH), β-mercaptoethanol, nicotine adenine dinucleotide (reduced) (NADH), nicotine adenine dinucleotide phosphate reduced salt (NADPH), *o*-phthalaldehyde (OPT), penicillin G, phloroglucinol (PG); *tert*-butylhydroperoxide (*t*-BOOH) and streptomycin were procured from Sigma Chemical Co. (Madrid, Spain), for cell culture studies. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water soluble analogue of vitamin E, was from Aldrich Chemical Co. (Guillingham, UK). The TPTZ (2,4,6-tripyridyl-s-triazine) was from Fluka chemicals (Madrid, Spain). The Bradford reagent was from BioRad Laboratories (Madrid, Spain). Glutathione-S-transferase (GST) assay kit was purchased from Biovision K260-100 (Mountain View, CA, USA). Other reagents were of analytical or chromatographic quality. Cell culture dishes were from Falcon (Cajal, Madrid, Spain).

2.2. Antioxidant capacity

The reducing power of phloroglucinol was determined by the ferric reducing/antioxidant power (FRAP) assay (Pulido et al., 2000). The FRAP value was calculated using the calibration curve of $FeSO_4$, a standard reducing agent allowing the transition from TPTZ-Fe(III) to TPTZ-Fe(II). Whereas the capacity of phloroglucinol to scavenge free radicals was assessed using the Trolox equivalent antioxidant capacity (TEAC) assay (Re et al., 1999); where the decolouration of ABTS by an antioxidant agent during the assay is followed against a control at 730 nm between 0 and 360 s, every 20 s. The absorbance read is compared to the one of a control solution, and then transformed into an inhibition percentage. The area under the curve between the control and the sample is calculated by the corresponding integral, and expressed as μM equivalent of Trolox per μM of phloroglucinol.

2.3. Cell culture

HepG2 cells were maintained in a humidified incubator containing 5% CO_2 and 95% air at 37 °C. They were grown in DMEM F-12 medium from Biowhitaker (Lonza, Madrid, Spain), supplemented with 2.5% Biowhitaker foetal bovine serum (FBS) for HepG2 with an additional 50 mg/L of each of the following antibiotics: gentamycin, penicillin and streptomycin (Sigma, Madrid, Spain). Plates were changed to FBS-free medium before the beginning of the assays as the serum might interfere in the running of the assays and affect the results. Moreover, a fairly good growth was observed in FBS-free medium (Alía et al., 2006).

2.4. Cell treatment conditions

Cells were incubated for 20 h with the various concentrations of phloroglucinol. To assess both direct and protective effects of phloroglucinol against oxidative stress, two types of experiments were designed (Goya et al., 2007). The direct effect of phloroglucinol on the cellular markers was assessed immediately after the incubation period, whereas for assessment of the protective effect, the incubation period was followed by a 3-h treatment in an oxidative environment with *t*-BOOH at 400 μM. For both assessments, a number of assays on various biomarkers were applied.

2.5. Evaluation of cytotoxicity

2.5.1. LDH leakage

Cells were plated in 60-mm diameter plates at a concentration of 1.5×10^6 cells per plate and the assay was carried out when plate coverage reached 70–80%. The day before the assay, concentrations of phloroglucinol diluted in serum free medium were added with a minimum of $n = 4$ per condition. After 20 h of incubation, culture medium and cells were collected separately and kept on ice. Cells were sonicated for 10 min in ice-bath prior to centrifugation (2515 g, 10 min, 4 °C). Culture medium and supernatant from cellular lysis were analysed respectively on both halves of 96-well plates. In each well 10 μL of sample and 200 μL reaction mix (2 mg/mL NADH in 1 mM KOH/0.08 M pyruvate/1.35 M Tris) were added. Absorbance ($\lambda = 340$ nm) was immediately measured at $t = 0$ and $t = 5$ min. The leakage was expressed as the ratio between the activity in the medium and that of the medium plus cell content (Alía et al., 2005).

