Absence of mutagenic activity in the bacterial reverse mutation assay with pulegone and peppermint oil

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Abstract
The essential oil of peppermint and one of its natural constituents, (R)-(−)-pulegone, are approved flavorings added to food worldwide. (R)-(−)-Pulegone and peppermint oil were tested separately in two independent bacterial reverse mutation assays according to Organisation for Economic Co-operation and Development Guideline 471. Both flavorings did not produce any evidence of mutagenicity up to cytotoxic concentrations in either the presence or the absence of exogenous metabolic activation.

Keywords
Ames assay, mutagenicity, pulegone, peppermint oil, bacterial reverse mutation, OECD 471

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Introduction
Herbs of the Mentha genus and their essential oils, including peppermint, spearmint, corn mint, and other related edible plants, are readily recognized for their familiar aroma.¹ Essential oils derived from plants belonging to the Mentha genus have a long history of use and are often used today as flavoring ingredients in foods and beverages.² In particular, peppermint oil is utilized for its familiar and characteristic minty, cooling organoleptic profile.³–⁵ Composition analysis conducted on peppermint oil and other Mentha-derived oils indicates that they consist of alicyclic ketones, related secondary alcohols, and other structurally similar substances, predominantly menthol-related and p-menthane-related constituents.³ The major constituents of these oils are responsible for their characteristic aroma. (R)-(−)-Pulegone (Figure 1) (hereafter referred to as pulegone) occurs naturally in Mentha herbs as an intermediate of menthol biosynthesis in peppermint leaves.³ It is a major constituent in pennyroyal oil and buchu leaves oil,⁶ but a minor constituent of peppermint oil and other mint essential oils.⁷ It is also known to occur naturally in foods, such as black currants, lemon peel, and rosemary.⁸–¹⁰

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The concern for potential genotoxicity of pulegone was raised following the results of genotoxicity studies conducted by the National Toxicology Program (NTP) in 2011. These studies included three bacterial reverse mutation studies in *Salmonella typhimurium* and *Escherichia coli* and an in vivo micronucleus study in mice. The NTP bacterial reverse mutation assays produced inconsistent findings in three independent studies, conducted at two separate research laboratories. Results were inconsistent between trials within the same study, between studies, and between laboratories. Furthermore, positive results were reported only in the *E. coli* strain WP2 uvrA/pKM101 at different concentrations between the two studies and in the *S. typhimurium* TA98 strain only in one out of the three studies. Meanwhile, the lack of mutagenicity for pulegone was reported in an earlier bacterial reverse mutation assays by Andersen and Jensen in four *S. typhimurium* strains, including TA98. In the NTP in vivo micronucleus assay conducted in male and female B6C3F1 mice, pulegone produced no increase in the frequency of micronucleated peripheral blood erythrocytes. No evidence of genotoxicity was also reported for a pulegone-rich essential oil in other in vivo micronucleus and comet assays or for the primary metabolite of pulegone, menthofuran, and for peppermint oil. The bacterial reverse mutation studies presented here for pulegone and pulegone-containing peppermint oil were conducted according to OECD Guideline 471 to independently investigate the concern of their mutagenic potential raised in the NTP in vitro mutagenicity assays. The lack of genotoxicity for either pulegone or pulegone-containing peppermint oil demonstrated by the results of the studies presented here in relation to the NTP findings is also discussed.

**Materials and methods**

**Study compliance**

The studies were conducted at BioReliance Corporation (Rockville, Maryland, USA) according to Organisation for Economic Co-operation and Development (OECD) Guideline 471 and BioReliance standard operating procedures. The studies were also compliant with US FDA Good Laboratory Practice (GLP) Regulations published in 21 CFR Part 58, which is also compatible with OECD Principles of Good Laboratory Practice.

**Test substances**

(R)-(5R)-Pulegone (IUPAC (5R)-5-methyl-2-propan-2-ylidenecyclohexan-1-one; MW 152.23; CAS No. 89-82-7) and peppermint oil (Mentha piperita L.; CAS No. 8006-90-4) were sourced from a flavor supplier specializing in mint and mint-derived products as clear, colorless liquids. The purity of pulegone test material was >94%. Gas chromatography–mass spectrometry analysis of the peppermint oil indicated that it was composed of 30.8% menthol, 27.7% menthone, 5.6% 1,8-cineole (eucalyptol), 4.7% menthyl acetate, 4.4% d-isomenthene, 4.3% menthofuran, 3.4% neomenthol, 2.9% limonene, 2.1% (R)-(+) pulegone, 1.8% β-pinene, 1.1% α-pinene, and other minor (<1%) reported constituents. The test substances were stored at ambient temperature and protected from light.

