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Methanolic extract of *Hypericum perforatum* cells elicited with *Agrobacterium tumefaciens* provides protection against oxidative stress induced in human HepG2 cells

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ABSTRACT

Hypericum perforatum L. (HP) is widely used in traditional medicine to treat several medical conditions since ancient times. In our group, this plant has been used for biotechnological production of bioactive phytochemicals. In the present study, the ability of a methanolic extract of HP cells elicited with *Agrobacterium tumefaciens* (AT) to induce intracellular antioxidant defenses of human HepG2 cells and to protect them against *tert*-butyl hydroperoxide-induced oxidative stress was tested. The elicited HP extract significantly prevented *tert*-butylhydroperoxide-induced cell death, glutathione (GSH) depletion, and DNA damage, in both pre- and co-incubation regimes, while the extract from control HP did not. When incubated alone, none of the extracts were cytotoxic or genotoxic. Interestingly, contrary to control HP extract, incubation of HepG2 cells with extract from elicited HP cells induced significantly GSH levels and several cytoprotective enzymes. These effects were associated with an increase of Nrf2 levels in nucleus, which may explain the cytoprotective action of the elicited HP extract in the pre-incubation regime. Taken together, our results suggest that elicitation of HP cells with AT is an interesting biotechnological approach for the production of cytoprotective and antioxidant compounds for pharmaceutical applications.

1. Introduction

Cells are continuously exposed to reactive oxygen species (ROS), of both endogenous and exogenous sources, and a complex antioxidant defense system protect them against toxic levels of ROS and to maintain cellular redox homeostasis. However, oxidative stress may arise when the antioxidant defense system is not able to properly respond to an overall increase of intracellular ROS. In this condition, damage to various cellular macromolecules, including lipids, proteins, and DNA can occur, inhibiting their normal function and ultimately causing cell death. Therefore, oxidative stress is thought to contribute to the pathogenesis of several diseases, including diabetes, liver, neurodegenerative, and cardiovascular diseases (Valko et al., 2007; Trachootham et al., 2008; Lima et al., 2011). Thus, much research have been focused in the search of antioxidants capable of reducing oxidative stress that, besides the ones produced by our cells (endogenous), are usually provided by our diet (exogenous) (Masella et al., 2005). Among these, plant bioactive compounds have been largely studied because they can act as direct antioxidants through scavenging ROS or inhibiting their formation and, also, as indirect antioxidants through upregulation of endogenous antioxidant defenses (Masella et al., 2005; Dinkova-Kostova and Talalay, 2008). This latter effect of phytochemicals is usually operated by activation of the nuclear factor erythroid 2-related factor (Nrf2)/antioxidant response element (ARE) pathway, a stress response regulated by cell's redox state (Surh et al., 2008).

Hypericum perforatum L. (HP), commonly known as St. John's wort, is widely used in traditional medicine to treat several medical conditions since ancient times. Nowadays, it remains a popular plant in phytomedicine for treatment of anxiety, depression, cuts, and burns (Guedes et al., 2012). Recent research suggests that this herb is also effective in treating other ailments, including cancer, inflammation-related disorders, bacterial, and viral diseases,

Abbreviations: ARE, antioxidant response element; AT, *Agrobacterium tumefaciens*; GSH, glutathione; HP, *Hypericum perforatum*; Nrf2, nuclear factor erythroid 2-related factor; ROS, reactive oxygen species; *t*-BOOH, *tert*-butyl hydroperoxide.

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acting also as an antioxidant and neuroprotective agent (Silva et al., 2004, 2005, 2008; Franchi et al., 2011; Klemow et al., 2011). The pharmacological properties attributed to this species may derive from its wide variety of biologically active metabolites, including naphthodianthrones, phloroglucinolds, phenolic acids, flavonoids, and xanthones (Nahrstedt and Butterweck, 2010). Several studies suggest that xanthones belong to the defense arsenal employed by HP to combat biological stress factors, including infection by pathogens (Crockett et al., 2011). In particular, we have shown recently that both biosynthesis of previously present xanthones and *de novo* production of new xanthones increased in HP cell suspension cultures elicited through co-cultivation with *Agrobacterium tumefaciens* (AT) (Franklin et al., 2009). Moreover, this rapid up-regulation of xanthone metabolism significantly increased the antiradical and antimicrobial properties of methanolic extracts from HP cells (Franklin et al., 2009).

Considering this metabolic shift of HP cells after elicitation with AT, in the present study, we evaluated the ability of methanolic extracts of control and elicited HP cells to protect human HepG2 cells against oxidative damage induced by *tert*-butyl hydroperoxide (*t*-BOOH), an organic peroxide widely used to induce oxidative stress (Buc-Calderon et al., 1991). *H. perforatum* extracts were pre- or co-incubated with the toxicant in HepG2 cells and cell viability, GSH levels, and DNA damage evaluated. In addition, the ability of HP extracts of induce intracellular antioxidant defenses of HepG2 cells was studied.