2.5.2. Crystal violet assay

Cells were seeded in 96-well plates with 100 μL of culture medium, conditions as described above for the LDH leakage assay. Cells were washed with 200 μL of PBS, and incubated with 50 μL crystal violet for 20 min at room temperature. The wells were then washed three times with 200 μL of distilled water. Finally, cell lysis was carried out in 100 μL 1% SDS and the absorbance was read at $\lambda = 560$ nm in a microplate reader (FL600, Bio-Tek, Winooski, VT, USA). Results were presented as a percentage of viable cells (Granado-Serrano et al., 2007).

2.6. Determination of reactive oxygen species generation

Cells were seeded in 24 multiwell-plates (2×10^5 cells per well), conditions were as described above for the LDH leakage assay. On the day of the assay, 10 μL of the fluorescent probe (DCFH-DA) was added under reduced light to each condition at a final concentration of 5 μM, and incubated for 30 min in the dark. The medium-plus condition was then discarded and cells were rinsed in 1 mL PBS. For the direct effect of phloroglucinol, the plate was immediately read for fluorescence (*t*-BOOH was used as a positive control), whereas for the protection effect, each well was filled with 0.5 mL *t*-BOOH (400 μM) in serum-free culture medium except the control, which was filled up with serum-free culture medium. The production of intracellular ROS was followed over 90 min and the control was attributed the index value of 100. A set of wells ($n = 4$) in each 24 multiwell-plate was treated with 400 μM *t*-BOOH, as a positive control. Both control and *t*-BOOH values were pooled from two multiwell plates to obtain average of $n = 8$. Fluorescence was read at Ex/Em = 485/530 nm (Alía et al., 2005) in a plate reader (FL600, Bio-Tek, Winooski, VT, USA).

2.7. Quantification of concentration of reduced glutathione (GSH)

The content of reduced GSH was quantified by the fluorometric assay of Hissin and Hilf (1976). The method is based on the reaction of the reduced GSH with *o*-phtalaldehyde (OPT) at pH 8.0. Cells were seeded onto 100-mm Petri dishes. Conditions were set as described for the LDH leakage assay ($n = 4$). The cells collected in PBS were centrifuged at 400*g*, for 10 min at 4 °C. The supernatant was discarded, while the pellet was resuspended in 250 μL TCA/EDTA (5 mM) and sonicated for 10 min in an ice bath. The result of the cell lysis was then centrifuged at 10,000*g*, for 10 min at 4 °C. The supernatant was kept frozen at −20 °C until analysis. The day of the assay, the supernatants from cellular lysis were thawed at room temperature and 50 μL of each sample were transferred in triplicate in a 96-multiwell plate. The reaction mix in each well consisted of 15 μL of 1 M NaOH, 175 μL sodium phosphate buffer/EDTA and 10 μL of a 10 mg/mL OPT solution. The fluorescence was read after 20 min in the dark (Ex/Em = 340/460 nm) in a plate reader (FL600, Bio-Tek, Winooski, VT, USA).

2.8. Determination of activity of antioxidant enzymatic defences

The determination of the glutathione peroxidase (GPx) activity was based on the oxidation of the reduced GSH by GPx, using *t*-BOOH as a substrate, coupled to the disappearance of NADPH by GR (Alía et al., 2006). GR activity was determined by following the decrease in absorbance due to the oxidation of NADPH utilised in the reduction of oxidised glutathione (Alía et al., 2006). The maximum absorption of NADPH is obtained at 340 nm. The determination of GST activity utilises monochlorobimane (MCB), a dye that responds to the presence of glutathione. The free form of MCB is almost non-fluorescent, whereas the dye fluoresces blue (Ex/Em = 380/461 nm) when it reacts with glutathione. GST catalyses the MCB-glutathione reactions and the fluorescence levels are proportional to the amounts of GST involved in the reaction.

2.9. Evaluation of malondialdehyde levels

Intracellular levels of malondialdehyde (MDA) were evaluated by high-performance liquid chromatography (HPLC) after collection of the cells, hydrolysis of MDA from intracellular proteins and derivatisation of MDA with