Based on solubility testing, ethanol (Sigma-Aldrich, St. Louis, MO) was chosen as the vehicle for dose preparations. The positive controls were all obtained from Sigma-Aldrich with purities of 97.5% and greater. The positive controls for treatments in the absence of metabolic activation were 2-nitrofluorene, sodium azide (SA), 9-aminoacridine, and methyl methanesulfonate. For treatments in the presence of metabolic activation, the positive control was 2-aminoanthracene. All positive controls were diluted in dimethylsulfoxide (Sigma-Aldrich), except SA, which was diluted in sterile water. Positive controls were stored at −10 to −30°C.

**Dose preparations.** Test substance dilutions and dose preparations were prepared immediately before use and delivered to the test system at room temperature under filtered light. The test substances were determined to be soluble in ethanol up to a concentration of approximately 500 mg/mL. Each test substance was weighed out and diluted in ethanol to a concentration of 100 mg/mL. Because the test solutions were used immediately after preparation, analyses to determine the concentration, homogeneity, and stability of the test substances in the vehicle (ethanol) were not performed in these studies. Although these analyses are not a requirement in the OECD Guideline, this was recorded as a deviation from the laboratory’s GLP.

**Test system**

The tester strains used were the *S. typhimurium* histidine-requiring strains TA98, TA100, TA1535, and TA1537 as described by Ames and co-authors and *E. coli* WP2 uvrA as described by Green and Muriel. The *S. typhimurium* tester strains were derived from Dr Bruce Ames’ cultures; *E. coli* tester strains were from the National Collection of Industrial and Marine Bacteria (Aberdeen, Scotland). *S. typhimurium* tester strains TA98 and TA1537 are

![Figure 1. Chemical structure of (R)-(+) pulegone.](image-url)
considered sensitive to mutagens that cause frameshift mutations, while TA1535 and *E. coli* WP2 uvrA are considered sensitive to base-pair substitution mutations, and TA100 is able to detect both frameshift and base-pair substitution mutations.\(^2\)

**Exogenous metabolic activation (S9).** Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats that were injected intraperitoneally with Aroclor\textsuperscript{TM} 1254 (200 mg/mL in corn oil) at a dose of 500 mg/kg, 5 days before sacrifice and was stored at \(-60^\circ\)C or colder until use. The S9 mixture prepared for addition was composed of 4 mM of \(^\beta\)-nicotinamide-adenine dinucleotide phosphate, 5 mM glucose-6-phosphate, 33 mM potassium chloride, 8 mM magnesium chloride, 100 mM phosphate buffer (pH 7.4), and 10\% (v/v) S9 homogenate.

**Experimental design.**

**Preparation of tester strains.** Overnight cultures were prepared by inoculating from the appropriate frozen permanent stock into a vessel, containing 30–50 mL of culture medium. To assure that cultures were harvested in late log phase, the length of incubation was controlled and monitored. Following inoculation, each flask was placed in a shaker/incubator programmed to begin shaking at 125–175 r/min and incubating inoculation, each flask was placed in a shaker/incubator programmed to begin shaking at 125–175 r/min and incubating at \(37 \pm 2^\circ\)C for approximately 12 h before the anticipated time of harvest. Each culture was monitored spectrophotometrically for turbidity and was harvested at a percent transmittance yielding a titer of greater than or equal to \(0.3 \times 10^9\) cells per milliliter. The actual titers were determined by viable count assays on nutrient agar plates.

**Scoring.** The condition of the bacterial background lawn was evaluated for evidence of test substance cytotoxicity by using a dissecting microscope. Precipitate was evaluated after the incubation period by visual examination without magnification. Cytotoxicity, defined as the reduction in the background lawn or the reduction (>50\%) of mean revertants, and degree of precipitation were scored relative to the vehicle control plate. As appropriate, colonies were enumerated either by hand or by machine (Sorcerer Colony Counter, Perceptive Instruments, Bury St Edmunds, UK).

