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Committee on Herbal Medicinal Products (HMPC)

Public statement on the use of herbal medicinal products[1] containing pulegone and menthofuran

Final

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[1] Throughout the document and unless otherwise specified, the term "herbal medicinal product" includes "traditional herbal medicinal product".

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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 pulegone and menthofuran. These constituents are found in herbal ingredients including peppermint oil (*Mentha x piperita* L.), mint oil (*Mentha canadensis* L., syn. *Mentha arvensis* var. *glabrata* (Benth.) Fern., *Mentha arvensis* var. *piperascens* Malinv. ex Holmes), pennyroyal oil (*Mentha pulegium* L. or *Hedeoma pulegoides* (L.) Pers).

The most important conclusions of the Public Statement in 2005 were the following: The reported NOEL of pulegone and menthofuran (20 mg/kg body weight (bw)/d) has not been determined with required accuracy, and remains uncertain. Despite that a TDI (tolerable daily intake) for pulegone and menthofuran has been set for food (0.1 mg/kg bw) and doses up to ca 2.3 mg/kg bw/day of pulegone[2] (exceeding the TDI for food) are commonly encountered in herbal medicinal products in Europe. No immediate actions were proposed, but HMPC recommended alerted pharmacovigilance and 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. In addition, HMPC recommended that a limit for menthofuran should be included in the monograph for mint oil of the European Pharmacopoeia[3] (HMPC, 2005).

Since 2005, a number of significant publications on pulegone and menthofuran have appeared in the scientific literature. The new data has raised concerns from a toxicological point of view and this has prompted the HMPC to re-assess all available data regarding their relevance for the safe human use of herbal medicinal products containing pulegone and menthofuran.

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 flavouring foods and drinks and for herbal medicinal products. Peppermint and Peppermint oil are also used as active ingredients in non-herbal medicinal products and are widely used as excipients in medicinal products.

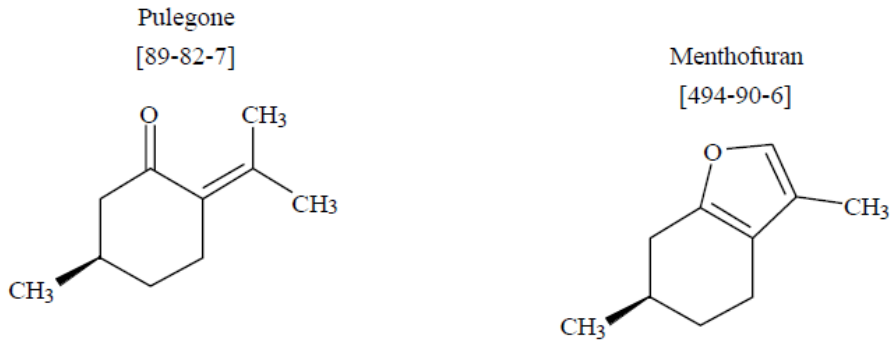

Fig: 1: structural formula of pulegone and menthofuran

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

Pulegone and menthofuran are significant constituents of several mint (*Mentha*) species and their derived volatile oils, including peppermint (*M. piperita*), spearmint (*M. spicata*), European pennyroyal (*M. pulegium*) and American pennyroyal (*H. pulegioides*). 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, 2015.

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, gelatines, and puddings) and of alcoholic and non-alcoholic beverages flavoured 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 (*M. piperita*) and mint oil (*M. canadensis*). Pennyroyal oil (*M. pulegium* or *H. pulegoides*) 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 known recommended daily dose in the EU is 1.2 ml peppermint oil (also reflected in the EU herbal monograph EMEA/HMPC/349466/2006); i.e. 1099 mg peppermint oil (based on relative density 0.916 - according Ph. Eur. 8.1 (2014)) contains maximum 32.97 mg pulegone and 87.92 mg menthofuran (according to Ph. Eur. 8.1 limits for pulegone and menthofuran in peppermint oil). For a 50 kg person this would correspond to a daily intake of 0.66 mg/kg bw of pulegone and 1.76 mg/kg bw of menthofuran. Clearly, this 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), the daily intake of pulegone could be calculated in the case of existing recommended daily dose in EU.

