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Public statement on the use of herbal medicinal products containing pulegone and menthofuran

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30 Churchill Place ● Canary Wharf ● London E14 5EU ● United Kingdom
Telephone +44 (0)20 3660 6000 **Facsimile** +44 (0)20 3660 5555
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Public statement on the use of herbal medicinal products containing pulegone and menthofuran

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1. Introduction (Problem statement)

The CHMP Herbal Medicinal Products Working Party and from 2004 on the Committee of Herbal Medicinal Products (HMPC), following the publication of the opinion of the Scientific Committee on Food (SCF) on pulegone and menthofuran, prepared a public statement reviewing the Scientific Committee on Food (SCF) opinion and recommending future action in relation to herbal medicinal products containing peppermint oil (*Mentha piperita* L.) or mint oil (*M. canadensis* L., syn. *M. arvensis var.piperascens* Malinv. ex Holmes) or pennyroyal oil (*M. pulegium* L.) or *Hedeoma pulegoides* (L.) Pers.

In the Public statement (EMEA/HMPC/138386/2005), the HMPC's conclusions concerning herbal medicinal products containing peppermint, mint oil and pennyroyal oil were as follows:

1. The first reports on brain toxicity of pulegone appear to have been erroneous.
2. Serious/lethal cases of intoxication from pennyroyal oil with a high content of pulegone indicate that pulegone is a hepatotoxin. A plausible mechanism for liver toxicity of pulegone and menthofuran has been proposed, which is supported by experimental data.
3. No approval of medicinal products containing pennyroyal oil appears to have been granted in EU and its use in unlicensed products should be discouraged.
4. The reported NOEL of pulegone and menthofuran (20 mg/kg bw/d) has not been determined with required accuracy, and remains uncertain. Despite that a TDI for pulegone and menthofuran has been set for food (0.1 mg/kg bw).
5. Doses up to ca 2.3 mg/kg bw/day of pulegone[1] (exceeding the TDI for food) are commonly encountered in herbal medicinal products in Europe. Pharmacovigilance has hitherto revealed no certain cases of liver toxicity in humans caused by peppermint oil or mint oil. Pharmacovigilance does not indicate that the use of herbal medicinal products in these doses is associated with liver disorders.
6. The therapeutic indications for peppermint oil and mint oil are mainly related to common cold and gastrointestinal disturbances and presumably the vast majority of these products are used in self-medication. An underreporting of side effects may be suspected.

The following regulatory actions were proposed:

No immediate actions are proposed, but alerted pharmacovigilance of peppermint oil and mint oil containing products is recommended.

An increased awareness in the medical community concerning high intake of peppermint oil and mint oil containing products as a potential cause of otherwise unexplained liver reactions would be desirable.

A limit for menthofuran should be included in the monograph for mint oil of the European Pharmacopoeia[2].

The use of penny royal oil should be discouraged.

Similar considerations should be given to other herbal products containing significant amounts of pulegone and menthofuran.

[1] 0.549 mg/kg bw of pulegone and 1.46 mg/kg bw of menthofuran (see the text below)
[2] since 2012, limits for pulegone and menthofuran exist – see text below.

There were toxicological assessments of pulegone and menthofuran, which were not referred to in the HMPC 2005 Public Statement. In preparation for the NTP study, a literature survey was performed in 1998 by the Integrated Laboratory Systems (R Tice). The survey noted hepatic injuries and fatal cases of pennyroyal oil ingestion. Most toxicology data were available from acute or subacute rodent studies which indicated relatively modest toxicity, mainly on the liver, and metabolic activation of pulegone and menthofuran as the most probable mechanism.

The Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in contact with Foods prepared and published an opinion on Pulegone and Menthofuran in flavourings and other food ingredients with flavouring properties (SPFA, 2005). The Panel concluded, in conformation with the opinion of the SCF (2002), that the data as a whole are not yet sufficient to establish a TDI for pulegone. Furthermore, The Panel wished the following studies to be provided within 2 years of the publication of this opinion:

1. Studies to establish a NOEL for (R)-(+)-menthofuran in 90-day oral toxicity study in rats;
2. Further genotoxicity studies on (R)-(+)-menthofuran and (R)-(+)-pulegone augmenting the database to comply with the SCF General Guideline for Food Additives (studies on mammalian cell gene mutation and chromosome aberration);
3. Further refinement of intake estimates from all dietary sources including actual usage levels and analytical data on concentrations in relevant products.

1.1. Pulegone and menthofuran in plants and plant preparations

Pulegone and menthofuran (Fig. 1) are major constituents of several plants and essential oils (e.g. peppermint, pennyroyal) used for flavoring foods and drinks and for herbal medicinal products.

