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Genotoxicity assessment of piperitenone oxide: An *in vitro* and *in silico* evaluation

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ABSTRACT

Piperitenone oxide, a natural flavouring agent also known as rotundifolone, has been studied for the genotoxicity assessment by an integrated *in vitro* and *in silico* experimental approach, including the bacterial reverse mutation assay, the micronucleus test, the comet assay and the computational prediction by Toxtree and VEGA tools. Under our experimental conditions, the monoterpene showed to induce both point mutations (i.e. frameshift, base-substitution and/or oxidative damage) and DNA damage (i.e. clastogenic or aneuploidic damage, or single-strand breaks). Computational prediction for piperitenone oxide agreed with the toxicological data, and highlighted the presence of the epoxide function and the α,β-unsaturated carbonyl as possible structural alerts for DNA damage. However, improving the toxicological libraries for natural occurring compounds is required in order to favour the applicability of *in silico* models to the toxicological predictions. Further *in vivo* evaluations are strictly needed in order to evaluate the role of the bioavailability of the substance and the metabolic fate on its genotoxicity profile. To the best of our knowledge, these data represent the first evaluation of the genotoxicity for this flavour compound and suggest the need of further studies to assess the safety of piperitenone oxide as a flavouring agent.

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1. Introduction

Piperitenone oxide (1-methyl-4-propan-2-ylidene-7-oxabicyclo [4.1.0]heptan-5-one; $C_{10}H_{14}O_2$; MW 166), also named rotundifolone, is a naturally occurring oxygenated monoterpene (Fig. 1). It was firstly isolated from *Mentha rotundifolia* Ehrh. and then found to be a major component (over 50%) of the essential oils from many *Mentha* species, including *M. suaveolens*, *M. spicata*, *Calamintha nepeta* and *C. incana* (Garzoli et al., 2015). It is currently used as a flavouring agent in different commercial products (viz. creams, lotions, detergents, and various other personal and household products). Furthermore, interesting biological activities have been highlighted over the years (Božović et al., 2015). Both the essential oil from *Mentha* spp. and piperitenone oxide have been found to possess antiparasitic activity (Matos-Rocha et al., 2013; De Sousa et al., 2016) and insecticidal properties against mosquitoes and weevils (Tripathi et al., 2004; Lima et al., 2014; Zekri et al., 2013). Conversely, the monoterpene weakly contributed to the cytotoxicity of the *Mentha villosa* essential oil against human cancer cell lines (Amaral et al., 2015). Interestingly, it exhibited antibacterial, antiviral and antifungal activities (Arruda et al., 2006; Pietrella et al., 2011; Civitelli et al., 2014). In addition, hypothensive, bradicardic and myorelaxant effects, likely due to the block of calcium current by inhibiting L-type Ca_v channels, were highlighted (Sousa et al., 1997; Silva et al., 2011). Piperitenone oxide and its structural analogues also exhibited antinociceptive properties, in which the epoxide group and the substituents on the ring carbon seem to play a pivotal role (De Sousa et al., 2007).

Being piperitenone oxide (FL no. 16.004; Flavouring Group Evaluation, FGE.213) classified as a flavouring agent used in foodstuffs, the European Commission asked the European Food Safety Authority (EFSA) Panel on Food Contact Materials, Enzymes,

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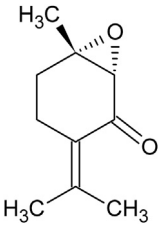

Fig. 1. Chemical structure of piperitenone oxide.

Flavourings and Processing Aids (CEF Panel) to give a scientific opinion on its implications and concerns for human health, by applying the procedure of Commission Regulation EC No 1565/2000 (EFSA CEF Panel, 2012). Particularly, due to the lack of supporting information provided by Flavour Industry, a toxicity assessment for piperitenone oxide has been reported to be pending (EFSA CEF Panel, 2014; 2015).

The safety evaluation of a fragrance material includes a broad range of toxicological information, both for the compound itself and for structurally related chemicals belonging to the same chemical group (Bickers et al., 2003). Among toxicological information, genotoxicity is a systemic consideration, as it can be related to carcinogenicity (Di Sotto et al., 2008). Normally, to evaluate a potential genotoxic risk due to a chemical exposition, *in vitro* assays for detecting point mutations (Ames test) and extended treatment (e.g., micronucleus assay, single cell gel electrophoresis assay or comet assay) are used in the first instance (EMEA, 2008; Di Sotto et al., 2013). If the results of these studies are positive, *in vivo* studies, for example a mammalian cytogenetic study, are performed. Recently, also a computational approach has been proposed by the regulatory Agencies to complete the toxicity profile of a compound by *in silico* predictions (EFSA, 2014).

In this context, in order to provide some toxicological data for the genotoxicity assessment of piperitenone oxide, present study was aimed at evaluating the ability of this flavouring compound to induce *in vitro* point mutations in bacteria by the Ames test, and in mammalian cells by both the micronucleus and comet assays, so allowing to detect different potential genotoxic endpoints. Particularly, the cytokinesis-block micronucleus technique, with an extended exposure treatment (24 h), was applied for detecting clastogenic and aneugenic effects (Kirsch-Volders et al., 2011; OECD, 2016); moreover, the alkaline protocol was used for the comet assay (Tice et al., 2000). For both tests, the extended treatment precludes the inclusion of the exogenous metabolic activator, due to the cytotoxicity of S9 mix and to the short half-life of the enzymatic system (Kirsch-Volders et al., 2011; OECD, 2016).

