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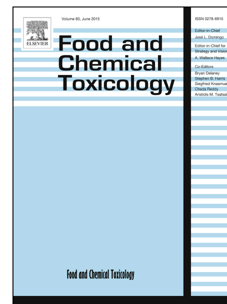

Genotoxicity assessment of piperitenone oxide: An *in vitro* and *in silico* evaluation

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

Di Sotto Antonella[a,*], Di Giacomo Silvia[a], Abete Lorena[a], Božović Mijat[b], Parisi Olga A.[c], Barile Fabio[d], Vitalone Annabella [a], Izzo Angelo A.[c], Ragno Rino[b], Mazzanti Gabriela[a]

[a] Department of Physiology and Pharmacology, Sapienza University of Rome, P.le Aldo Moro 5, 00185 Rome, Italy

[b] Department of Drug Chemistry and Technologies, Sapienza University of Rome, P.le Aldo Moro 5, 00185 Rome, Italy

[c] Department of Pharmacy, University of Naples Federico II, Via D. Montesano 49, 80132, Naples, Italy

[d] C4T, Colosseum Combinatorial Chemistry Centre for Technology, University of Rome "Tor Vergata", Via della Ricerca Scientifica, 00133 Rome, Italy

*Corresponding author. Department of Physiology and Pharmacology “V. Erspamer”, Sapienza University of Rome, P.le Aldo Moro, 5, 00185 Rome, Italy

E-mail address: antonella.disotto@uniroma1.it (A. Di Sotto)

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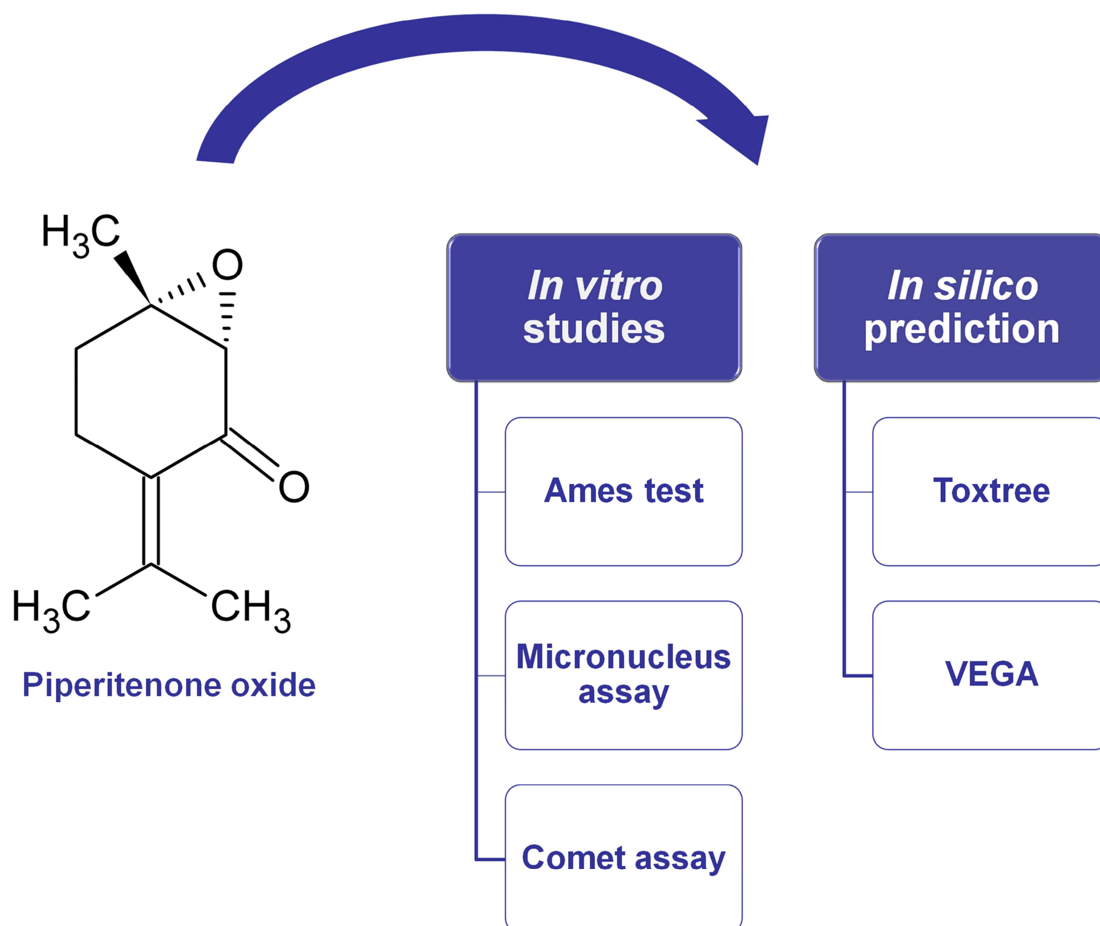


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1 **1. Introduction**

2 Piperitenone oxide (1-methyl-4-propan-2-ylidene-7-oxabicyclo[4.1.0]heptan-5-one;
3 $C_{10}H_{14}O_2$; MW 166), also named rotundifolone, is a naturally occurring oxygenated
4 monoterpene (Figure 1). It was firstly isolated from *Mentha rotundifolia* Ehrh. and then found
5 to be a major component (over 50%) of the essential oils from many *Mentha* species,
6 including *M. suaveolens*, *M. spicata*, *Calamintha nepeta* and *C. incana* (Garzoli et al., 2015).
7 It is currently used as a flavouring agent in different commercial products (viz. creams,
8 lotions, detergents, and various other personal and household products). Furthermore,
9 interesting biological activities have been highlighted over the years (Božović et al., 2015).
10 Both the essential oils from *Mentha* spp. and piperitenone oxide only have been found to
11 possess antiparasitic activity (Matos-Rocha et al., 2013; de Sousa et al., 2016) and insecticidal
12 properties against mosquitoes and weevils (Tripathi et al., 2004; Lima et al., 2014; Zekri et al.,
13 2013). Conversely, it weakly contributed to the cytotoxicity of the *Mentha villosa* essential oil
14 against human cancer cell lines (Amaral et al., 2015). Interestingly, this monoterpene
15 exhibited antibacterial, antiviral and antifungal activities (Arruda et al., 2006: Civitelli et al.,
16 2014). In addition, hypothensive, bradicardic and myorelaxant effects, likely due to block of
17 calcium current by inhibiting L-type $Ca_v$ channels, were highlighted (Sousa et al., 1997;
18 Guedes et al., 2004; Silva et al., 2011). Piperitenone oxide and its structural analogues also
19 exhibited antinociceptive properties, in which the epoxide group and the substituents on the
20 ring carbon seem to play a pivotal role (De Sousa et al., 2007).

