Food and Chemical Toxicology 105 (2017) 300–307

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Evaluation of cytotoxic, apoptotic, mutagenic, and chemopreventive activities of semi-synthetic esters of gallic acid

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ARTICLE INFO

Article history:
Received 16 January 2017
Received in revised form
3 April 2017
Accepted 24 April 2017
Available online 25 April 2017

Keywords:
Alkyl gallates
Toxicity
Micronucleus
DNA damage
Cell death
Chemoprotective

ABSTRACT

Gallic acid and its derivatives are phenolic compounds widely used as food supplements in the form of capsules, liquid extracts, and ointments owing to their good antioxidant properties. Besides, these compounds are potent inhibitors of fungi, bacteria, and some viruses and possess strong antiproliferative and chemopreventive activities. However, gallic acid derivatives are also known to exert harmful effects like mutagenicity and cytotoxicity. The present study aimed to understand and explore the toxicological risks of these compounds. For this, a series of alkyl gallates with side chains varying from five to eight carbons (pentyl, hexyl, heptyl, and octyl gallates) were evaluated for their cytotoxic and pro-apoptotic potential. In addition, the genotoxic effects of alkyl gallates were measured in HepG2 cells using the single cell gel electrophoresis (SCGE)/comet assay and the cytokinesis-blocked micronucleus (CBMN) test. In both the tests, the substances did not induce any significant differences when compared to the control group. In addition, alkyl gallates exhibited a chemopreventive effect, thereby considerably reducing the mutagenicity caused by H2O2. In conclusion, our results suggest that alkyl gallates are non-genotoxic, non-mutagenic, and pro-apoptotic agents, which may serve as suitable and promising candidates for preventing chemically-induced chromosomal damage.

1. Introduction

Cancer is considered as one of the leading causes of morbidity and mortality, thereby posing a major challenge to the health professionals worldwide (Stewart and Wild, 2014). Oxidative stress, a term used to describe the imbalance between the generation of harmful free radicals and their elimination by the protective machinery of the body is an aspect common to all types of cancers (Noda and Wakasugi, 2001). A significant number of new drugs and pharmaceuticals have been developed displaying protection against the deleterious effects of oxidative stress; however, most of these drugs are also associated with adverse side effects, such as cytotoxicity, mutagenicity, and carcinogenicity (Garattini and Bertele, 2002; Bagchi et al., 2014).

In an attempt to develop a safe and non-toxic treatment for cancer with lower side effects, a special attention has been diverted to antioxidants extracted from plants and their semi-synthetic derivatives. Among them, gallic acid (GA), a natural plant triphenol and its derivatives have been demonstrated by several investigators to possess strong antioxidant activity (Aruoma et al., 1993; Morley et al., 2005; Savi et al., 2005; Locatelli et al., 2009; Merkl et al., 2010; Morais et al., 2010).

The gallic acid (3,4,5-trihydroxybenzoic acid) is a naturally occurring phenol present in plants and obtained by the hydrolysis of tannins (Inoue et al., 1995). Its alkyl esters, especially propyl gallate, octyl gallate, and lauryl gallate, are currently utilized as antioxidant additives to prevent changes in food flavor and nutritional values and increase the shelf-life of lipid-based foods by serving as chain-breaking inhibitors of oxygen-induced lipid peroxidation. The same property is exploited by the cosmetic and

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http://dx.doi.org/10.1016/j.fct.2017.04.033
0278-6915/

pharmaceutical industries (Aruoma et al., 1993; Fujita and Kubo, 2002; Kubo et al., 2002), where these compounds are employed as natural coloring agents and for drug formulations. The diverse range of properties of gallic acid and its derivatives has been associated with a wide variety of biological activities, including antifungal (Fujita and Kubo, 2002; Hsu et al., 2009), antibacterial (Kubo et al., 2002; Kubo et al., 2004; Silva et al., 2013), antiviral (Kratz et al., 2008), antiherpetic (Savi et al., 2005), antiproliferative (Dodo et al., 2008; Locatelli et al., 2008; Locatelli et al., 2009), and chemopreventive (Morley et al., 2005; Pandır, 2015).

Contrary to their versatile and beneficial properties, alkyl gallates have also exhibited controversial results. Some studies have demonstrated that gallates could induce DNA damage and mutagenicity (Tayama and Nakagawa, 2001; Furukawa et al., 2003; Savi et al., 2005), and their antioxidant potential can under certain conditions turn prooxidant (Strlic et al., 2002; Furukawa et al., 2003). For these reasons, the safety and biological effects of alkyl gallates would benefit of further toxicological investigations.

Toxicological risk assessment is considered as an essential part of a drug design initiative to identify compounds that are most likely to be detrimental to the human health. The evaluation of toxicological risk involves hazard identification, dose-response assessment, exposure assessment, and risk characterization. Both *in vivo* and *in vitro* toxicity methods are employed for a comprehensive and valid analysis of the risks associated with the exposure to potentially harmful substances. *In vitro* assay systems provide an important contribution in elucidating the mechanisms of toxicity, carcinogenicity, and metabolism of drugs and chemicals. Thus, they serve as an indispensable tool for identifying potentially hazardous compounds at early stages of drug design (Davila et al., 1998).