In addition, a computational evaluation of the piperitenone oxide genotoxicity potential has been performed by using the freely available *in silico* Toxtree (Estimation of Toxic Hazard - A Decision Tree Approach) and VEGA tools, based on toxicity and QSAR database respectively. In fact, Toxtree can estimate toxic hazards using a decision tree-based approach (Patlewicz et al., 2008). For evaluating a potential mutagenicity, the decision tree is based on the Benigni/Bossa rules and on the structural alerts for genotoxic carcinogens available in the literature (Benigni and Bossa, 2011). VEGA is a QSAR statistical model which relates the chemical structure to mutagenicity by mathematical relationships (Bakhtyari et al., 2013). For the mutagenic predictions, CAESAR (developed by Polytechnic of Milan, Milan, Italy), SarPy/IRFMN (developed by Polytechnic of Milan and "Mario Negri" Institute, Milan, Italy), ISS (developed by Superior Institute of Health, Rome, Italy) and KNN/Read-Across (developed by "Mario Negri" Institute, Milan, Italy) tools were applied.

2. Materials and methods

2.1. Extraction and purification of piperitenone oxide

Piperitenone oxide has been isolated from the *Mentha suaveolens* L. (Fam. *Lamiaceae*) essential oil, obtained by 4-h hydrodistillation of the mintleaves in Clevenger-type apparatus, as previously described (Angiolella et al., 2010). The analysis of the essential oil was performed by gas chromatography and mass spectroscopy (DMePe BETA PS086, 0.25 mm film, 25 m column length, 0.25 mm diameter, operating temperature of 220 °C, elution with helium) and the constituents were identified by comparison with the NIST 08 Mass Spectral Library. Piperitenone oxide was the major constituent of the essential oil, with an amount of 80–90%. It was purified by serial column chromatographies (CC), by elution with $CHCl_3$/n-Hexane (1:1) on silica gel 60. After three repeated CC, piperitenone oxide was obtained at higher than 97% purity (Figs. S1 and S2). Further attempts did not allow to increase the purity of the compound.

2.2. Chemicals and media

All the substances, including the mutagens 2-nitrofluorene (2NF; 98% purity), 2-aminoanthracene (2AA; 96% purity), 2-aminofluorene (2AF; 98% purity), sodium azide (SA; > 99.5% purity), methyl methanesulfonate (MMS; 99% purity) and benzo[a]pyrene (BaP; > 96% purity), the stains May-Greunwald and Giemsa and the chemicals 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; ≥ 97.5% purity), cytochalasin B (≥ 98% purity), glucose-6-phosphate (G6P; ≥ 98% purity) and nicotinamide adenine dinucleotide phosphate (NADP; ≥ 98% purity) were purchased from Sigma-Aldrich Co (St. Louis, MO, USA). Ethidium bromide solution was purchased from Invitrogen, Life Technologies (Monza, Italy). All the other reagents used for the comet assay were obtained from Microtech Srl (Naples, Italy). S9 fraction (the liver postmitochondrial supernatant of rats treated with the mixture phenobarbital/β-naphthoflavone to induce the hepatic microsomal enzymes) was purchased from Moltox (Molecular Toxicology, Boone, NC, USA).

To perform the assays, piperitenone oxide, 2NF, 2AA and BaP were dissolved in DMSO, while SA and MMS in deionised water. The S9 mixture was prepared just before use by adding: phosphate buffer (0.2 M) 500 μL, deionised water 130 μL, KCl (0.33 M) 100 μL, $MgCl_2$ (0.1 M) 80 μL, S9 fraction 100 μL, glucose-6-phosphate (0.1 M) 50 μL and NADP (0.1 M) 40 μL. The mixture was kept on ice during testing.

2.3. Bacterial reverse mutation assay

A set of different strains, whose genotype is described in Table 1, was used. In particular, *Salmonella typhimurium* TA1535 and TA1538 were kindly provided by Prof.ssa P. Hrelia, Department of Pharmacology, University of Bologna, while *S. typhimurium* TA98 and TA100, and *Escherichia coli* WP2, WP2*uvr*A, and WP2*uvr*A/pKM101 were supplied by the Research Toxicological Centre (Pomezia, Rome, Italy). For each strain, the genotype characters were confirmed by the Strain Check Assay (Di Sotto et al., 2014), so the permanent cultures were prepared and then frozen. The working cultures, prepared from the permanent ones, were incubated overnight (16 h) at 37 °C, to reach a concentration of approximately 1×10^9 bacteria/mL. In each experiment the number of viable cells for each strain was determined.

Preliminarily, in order to establish the highest concentration to use in the following assays, the solubility of piperitenone oxide in the final mixture has been evaluated. Starting from the highest

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