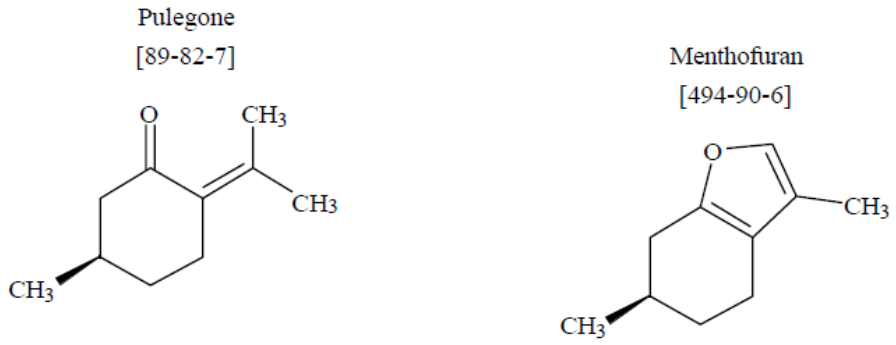

Fig: 1: structural formula of pulegone and menthofuran

Pulegone and menthofuran are significant constituents of several mint (*Mentha*) species and their derived volatile oils, including peppermint (*Mentha piperita*), spearmint (*Mentha spicata*), European pennyroyal (*Mentha pulegium* L.*)* and American pennyroyal (*Hedeoma pulegioides* L.). Pulegone is the major component of the volatile oils of European and American Pennyroyal where it comprises 85–97% (w/v) and about 30% (w/v) of the respective oil. In different varieties of *M. piperita* oils and *M. arvensis* oils pulegone and menthofuran are found in ranges of 0.5–4.6% and 1-9%, respectively. For further information, see SPFA, 2005; IARC, 2014.

1.2. Exposure to pulegone and menthofuran

It is of importance to keep in mind that exposure to pulegone leads also to the exposure to menthofuran, which is a major metabolite of pulegone in the body. Pulegone and menthofuran display qualitative similar hepatotoxicities in rodents and thus it is reasonable that these substances are evaluated together.

Exposure to pulegone and menthofuran is primarily through ingestion of food products (e.g., frozen dairy dessert, candy, baked goods, gelatins, and puddings) and of alcoholic and nonalcoholic beverages flavored with spearmint oil, peppermint oil, or synthetic pulegone. Pulegone was not detected in meat products, processed fruit, confectioner frosting, jams, or jellies.

Herbal medicinal products have been produced from peppermint oil (*Mentha piperita* L.) and mint oil (*M. canadensis* L., syn. *M. arvensis var piperascens* Malinv. Ex Holmes). Pennyroyal oil (*M. Pulegium* L. or *Hedeoma pulegoides* (L.) Pers) has also been used as a fragrance agent and as an herbal medicine to induce menstruation and abortion. It is not used anymore.

The highest recommended daily dose in the EU is 1.2 ml peppermint oil i.e. 1080-1099 mg peppermint oil (based on relative density 0.9-0.916 g/cm^3 according Ph. Eur. 8.1 (2014)), which contains maximum 32.4-32.97 mg pulegone and 86.4-87.92 mg menthofuran (according to Ph. Eur. 8.1 limits for pulegone and menthofuran in peppermint oil). For a 60 kg person this would correspond to a daily intake of 0.540-0.549 mg/kg bw of pulegone and 1.44-1.46 mg/kg bw of menthofuran. Clearly, this recommended daily dose of peppermint oil in herbal medicinal products results in an intake of pulegone/menthofuran that exceeds the TDI (0.1 mg/kg) set for food by the Committee of Experts on Flavouring Substances (CEFS).

By analogy, based on Ph. Eur. 8.1 limits for mint oil, partly dementholised (maximum 2.5% of pulegone contents and relative density 0.888-0.910 g/cm^3), the daily intake of pulegone could be calculated in the case of existing recommended daily dose in EU.

In addition to the use in medication, humans are exposed to pulegone as part of the essential oil in flavourings, confectionery, and cosmetics (Karousou *et al*., 2007; Barceloux, 2008). According to JECFA, the estimated per capita intake of pulegone is reported as 2 µg/day and 0.04 µg/kg bw/day for Europe, and 12 µg/day and 0.03 µg/kg bw/day for the USA (IPCS, 2001).

The Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in contact with Foods (SPFA, 2005) noted that in certain cases the maximum permitted levels of pulegone in food may lead to high intakes in subjects consuming regularly mint flavoured beverages or confectionery. For example, 500 ml/day of mint flavoured beverage and 100 g/day of mint confectionery could lead to intakes of respectively 4.2 mg/kg bw and 1.2 mg/kg bw for a 30 kg child (SPFA, 2005).

In conclusion, humans are exposed to pulegone and menthofuran in herbal medicinal products and food, and as part of the essential oil in flavourings, confectionery, and cosmetics. Estimates of per capita intakes are widely variable (see above) and thus are difficult to take into consideration in an overall exposure assessment.

1.3. Regulatory status

There are currently no limits for pulegone and menthofuran in the area of medicinal products apart from some quality criteria for herbal substances such as Ph. Eur. monographs (see 1.2).

Limits in the use of pulegone in food products have been issued for different applications. According to regulations EC1334/2008, the use of pulegone in food and beverages has limits set of: 100 mg/kg for

mint/peppermint containing alcoholic beverages; 20 mg/kg for mint/peppermint containing non-alcoholic beverages; 2000 mg/kg for micro breath freshening confectionery; 350 mg/kg for chewing gum; and 250 mg/kg for mint/peppermint containing confectionery, except the micro breath. As a pure ingredient, pulegone may not be added to foodstuffs. According to the Committee of Experts on Flavoring Substances (CEFS), provisional consumption limits were established for pulegone at 20 mg/kg in food and beverages (European Commission, 2002; 2008).

In cosmetic formulations, the concentration of pulegone should not exceed 1% (Nair, 2001).

In the USA, pulegone is not authorized as a synthetic flavouring substance (DHHS-FDA, 2012).

2. Discussion

Since 2005, a number of significant publications on pulegone and menthofuran have appeared in the scientific literature.