21 Being piperitenone oxide (FL no. 16.004; Flavouring Group Evaluation, FGE.213)
22 classified as a flavouring agent used in foodstuffs, the European Commission asked the
23 European Food Safety Authority (EFSA) Panel on Food Contact Materials, Enzymes,
24 Flavourings and Processing Aids (CEF Panel) to give a scientific opinion on its implications
25 and concerns for human health, by applying the procedure of Commission Regulation EC No

1 1565/2000 (EFSA, 2012). Particularly, due to the lack of supporting information provided by
2 Flavour Industry, a toxicity assessment for piperitenone oxide has been reported to be pending
3 (EFSA, 2014 and 2015).

4 The safety evaluation of a fragrance material includes a broad range of toxicological
5 information, both for the compound itself and for structurally related chemicals belonging to
6 the same chemical group (Bickers et al., 2003). Among toxicological information,
7 genotoxicity is a systemic consideration, as it can be related to carcinogenicity (Di Sotto et al.,
8 2008). Normally, to evaluate a potential genotoxic risk due to a chemical exposition, *in vitro*
9 assays for detecting point mutations (Ames test) and extended treatment (e.g., micronucleus
10 assay, single cell gel electrophoresis assay or comet assay) are used in the first instance
11 (EMEA, 2008; Di Sotto et al., 2013). If the results of these studies are positive, *in vivo* studies,
12 for example a mammalian cytogenetic study, are performed. Recently, also a computational
13 approach has been proposed by the regulatory Agencies to complete the toxicity profile of a
14 compound by *in silico* predictions (EFSA, 2014).

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

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1 In addition, a computational evaluation of the piperitenone oxide genotoxicity potential has
2 been performed by using the freely available *in silico* Toxtree (Estimation of Toxic Hazard -
3 A Decision Tree Approach) and VEGA tools, based on toxicity and QSAR database
4 respectively. In fact, Toxtree can estimate toxic hazards using a decision tree-based approach
5 (Patlewicz, 2008). For evaluating a potential mutagenicity, the decision tree is based on the
6 Benigni/Bossa rules and on the structural alerts for genotoxic carcinogens available in the
7 literature (Benigni and Bossa, 2011). VEGA is a QSAR statistical model which relates the
8 chemical structure to mutagenicity by mathematical relationships (Bakhtyari et al., 2013). For
9 the mutagenic predictions, CAESAR (developed by Polytechnic of Milan, Milan, Italy),
10 SarPy/IRFMN (developed by Polytechnic of Milan and "Mario Negri" Institute, Milan, Italy),
11 ISS (developed by Superior Institute of Health, Rome, Italy) and KNN/Read-Across
12 (developed by "Mario Negri" Institute, Milan, Italy) tools were applied.
13
14 **2. Materials and methods**
15 *2.1. Extraction and purification of piperitenone oxide*
16 Piperitenone oxide has been isolated from the *Mentha suaveolens* L. (Fam. *Lamiaceae*)
17 essential oil, obtained by four-hour hydrodistillation of the mintleaves in Clevenger-type
18 apparatus, as previously described (Angiolella et al., 2010). The analysis of the essential oil
19 was performed by gas chromatography and mass spectroscopy (DMePe BETA PS086, 0.25
20 mm film, 25 m column length, 0.25 mm diameter, operating temperature of 220 °C, elution
21 with helium) and the constituents were identified by comparison with the NIST 08 Mass
22 Spectral Library. Piperitenone oxide was the major constituent of the essential oil, with an
23 amount of 80-90%. It was purified by serial column chromatographies (CC), by elution with
24 $CHCl_3$/n-Hexane (1:1) on silica gel 60. After three repeated CC, piperitenone oxide was

obtained at higher than 97% purity (Figures 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

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1 Toxicological Centre (Pomezia, Rome, Italy). For each strain, the genotype characters were
2 confirmed by the Strain Check Assay (Di Sotto et al., 2014), so the permanent cultures were
3 prepared and then frozen. The working cultures, prepared from the permanent ones, were
4 incubated overnight (16 h) at 37 °C, to reach a concentration of approximately $1\times10^9$
5 bacteria/mL. In each experiment the number of viable cells for each strain was determined.
6 Preliminarily, in order to establish the highest concentration to use in the following assays,
7 the solubility of piperitenone oxide in the final mixture has been evaluated. Starting from the
8 highest soluble concentration, the solutions of test substance were prepared by serial dilution
9 in DMSO (dilution factor 1:2). These dilutions were then studied by the cytotoxicity test, in
10 order to determine the highest nontoxic concentration to test in the mutagenicity assay.
11 Cytotoxicity was evaluated as reduction in the number of revertant colonies and as change of
12 the auxotrophic background lawn in comparison with the control plates.
13 Mutagenicity of piperitenone oxide was assayed by the pre-incubation method, according
14 to the OECD guideline 471 (1997) with minor changes (Di Sotto et al., 2008). The vehicle
15 DMSO (2% v/v) was used as the negative control. In turn, the mutagens 2NF (2 µg/plate for
16 TA1538 and TA98 without S9), SA (1 µg/plate for TA1535 and TA100 without S9), MMS
17 (500 µg/plate for WP2, WP2*uvr*A and WP2*uvr*A/pKM101 without S9), 2AF (10 µg/plate for
18 WP2 with S9) and 2AA (1 µg/plate for TA98 and TA100 with S9; 10 µg/plate for WP2*uvr*A
19 and WP2*uvr*A/pKM101 with S9), were used as positive controls, in order to verify the
20 bacteria susceptibility to known genotoxic damages. These concentrations of mutagens,
21 obtained from the linear part of the concentration-response curve, were chosen as they
22 increased the number of revertant colonies at least two-folds above the control value. The
23 experiments were repeated at least twice and each concentration was tested in triplicate.
24 To perform the test, an overnight culture (100 µL) was added with test compound (50 µL)
25 and S9 mixture or phosphate buffer (0.1 M; 500 µL). Each mixture was gently vortexed in a

1 sterile tube; then it was pre-incubated under shaking at 37 °C for 30 min. After pre-incubation,
2 the tubes were added with top agar (2 mL), containing 10% of histidine/biotin (0.5 mM) for *S.*
3 *typhimurium* strains and 10% of tryptophan (0.5 mM) for *E. coli* ones. Then the mixture was
4 gently vortexed and poured onto a minimal agar plate. The plates were incubated at 37 °C for
5 72 h and then examined. The histidine or tryptophan independent revertant colonies and the
6 viable cells were scored and the bacterial background lawn was observed. A positive response
7 in the mutagenicity assay was defined as an increase (at least two-fold above the control) in
8 the histidine- or tryptophan-independent revertant colonies in each strain.

