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Genotoxicity assessment of propyl thiosulfinate oxide, an organosulfur compound from *Allium* extract, intended to food active packaging

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ABSTRACT

Essential oils from onion (*Allium cepa* L.), garlic (*Allium sativum* L.), and their main components, such as propyl thiosulfinate oxide (PTSO) are being intended for active packaging with the purpose of maintaining and extending food product quality and shelf life. The present work aims to assess for the first time the potential mutagenicity/genotoxicity of PTSO (0–50 μM) using the following battery of genotoxicity tests: (1) the bacterial reverse-mutation assay in *Salmonella typhimurium* (Ames test, OECD 471); (2) the micronucleus test (OECD 487) (MN) and (3) the mouse lymphoma thymidine-kinase assay (OECD 476) (MLA) on L5178Y*Tk* $^{+/-}$, cells; and (4) the comet assay (with and without Endo III and FPG enzymes) on Caco-2 cells. The results revealed that PTSO was not mutagenic in the Ames test, however it was mutagenic in the MLA assay after 24 h of treatment (2.5–20 μM). The parent compound did not induce MN on mammalian cells; however, its metabolites (in the presence S9) produced positive results (from 15 μM). Data from the comet assay indicated that PTSO did not induce DNA breaks or oxidative DNA damage. Further *in vivo* genotoxicity tests are needed to confirm its safety before it is used as active additive in food packaging.

1. Introduction

Synthetic food additives are generally recognized as safe (GRAS) and have a beneficial effect on food preservation. However, consumers have become more conscious about potential health problems associated with these substances and demand alternative compounds or extracts from natural sources (Cao et al., 2013; Echegoyen and Nerin, 2015). In this regard, the "green" consumerism has lead to the renewal of scientific view regarding essential oils and natural extracts (Sinha et al., 2014) because these natural compounds have potentially lesser harmful activity than synthetic compounds providing long-term benefits without any side effect (Espin et al., 2007).

Essential oils (EOs) derived from plants and natural extracts are widely used for their reported biological activities, including antioxidant, anticancer, and antimicrobial attributes (Bakkali et al., 2008; Seow et al., 2014). Several experimental studies have shown that garlic (*Allium sativum*) and onion (*Allium cepa*) extracts exhibit antioxidant, antibacterial and antifungal properties and they have been traditionally used as flavouring agents in gastronomy (Corzo-Martínez et al., 2007) and also in the treatment and prevention of different diseases (including preclinical studies against cancer) (Amagase, 2006; Milner, 2006). Many of these biological effects are largely attributed to the thiosulfinates, that are volatile sulfur compounds (Corzo-Martínez et al., 2007). Organosulfur compounds scavenge oxidizing agents, inhibit the oxidation of fatty acids, thereby preventing the formation of pro-inflammatory messengers, and inhibit bacterial growth, via interaction with sulfur-containing enzymes (Wilson and Demmig-Adams, 2007).

The purity and composition of EOs and extracts from *Allium* species are directly related to the vegetative cycle station, soil composition, season of harvest or time of storage (Bakkali et al., 2008). Moreover, these substances are very unstable and under differing conditions thiosulfinates can decompose to form additional sulfur constituents (Benkeblia and Lanzotti, 2007). This variability in the extract contents could affect their biological properties. In order to avoid these problems, propyl propane thiosulfonate (PTSO), an

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organosulfur compound obtained by decomposition of initial compounds present in *Allium* sp, has been stabilized and characterized by DOMCA Research Center (DMC, Granada, Spain). Although little is known about PTSO, its antimicrobial and antioxidant properties have been previously studied (Peinado et al., 2012, 2013; Ruiz et al., 2010), as well as of the commercial formula Proallium AP® (Ruiz et al., 2010). In this regard, PTSO was able to inhibit the growth of Gram-negative and Gram-positive bacteria as well as moulds and yeast (Llana-Ruiz-Cabello et al., 2015a).

To improve half-life of perishable products several strategies have been developed by the food industry. One alternative to the direct addition of EOs in food, that could affect the acceptability of product, is active packaging where volatile compounds create a protective atmosphere (Nerin et al., 2006). EOs from onion (*A. cepa* L.) and garlic (*A. sativum* L.) are currently being used in "active packaging" (Llana-Ruiz-Cabello et al., 2015b) with the purpose of maintaining and extending food product quality and shelf life (Lee et al., 2015). In this way, Proallium AP®, with a concentration of active PTSO of 14.5%, has been incorporated to polymeric matrices to act protecting the food inside the package (Llana-Ruiz-Cabello et al., 2015c); nevertheless the evaluation of its safety is needed. Moreover, the growing interest in the new uses of EOs in general, makes it necessary to assess their genotoxic potential and identify the mutagenic components (Evandri et al., 2005; Llana-Ruiz-Cabello et al., 2015b).

