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In vitro toxicological assessment of an organosulfur compound from *Allium* extract: Cytotoxicity, mutagenicity and genotoxicity studies

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ABSTRACT

Garlic (*Allium* sativum) and onion (*Allium* cepa) are being used in the food industry as flavoring but also for their antimicrobial activities. These activities are mainly derived from the organosulfur compounds (OSCs). Propyl propane thiosulfinate (PTS) is an OSC with potential use in the active packaging, but its safety should be guaranteed before being commercialized. The aim of this work was to investigate for the first time the cytotoxicity of PTS as well as its *in vitro* mutagenic/genotoxic potential using the following battery of genotoxicity tests:(1)the bacterial reverse-mutation assay in *S. typhimurium* (Ames test, OECD 471, 1997); (2) the micronucleus test (MN, OECD 487, 2016); (3) the mouse lymphoma thymidine-kinase assay (MLA, OECD 476, 2015), and (4) the comet assay (standard and modified with restriction enzymes). The results revealed that PTS was not mutagenic neither in the Ames test nor in MLA. However, genotoxic effects were recorded in the MN test on mammalian cells (L5178YTk$^{+/-}$cells) after PTS exposure at the highest concentration tested (17.25 μM) without S9, and also its metabolites (+S9, from 20 μM). Moreover, in the comet assay, PTS induced DNA breaks damage in Caco-2 cells at the highest concentration tested (280 μM) but it did not induce oxidative DNA damage.

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1. Introduction

Garlic, *Allium sativum* L., is considered one of the twenty most important vegetables with various uses throughout the world, not only for culinary purposes but also as an ingredient of traditional and modern medicine (Martins et al., 2016). In addition, onion, *Allium cepa*, has similar applications (Corzo-Martínez et al., 2007). They both exhibit a peculiar odour, which is associated with the presence of organosulfur compounds (OSCs), which are also responsible of their beneficial properties (Lekshmi et al., 2015). Essential oils (EOs) from garlic and onion have been studied for their use in the food industry (Mnayer et al., 2014). In fact, onion EO has been pointed out as a potential source of natural antimicrobial and antioxidant agents to be applied in food systems, due to its interesting properties (Benkeblia et al., 2004; Ye et al., 2013; Prakash et al., 2015). In this regard, the toxic assessment of extracts from garlic and onion, as well as their components, need to be addressed. The toxic assessment of OSCs is of great interest because their content can vary substantially depending on the different conditions (Benkeblia and Lanzotti, 2007). Previous works from our research group have studied the cytotoxicity and mutagenicity/genotoxicity of several OSCs such propyl propane thiosulfonate (PTSO) (Llana-Ruiz-Cabello et al., 2015a; Mellado-García et al., 2015), dipropyl sulfide (DPS) and dipropyl disulfide (DPDS) (Llana-Ruiz-Cabello et al., 2015b). The obtained results evidenced a lack of toxic effects at the concentration ranges intended to be used for food packaging. Nevertheless other authors have reported both genotoxic (Musk et al., 1997) and antigenotoxic effects (Guyonnet et al., 2000, 2001; Belloir et al., 2006; Arranz et al., 2007; Chiu et al., 2016) for some OSCs. The contradictory results reported for these OSCs (Llana-Ruiz-Cabello et al., 2015c) made necessary to evaluate the toxicological profile of them before their use in food industry, particularly to intended to be incorporated in food contact materials (FCM).

Despite the beneficial effects attributed to *Allium* plants, growing awareness exist on the hazards associated with the use of plants and their extracts as antibiotic and chemical feed additives (Wallace, 2004), as well as actives in food packaging (Llana-Ruiz-

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Cabello et al., 2015c; Maisanaba et al., 2016). Among the OSCs, propyl propane thiosulfinate (PTS) is an organosulfur compound obtained by decomposition of initial components present in *Allium* plants, that has been stabilized and characterized by DOMCA Research Center (DMC, Granada, Spain). This compound has previously shown to have beneficial effects on goats as methanogenesis inhibitor in the rumen (Martínez-Fernández et al., 2013, 2015). PTS is considered as a secondary metabolite of garlic, derived of the formation of the sulfenic acids, which nowadays has been studied for evaluate its antimicrobial activity against the faecal microbiota of pigs (Ruiz et al., 2015), or as dose-dependently killer of invasive sporozoites and stimulating of higher spleen cell proliferation in chickens (Kim et al., 2013). Nevertheless, little is known about its safety for human consumption.

According to the Guidelines of the Scientific Committee on Food for Safety Assessment of Substances Used in FCM, information on the genotoxic potential is a key component in the risk assessment of substances used in active packaging (EFSA, 2011; 2016). In the new European Food Safety Authority (EFSA) Scientific Committee recommendations on genotoxicity testing strategies, a basic battery of two *in vitro t*ests, a bacterial reverse mutation assay (OECD 471, 1997) and an *in vitro* mammalian cell micronucleus test (MN) (OECD 487, 2016) are recommended (EFSA, 2016). The bacterial reverse-mutation assay in *Salmonella typhimurium* (Ames test) is a short-term mutation study specifically designed to detect a wide range of chemical substances that can produce genetic damage leading to gene mutation. It is rapid and relatively easy to perform (Mortelmans and Zeiger, 2000). Concerning the MN test using mammalian cells, it is one of the preferred methods for assessing chromosome damage because they enable both chromosome loss and chromosome breakage, together with chromosome non-disjunction to be measured reliably (Fenech, 2000; EFSA, 2016). In the case of inconclusive, contradictory or positive results from these two *in vitro* tests, it may be appropriate to conduct further *in vitro* tests to optimize any subsequent *in vivo* testing, or to provide additional useful mechanistic information (EFSA, 2011; 2016). In this sense, the *in vitro* mammalian cell gene mutation assay (MLA) (OECD 476, 2015) is an assay commonly used, to evaluate the mutagenicity of chemical and physical agents This can detect a wide range of genetic alterations, including both point and chromosomal, gene, base pair substitutions and frame-shift mutations (Wang et al., 2009). Finally, the effect on DNA damage can be studied through the comet assay which is a reproducible and sensitive test for the detection of DNA damage (mainly DNA breaks) in eukaryotic cells. Incorporation of oxidative DNA damage repair enzymes (for example, formamidopyrimidine DNA glycosylase (FPG) and endonuclease III (EndoIII)) in the standard alkaline comet assay procedure allows the detection and measurement of oxidatively DNA damaged (Collins, 2004; Pu et al., 2016).