9 *2.4. HepG2 cell line*

10 The liver cancer cells HepG2 (American Type Culture Collection, Milan, Italy) were grown
11 under standard conditions (37 °C and 5% $CO_2$) in Dulbecco's modified Eagle's medium
12 (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL
13 streptomycin and 2mM L-glutamine in 75 $cm^2$ flasks, and subcultured every 4 days, renewing
14 growth medium every 2-3 days.

15 The cytotoxicity of PO on HepG2 cells was previously evaluated by both MTT
16 tetrazolium salt colorimetric assay (Di Sotto et al., 2014) and neutral red uptake assay
17 (Aviello et al., 2010). A vehicle control, corresponding to 100% cell viability and a standard
18 cytotoxic agent (doxorubicin, 10 μg/mL) were also included in the experiments. The results
19 were expressed as percentage of cell viability (about three experiments including 8-10
20 replicates for each treatment) with respect to the vehicle. Genotoxicity was assayed starting
21 from the highest concentration at which neither necrosis nor cytotoxic or cytostatic effects
22 were observed, according to previous published methods (Di Sotto et al., 2014).

23 *2.5. Micronucleus assay*

24 The cultured cells were treated for 24 h at 37 °C with the test substance, then supplemented
25 with cytochalasin-B (4.5 μg/mL) for other 24 h. The vehicle DMSO was used as the negative

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1 control, while BaP (25 µM) as the positive control. At the end of the incubation time, the cells
2 were collected, treated for 20 minutes with an ice-cold hypotonic solution of KCl 0.075 M,
3 and then fixed in acetic acid: ethanol (1:3). After fixation, the cells were put directly onto
4 slides, air-dried, and stained with conventional May-Grünwald-Giemsa stain. All slides were
5 coded and analysed by a Zeiss Axioplan light microscope at 1000x magnification under oil
6 immersion. For each treatment, at least 1000 cells were scored for the presence of one, two,
7 three or more nuclei and the nuclear division index (NDI) was determined according to the
8 OECD guideline 487 (2016) with minor changes (Di Sotto et al., 2011). Furthermore, at least
9 2000 binucleated (BNCs) cells were examined for the presence of micronuclei. A positive
10 response was defined as a statistically significant increase of the MN frequencies in the
11 treated cultures with respect to the vehicle control.

12 *2.6. Comet assay*

13 DNA damage was evaluated by the alkaline (pH > 13) Comet assay (Di Sotto et al., 2014).
14 HepG2 cells were seeded in 6 well-plates ($50 \times 10^4$ cells per well) and allowed to adhere for
15 48 h. Thereafter, the cells were incubated with nontoxic concentrations of piperitenone oxide
16 (30, 60, 90 and 150 µM) for 24 h at 37 °C, and subsequently they were trypsinized to obtain a
17 suspension of $2.2 \times 10^4$ cells/ml. The vehicle DMSO was used as the negative control, while
18 hydrogen peroxide ($H_2O_2$, 75 µM) as the positive control. The aliquots of cell suspension
19 were centrifuged at 1300 × g for 5 min, then the pellets were collected, mixed with 0.85% low
20 melting point agarose and laid on pre-treated glass slides (Trevigen, TEMA Ricerca S.r.l.,
21 Bologna, Italy). The slides were then suspended, at 4 °C for 1 h (pH = 10), in NaCl (2.5 M),
22 $Na_2EDTA \times 2H_2O$ (100 mM), Tris (10 mM) and Triton X-100 (1% v/v), and electrophoresed
23 in alkaline buffer (300 mM NaOH, 1 mM $Na_2EDTA$, pH > 12-13) at 26 V, and 300 mA for
24 20 min. After neutralization in Tris-HCl (0.4 M, pH 7.5), the gels were stained with ethidium
25 bromide (50 µM). The images were acquired using a Leica DCF 340 FX microscope and

analysed with the Casp Semi-automatic Software (http://www.casp.of.pl). The analysis parameters were selected and defined as follow: head center threshold (HCT), 0.8; tail threshold (TT), 0.05; head threshold (HT), 0.05; comet threshold, 0.05. The DNA tail was evaluated as the outcome of the assay and expressed as percentage.

*2.7. In silico predictions*

For the present investigation, Toxtree 2.6.1.3 application (available at http://toxtree.sourceforge.net), developed by Ideaconsult Ltd (Sofia, Bulgaria) and the quantitative structure-activity relationship (QSAR) modelling VEGA 1.1.1 software (available at http://www.vega-qsar.eu), produced by Mario Negri Institute for Pharmacological Research (Milan, Italy), were used. For discriminating between mutagenic and nonmutagenic compounds, both softwares include SAR models, designed on the basis of the mutagenicity of some chemicals in the Ames assay, using *Salmonella typhimurium* TA100 strain in the presence of the S9 metabolic activation. To run the analysis, the molecular structure of piperitenone oxide was inserted, using the Simplified Molecular Input Line Entry Specification (SMILES) system O=C1C(=C(C)C)CCC2(OC12)(C), according to the PubCHEM descriptors (available at https://pubchem.ncbi.nlm.nih.gov/compound/442497). The toxicity predictions were then carried out by means of existing rules, available in those softwares. For Toxtree, the presence of a structural alert was associated with a positive Ames mutagenicity result. VEGA classified chemicals as mutagenic, nonmutagenic, and suspicious mutagenic (when a high degree of prediction uncertainty was assumed).

*2.8. Statistical analysis*

Data are expressed as the mean ± SEM of 6-8 experiments. Statistical analysis was performed with GraphPad Prism™ (Version 5.00) software (GraphPad Software, Inc., San Diego, California, USA). To determine the statistical significance, the Student's t-test was used for comparing a single treatment mean with the control mean, and the one-way analysis of

variance (ANOVA) followed by the Dunnett's multiple comparisons post-test was used for the analysis of multiple treatment means. A $p < 0.05$ value was considered as statistically significant.

For the Ames test, the sample was compared to the vehicle control by its mutagenic index value, calculated as $n/c$, where $n$ and $c$ were the number of revertant colonies for the sample and the vehicle, respectively. The mutagenic potency of the sample in each strain was expressed as induction factor (IF) and calculated as ln $(n - c)/c$. A Maximum Induction Factor (MIF) higher than 1.5, indicated that the sample was significantly genotoxic; if MIF does not achieve 1.5, but the dose-response relationship was still observed, the sample was probably genotoxic; otherwise, the sample was considered as not genotoxic (Masood and Malik, 2013).