According to the Guidelines of the Scientific Committee (SCF) on Food for Safety Assessment of Substances Used in Food Contact Materials (FCM), information on the genotoxicity is a key component in the risk assessment of substances used in active packaging (EFSA, 2011). Several studies have been carried out with the aim to identify organosulfur compounds which inhibit the mutagenic and genotoxic activities of chemical carcinogens in *in vitro* models (Arranz et al., 2007; Belloir et al., 2006; Chung, 1999; Garcia et al., 2008; Guyonnet et al., 2000, 2001; Le Bon and Siess, 2000; Lohani et al., 2003; Nkrumah-Elie et al., 2012; Sheen et al., 2001; Shukla et al., 2003; Yamazaki et al., 1992). However, studies concerning the mutagenicity/genotoxicity of garlic and onion extracts or their active compounds are lacking (Llana-Ruiz-Cabello et al., 2015b). Musk et al. (1997) found that diallyl sulfide (DAS) and diallyl disulfide (DADS) induced chromosome aberrations (CA) and sister chromatid exchanges (SCE) in a Chinese hamster ovary cell line (CHO). Moreover, the mutagenicity of dipropyl sulfide (DPS) and dipropyl disulfide (DPDS), as well as their mixture, showed lack of mutagenicity in the range of concentrations assayed (0–200 μM) with and without S9 metabolic activation (Llana-Ruiz-Cabello et al., 2015d). According to EFSA (2011), a step-wise approach for the generation and evaluation of data on genotoxic potential of substances in food and feed is recommended. This approach starts with a basic battery of two *in vitro* tests, comprising (1) a bacterial reverse mutation assay (OECD TG 471) and (2) an *in vitro* micronucleus test (OECD TG 487). The bacterial reverse-mutation assay in *Salmonella typhimurium* (Ames test), the most widely used to detect gene mutations, is rapid, inexpensive and relatively easy to perform. The micronucleus test (MN) using mammalian cells is one of the preferred methods for assessing chromosome damage, because it is the only *in vitro* test that can efficiently detect both clastogens and aneugens (EFSA, 2011). In the case of inconclusive, contradictory or positive results from these two *in vitro* tests, it may be appropriate to conduct further tests *in vitro* to optimize any subsequent *in vivo* testing, or to provide additional useful mechanistic information (EFSA, 2011). In this sense, the *in vitro* mammalian cell gene mutation assay (MLA) is commonly used, and it can detect gene mutations, including base pair substitutions and frame-shift mutations (Wang et al., 2009). Moreover, the alkaline version of the comet assay which detects DNA strand breaks (Collins et al., 1997), is probably the most widely used test to detect potential genotoxicity of EOs and their main components (Llana-Ruiz-Cabello et al., 2015b). It is important to emphasize that a simple modification of the comet assay, incorporating DNA digestion with lesion-specific enzymes, such as Endonuclease III (Endo III) and formamidopyrimidine DNA glycosylase (FPG), allows the measurement of oxidized pyrimidines and oxidized purines, respectively (Collins, 2004).

Taking into account these facts, the aim of this study was to investigate for the first time the potential *in vitro* mutagenicity/genotoxicity of PTSO at relevant concentrations, according to their possible use in food packaging, using the following battery of genotoxiciyt tests: (1) the bacterial reverse-mutation assay in *S. typhimurium* (Ames test, OECD 471, 1997); (2) the micronucleus test (OECD 487, 2014) (MN); (3) the mouse lymphoma thymidine-kinase assay (OECD 476) (MLA), and (4) the comet assay (with and without EndoIII and FPG enzymes). MN and MLA assays used the L5178Y*Tk* $^{+/-}$ mouse lymphoma cell line, while the intestinal Caco-2 cell line was used for the comet assay.

2. Materials and methods

2.1. Supplies and chemicals

Culture medium, foetal bovine serum and cell culture reagents were obtained from BioWhittaker (Madrid, Spain). PTSO 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), trifluorothymidine (TFT, ≥ 99%purity; CAS No. 70-00-8), thymidine (CAS No. 4449-43-8), methotrexate (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) and trypan blue solution 0.4% (CAS No. 72-57-1) were purchased from Sigma–Aldrich (Madrid, Spain), RPMI 1640 medium, horse serum, L-glutamine solution (CAS No. 56-85-9), sodium pyruvate solution (CAS No. 113-24-6), penicillin/streptomycin solution and amphotericin B solution (CAS No. 1397-89-3) were from Gibco (Biomol, Sevilla, Spain). 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

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

For the MN and MLA tests L5178Y *Tk* $^{+/-}$ mouse lymphoma cells were used for both assays and were originally provided by Dr. Olivier Gillardeux (Safoni-Synthélabo, Paris, France). Cells were confirmed as free of mycoplasma by PCR. L5178Y *Tk* $^{+/-}$cells were cultured in suspension in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 g/mL streptomycin, 1 mM sodium pyruvate, and 2.5 g/mL amphotericin B. Cells were routinely diluted at 2×10^5 cells/mL each day to prevent overgrowth. Cell density was determined with an automated cell counter (Invitrogen®, Thermofisher, MA, USA). Cultures were maintained in a humidified incubator with 5% CO_2 at 37 °C. To perform the assays, cells were seeded at 2×10^5 cells/mL and 1×10^7 cells/mL for the MN and MLA tests, respectively.

The Caco-2 cell line, used for the comet assay, derived from a human colon carcinoma (ATCC© HTB-37) was maintained at 37 °C