2.1. Toxicokinetics of pulegone and menthofuran

There are no formal toxicokinetic studies performed in humans, There are few studies in which serum levels and/or urinary excretion of the parents and metabolites has been analysed (see below) and tentative metabolic pathways have been uncovered to a considerable extent (see below). Pulegone and menthofuran are absorbed from the gastrointestinal tract, but there are no studies available to estimate oral bioavailability. There are no studies on dermal penetration, but the use of pulegone as a dermal absorption enhancer seems to suggest that it may be absorbed. There are no inhalation studies available.

***In vivo* human observations**

In an *in vivo* study by Engel (2003), 0.5 mg/kg body weight (bw) of (*R*)-(+)-pulegone or 1 mg/kg bw of (*S*)-(-)-pulegone were administered orally to six human volunteers. Six metabolites were identified in the urine. The major metabolite of (*R*)-(+)-pulegone was 10-hydroxypulegone. Another major metabolite, 9-hydroxy-*p*-menthan-3-one, is formed through the oxidation of 10-hydroxypulegone via the reduction of the exocyclic double bond. Menthofuran and its metabolites were found in relative small amounts in the urine. However, menthofuran was present in the serum of two individuals, hours after ingestion of a large amount of pennyroyal oil (Andersson *et al*., 1996). In a fatally poisoned patient 18 ng/mL of pulegone and 1 ng/mL of menthofuran were found in serum analysed at 26 hours post-mortem, 72 hours following acute ingestion. In another case, 40 ng/ml menthofuran were found in serum with no detectable pulegone levels, 10 hours after ingestion.

Toxicokinetics in experimental animals *in vivo*

Several studies on metabolism and urinary and bile excretion of C^{14}-labelled pulegone have been performed in rats and mice. Doses ranged from 0.8 mg/kg bw intravenously to 8-250 mg/kg bw by gavage (Thomassen *et al.,* 1991; Chen *et al.,* 2001; 2003a; 2003b). The half-life of pulegone after the iv administration was about 2 hours. After gavage administration in mice, clearance of pulegone was practically complete in 24 hours whereas in rats about 60-80% of the dose was excreted in 24 hours (Chen *et al.,* 2003b). In rats, 45-60% of the dose was excreted via urine during the first 24 hours and 5-14% in the period of 24-72 hours. Biliary conjugates, principally glucuronide or glutathione conjugates of hydroxylated pulegone or reduced pulegone, accounted for about 3% of the dose (Thomassen *et al.,* 1991). Tissue levels of pulegone-derived radioactivity were highest in the liver of both species and both sexes, but high levels were also observed in male rat kidney (Chen *et al*., 2003a).

2.2. Metabolism of pulegone and menthofuran

Generally, the metabolism of pulegone and menthofuran has been elucidated in a considerable detail in *in vivo* and vitro studies (Fig. 2). Pathways leading to metabolic activation, covalent binding and hepatic effects have been investigated also in various *in vivo* animal studies.

The metabolism of pulegone is rather complex in terms of pathways and metabolites, but it could be classified into several major metabolic pathways (Thomassen *et al*., 1990; Speijers, 2001; Chen *et al.,* 2011):

1. the pathway leading to the formation of menthofuran involving the 9-hydroxylation with a subsequent reduction of carbon-carbon double bond and furan ring formation (Gordon *et al*., 1987; Madyastha & Raj, 1992;1993);
2. reduction of pulegone to menthone and isomenthone followed by hydroxylation in ring or side chain and subsequent conjugation with glucuronic acid (SPFA 2005);
3. hydroxylation at C-5 or methyl (9- or 10-) to hydroxylated metabolites, followed by conjugation with glucuronic acid or with glutathione (GSH); the conjugates being further metabolized;
4. the formation of piperitenone (*p*-mentha-1,4(8)-dien-3-one) after 5-hydroxylation followed by dehydration (Speijers 2001); piperitenone is further metabolized by ring and side-chain hydroxylations (4, 5, 7, 10-positions).

It should be noted that the order of metabolic reactions in the above pathways may not be obligatory, but for example reduction of pulegone may follow hydroxylation or vice versa. More distal metabolites are nevertheless identical.