## 3. Results

### *3.1. Bacterial reverse mutation assay*

Piperitenone oxide did not give any precipitate up to the highest concentration of 60 μmol/plate (corresponding to 22.6 mM), while produced cytotoxic effects up to the concentration of 6 μmol/plate in all strains tested (Table S1). Mutagenicity was evaluated at nontoxic concentrations (from 0.6 to 5.1 μmol/plate, 1:1.4 dilution factor). When tested in *S. typhimurium his*D3052 strains, despite a lack of mutagenicity in the precursor TA1538, the substance produced a statically significant and concentration dependent mutagenic effect in TA98 in the presence of the metabolic activator S9, with a mutagenic index of 2.78 ± 0.18 (MIF 0.57 ± 0.05) at the highest concentration tested (Figure 2). In *S. typhimurium his*G46 strains, the monoterpene was mutagenic both in the precursor TA1535 and in TA100 in the absence of S9, with a mutagenic index of 3.14 ± 0.12 (MIF 0.81 ± 0.15) and 3.78 ± 0.21 (MIF 1.01 ± 0.10) at the highest concentration tested, respectively (Figure 3). In the presence of metabolic activator, piperitenone oxide lost the mutagenicity in TA1535, while a strong increase of the revertant colonies number of TA100 was induced, reaching a mutagenic index



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1 of 4.60 ± 0.30 (MIF 1.27 ± 0.08) at the highest concentration tested (Figure 3). For *E. coli*
2 strains, no mutagenic effects were produced in the wild type WP2 both in the absence and
3 presence of S9. Conversely, a statically significant and concentration dependent mutagenic
4 effect was induced in WP2*uvr*A/pKM101 in the absence of the metabolic activator, reaching a
5 mutagenic index of 2.24 ± 0.15 (MIF 0.21 ± 0.05) at the highest concentration tested.
6 Analogously, PO showed to be mutagenic in both WP2*uvr*A and WP2*uvr*A/pKM101 in the
7 presence of S9: the mutagenic index reached the values of 2.83 ± 0.13 (MIF 0.60 ± 0.02) and
8 5.61 ± 0.32 (MIF 1.53 ± 0.02), respectively (Figure 4). A positive mutagenic effect was also
9 produced by all the mutagens used in our experiments, showing that the system was suitable
10 for detecting mutagenic species (Table S2).
11 *3.2. Cytotoxicity evaluation*
12 When tested on the HepG2 cells, PO did not induce cytotoxic effects up to the concentration
13 of 300 μM, both in the MTT and Neutral Red assays (Figure S3); conversely, at higher
14 concentrations, a less than 70% cell viability was found. The vehicle control DMSO (0.1%
15 v/v) did not affect the cell growth.
16 *3.3. Micronucleus assay*
17 On the basis of the cytotoxicity assays, the effect of PO on the micronuclei frequency was
18 evaluated at the concentrations of 10, 30, 60, 90 and 150 μM. In our experimental conditions,
19 the substance induced a statistically significant increase in the MN frequency in comparison
20 with the vehicle control. A maximum two-fold increase of the MN frequency was reached at
21 the concentration of 90 μM. For each treatment, the Nuclear Division Index (NDI) was
22 similar to that of the vehicle control. Analogously, the positive control BaP (25 μM)
23 significantly increased (about two-fold) the MN frequency with respect to the vehicle,
24 showing that the cells were suitable to detect a genotoxic damage (Table 2).
25 *3.4. Comet assay*



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1 Taking into account the results of the cytotoxicity assays, the PO ability to induce DNA tail in
2 the comet assay was evaluated at the concentrations of 30, 60, 90 and 150 µM. Starting from
3 the concentration of 60 µM, the substance induced a statistically significant increase of the
4 percentage of DNA-tail (about 2.2 folds) with respect to the vehicle control, suggesting its
5 ability to affect the DNA integrity and to produce genotoxic effects. A maximum three-fold
6 increase of the DNA-tail was reached at the concentration of 150 µM. Analogously, the
7 positive control induced a statistically significant increase (about 2.8 folds) of the DNA tail
8 with respect to the vehicle, showing that the cells were suitable to detect this kind of DNA
9 damage (Figure 5).

10 *3.5. In silico*

11 When the potential mutagenicity of PO was evaluated by Toxtree, two structural alerts,
12 including the epoxide and the azaridine functions, were highlighted (Figure S4).
13 When PO was analysed by the Vega software, a possible mutagenic potential was highlighted
14 applying the CAESAR, SarPy/IRFMN and ISS tools. Only the KNN/Read-Across method
15 suggested a lack of mutagenicity, although a not optimal accuracy of the prediction for similar
16 molecules, found in the training set, was reported. The applicability domain of the VEGA
17 analysis was in general not reliable, due to the not adequacy of the experimental value
18 available for similar compounds in the training set. Although this prediction possessed these
19 critical points, in agreement with the Toxtree evaluation, the QSAR analysis reported the
20 presence of the epoxide and aziridine functions and the α,β-unsaturated carbonyl as possible
21 reactive sites (Figure S5).
22

23 **4. Discussion**

24 Piperitenone oxide is a natural fragrance, commonly used as food and cosmetic additive in
25 commercial products. Due to its natural origin and the low amount used as flavouring agent, a

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relatively scanty and safe consumer exposure has been expected, then its toxicity has been perceived as of minor concern. However, a risk of overexposure due to its natural presence in several essential oils and its widespread use as flavouring additive should not be excluded.

Recently, in order to characterize the safety profile of flavouring materials and because of the lack of information provided by Flavour Industry, the EFSA scientists required additional toxicity data for piperitenone oxide, particularly for the genotoxicity assessment (EFSA, 2014 and 2015).

In the present study, this point has been assessed by an integrated experimental approach: the bacterial reverse mutation assay (to study point mutations in bacteria), the micronucleus test (to evaluate clastogenicity and aneuploidogenicity in HepG2 cells), and the comet assay (to highlight primary DNA damage, including repair-effects and repairable DNA damages). The combination of the bacterial reverse mutation assay and the micronucleus test has been shown to be very suitable for revealing potential genotoxic carcinogens, so that, when results of both tests are negative, a further in vivo test may not be necessary (Kirkland et al., 2011). Nevertheless, the micronucleus assay highlights only a little amount of DNA damages, that occurs in the interphase, and that will lead to fixed chromosome abnormalities after a passage through mitosis (Tafazoli and Volders, 1996). In this context, including the comet assay increases the sensitivity of the experimental system, because it also reveals very early damages (i.e. DNA double- and single-strand breaks, alkaline labile and transient repair sites, DNA crosslink and oxidative damage) (Collins et al., 2014).

At last, an *in silico* prediction, based on both toxicology database and QSAR measurements, has been carried out, thus allowing to relate the biological data to the chemical structure and to highlight possible reactive sites.

Under our experimental conditions, piperitenone oxide showed to produce point mutations in the Ames test, both in the absence and presence of the exogenous metabolic activation system.