**Preliminary toxicity experiments.** In the preliminary toxicity/range-finding experiments, *S. typhimurium* strains TA98, TA100, TA1535, and TA1537 and *E. coli* strain WP2 uvrA were exposed to the vehicle alone and 10 concentrations of the test substance (either pulegone or peppermint oil) through the plate incorporation method\(^2\) in the presence and absence of metabolic activation (S9). Concentrations for the mutagenicity assays were selected based on the range of cytotoxicity recorded in the preliminary test. For the preliminary toxicity experiment of pulegone and peppermint oil, the concentrations tested were 0 (control), 6.67, 10.0, 33.3, 66.7, 100, 333, 667, 100, 3333, and 5000 \(\mu\)g/plate.

**Main mutagenicity experiments.** Based on the preliminary toxicity experiments, the concentrations selected for the mutagenicity experiments for both test substances were 15.0, 50.0, 150, 500, 1500, and 5000 \(\mu\)g/plate, using approximate half-log spacing. Each of the concentrations were tested in triplicate using the plate incorporation method.

**Results**

**Pulegone mutagenicity experiments**

**Preliminary toxicity experiment.** In the preliminary toxicity/range-finding experiment for pulegone, no precipitate was observed for any of the concentrations tested. Cytotoxicity was observed at 5000 \(\mu\)g/plate in all tester strains in the absence and/or presence of S9, and at 3333 \(\mu\)g/plate in TA100 in the absence and presence of S9 and in TA1535 in the absence of S9. Only moderate toxicity was observed at 5000 \(\mu\)g/plate in most tester strains in the range-finding experiment. Marked cytotoxicity at 5000 \(\mu\)g/plate was observed only in TA100 and TA1535 with S9 and slight or moderate cytotoxicity at the next highest concentration of 3333 \(\mu\)g/plate. The results of the preliminary toxicity experiment are presented in Table 1.

**Main mutagenicity experiment.** The results of the main mutagenicity experiment on pulegone are presented in Table 2. No precipitate was observed in any of the concentrations tested. The highest test concentration for the main study was maintained at 5000 \(\mu\)g/plate, since only slight to moderate toxicity was observed at the next highest test concentration in most strains in the range-finding experiment. In the main experiment, marked cytotoxicity was observed at 5000 \(\mu\)g/plate only in TA100 without and with S9 and in TA1535 with S9 and only moderate cytotoxicity in all other tester strains in the absence and presence of S9. No cytotoxicity was observed at the next highest test concentration (1500 \(\mu\)g/plate) except for slight cytotoxicity in TA100 in the absence of S9. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

**Peppermint oil mutagenicity experiments**

**Preliminary toxicity experiment.** In the preliminary toxicity/range-finding experiment for peppermint oil, no precipitate was observed for any of the concentrations tested. Cytotoxicity was observed at 3333 and 5000 \(\mu\)g/plate in all tester strains in the absence and presence of S9. Marked cytotoxicity was observed at 5000 and 3333 \(\mu\)g/plate for TA100 and TA1535 without S9 and in TA100, TA1535, and TA1537 with S9. Moderate to slight cytotoxicity was observed at 3333 \(\mu\)g/plate in TA1537 without S9 and in WP2 uvrA in the absence and/or presence of S9 and at 1000 \(\mu\)g/plate in TA100 and TA1535 in the absence and presence of S9. Slight cytotoxicity was also observed in TA1537 at 1000 \(\mu\)g/plate and in TA100 and TA1535 at 667 \(\mu\)g/plate in
the absence of S9. The results of the preliminary toxicity experiment are presented in Table 3.

**Main mutagenicity experiment.** The results of the main mutagenicity experiment on peppermint oil are presented in Table 4. No precipitate was observed in any of the concentrations tested. Marked cytotoxicity was observed at 5000 µg/plate in all strains and tested conditions. Moderate cytotoxicity was observed at 1500 µg/plate in TA100 and TA1535 in the absence and presence of S9 and in TA1537 in the presence of S9. Slight cytotoxicity was observed at 1500 µg/plate in TA98 and TA1537 in the absence of S9 and in WP2 uvrA in the presence of S9. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