In addition to the use of peppermint oil or herbal preparations of peppermint leaves in medications as active substance or excipients, 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 prepared and published an opinion on Pulegone and Menthofuran in flavourings and other food ingredients with flavouring properties (SPFA, 2005). It was 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). 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 studies to establish a NOEL for (R)-(+)-menthofuran in 90-day oral toxicity study in

rats, 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), and further refinement of intake estimates from all dietary sources including actual usage levels and analytical data on concentrations in relevant products.

In conclusion, humans are exposed to pulegone and menthofuran in medicinal products and in 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

So far, there have been no limits for pulegone and menthofuran in the medicinal products area apart from quality criteria for herbal substances such as in 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, a joint MDI (maximum daily intake) for pulegone and menthofuran of 0.1 mg/kg bodyweight is set, and the following limits in foodstuffs are proposed: Menthofuran: foods and beverages in general: 20 mg/kg. Exceptions: Mint/peppermint flavoured alcoholic beverages 100 mg/kg, Mint/peppermint flavoured confectionery 200 mg/kg, Mint/peppermint flavoured chewing gum 1000 mg/kg. Pulegone: foods and beverages in general: 20 mg/kg. Exceptions: Mint/peppermint flavoured alcoholic beverages 100 mg/kg, Mint/peppermint flavoured confectionery 100 mg/kg, Intensely strong mint/peppermint flavoured confectionery 200 mg/kg, Mint/peppermint flavoured chewing gum 30 mg/kg. As a pure ingredient, pulegone and menthofuran shall not be added to foodstuffs. According to the Committee of Experts on Flavouring Substances (CEFS), provisional consumption limits were established for pulegone at 20 mg/kg in food and beverages (Council of Europe 2005; 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

2.1. Toxicokinetics of pulegone and menthofuran

There are no formal toxicokinetic studies performed in humans, but there are few studies in which serum levels and/or urinary excretion of the parents and metabolites have 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 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 (i.v.) 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 i.v. 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 in-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. 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.