1 In order to characterize the possible mechanism of mutagenicity, seven bacterial strains,
2 sensitive to different mutational events due to their specific genotypes, were used. Particularly,
3 *S. typhimurium* TA1538 and TA98 are characterized by the -1 frameshift deletion *his*D3052,
4 which affects the reading frame of a nearby repetitive –C–G– sequence and can be reverted by
5 frameshift mutagens. TA1535 and TA100 contain the marker *his*G46, which results from a
6 base-pair substitution of a leucine (GAG/CTC) by a proline (GGG/CCC): this mutation is
7 reverted by mutagens causing base-substitutions at G-C base pairs (Di Sotto et al., 2008).
8 Furthermore, the *E. coli* WP2 strains carry a tryptophan-dependence due to an ochre (UAA)
9 nonsense mutation in the *trp*E65 gene, induced by mechanisms of misreplication or misrepair.
10 Such dependence can be reversed by mutagens causing base change or oxidative damage
11 (such as free radical generators and cross-linking agents) and which preferentially attack the
12 A-T base pairs (Di Sotto et al., 2012). The presence of the pKM101plasmid (namely R factor)
13 in TA98, TA100 and WP2*uvr*A/pKM101 strains greatly increases their sensitivity to
14 mutagens, because it is believed to code for the error-prone DNA repair enzymes (Di Sotto et
15 al., 2012).
16 Taking into account these bacterial features, our results highlighted that the PO mutagenicity,
17 in the absence of S9, was likely due to base-change mechanisms, as found in both the wild-
18 type TA1535 strain and in the pKM101-derived TA100 and WP2uvrA/pKM101. In the
19 presence of S9, both frameshift and base-substitution mutations are induced by the
20 monoterpene in the pKM101-derived TA98, TA100 and WP2uvrA/pKM101 strains and in
21 WP2uvrA. This behaviour suggests that some unknown mutagenic metabolites of
22 piperitenone oxide, carrying a different genotoxic profile, could be produced by the CYP-
23 mediated biotransformations.
24 Our hypothesis has been also strengthened by the results obtained in the mammalian HepG2
25 cells by the micronucleus and comet assays. HepG2 are metabolically competent cells and

1 have been found to retain the activities of various phase I and phase II enzymes which play a
2 crucial role in the activation/detoxification of genotoxic procarcinogens. Also, these cells
3 seem to reflect the in vivo metabolism of such compounds better than the experimental
4 models with metabolically incompetent cells and exogenous activation mixtures (Knasmüller
5 et al., 1998). In these cells, the tested substance has been highlighted to affect the genome
6 integrity, inducing both early and fixed DNA-damage, being increased both the percentage of
7 DNA-tail and the frequency of micronuclei.
8 The consistency between results in bacteria and in mammalian cells is an important goal, as
9 the genotoxicity was evaluated in term of different endpoints, in particular point mutations
10 (i.e. frameshift, base-substitution and/or oxidative damage) and chromosomal damage (i.e.
11 clastogenic or aneuploidic damage, or single-strand breaks). To the best of our knowledge,
12 these data represent the first evaluation of the genotoxicity for this flavour compound.
13 Computational prediction for piperitenone oxide are in agreement with the biological data,
14 and highlighted the presence of different structural alerts, including the epoxide function and
15 the α,β-unsaturated carbonyl, which could be responsible for the DNA damage. Provision for
16 possible metabolic transformations, carried out by SMARTCyp in ToxTree (data not shown),
17 highlighted that the carbons 3 and 4 represent the two major sites of the CYP-mediated
18 metabolism. They can undergo epoxidation, thus forming a new epoxide-based DNA reactive
19 function in the metabolite and increasing the genotoxicity power of the precursor compound.
20 Epoxides are ubiquitous compounds, occurring both naturally and as a consequence of
21 industrial processes or of food cooking. Depending on their instability, they are considered as
22 reactive electrophilic intermediates, which are able to form covalent adducts with cellular
23 macromolecules, including proteins and DNA. For instance, some structurally simple
24 epoxides, such as styrene-7,8-oxide and aflatoxin B1-oxide, were shown to be genotoxic in
25 many *in vitro* systems (Laffon et al., 2003; Martin and Garner, 1977).

1 Several factors, including the reactivity and the ability to enter the body or to be produced *in*
2 *vivo* from a precursor, may affect their binding ability. Some epoxides may be only transient
3 intermediates (they did not bind to cell components, because are metabolised immediately by
4 epoxide hydrolase or by glutathione transferase), whereas others are so stable to be excreted
5 as epoxides. For example, diepoxybutane and glycidaldehyde resulted carcinogenic in
6 mammalian models, while negative or inconclusive evidences have been found for limonene
7 mono- and dioxide and for cyclohexene epoxide (Manson, 1980). Van Duuren (1967)
8 highlighted that diepoxides are more often carcinogenic than monofunctional epoxides, and
9 that some monoepoxides found to be carcinogenic have an additional reactive site. The
10 flexibility of the reactive centres seems to be also important feature for carcinogenicity (Van
11 Duuren, 1967).
12 In line with these evidences and according to the *in silico* predictions, the epoxide function of
13 piperitenone oxide is accompanied by an additional reactive site, characterized by the α
14 carbonyl group. This is also in conjugation with an α,β-unsaturated function, which makes the
15 α-carbon positively polarized and consequently a suitable site for a nucleophilic attack (Feron
16 et al., 1991). Although the presence of a vicinal methyl group may hinder the epoxide
17 reactivity by electron release (Parker, 1959), the α,β-unsaturated carbonyl group makes the
18 molecules low stable, thus allowing it reacts with electron-rich biological macromolecules (i.e.
19 DNA and proteins) and induces adverse health effects, including point mutations and
20 chromosomal breaks (Feron et al.,1991; Patlewicz et al., 2002).
21 VEGA tools reported a major limit for the QSAR analysis on piperitenone oxide, which is
22 represented by the lacking or low information about the genotoxicity of similar structural
23 compounds. In fact, a lot of known and unknown natural compounds are only scantily
24 investigated for their toxicological effects as their toxicity is perceived of minor concern: this
25 increase the uncertainty of the safe exposure for human and environmental health.