**Discussion and conclusion**

Herbs of the *Mentha* genus and their essential oils owe their familiar aroma to menthol and related substances, of which pulegone is a minor constituent. The reverse mutation studies presented here for pulegone and peppermint oil that contains pulegone were conducted according to OECD Guideline 471 to independently investigate the concern for genotoxicity of pulegone following the ambiguous results

| Strain | TA98 | TA100 | TA1535 | TA1537 | WP2 uvrA |
|--------|------|-------|--------|--------|---------|
| Concentration/plate (µg) | S9 | +S9 | S9 | +S9 | S9 | +S9 | S9 | +S9 |
| 5000 | 24b | 25 | 78c | 0d | 1c | 0d | 9c | 7b | 31b | 14b |
| 3333 | 14 | 25 | 88b | 83c | 10 | 14c | 10 | 9 | 21 | 22 |
| 1000 | 30 | 21 | 68 | 88 | 11 | 19 | 12 | 14 | 35 | 31 |
| 667 | 21 | 26 | 88 | 79 | 14 | 10 | 5 | 9 | 5 | 43 |
| 333 | 22 | 32 | 85 | 78 | 16 | 16 | 6 | 7 | 40 | 21 |
| 100 | 12 | 35 | 80 | 88 | 7 | 16 | 9 | 6 | 37 | 25 |
| 66.7 | 15 | 20 | 87 | 95 | 12 | 7 | 7 | 10 | 45 | 43 |
| 33.3 | 15 | 28 | 106 | 87 | 11 | 9 | 6 | 4 | 41 | 22 |
| 10.0 | 14 | 25 | 77 | 84 | 9 | 15 | 5 | 5 | 32 | 45 |
| 6.67 | 15 | 27 | 85 | 95 | 11 | 5 | 5 | 10 | 33 | 26 |
| Ethanol | 14 | 27 | 94 | 82 | 12 | 9 | 9 | 3 | 32 | 38 |

*aNo concurrent positive controls were run in the preliminary toxicity experiment.

bSlightly reduced background.

cModerately reduced background.

dExtremely reduced background.

**Table 1.** Mean revertant counts with (+S9) and without (–S9) metabolic activation in the preliminary toxicity experiment on pulegone.

| Strain | TA98 | TA100 | TA1535 | TA1537 | WP2 uvrA |
|--------|------|-------|--------|--------|---------|
| Concentration/plate (µg) | S9 | +S9 | S9 | +S9 | S9 | +S9 | S9 | +S9 | S9 | +S9 |
| 5000 | 13a | 23b | 0c | 0d | 4c | 0c | 4c | 7a | 2a | 8a |
| 1500 | 23 | 30 | 70b | 63 | 15 | 16 | 2 | 7 | 13 | 15 |
| 500 | 19 | 29 | 92 | 77 | 12 | 15 | 9 | 9 | 23 | 21 |
| 150 | 21 | 29 | 101 | 75 | 16 | 16 | 6 | 6 | 24 | 27 |
| 50.0 | 19 | 25 | 92 | 102 | 14 | 20 | 7 | 8 | 24 | 27 |
| 15.0 | 25 | 28 | 81 | 79 | 13 | 16 | 8 | 8 | 27 | 23 |
| Ethanol | 22 | 27 | 91 | 82 | 15 | 19 | 7 | 7 | 27 | 26 |
| Positive control | 106 | 241 | 703 | 447 | 686 | 138 | 558 | 50 | 324 | 343 |

2-NF: 2-nitrofluorene; SA: sodium azide; 9-AAD: 9-aminoacridine; MMS: methyl methanesulfonate; 2-AA: 2-aminoanthracene.

*aModerately reduced background.

bSlightly reduced background.

cExtremely reduced background.

dTA98 (–S9): 2-NF, 1 µg/plate; TA100, TA1535 (–S9): SA, 1 µg/plate; TA1537 (–S9): 9-AAD, 75 µg/plate; WP2 uvrA (–S9): MMS, 1000 µg/plate; TA98, TA1535 (+S9): 2-AA, 1 µg/plate; TA100, TA1537 (+S9): 2-AA, 2 µg/plate; WP2 uvrA (+S9): 2-AA, 15 µg/plate.
of the 2011 NTP bacterial reverse mutation studies. No indication of mutagenicity was observed for either pulegone or pulegone-containing peppermint oil with any of the tester strains in either the presence or absence of S9 activation.