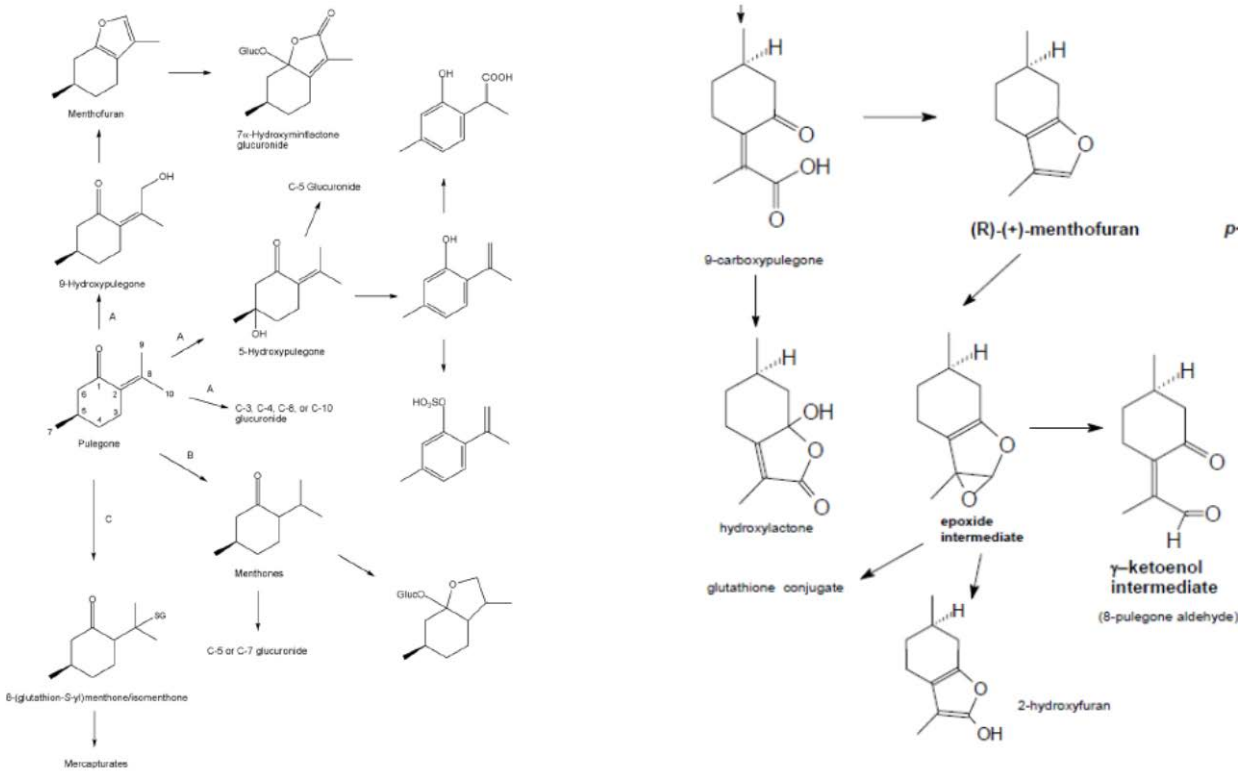

Fig. 2: Principal metabolic pathways of pulegone (left) (modified from NTP, 2011) and menthofuran (right) (modified from SPFA, 2005). A = hydroxylation followed by conjugation or further metabolism. B = reduction followed by hydroxylation/glucuronidation. C = glutathione conjugation and metabolism to mercapturic acids.

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 phases 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 the menthofuran pathway 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 *et al*., 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 *et al*., 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 p-cresol (or any of the other reported unusual oxidative metabolites of menthofuran) was 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 re-examined 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 mitochondrial 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

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).

A near fatal case due to suicidal ingestion of peppermint oil was reported recently (Nath *et al*., 2012). The patient ingested an unknown amount of peppermint oil (content of pulegone and menthofuran unknown) and came in a comatosed state and was in shock. After management with mechanical ventilation and ionotropes, her vital parameters reached normal within 8 hours and she became conscious by 24 hours. No evidence of nephrotoxicity (normal serum creatinine and absence of dyselectrolytemia) or hepatotoxicity (normal SGOT and SGPT) was observed.

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

2.5. Subchronic and chronic toxicity and carcinogenicity of pulegone

Subchronic studies in rats and mice

In a 3-month study performed by the National Toxicology Program (NTP, 2011), groups of 10 male and 10 female F344/N rats were administered 0, 9.375, 18.75, 37.5, 75, or 150 mg pulegone/kg bw 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 (relative liver and kidney weights, incidences of bone marrow hyperplasia) were regarded toxicologically significant. The NTP study scientists did not establish NOAEL values.

In a companion 3-month study in B6C3F1 mice with the same dosages the only treatment-related observations were the increase of liver weight and glutathione levels at the highest dose of 150 mg/kg bw in males and at the 2 highest doses of 75 and 150 mg/kg bw in females (NTP, 2011).

In an earlier 28-day study (Thorup *et al*., 1983), male and female SPF rats were treated by gavage with pulegone. The animals treated at 80 or 160 mg/kg per day had atonia, decreased blood creatinine content, lower terminal body weights, and histopathologic changes in the liver, while the lowest dose tested, 20 mg/kg per day, did not produce these effects. In another 28-day study, 0 or 160 mg/kg pulegone was given orally by gavage to groups of 28 female Wistar rats; treatment-related increases in plasma glucose, alkaline phosphatase, and ALT were observed along with a decrease in plasma creatinine (Mølck *et al*., 1998). A NOAEL was set with 20 mg/kg bw/day of pulegone.

2-Year studies in rats and mice (NTP, 2011)

Pulegone dissolved in corn oil was administered intragastrically to groups of 50 male and female F344/N rats and B6C3F1 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).