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1 Furthermore, being this monoterpene widely contained in different essential oils, it is not easy
2 to define an expected exposure for humans and to perform a genotoxicity risk evaluation.
3 Kroes et al. (2004) recommended that when a genotoxicity hazard is hypothesized, the
4 maximum limit of 0.15 × 10-3 mg/day should be respected for a safe human exposure.
5 Therefore, results on piperitenone oxide suggest the importance to enrich the toxicological
6 knowledge about natural compounds and to give new SAR data for obtaining more complete
7 libraries. This allows to improve the applicability of *in silico* models to the toxicity evaluation
8 of a lot of natural substances, with great advantages in terms of time and economic
9 expenditure.
10 In conclusion, although the consistency between data from biological and computational data
11 suggest a possible genotoxicity risk due to the exposure to piperitenone oxide, further *in vivo*
12 studies are strictly needed in order to evaluate the role of the bioavailability of this substance
13 and its metabolic fate on the genotoxic profile. Our findings improve the knowledge on the
14 toxicity of piperitenone oxide, which has been very scantily investigated up to now, and
15 represent a starting point to characterize its safety as flavouring/fragrance ingredient.
16

17 **Author contributions**

18 Study design: Di Sotto A. and Mazzanti G.; extraction and purification of piperitenone oxide:
19 Rino Ragno, Mijat Bozovic; chemical analysis of the test substance: Fabio Barile; *in vitro*
20 experiments: Di Sotto A., Di Giacomo S., Abete L., Parisi O.A., Vitalone A.; *in silico*
21 genotoxicity predictions: Di Sotto A.; writing the manuscript: Di Sotto A., Di Giacomo S.,
22 Izzo A.A., Mazzanti G.; Final approval: all authors.
23

24 **Conflict of interest**

25 The authors declare no conflict of interest.

1

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4 The Authors thank Prof. Luca Romanelli for him valuable suggestions and advice.

5

6 **Transparency document**

7

8 **Appendix A. Supplementary data**

9

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1 **Legend to figures**

2 **Figure 1.** Chemical structure of piperitenone oxide.

3 **Figure 2.** Effect of piperitenone oxide on the number of revertant colonies of *Salmonella*
4 *typhimurium* TA1538 and TA98 strains, in the absence (continuous line) and presence
5 (broken line) of S9. Values represent the number of revertant colonies expressed as means ±
6 SEM (n = 6 plates).

7 **Figure 3.** Effect of piperitenone oxide on the number of revertant colonies of *Salmonella*
8 *typhimurium* TA1535 and TA100 in the absence (continuous line) and presence (broken line)
9 of S9. Values represent the number of revertant colonies expressed as means ± SEM (n = 6
10 plates).

11 **Figure 4.** Effect of piperitenone oxide on the number of revertant colonies of *Escherichia coli*
12 WP2, WP2uvrA and WP2uvrA/pKM101 strains in the absence (continuous line) and presence
13 (broken line) of S9. Values represent the number of revertant colonies expressed as means ±
14 SEM (n = 6 plates).

15 **Figure 5.** Effect of piperitenone oxide on DNA integrity of HepG2 cells evaluated in term of
16 DNA-tail (%)by the comet assay. Values represent the mean ± S.E.M. (n= 3). $H_2O_2$, 75 μM. $^{*}$
17 and $^{***}$ $p < 0.05$ and $p < 0.001$ respectively vs. control.

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Table 1. Genotype characteristics of the strains tested.

| | *Salmonella typhimurium* | | | | *Escherichia coli* | | |
|---|---|---|---|---|---|---|---|
| Point mutation | TA1538 | TA98 | TA1535 | TA100 | WP2 | WP2*uvr*A | WP2*uvr*A/pKM101 |
| | *his D3052* | *his D3052* | *his G46* | *his G46* | *trp E56* | *trp E56* | *trp E56* |
| *missense* | | | + | + | + | + | + |
| *frameshift* | + | + | | | | | |
| | | | | | | | |
| *bio* | + | + | + | + | | | |
| *rfa* | + | + | + | + | | | |
| *uvr*A | | | | | | + | + |
| *uvr*B | + | + | + | + | | | |
| | | | | | | | |
| pKM101 | | + | | + | | | + |

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Table 2. Mean frequency of micronuclei (MN) in binucleated cells (BNCs) and nuclear division index (NDI) in HepG2 cells treated with piperitenone oxide (PO). Value represents the mean ± SEM (n = 6).

| Treatment | [μM] | MN/1000 BNCs [a] (mean ± SE) | NDI (mean ± SE) |
|---|---|---|---|
| PO | 10 | 28.9 ± 3.3 | 1.59 ± 0.09 |
| | 30 | 29.4 ± 2.2 | 1.44 ± 0.07 |
| | 60 | 46.1 ± 3.4** | 1.48 ± 0.05 |
| | 90 | 53.9 ± 2.3** | 1.47 ± 0.03 |
| | 150 | 48.2 ± 3.2** | 1.51 ± 0.05 |
| Vehicle [b] | | 26.7 ± 3.5 | 1.56 ± 0.06 |
| Benzo[a]pyrene | 25 | 63.2 ± 8.2** | 1.58 ± 0.03 |

[a] For each treatment, MN frequency was determined by scoring at least 1000 binucleated HepG2 cells (BNCs). [b] DMSO 1%. ** Denote a significant difference from the vehicle ($p$ < 0.01).

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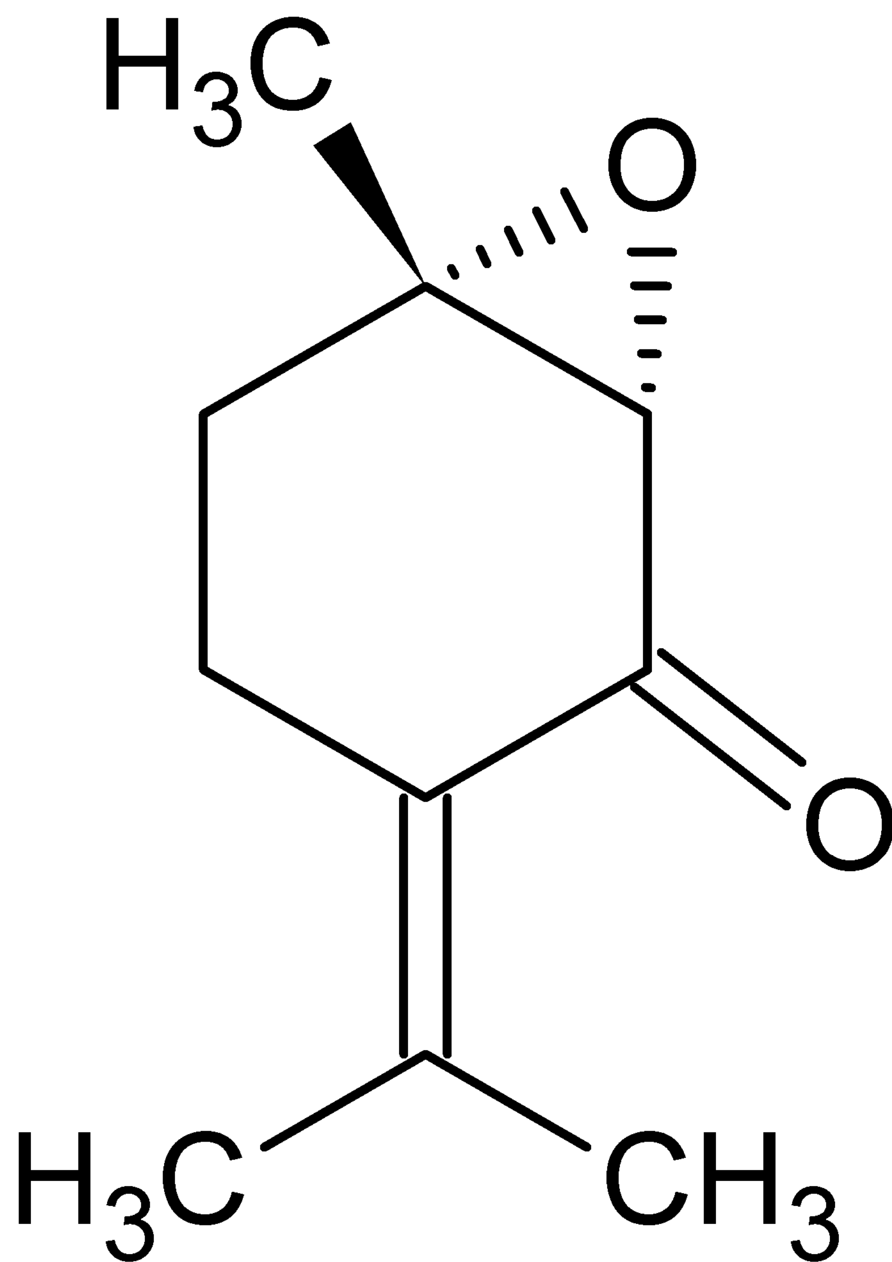