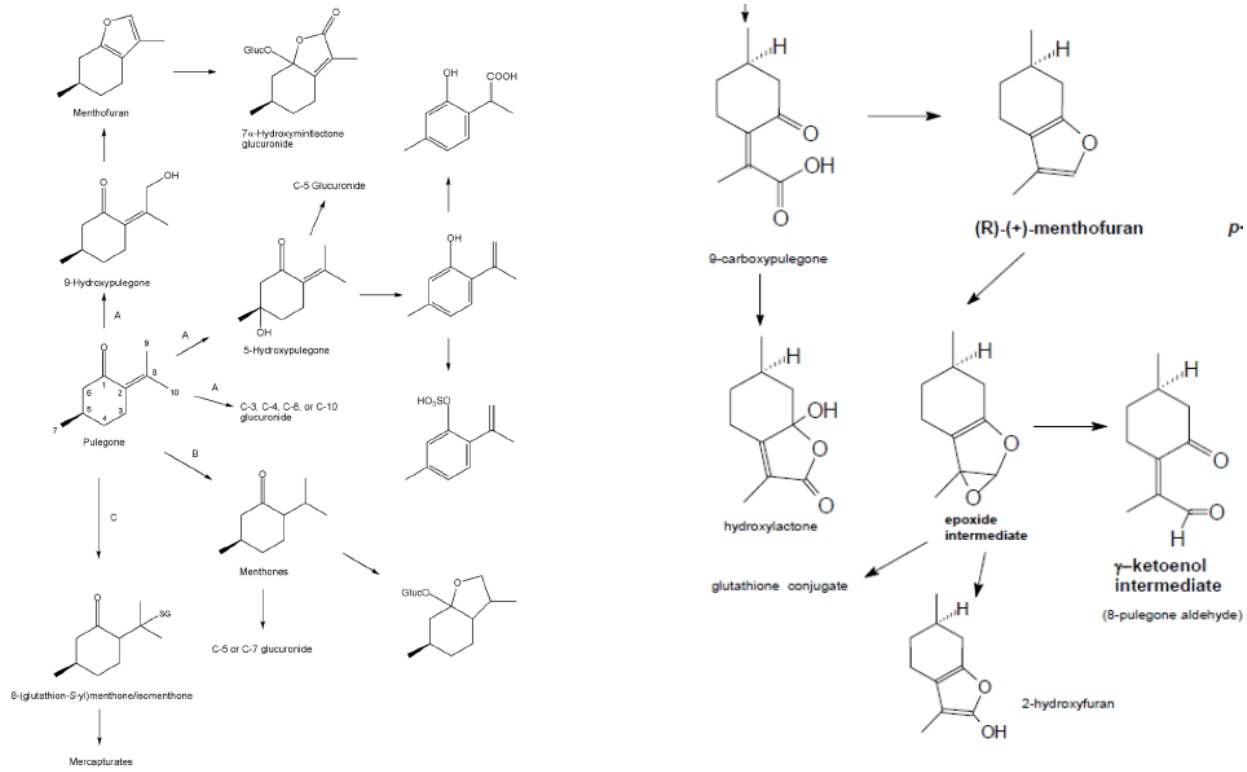

Fig. 2: Principal metabolic pathways of pulegone (left) and menthofuran (right) (modified from SPFA, 2005)

Many of the metabolites of pulegone are derived from menthofuran and piperitenone. In *in vivo* rodent studies on pulegone metabolism, a total of approximately fourteen phase I metabolites exist, with approximately ten identified phase II metabolites (Thomassen *et al.,* 1991; Chen *et al.,* 2001; Zhou *et al.*, 2005). Administration of menthofuran to rats in doses of 6 or 60 mg/kg bw yielded 3 sulphonic acid metabolites and several glucuronide conjugates of hydroxylated mint lactones. Four of the metabolites were identical to pulegone metabolites (Chen *et al*., 2003b).

There is some evidence that in the metabolism of pulegone, conjugation reactions predominate over menthofuran partway at lower doses of pulegone (Chen *et al*., 2001), i.e. the formation of menthofuran would not be significant at lower, more "realistic" doses. Also the only available human study (Engel, 2003) seems to point to a similar scenario. However, because of many competing and interlinked pathways affecting the formation and degradation of toxicologically important metabolites, it is difficult to make any firm conclusions about this possibility.

Although rodent P450 or other enzymes metabolizing pulegone or menthofuran have not been directly identified, *in vivo* studies with inducers and inhibitors and *in vitro* studies employing microsomes from variously treated animals suggest that phenobarbital-induced enzymes are involved in the pathway(s) leading to increased hepatotoxicity whereas methylcholanthrene-induced enzymes protect against hepatotoxicity.

Studies with the expressed human CYP enzymes and human liver microsomes indicate that pulegone is metabolized by human liver CYP2E1, CYP1A2, and CYP2C19 to menthofuran (Khojasteh-Bakht *et al*., 1999). Menthofuran was metabolized by the same human liver CYPs involved in the metabolism of pulegone and additionally by CYP2A6. Menthofuran inhibits human CYP2A6 irreversibly, possibly by covalent adduction (Khojasteh-Bakht et al., 1998).

2.3. Bioactivation of pulegone and menthofuran

An extensive series of *in vitro* and *in vivo* studies in rodents (and *in vitro* studies with human liver preparations) employing inducers (phenobarbital, methylcholanthrene) and inhibitors of drug metabolism and P450 enzymes, as well as depletors of glutathione have amply demonstrated that bioactivation and covalent binding of pulegone via its metabolite(s) is a prerequisite for its hepatotoxic action (for a review, see Nelson, 1992; SCF, 2002). Most probably the principal pathway to bioactivation is the conversion of pulegone to menthofuran. Subsequently, a gamma-ketoenal (pulegone 8-aldehyde) is generated as a major electrophilic metabolite from both pulegone and menthofuran (Thomassen *et al*., 1988, 1992; Nelson, 1992; Speijers, 2001). This reactive enonal may be derived directly from oxycarbonium ions formed in the CYP-mediated oxidation of menthofuran, or from an epoxyfuran intermediate (Thomassen *et al.,* 1992; Nelson *et al*., 1992). Mintlactones are formed as stable products of the γ-ketoenal, but also may be formed by direct proton loss from an oxycarbonium ion (Chen *et al*., 2011). Additionally, p-cresol is also generated via pulegone metabolism (Madyastha & Raj, 1991) and also depletes glutathione with minor hepatotoxic effects (Chen *et al*., 2011). It is possible that other additional pathways for bioactivation are operative (see Nelson et al., 1992; SCF, 2002).