The NTP genotoxicity testing for pulegone included three bacterial reverse mutation studies and an in vivo micronucleus induction study in B6C3F1 mice. In the first NTP bacterial reverse mutation study conducted at an independent contract laboratory, *S. typhimurium* strains TA100, TA1535, TA98, and TA97 were incubated with pulegone in the presence and absence of liver S9 fractions derived from either Aroclor 1254-induced Syrian hamster or Sprague-Dawley rat (10% or 30% v/v). When tested up to a maximum concentration of 3333 μg/plate, pulegone did not cause any increases in the frequency of revertant colonies under any of the treatment conditions. The preliminary range-finding test for this assay also did not show any evidence of increased mutation frequency. In the second NTP bacterial reverse mutation study, pulegone was tested up to cytotoxic concentrations in *S. typhimurium* strains TA98 and TA100, as well as *E. coli* WP2 uvrA at a different (external) research laboratory in the presence and absence of rat liver S9 (10% v/v).

### Table 3. Mean revertant counts with (+S9) and without (−S9) metabolic activation in the preliminary toxicity experiment on peppermint oil.

| Strain | TA98 | TA100 | TA1535 | TA1537 | WP2 uvrA |
|--------|------|-------|--------|--------|---------|
| Concentration/plate (μg) | −S9 +S9 | −S9 +S9 | −S9 +S9 | −S9 +S9 | −S9 +S9 |
| 5000   | 11b  | 0c    | 0c     | 0c     | 0c      |
| 3333   | 25b  | 29b   | 0c     | 0c     | 5b      |
| 1000   | 21   | 22    | 59b    | 97b    | 10b     |
| 667    | 16   | 31    | 80d    | 95     | 14d     |
| 333    | 22   | 30    | 80     | 83     | 9       |
| 100    | 16   | 36    | 80     | 87     | 11      |
| 66.7   | 22   | 26    | 97     | 88     | 6       |
| 10.0   | 17   | 18    | 96     | 84     | 9       |
| 6.67   | 13   | 24    | 68     | 90     | 8       |
| Ethanol| 21   | 29    | 80     | 87     | 10      |

*No concurrent positive controls were run in the preliminary toxicity experiment. Moderately reduced background. Extremely reduced background. Slightly reduced background.*

### Table 4. Mean revertant counts with (+S9) and without (−S9) metabolic activation in the main mutagenicity experiment on peppermint oil.

| Strain | TA98 | TA100 | TA1535 | TA1537 | WP2 uvrA |
|--------|------|-------|--------|--------|---------|
| Concentration/plate (μg) | −S9 +S9 | −S9 +S9 | −S9 +S9 | −S9 +S9 | −S9 +S9 |
| 5000   | 0a   | 0a    | 0a     | 0a     | 0a      |
| 1500   | 15b  | 29c   | 73c    | 10c    | 9c      |
| 500    | 15   | 24    | 66     | 64     | 9       |
| 150    | 20   | 24    | 78     | 77     | 9       |
| 50.0   | 19   | 24    | 76     | 71     | 8       |
| 15.0   | 19   | 32    | 83     | 69     | 12      |
| Ethanol| 18   | 27    | 78     | 74     | 11      |
| Positive control | 134  | 209   | 731    | 899    | 595     |