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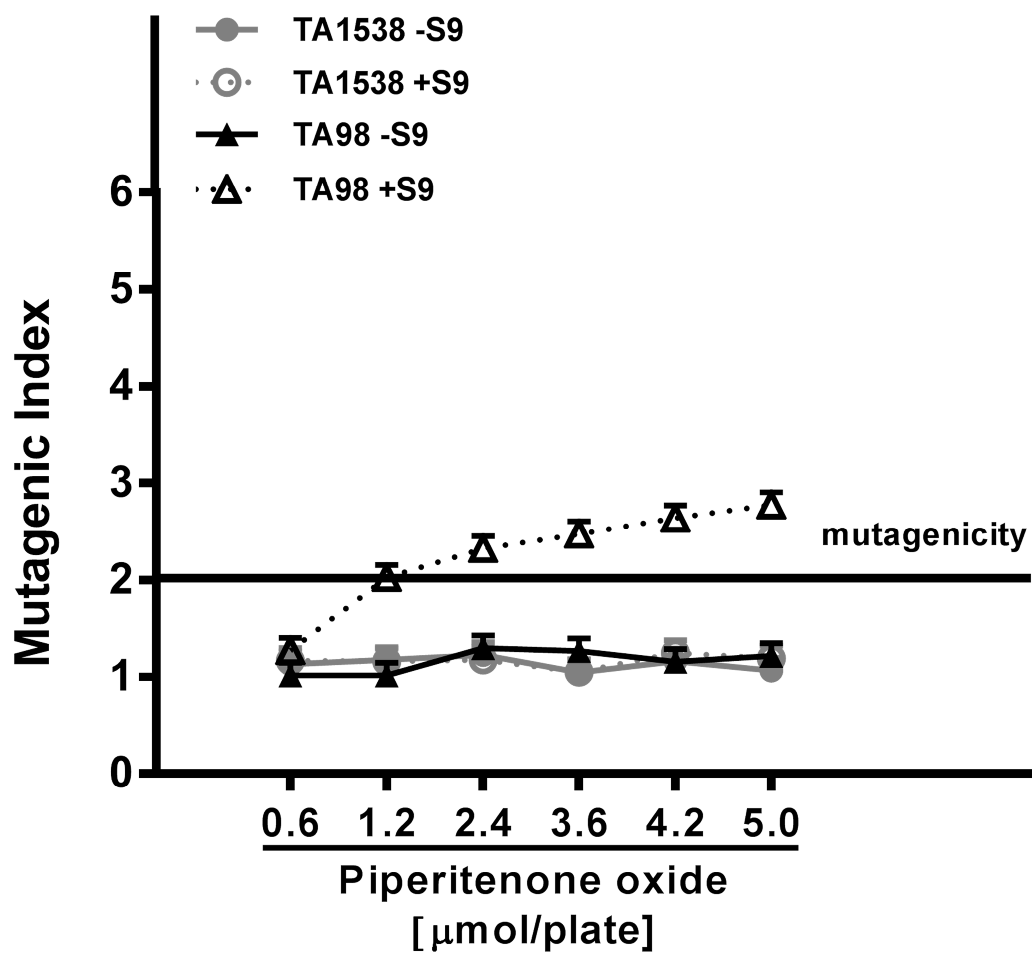


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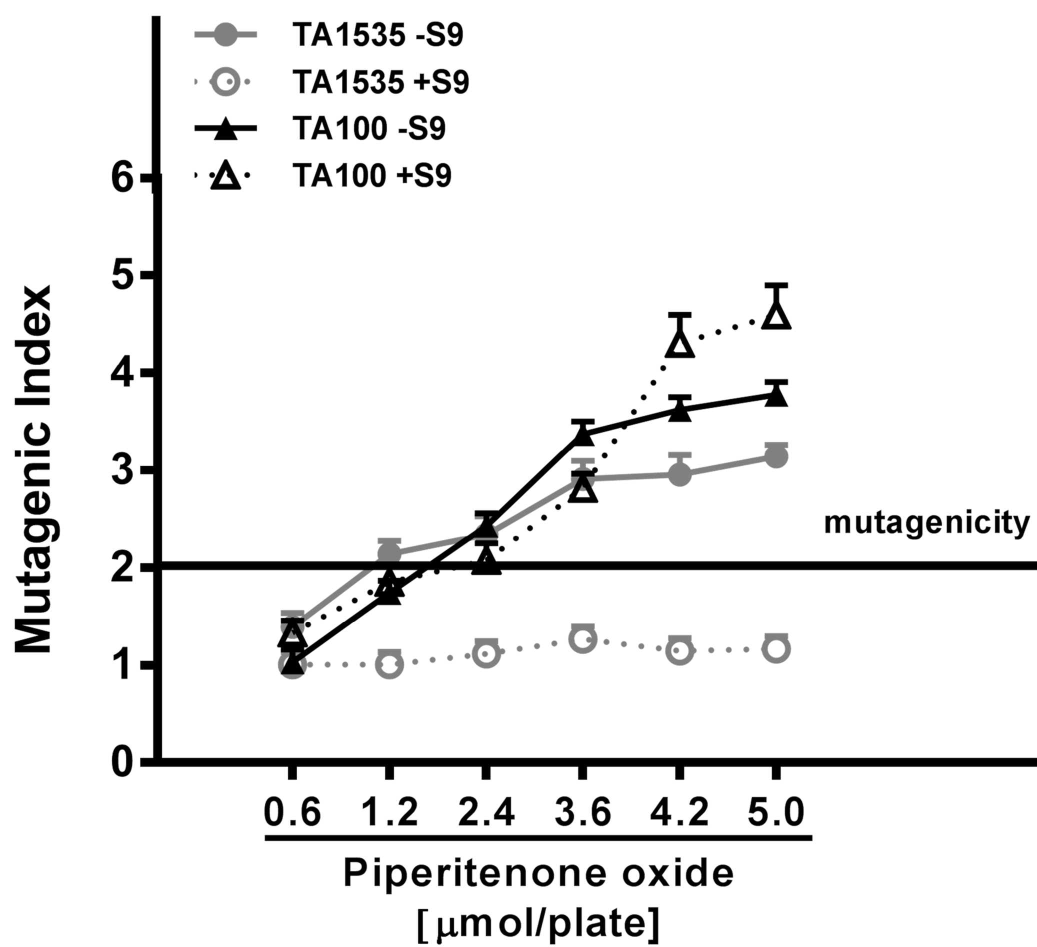