2. Material and methods

2.1. Chemicals and antibodies

Minimum essential medium eagle (MEM), antibiotic-antimycotic solution, HEPES, *tert*-butyl hydroperoxide (*t*-BOOH), anti-β-actin antibody, and all other not specified reagents were from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Biochrom AG (Berlin, Germany). SYBR Gold nucleic acid gel stain was purchased from Invitrogen (Paisley, UK), and complete protease inhibitor cocktail from Roche (Penzberg, Germany).

Antibodies against NQO1, GCLC, Histone H1, and secondary antibody goat anti-mouse IgG-HRP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-GCLM and anti-PRDX4 antibodies developed by the Clinical Proteomics Technologies for Cancer were obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD, and maintained by the University of Iowa, Department of Biology, Iowa City, IA, USA. Anti-HO-1 antibody was from Enzo Life Sciences (Farmingdale, NY, USA), anti-Caspase-3 from EMD Millipore Corporation (Billerica, MA, USA), anti-Nrf2 from Novus Biologicals (Littleton, CO, USA), and secondary antibody goat anti-rabbit IgG-HRP from Cell Signaling Technology (Beverly, MA, USA).

2.2. Extracts from H. perforatum cells

The extracts from HP cells were the same used in the study by Franklin et al. (2009). In brief, HP cell suspension cultures (established in Dias et al., 2001) were maintained and elicited with AT. Control and elicited HP cells were harvested by vacuum filtration and freeze-dried in a lyophilizer (Alpha 2–4 LD plus, Christ, Osterode am Harz, Germany). Equal quantity of dry biomass from control and elicited cells were extracted in 90% MeOH under dark. Extracts were dried in a rotary evaporator and frieze-dried powders were redissolved in dimethyl sulfoxide (DMSO) at 20 mg/mL. Similarly, AT cells were harvested by centrifugation (10,000 × *g*), freeze-dried and extracted in methanol as above. The extracts' stock solutions were kept at −20 °C in aliquots until use in the cell culture experiments. DMSO alone was used in control conditions at 0.5% (v/v).

The HP cells extracts were previously analyzed by HPLC (Franklin et al., 2009), and it was found that xanthone content increased significantly whereas flavonoids remained unchanged after HP elicitation with AT. In detail, flavonoids were present in about 0.9 mg/g of HP cells biomass dry weight with and without AT elicitation; xanthones were present at 0.3 mg/g in HP control cells and at 4.1 mg/g in elicited HP cells, being the major one the 1,3,6,7-tetrahydroxy-8-prenylxanthone representing 23% of the total xanthones (Franklin et al., 2009).

2.3. Cell culture and experimental conditions

The human hepatocellular carcinoma cells HepG2 (HB-8065) were purchased from American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in MEM supplemented with 10% FBS, 1% antibiotic antimycotic solution, 1 mM sodium pyruvate, 10 mM HEPES, and 1.5 g/L sodium bicarbonate, at 37 °C in a humidified incubator containing 5% CO_2.

For experiments, cells were seeded at 2×10^5 cells/mL 48 h before incubation with HP extracts or AT extract with or without *t*-BOOH as specified below. The AT extract was used to control the possible presence of any bioactive compound present in this bacteria that would be extracted from the biomass of HP cells elicited with AT. The tested concentration of AT extract was normalized considering the extraction yield and the relative biomass of AT present in the elicited HP cells. Therefore, when the tested concentration for HP extracts was 100 μg/mL, for AT it was 25 μg/mL.

To study the effects of extracts against *t*-BOOH toxicity, two incubation regimes were used, described as follow. In the co-incubation regime, HepG2 cells were incubated with extracts and 800 μM *t*-BOOH for 4 h. In the pre-incubation regime, HepG2 cells were incubated with extracts for 6 h, followed by a recovery period of 16 h in fresh medium without extracts to let antioxidant defenses to be synthesized, and, then, cells were incubated with 800 μM *t*-BOOH for 4 h. In the assessment of DNA damage induced by *t*-BOOH the concentration used of the toxicant was 200 μM for 1 h.

2.4. LDH leakage assay

In order to determine the effect of HP extracts and *t*-BOOH on cell viability the lactate dehydrogenase (LDH) leakage assay was used as previously described (Lima et al., 2005). Briefly, LDH activity was measured at 30 °C by quantification of NADH (0.28 mM) consumption by continuous spectrophotometry (at 340 nm) on a microplate reader (SpectraMax 340pc, Molecular Devices, Sunnyvale, CA, USA) using pyruvate (0.32 mM) as substrate in 50 mM phosphate buffer (pH 7.4). LDH leakage was calculated using the following equation: LDH leakage (%) = 100 × extracellular LDH/total LDH.

2.5. Glutathione content

The effect of HP extracts and *t*-BOOH on glutathione (GSH) content was determined by the DTNB-GSSG reductase recycling assay, as previously described (Lima et al., 2004). Briefly, after protein precipitation with 5-sulfosalicylic acid, samples were centrifuged and supernatants were used for measurement of GSH following the DTNB oxidation at 415 nm and compared with a standard curve. The results were expressed as nmol GSH/mg of protein.

2.6. Comet assay

In order to assess the effects of HP extracts against *t*-BOOH-induced DNA damage, the alkaline version of the single cell gel