Chronic toxicity

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). Hyaline glomerulopathy was seen at the dose of 37.5 mg/kg bw of pulegone in male and female rats and in male and female mice. No toxicologically significant effects were observed in male rats at the dose of 18.75 mg/kg bw; thus the NOAEL was 18.75 mg/kg bw. Visual dose-response inspections[4] 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.

Liver was another major target organ for chronic toxicity. In rats, incidences of diffuse hepatocyte cellular alteration were significantly increased in 37.5 mg/kg and 75 mg/kg stop-exposure males and 75 mg/kg and 150 mg/kg stop-exposure females. There were significant increases in fatty change, bile duct cyst, hepatocyte necrosis, oval cell hyperplasia, bile duct hyperplasia, and portal fibrosis. In the mouse liver, in addition to neoplastic changes, several non-neoplastic lesions were significantly increased, primarily in the 75 and 150 mg/kg groups. These non-neoplastic lesions included clear cell, eosinophilic, and mixed cell foci; focal fatty change; centrilobular hepatocyte hypertrophy; intravascular hepatocyte; necrosis; pigmentation; bile duct cyst and hyperplasia; and oval cell hyperplasia. Most of these non-neoplastic alterations were dose-dependent.

Carcinogenicity

There were statistically increased incidences of several neoplasms. Female rats receiving pulegone had increased incidences of urinary bladder tumours. A relationship of these tumours with hyaline glomerulopathy and progressive kidney damage was speculated upon by the authors of the NTP studies. This is further discussed below (see section 2.8.). The relevance to human is not known; however urinary tract carcinogenesis in humans has been associated with other genotoxic carcinogens. Male and female mice had increased incidences of benign and malignant tumours 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.

[4] No formal analysis of benchmark dose limit values was performed.

2.6. Genotoxicity of pulegone and menthofuran

Genotoxicity of pulegone

SCF (2002) summarised 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).

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 (2015) considers pulegone non-mutagenic in these standard tests.

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

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 (see below).

The overall conclusion on the basis of the above considerations remains that despite some marginal positive observations genotoxicity of pulegone has not been convincingly demonstrated.

Genotoxicity of pulegone-containing herbal preparations

Some recent genotoxicity studies of pulegone-containing herbal preparations have been provided to the HMPC.

Three genotoxicity studies with peppermint oil (Ph. Eur. grade, contents of menthofuran and pulegone 3.7% and 0.9%, respectively) have been conducted, an in-vitro bacterial reverse mutation study (Ames test), an in-vitro mammalian cell gene mutation study (mouse lymphoma assay), and an in-vivo mammalian study (rat bone marrow micronucleus test) (Tillotts Pharma AG, 2012, 2013a, 2013b). The results of all studies were negative. The rat bone marrow micronucleus test provided unequivocal evidence of a lack of genotoxicity for peppermint oil when administered orally. The highest dose of 1350 mg/kg bw/day for 2 days corresponded to 62 mg/kg bw/day of pulegone and menthofuran. Overall, the three tests demonstrated that peppermint oil shows no potential for genotoxic effects. However, the appropriateness of the tests remains in doubt (see above).

Escobar *et al*. (2015) examined the genotoxicity of the essential oil (peperina oil) of *Minthostachys verticillata*, containing 64.7% pulegone. Groups of 10 Wistar rats were treated for 90 days by 0, 70, 260 and 460 mg/kg of the essential oil in feed, corresponding to the pulegone doses of 45.2, 168 and 297 mg/kg bw per day. The micronucleus assay in peripheral erythrocytes and the Comet assay on lymphocytes were performed. No genotoxicity even at the highest dose was observed.