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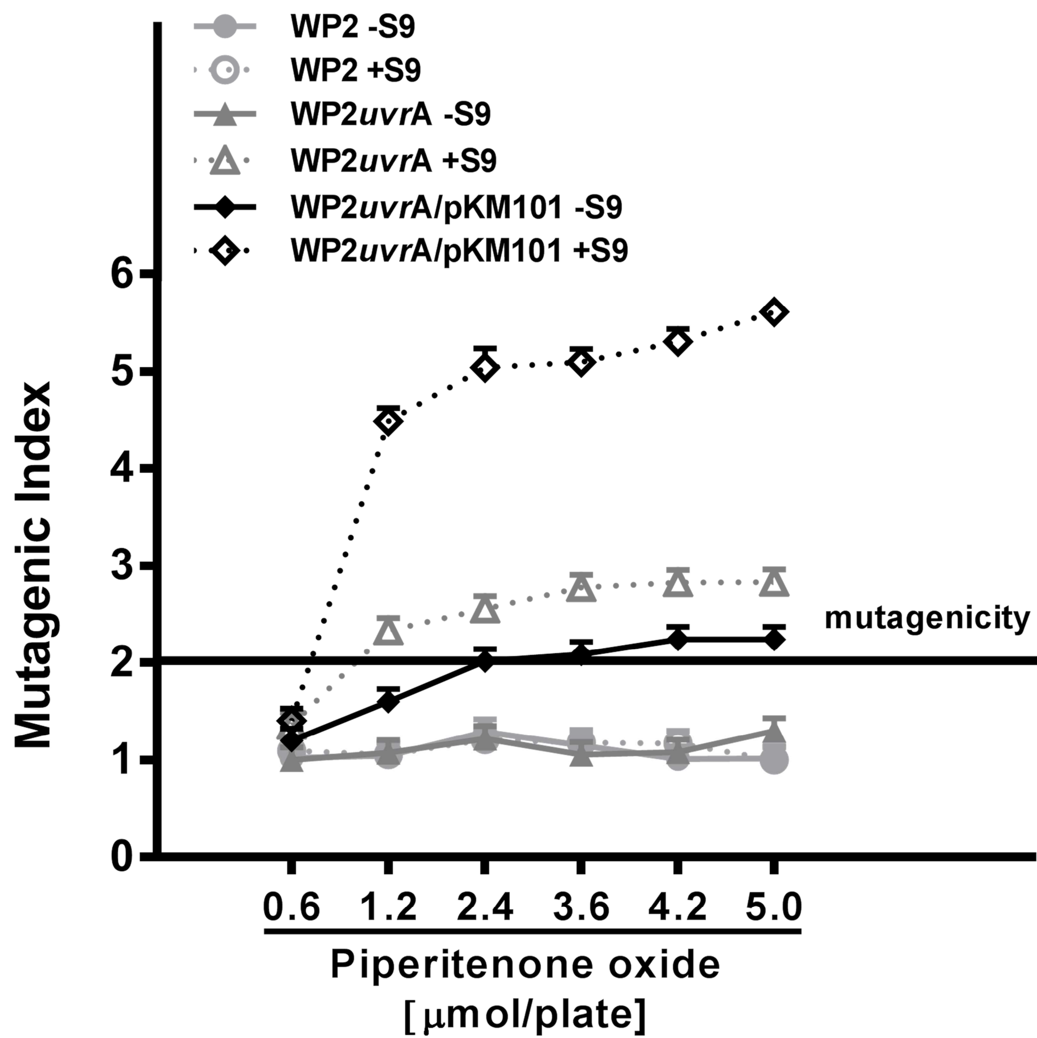


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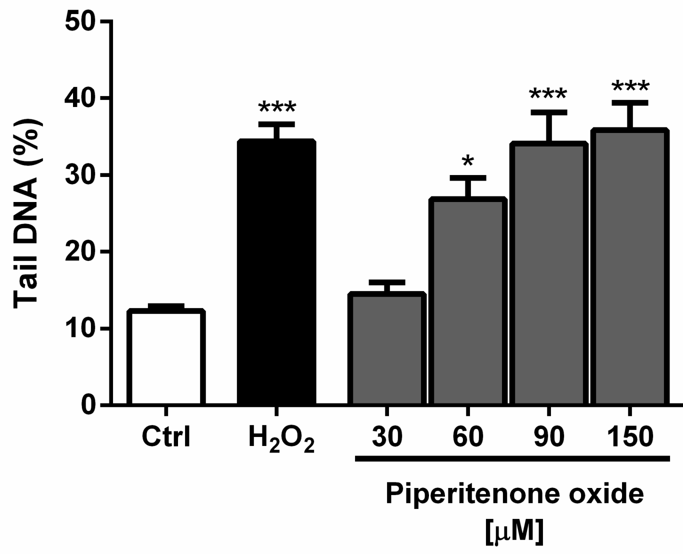


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HIGHLIGHTS

- An integrated *in vitro* and *in silico* genotoxicity assessment for the natural flavouring agent piperitenone oxide.
- Experiments carried out *in vitro* by Ames test, micronucleus and comet assays, and i*n silico* by Toxtree and VEGA tools.
- Test substance both point mutations and DNA damage, including micronuclei and single-strand breaks.
- Epoxide and α,β-unsaturated carbonyl found as structural alerts for mutagenicity at computational analysis.

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