The predictive *in vitro* assay is advantageous in terms of providing useful ways to prioritize screening and reduce the assay requirements. It also offers a clear, next step in exploring the biological effects of a compound. *In vitro* assays are less complex in the number of factors that would influence a positive readout, making the successful development of an *in silico* model more likely (Greene and Naven, 2009).

The contradicting results obtained so far with gallates indicate that the knowledge of the effects (beneficial or adverse) has largely remained elusive. With an aim to ensure public safety, the present study assessed alkyl gallates for their cytotoxicity and pro-apoptotic activities, as well as their genotoxic and mutagenic potential. Further, the chemopreventive activity of alkyl gallates on DNA damage induced by H_2O_2 (a direct-acting mutagen) was investigated. The results of the present study would add beneficial data to the knowledge of alkyl gallates as non-genotoxic, non-mutagenic, pro-apoptotic, and suitable agents for preventing various types of cancer.

2. Methodology

2.1. Chemicals

Alkyl gallates with side chains varying from five to eight carbons (pentyl, hexyl, heptyl, and octyl gallates) were synthesized as described by (Silva et al., 2013).

2.2. Cell culture

The following cell lines were used in the study: HepG2; human liver carcinoma cell line (ATCC, HB8065), DU-145; human prostate cancer cell line (ATCC, HTB-81), MDA-MB-231; human breast carcinoma cell line (ATCC, HTB-26), MRC-5; human lung fibroblast cell line (ATCC, CCL-171), and A549; human lung adenocarcinoma epithelial cell line (ATCC, CCL-185). The cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and grown in DMEM or Ham's F10 (A549 cell line) medium supplemented with 10% FBS, 1% penicillin (100 U/mL), and streptomycin (100 μg/mL). The cells were maintained in a humidified environment at 37 °C with 5% CO_2 and sub-cultured twice per week. The viability of the cells was checked before conducting the experiments. In brief, a freshly prepared solution of Trypan blue (0.05%, 10 μL) in distilled water was mixed with 10 μL of cellular suspension for 5 min, spread onto a microscope slide and covered with a coverslip. The non-viable cells appeared blue under the microscope.

2.3. Cytotoxicity tests

The cytotoxicity was measured fluorometrically by the resazurin reduction assay. Resazurin is a non-toxic and non-fluorescent blue reagent that is irreversibly reduced to a fluorescent and pink-colored resorufin by the viable cells undergoing active metabolism. Nonviable cells rapidly lose the metabolic capacity to reduce resazurin and thus do not produce a fluorescent signal. Therefore, resazurin serves as a cell permeable redox indicator for monitoring the number of viable cells.

For the resazurin reduction assay, 2.5×10^4 cells/well were seeded into a 96-well cell culture plate (Costar®, Cambridge, MA, USA) in a total volume of 100 μL for 24 h. The cells were then treated with the following alkyl gallates: pentyl, hexyl, heptyl, and octyl gallates at concentrations ranging from 1.6 to 200 μg/mL. After 24 h incubation, the medium was removed, and 50 μL of resazurin (Sigma-Aldrich®, St. Louis, MO, USA) prepared in 0.01% w/v of DMEM was added to each well, and the plates were incubated at 37 °C for 3 h. The fluorescence was measured on Synergy H1 microplate reader (BioTek®, Winooski, VT, USA) using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The untreated cells constituted the negative control (viable cells), whereas those treated with 1% dimethyl sulfoxide (DMSO) and 5 μg/mL of doxorubicin (Sigma-Aldrich®, St. Louis, MO, USA) constituted the vehicle and positive controls (dead cells), respectively. Three independents assays were performed. The IC_{50} value that represents the sample concentration required to inhibit 50% of cell proliferation was calculated from a calibration curve by regression analysis.

2.4. Detection of apoptosis

Nuclear morphology was assessed by staining the cells with Hoechst 33342 and propidium iodide (PI). HepG2 cells were cultured at a density of 2.5×10^4 cells/well in a 96-well plate followed by incubation in the culture medium for 24 h at 37 °C with 5% CO_2. The culture medium was replaced with 100 μL of medium containing the IC_{50} concentration of the alkyl gallates (pentyl, hexyl, heptyl, and octyl gallates) previously determined by the resazurin reduction assay and incubated for 24 h. The untreated cells, 1% DMSO, and 0.5 μg/mL of doxorubicin were utilized as negative, vehicle, and positive controls, respectively. To stain the DNA, the cells were incubated with Hoechst 33342 (10 μM) and PI (10 μM) diluted in phosphate-buffered saline (PBS) for 10 min at room temperature. The cells were then observed by the IN Cell Analyzer 1000 (GE Healthcare®, USA). Based on the staining and the level of DNA condensation, the nuclei were categorized into four types, namely intact blue nuclei, condensed/fragmented blue nuclei, condensed/fragmented pink nuclei, and intact pink nuclei. These categories corresponded to viable, early apoptotic, late apoptotic, and necrotic cells, respectively. A total of 500 cells were counted, and the percentage of viable, apoptotic, and necrotic cells was calculated. The results were expressed as the mean ± standard