In the present work, a specifically toxicological approach has been carried out on PTS for the first time. The cytotoxicity of PTS was studied in the Caco-2 cell line by means of three endpoints: total protein content (TP), neutral red uptake (NRU) and MTS tetrazolium salt reduction, as well as in L5178Y $Tk^{+/-}$ by the trypan blue exclusion test. Afterwards, an *in vitro* mutagenicity and genotoxicity assessment of PTS has been performed using a prokaryotic system for the Ames test, and two different mammalian cell lines, L5178Y $Tk^{+/-}$ (for MLA and MN tests) and Caco-2 cells (for the standard and enzyme-modified comet assay).

2. Materials and methods

2.1. Supplies and chemicals

Culture mediums (Minimum essential medium (MEM) and RPMI 1640 medium), cell culture reagents L-glutamine solution (CAS No. 56-85-9), sodium pyruvate solution (CAS No. 113-24-6), penicillin/streptomycin solution, gentamicin solution (CAS No 1405-41-0) and amphotericin B solution (CAS No. 1397-89-3), foetal bovine serum (FBS) and horse serum were obtained from Gibco (Biomol, Sevilla, Spain). PTS (95.5% purity) was kindly supplied by DOMCA S.L. (Granada, Spain). Cyclophosphamide (CP, CAS No. 6055-19-2), mitomycin C (MMC, CAS No. 50-07-7), methyl methanesulfonate (MMS, 99% purity; CAS No. 66-27-3), hypoxanthine (99% purity; CAS No. 68- 94-0), thiazolyl blue tetrazolium bromide (MTT, 99.7% purity; CAS No. 298-93-1), MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium salt), and tetrazolium compound (CAS No. 351330-42-2) were purchased from Promega (Biotech Ibérica, Madrid, Spain); Coomassie Brilliant Blue G-250 (CAS No. 6104-59-2) was purchased from (BioRad, Madrid, Spain), trifluorothymidine (TFT, ≥ 99% purity; CAS No. 70-00-8), thymidine (CAS No. 4449-43-8), THMG medium (thymidine 9 μg/mL, methotrexate 0.3 μg/mL, hypoxantine 15 μg/mL, glycine 22.5 μg/mL) (CAS No. 59-05-2), glycine (≥99% purity; CAS No. 56-40-6), cythochalasin B (Cyt-B, 98%, CAS No. 14,930-96-2), giemsa stain (CAS No. 51,811-82-6), dimethyl sulfoxide (DMSO) (CAS No. 67-68-5), 2-nitrofluoerene (2-NF) (CAS No. 607-57-8), sodium azide (NaN_3) (CAS No. 26628-22-8), 2-aminofluorene (2-AF) (CAS No. 153-78-6), Neutral Red (CAS No. 553-24-2), and trypan blue solution 0.4% (CAS No. 72-57-1) were purchased from Sigma–Aldrich (Madrid, Spain). S9 fraction was purchased from Moltox (Trinova, Biochem, Germany). Endo III (EC 3.1.21.5) was purchased from C-viral S.L. (Sevilla, Spain), and FPG (EC 3.2.2.23) from Sigma-Aldrich (Madrid, Spain).

2.2. Cells and culture conditions

Caco-2 cells, used for the cytotoxicity and for the standard and enzyme-modified comet assays, are derived from a human colon carcinoma (ATCC© HTB-37). They were maintained at 37 °C in a humidified incubator gassed with 5% CO_2 in air at 95% relative humidity (CO_2 incubator, NuAire, Spain), in MEM supplemented with 1% non-essential amino acids (NEAA), 2 mM L-glutamine, 1.25 μg/mL fungizone, 1 mM pyruvate, 50 μg/mL gentamicin and 10% FBS. Cell viability and cell number were determined with the trypan blue exclusion test.

Five *S. typhimurium* histidine-auxotrophic strains (TA97A, TA98, TA100, TA102 and TA104) were used for the Ames test, according to Organisation for Economic Cooperation and Development recommendations (OECD 471, 1997).

For the MN and MLA tests, L5178Y $Tk^{+/-}$ mouse lymphoma cells were used. This cell line was originally provided by Dr. Olivier Gillardeux (Safoni-Synthélabo, Paris, France). L5178Y $Tk^{+/-}$ cells were cultured according to Mellado-García et al. (2015). Cultures were maintained in a humidified incubator with 5% CO_2 at 37 °C.

2.3. Test solutions

The concentration ranges of PTS were selected according the results obtained in the cytotoxicity assays carried out in Caco-2 and L5178Y $Tk^{+/-}$ cells. Stock solution of PTS (400 mM) was prepared in DMSO, and the different exposure concentration solutions were made by dilution in MilliQ sterile water (Ames test), RPMI 1640 medium (MN and MLA assays) or MEM medium (cytotoxicity, standard and modified comet assays). To avoid the toxic effects for the cells, the final percentages of DMSO were always less than 0.1% in all the assays.

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