In a recent experimental study, several oxidative metabolites of menthofuran were characterized in rat and human liver microsomes and in rat liver slices exposed to cytotoxic concentrations of menthofuran (Khojasteh *et al*., 2010). Metabolites that were identified were monohydroxylation products of the furanyl and cyclohexyl groups, mintlactones and hydroxymintlactones, a reactive γ-ketoenal, and a glutathione conjugate. A similar spectrum of metabolites was found in urine 24 hours after the administration of hepatotoxic doses of menthofuran to rats. In no case was p-cresol (or any of the other reported unusual oxidative metabolites of menthofuran) detected above background

concentrations that were well below concentrations of p-cresol that cause cytotoxicity in rat liver slices. Thus, the major metabolites responsible for the hepatotoxic effects of menthofuran appear to be a γ-ketoenal and/or epoxides formed by oxidation of the furan ring. This is in contrast with earlier evidence that p-cresol and other unusual oxidative products are metabolites of menthofuran in rats and that p-cresol may be responsible in part for the hepatotoxicity caused by menthofuran (Madyastha & Raj, 1991).

Adducts of pulegone and menthofuran

As a final step in the bioactivation, reactive metabolites are bound covalently to cellular macromolecules or trapped by small-molecular scavengers such as glutathione. In a recent study, at least 10 GSH-conjugates and one semicarbazide adduct of pulegone based on variable parent structures were detected by LC-MS analyses in *in vitro* incubations with human liver microsomes. Furthermore, 7 GSH-conjugates and 1 CN and 3 semicarbazide-trapped reactive metabolites derived from menthofuran were detected in similar incubations (Rousu *et al*., 2009).

A novel approach based upon metabolomic technologies to screen CN- and semicarbazide-trapped reactive metabolites has been recently developed; the bioactivation of pulegone was reexamined by using this metabolomic approach and a large number of trapped reactive metabolites, GSH-conjugates and aldehydes, including gamma-ketoenal (pulegone 8-aldehyde), were readily identified (Li *et al*., 2011). Khojasteh *et al*., (2012) detected 10 rat liver proteins spots by an antiserum developed to detect protein adducts resulting from menthofuran bioactivation. Four of them were identified by LC-MS/MS analysis of tryptic peptides as serum albumin, mitochondrial acetaldehyde dehydrogenase, cytoplasmic malate dehydrogenase and subunit d of mitochonfrial ATP synthase.

The overall consensus on bioactivation of pulegone and menthofuran is that metabolic pathways leading to reactive metabolites have been elucidated to a considerable detail and the most probable hepatotoxic metabolite is derived from menthofuran, although some additional toxic metabolites may contribute to hepatotoxicity. Pulegone and menthofuran have been used as illustrative examples of metabolic bioactivation of herbal components (Zhou *et al*., 2007; Chen *et al*., 2011).

2.4. Human toxicity

No new information is available. Thus, the following is based on the previous HMPC public statement (2005).

A literature review of cases of human intoxication with pennyroyal oil (pulegone content 62-97%) indicate that ingestion of 10 ml (corresponding to ca 5.4-9 g pulegone, ca 90-150 mg/kg bw for a 60 kg person; calculated with a relative density of 0.9 as for peppermint oil) resulted in moderate to severe toxicity and ingestion of greater than 15 ml (corresponding to ca. 8-13 g pulegone, ca 130-215 mg/kg bw for a 60 kg person) resulted in death. The clinical pathology was characterised by massive centrilobular necrosis of the liver, pulmonary oedema and internal haemorrhage (SCF, 2002).

No confirmed cases of liver damage caused by peppermint oil or mint oil have been reported.

2.5. Subchronic and chronic toxicity and carcinogenicity of pulegone (NTP 2011)

3-Month study in rats and mice

Groups of 10 male and 10 female rats were administered 0, 9.375, 18.75, 37.5, 75, or 150 mg pulegone/kg body weight in corn oil by gavage, 5 days per week for 14 weeks. No treatment-related

mortality was observed. At the two highest doses (75 and 150 mg/kg) several adverse effects could be observed: weight reduction, increased absolute and relative liver and kidney weights, hyaline glomerulopathy, bile duct hyperplasia, hepatocyte hypertrophy and many others. Some of these effects were seen also in lower doses; NOAEL values are either 18.75 or 37.5 mg/kg depending on whether some small but significant tissue weight changes were regarded toxicologically significant.

In a similar 3-month study in mice the only treatment-related observations were the increase of liver weight and glutathione levels at the highest dose in males and at the 2 highest doses in females.

2-Year studies in rats and mice

Pulegone dissolved in corn oil was administered intragastrically to groups of 50 male and female rats and mice for up to two years. Male rats received 18.75, 37.5, or 75 mg of pulegone per kg of body weight five times per week; female rats and male and female mice received 37.5, 75, or 150 mg/kg five days per week. Control animals received corn oil. After 60 weeks many of the male rats receiving 75 mg/kg and female rats receiving 150 mg/kg had died, so the surviving animals from those groups received corn oil for the duration of the study (stop-exposures).

A highly unusual effect, hyaline glomerulopathy, was the most conspicuous non-neoplastic finding in both rats and mice. Kidney damage seemed also to be behind the progressive morbidity and mortality of rats at the highest dose (stop-exposure groups). No NOAEL values could be determined, because hyaline glomerulopathy was seen also at the lowest dose of pulegone in female rats and in male and female mice. Thus the lowest LOAEL was 18.75 mg/kg bw. Visual dose-response inspections[3] suggest that benchmark dose limit of 10% response varied from <10 mg/kg bw (female mice) to about 45 mg/kg bw (male mice). Values for rats were of the order of 20 to 30 mg/kg bw.

Carcinogenicity

There were statistically increased incidences of several neoplasms. Female rats receiving pulegone had increased incidences of urinary bladder tumors. Relationship of these tumours with hyaline glomerulopathy and progressive kidney damage is suggestive, but not proven. Also the relevance to human is not known, although urinary tract carcinogenesis in humans has been associated with other genotoxic carcinogens. Male and female mice had increased incidences of benign and malignant tumors of the liver, and female mice also had a small increase in rare bone lesions (osteoma or osteosarcoma).