2-NF: 2-nitrofluorene; SA: sodium azide; 9-AAD: 9-aminoacridine; MMS: methyl methanesulfonate; 2-AA: 2-aminoanthracene. Extremely reduced background. Slightly reduced background. Moderately reduced background. TA98 (−S9): 2-NF, 1 μg/plate; TA100, TA1535 (−S9): SA, 1 μg/plate; TA1537 (−S9): 9-AAD, 75 μg/plate; WP2 uvrA (−S9): MMS, 1000 μg/plate; TA98, TA1535 (+S9): 2-AA, 1 μg/plate; TA100, TA1537 (+S9): 2-AA, 2 μg/plate; WP2 uvrA (+S9), 2-AA, 15 μg/plate.
laboratory, pulegone was tested at concentrations up to 1500, 2500, and 3500 μg/plate for TA100, TA98, and WP2 uvrA/pKM101, respectively. Slight (less than twofold) increases were observed in one of the three trials in WP2 uvrA/pKM101 at the highest two concentrations of 2500 and 3500 μg/plate, in the presence of S9 activation; however, as these slight increases were small in magnitude and were not reproducible in the other two trials where the same concentrations were cytotoxic, the study was evaluated as negative. In the third NTP bacterial reverse mutation study, the same lot of pulegone (purity of 96%) that was tested in the NTP toxicity and carcinogenicity studies was tested at a second external research laboratory at concentrations up to 1500 μg/plate. In this study, increases in revertant frequencies (greater than twofold) were reported for WP2 uvrA/pKM101 in the presence of rat liver S9 in two trials at the two highest non-cytotoxic concentrations of 500 and 750 μg/plate. Unlike the previous two studies, increases in mutation frequency were reported also in S. typhimurium TA98 in the third study, at the highest non-cytotoxic concentrations of 500 and 750 μg/plate in the presence of S9 (greater than twofold at both concentrations in one trial and only at the highest in the second trial). Lack of mutagenicity for pulegone was also reported in an earlier bacterial reverse mutation assay in four S. typhimurium strains (TA98, TA100, TA1535, TA1537), albeit at concentrations of 6.4, 32, 160, and 800 μg/plate, either with or without metabolic activation with S9.12

In the in vivo micronucleus assay in male and female B6C3F1 mice conducted by the NTP, pulegone did not increase the frequency of micronucleated peripheral blood erythrocytes when administered at dose levels of 9.375, 18.75, 37.5, 75, or 150 mg/kg bw/day by gavage for 3 months.11 In another recent in vivo study, an essential oil containing 65% pulegone was tested in a bone marrow micronucleus assay in rat and a modified comet assay in blood lymphocytes at dietary concentrations of 70, 260, and 460 mg/kg bw/day (equivalent to 45.5, 169, and 299 mg pulegone/kg bw/day, respectively) and was negative for induction of micronuclei in bone marrow erythrocytes and for DNA damage in blood cells.13

Previous genotoxicity studies also support the lack of genotoxicity for the primary metabolite of pulegone, menthofuran,14–16 and for peppermint oil,12,17,18 including a lack of mutagenicity in various S. typhimurium strains, all in the presence and absence of metabolic activation (S9). In addition, peppermint oil was negative for mutagenicity in an in vitro mammalian cell mutagenicity assay.27 Among the in vivo genotoxicity evidence, negative results were obtained in the NTP bone marrow micronucleus study and in a combined comet and micronucleus study with pulegone-containing essential oil,13 while menthofuran, the known hepatotoxic constituent of peppermint oil and pulegone metabolite, was weakly positive in the liver comet assay at cytotoxic dose levels (based on the increased frequency of ghost cells at the same dose levels and clear systemic toxicity at the highest dose).16 This latter finding contributes to the evidence that genotoxicity associated with pulegone and/or its metabolite is secondary to cytotoxicity in vitro and is consistent with tumor growth in vivo at doses associated with hepatotoxicity.

When these experiments are considered collectively, the only positive finding for pulegone was found in the third reverse mutation study conducted by the NTP, a finding inconsistent with the other genotoxicity assays conducted by NTP, as well as all other genotoxicity and mutagenicity assays.11–13 The validity and significance of the positive result that was reported with pulegone in E. coli strain WP2 uvrA/pKM101, with activation, in the third NTP mutagenicity study,11 is questionable, as the concentrations at which higher number of revertants were reported were within the range of the previous two NTP assays in which the material was clearly negative. In addition, the positive result in TA98 strain is inconsistent with the previous two NTP bacterial reverse mutation assays. These NTP mutagenicity studies performed in two separate research laboratories with different results demonstrate the lack of confidence in the reproducibility of the results. Furthermore, the discrepancy in the results of the third assay compared to the previous two assays sponsored by NTP was not explained by protocol differences or any other speculation in the final NTP report. The results of the studies presented here showed no evidence for mutagenicity for pulegone and pulegone-containing peppermint oil and further strengthen the overall weight of evidence that they are not mutagenic or genotoxic.

**Author contributions**

Mses. Harman and Lu and Drs Bastaki and Taylor researched, compiled, and interpreted available study data and drafted the manuscript. Drs Aubanel, Bialk, Choi, Demyttenaere, Diop, Etter, Han, Krammer, Schnabel, and Wilson reviewed and provided comments on the content and interpretation of the manuscript.

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