A new test on mutagenicity using a preparation of mint oil (JHP Rödler, 95% mint oil (partly dementholised) and 5% ethanol 96% was performed according to latest standards (OECD guidelines 474 and 489). The methods used were an in-vivo mammalian alkaline COMET assay and Micronucleus test. The mint oil was dissolved in sesame oil and doses of 250, 500 and 1000 mg/kg per day of mint

oil, corresponding maximally to 5.91, 11.81 and 23.62 mg/kg bw pulegone, were administered to the CD/ Crl: CD(SD)) rats p.o. 3 times at 0, 24, and 45 hours. However, since the specification of mint oil in the Ph. Eur. includes also batches which do not contain pulegone at all, the exposure of the animals to pulegone cannot be regarded as proven. Furthermore, the Ph. Eur. specification does not contain menthofuran at all. The negative control was sesame oil, a positive control group received ethyl methyl sulfonate. For the COMET assay liver and mucosa cells collected 3 hours after the last dose (i.e. 48 hours after the first administration) were examined. JHP Rödler did not increase DNA tail intensity at any dose compared to vehicle control. For the micronucleus assay erythrocytes were evaluated 48 hours post the first exposure. The mint oil did not increase the incidence of micro nucleated polychromatic erythrocytes at any dose. The mint oil has also been examined in the AMES-test, however, due to the antimicrobial effects the dose range with no interference from antibacterial effects on the *Salmonella* strains was a concentration of up to 100 µg/plate. No mutagenicity was detected under these conditions.

However, the interpretation of the results remains ambiguous because of rat strain differences and the uncertainty and adequacy of pulegone/menthofuran exposure (0 mg pulegone/kg up to max. 23.62 mg pulegone/kg). Speculation on matrix effect resulting in an absence of genotoxicity by pulegone remains rather hypothetical.

The in-vivo rat Comet / micronucleus test with peppermint oil, pulegone and menthofuran

To address the above mentioned problems in the available genotoxicity tests, i.e. their appropriateness vis-à-vis short-lived liver-produced reactive metabolites and effect organotrophy found in NTP study and the IARC evaluation and classification, an industrial consortium conducted a GLP study (Boehringer Ingelheim International GmbH, 2015) in which oral doses of 187.5, 375 and 750 mg/kg peppermint oil (Ph. Eur. quality, containing 1.9% pulegone and 3.7% menthofuran; i.e. in the medium range of pharmacopoeial specification for pulegone: max 3%; menthofuran: 1-8%), or 75, 150 and 300 mg/kg pulegone or menthofuran were given 3 times to 5 female Crl:CD (SD) rats, respectively. The highest doses were deemed to be maximum tolerated doses on the basis of considerable toxicity signs observed. Female animals were examined due to the presence of sex-specific urinary bladder neoplasms in the NTP pulegone study. The combined bone marrow micronucleus test and the Comet assay with liver, kidney and urinary bladder urothelium as target organs were performed according to appropriate OECD guidelines. In the Comet Assay, there was no dose-dependent increase in DNA strand breaks in the liver, kidney and urinary bladder when rats were orally treated with peppermint oil or pulegone. Menthofuran exposure did also not result in Comet induction in kidney and urinary bladder. However, statistically significant slight increases in the median tail intensity were observed in the liver cells of animals treated with 150 and 300 mg/kg/day menthofuran. The dose-dependent increase in hedgehog "ghost" cells observed in animals administered menthofuran across all tissues (predominantly liver) indicates cytotoxicity. In the micronucleus analysis, peppermint oil, pulegone and menthofuran did not cause any increase in the induction of micro nucleated polychromatic erythrocytes or bone marrow cell toxicity. The positive controls EMS and cyclophosphamide showed the expected response and sensitivity of the assay.

Conclusions on genotoxicity of pulegone and menthofuran

An analysis of the totality of above mentioned genotoxicity studies suggests that pulegone is devoid of genotoxic potential also in those studies in which the production of short-lived reactive intermediates and their scavenging by cellular protection mechanisms has been taken into consideration. A slight increase in tail intensity by high-dose menthofuran in the Comet assay is most likely due to cytotoxicity. Despite some (weak) positive findings in some studies the overall conclusion is that pulegone and menthofuran do not possess genotoxic potential.