As to the possible mechanisms of carcinogenesis, the authors of the NTP study are of the opinion that pulegone acts as a genotoxic carcinogen in the female rat bladder. Although pulegone has not been uniformly positive in genotoxicity studies (see below), the evidence of the extensive formation of reactive metabolites from pulegone both *in vitro* and *in vivo* is convincing. Thus, despite equivocal outcome of the Ames test, the authors of the NTP studies considered it reasonable to believe that pulegone is metabolically activated to reactive intermediates which bind to DNA and other macromolecules and initiate carcinogenic process. On the other hand, reactive metabolite-associated cytotoxicity and regenerative proliferation was thought to be a likely mechanistic background for the liver tumorigenesis in male and female mice (NTP, 2011).

2.6. Genotoxicity of pulegone and menthofuran

SCF (2002) summarized the genotoxicity studies. Pulegone was negative in the Ames assay using Salmonella typhimurium strains TA1537, TA1535, TA100, TA 98 and TA97 with and without metabolic activation at concentrations of up to 800 µg/plate (Andersen & Jensen, 1984).

[3] no formal analysis of benchmark dose limit values was performed.

Neither (R)-(+)-pulegone nor (R)-(+)-menthofuran were mutagenic in the Ames assay using S. typhimurium TA100 and TA98 at concentrations of up to 1000 µg/plate, with and without metabolic activation (Council of Europe, 1999).

In connection with the NTP study (2011), 3 independent Ames tests were carried out. Pulegone was negative in two Ames tests (the first study: TA97, TA98, TA100, TA1535, 10 or 30% rat or hamster S9; the second study: TA98, TA100, E.coli WP2uvrA/pKM101, rat S9) and in the third study pulegone was marginally positive (500 µg/plate and higher) in TA98 and E.coli WP2uvrA/pKM101 with rat S9. However, the IARC Working Group (2014) considers pulegone non-mutagenic in these standard tests.

In vivo micronucleus test in mice was negative with pulegone (9.375 to 150 mg/kg).

The overall conclusion on the basis of the above genotoxicity studies remains that despite some marginal positive observations genotoxicity of pulegone has not been demonstrated and furthermore, tests are not up to the current standards.

2.7. Mode of action considerations

In chronic studies (3-month, 2-year) in rats and mice the principal target organs were liver and kidney. Neoplasms were observed in female rats (kidney) and male and female mice (liver). It has been suggested that bioactivation of pulegone (and menthofuran) to reactive metabolites is behind liver and kidney effects, histological and functional changes, frank injury and neoplasms. While non-neoplastic injuries have been linked directly with bioactivation, the role of bioactivation in genotoxicity and carcinogenesis remains uncertain, because *in vitro* and *in vivo* genotoxicity studies with pulegone have generally been negative. However, it is questionable whether conventional genotoxicity tests performed with pulegone and menthofuran are appropriate to demonstrate the genotoxic potential of pulegone-associated liver-derived metabolites. It seems likely that liver-produced short-lived reactive metabolites may not reach the DNA in the conventional Ames test or bone marrow in the *in vivo* mouse micronucleus test. More appropriate tests to assess the potential genotoxicity of pulegone are probably the Comet assay or a transgenic gene mutation assay for both liver and bladder. Without such data it is not possible to conclude definitely on the genotoxic potential of pulegone and its metabolites.

Recently, the International Agency of Research on Cancer (IARC, 2014) has evaluated pulegone and has classified pulegone as a group 2B carcinogen, i.e. possibly carcinogenic to humans (Grosse *et al*., 2013). The IARC Working Group concluded that pulegone was not mutagenic in standard bacterial assays, either with or without exogenous metabolic activation. Regarding a potential mechanism of action the Working Group concluded that studies in humans and rodents indicate that some of the pulegone metabolites deplete hepatic levels of glutathione and can bind to cellular proteins. This may result in chronic regenerative cell proliferation, which may be related to the carcinogenicity observed in the liver and other organs in experimental animals. (IARC, 2014).

Regenerative cell proliferation can also be considered as a plausible mechanism of action for pulegone-induced bladder tumours in female rats. A recent mechanistic study (Da Rocha *et al*., 2012) bladders from treated rats showed superficial cell layer necrosis and exfoliation and a significant increase in cellular proliferation in the high dose group (150 mg/kg bw). Urine of treated animals contained pulegone, piperitone, piperitenone and menthofuran; piperitone was present at cytotoxic levels in the high-dose group.

Even if pulegone would prove to be genotoxic in future appropriate genotoxicity assays, it is plausible that at lower realistic exposure levels cellular protective mechanisms, trapping by glutathione and other scavengers of reactive metabolites, would constitute a practical threshold below which no

genotoxicity would become manifest. Consequently, in this case a scenario to be used in risk assessment would be the threshold-based limit use of an uncertainty factor.

2.8. Relevance of experimental toxicities for human risk assessment

Are the tumours observed in animal experiments relevant for human risk assessment?

Hepatocellular tumours, especially adenomas, are often regarded rodent (mouse)-specific tumours especially if a rodent-specific mechanism of action (liver enzyme induction) could be elicited. There are no studies on liver enzyme induction in mice or rats, and evidence for genotoxicity of pulegone and menthofuran is essentially negative and also less than satisfactory (but see some reservations above). The type of bladder tumours in rats seem to be very rare and their possible association with hyaline nephropathy, a very rare human condition, seem to suggest that these tumours are not relevant for human risk assessment. Consequently, tumours in animals seem not to be relevant for human risk assessment.

Is the mode of action for tumour formation relevant for human risk assessment?

For pulegone and menthofuran, metabolic activation pathway and adduct formation with trapping agents such as GSH and proteins are demonstrated in animals and a similar pathway is operative in human *in vitro* systems. There is no studies on DNA adducts of pulegone or menthofuran. The IARC working group (IARC, 2014) concluded that there is no evidence of genotoxicity for pulegone (and presumably also for menthofuran, its major metabolite) and the Working Group regarded chronic regenerative cell proliferation as a possible mechanism of action for observed rodent cancers. Furthermore, pulegone and its metabolites have been demonstrated to cause bladder cytotoxicity and cell proliferation, suggesting a non-genotoxic mechanism of action. Consequently, pulegone is likely a non-genotoxic carcinogen in rodents and there exists a threshold for its carcinogenic action. Consequently, tumour findings in animals as such are not relevant for carcinogenicity risk assessment for humans, but naturally they can be used to determine NOAEL or BMDL values, if applicable.

Are toxicokinetic data (metabolic behaviour, activation etc) conducive to extrapolation of animal data to humans?

Although metabolism and toxicokinetics of pulegone and menthofuran have not been adequately elucidated in humans, there is evidence that at least metabolic routes are qualitatively similar in humans and rodents. There are some older evidence that at low, realistic exposures of humans to pulegone, menthofuran is not an important metabolite, thus suggesting a dose-dependent metabolic activation (Engel, 2003). In this study, the single pulegone dose administered was more similar to dietary exposure i.e. ~500 µg/kg bw. (Engel, 2003). However, the significance of this study in proving that at lower doses the conversion or pulegone to menthofuran is proportionally lower than in higher doses seems rather questionable. More definitive studies are needed.

2.9. Summary of weight-of-evidence toxicity risk assessment of pulegone and menthofuran

A modified weight-of-evidence (WoE) assessment is formally presented in Table 1 taking into account the findings and argumentations above.

Table 1: Summary of weight-of-evidence (WoE) evaluation of genotoxicity and carcinogenicity of pulegone (and menthofuran)

Structure/grouping	related compounds (isopulegone etc) are also hepatotoxic; carcinogenicity is not known
Computational models	no studies available
Metabolic activation	Convincing evidence for the activation pathways via oxidation in rodent and human *in vitro* systems and in rodents *in vivo*
Covalent binding	Covalent binding to cell proteins and small-molecular trapping agents demonstrated
DNA binding *in vitro*	No information
DNA binding *in vivo*	No information
Genotoxicity *in vitro*	Generally negative; few positive findings in the Ames test, whichNTP considers significant, i.e.pulegone is genotoxic The IARC working Group regards pulegone as non-genotoxic Current conclusion: genotoxic potential cannot be evaluated
Genotoxicity *in vivo*	Micronucleus tests consistently negative, but may not be appropriate for pulegone-type compound
Carcinogenicity in rodents	Clear evidence of carcinogenicity in male and female mice (liver) and in female rats (bladder)
Human information	Metabolic activation pathway present Metabolic activation in human *in vitro* systems is qualitatively similar to the one in rodents
Other information	Some evidence of non-linearity of metabolic activation and adduct formation
WoE conclusion	IARC: class 2B (possibly carcinogenic to humans) NTP proposal to be included in the Report of Carcinogens (RoC) Mechanism of action uncertain (NTP considers genotoxicity as a MoA of bladder tumours and regenerative cell proliferation as a MoA of liver tumours; IARC consider cell proliferation driven by metabolic activation and GSH depletion as a possible mechanism of action) Tentative decision: toxicity and carcinogenicity of pulegone have a (practical) threshold

2.10. Determination of the limit value

The NTP study showing carcinogenicity of pulegone and the recent IARC classification of pulegone as a 2B carcinogen, possibly carcinogenic to humans, have raised concerns about the implications for public health of intake of preparations containing pulegone and menthofuran. The uncertainties about the genotoxic potential highlight the urgent need for comprehensive and reliable genotoxicity studies on pulegone. However, the IARC Working Group concluded that according to the available evidence, pulegone is not mutagenic and consequently, pulegone is a non-genotoxic carcinogen. Furthermore, tumours found in rodent studies probably may be considered not relevant for humans. Even if the future studies would demonstrate the genotoxic potential of pulegone, efficient scavenging of potentially DNA-reactive metabolites at lower pulegone exposures is likely to create a practical

threshold. Consequently, the risk assessment scenario adopted below is based on the above considerations. The limit value should be reviewed when adequate genotoxicity and other studies are available.

The value of 20 mg/kg bw per day, based on the NTP chronic study, is taken as a LOAEL value. It is possible to use a safety factor of 300 (not 100, because of LOAEL was the lowest significant effect level). Consequently the acceptable exposure would be 0.07 mg/kg bw per day, which is close to the current ADI value of 0.1 mg/kg bw per day. The daily dose for an adult of 50 kg body weight would thus be 3.5 mg/person/day.

It is also possible to use other safety or uncertainty factors, depending on particular considerations about relevancy of tumours to humans, details of exposure characteristics and toxicokinetics of pulegone or for other reasons. Relevancy of tumours to humans should be thoroughly evaluated in the light of the IARC monograph on pulegone. Bioavailability from various formulations and possible non-linearities in the formation of reactive metabolites from pulegone and menthofuran may modify actual exposures and consequently limit values.

3. Conclusions and recommendations of the HMPC

3.1. Toxicological conclusions

1. On the basis of recent rodent chronic studies (NTP, 2011), target organs for pulegone and menthofuran are liver and kidney and a plausible mechanism for toxicity is the formation of reactive metabolites, which is also supported by recent *in vitro* experimental data.
2. Neoplasms were observed in female rats (kidney) and male and female mice (liver) in the NTP study. While non-neoplastic injuries have been linked directly with bioactivation, its role in genotoxicity and carcinogenesis remain uncertain, because *in vitro* and *in vivo* genotoxicity studies with pulegone and menthofuran have generally been negative. More detailed genotoxicity studies on pulegone and menthofuran and on preparations containing these ingredients are needed for proper risk assessment.
3. Relevance of rodent neoplasms to human carcinogenesis remain uncertain because target rodent neoplasms (bladder in female rats, liver in mice) are often not relevant to human situation and mode of action of tumorigenesis has not been adequately resolved. Assessment of human relevance of tumours observed in the NTP carcinogenicity study on pulegone is of primary priority.
4. There are no new data on pharmacovigilance, but prevailing opinion is that no certain cases of liver toxicity in humans have appeared by peppermint oil or mint oil.
5. As an interim recommendation, the HMPC suggests that an acceptable exposure limit is 0.07 mg/kg bw per day, which is close to the current ADI value of 0.1 mg/kg bw per day. This limit value should be reviewed when adequate genotoxicity studies are available and relevance of rodent tumours to human carcinogenicity has been assessed.

3.2. Recommended limit values

Because of uncertainties about the mode of action regarding carcinogenicity of pulegone (and menthofuran), the limit values to pulegone and menthofuran (for regulatory purposes, pulegone and menthofuran should be taken together, pulegone + menthofuran) should be regarded as provisional

and exposure to them should be kept as low as practically achievable. In the evaluation of herbal medicinal products containing pulegone and menthofuran Member States should take steps to ensure that the public are protected from exposure and the following thresholds should be applied.

Oral use

The value of 20 mg/kg bw per day, based on the NTP chronic study, is taken as a LOAEL value. It is possible to use a safety factor of 300 (not 100, because of LOAEL was the lowest significant effect level). Consequently the acceptable exposure is 0.07 mg/kg bw per day, which is close to the current ADI value of 0.1 mg/kg bw per day. The daily dose for an adult of 50 kg body weight[4] is thus 3.5 mg/person/day.

The intake (pulegone + menthofuran) of 3.5 mg/person/day (even if the limit presents the overall intake from all sources) can be accepted for herbal medicinal products as short-term intake (maximum 14 days).

Dietary background

The potential daily intake of pulegone and menthofuran via food cannot be ignored especially as consumers/patients are not able to avoid them. Although comprehensive estimates of pulegone and menthofuran intake via food and other products are not available, improved estimates should be taken into consideration when available.

Sensitive groups: Children

If children are included in the usage of certain products the daily amount of pulegone + menthofuran has to be adjusted to the body weight of the age group: e.g. body weight of 20 kg would lead to an acceptable daily intake of 1.5 mg/day.

Pregnant and breast feeding woman

Sensitive groups such as pregnant and breast feeding woman are also covered by the limit calculated above. If these limits are complied with, the chapter 4.6 of the SmPC of the products concerned should be phrased according to the 'Guideline on risk assessment of medicinal products on human reproduction and lactation: from data to labelling' (EMEA/CHMP/203927/2005).

Cutaneous use

No quantitative data concerning absorption of pulegone and menthofuran through the skin exist although it is known that pulegone has been used as a "penetration enhancer". It is to ensure that the sum of pulegone and menthofuran within the daily dose is <3.5 mg for adults. The short term use (maximum 14 days) is restricted to intact skin.

Higher contents within the products would be possible if for the relevant product (means the relevant matrix, because absorption might be greatly influenced by the excipients, for instance essential oils as enhancers) low absorption rates can be shown, not exceeding the daily intake of 3.5 mg for adults.

Sensitive groups: Children

If children are included in the usage of certain products the daily amount has to be adjusted to the body weight of the age group: e.g. body weight of 20 kg would lead to an acceptable daily intake of 1.5 mg/day.

[4] For ~18% (average) of the European population the body weight is given with less than 60 kg [EUROPEAN COMMISSION 2006]. These number would increase to up to 30%, if only taking into account woman. Therefore the calculation is linked to a body weight of 50 kg.

Pregnant and breast feeding woman

Sensitive groups such as pregnant and breast feeding woman are also covered by the limit calculated above. If these limits are complied with, the chapter 4.6 of the SmPC of the products concerned should be phrased according to the 'Guideline on risk assessment of medicinal products on human reproduction and lactation: from data to labelling' (EMEA/CHMP/203927/2005).

3.3. Proposal for regulatory actions

- Improved estimates for dietary (background) exposures to pulegone and menthofuran are necessary for the comprehensive assessment and threshold values of pulegone and menthofuran.
- Focussed pharmacovigilance of peppermint oil and mint oil containing products is recommended.
- An increased awareness in the medical community concerning high intake of peppermint oil and mint oil containing products as a potential cause of otherwise unexplained liver reactions would be desirable.
- Companies marketing products containing pulegone and menthofuran should check and provide data whether their products are complying with the above mentioned exposure limit.

Proposals are preliminary. Further proposals will be concluded after final decision about recommended limit values.

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