2.7. Mode of action considerations about carcinogenicity

In chronic studies (3-month, 2-year) in rats and mice the principal target organs were liver and kidney. It seems probable that bioactivation of pulegone (and menthofuran) to reactive metabolites is behind non-neoplastic liver and kidney effects, histological and functional changes, and frank injury. The role of bioactivation in carcinogenesis remains to be defined, because in-vitro and in-vivo genotoxicity studies with pulegone have generally been negative and a lack of genotoxicity has been demonstrated also in the most recent studies which address the appropriateness of the test (see above 2.6.). Recently, the International Agency of Research on Cancer (IARC, 2015) 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, 2015).

Regenerative cell proliferation as a plausible mechanism of action for pulegone-induced bladder tumours in female rats has received rather convincing evidence from a recent mechanistic study (Da Rocha *et al*., 2012), in which 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. Also the recently performed Comet assay did not demonstrate any DNA damage (strand breaks) in liver cells from pulegone or menthofuran-treated rats (Boehringer Ingelheim International GmbH, 2015).

Indirect clues to the genotoxic potential of a substance can be found in rodent toxicity and carcinogenicity studies. Tumours caused by a genotoxic compound usually appear at an earlier age of onset compared with spontaneously developing tumours and tumours caused by non-genotoxic mechanisms (Greaves, 2012). Furthermore, genotoxic carcinogens tend to produce tumours at multiple sites, with an increased malignancy (i.e. the presence of metastases distant from the primary tumour site) compared with spontaneously developing tumours (Gold *et al*., 2001). A genotoxic hepatocarcinogen would most likely produce pre-neoplastic changes (foci of hepatocellular alteration) in repeat-dose toxicity studies, sometimes as early as after 2-3 weeks of dosing (Williams, 1997).

At least the following findings in the NTP pulegone toxicity and carcinogenicity studies are not in line with genotoxicity as a mechanism of tumour formation:

- No pre-neoplastic liver changes (foci of hepatocellular alteration) were observed in the 3-months rat and mouse repeat-dose toxicity studies.
- No liver tumours occurred in the rat 2-year carcinogenicity study, despite the occurrence of a spectrum of hepatotoxic changes, presumably linked to reactive metabolites (e.g. fatty change, hepatocyte necrosis, oval cell hyperplasia).
- Liver tumours in pulegone-treated mice did not appear earlier than those in the vehicle controls. The first incidence (all liver tumours combined) in males/females was noted at days 479/663, 428/645, 588/631 and 638/566 for vehicle, 37.5, 75 and 150 mg/kg/day groups, respectively. In males, tumours at 75 and 150 mg/kg/day occurred later than those in the control group.
- Apart from an increased incidence of osteoma /osteosarcoma in female mice at 75 mg/kg/day (considered by the NTP to be an equivocal finding, possibly linked to secondary

hyperparathyroidism due to renal lesions) there were no other tumours observed in the 2-year mouse study.

- There was a slight, non-dose related increased incidence of lung metastases originating from hepatocellular carcinomas in male mice; however, this was not the case in females and the total number of animals with metastatic neoplasms did not differ significantly between pulegone-treated mice and vehicle controls.
- In rats, the only treatment-related neoplastic finding was urinary bladder papilloma and carcinoma in females at 150 mg/kg/day. The urinary bladder carcinoma in females did not produce any metastases.

In summary, on the basis of all the above mentioned arguments, pulegone did not produce tumours of a character and pattern typical for a genotoxic carcinogen. Also a remaining uncertainty regarding short-lived, potentially DNA-reactive liver metabolites seems to be resolved by the in-vivo Comet assay (Boehringer Ingelheim International GmbH, 2015). Thus it is justified to use a threshold approach when calculating limits of pulegone in medicinal products. This is in line with the revised version of IHC M7, which opens up for a practical threshold also for DNA-reactive compounds whose effects may be modulated by rapid detoxification or effective repair of induced damage.

In conclusion, the mode-of-action of pulegone in producing tumours in experimental animals is most likely the regenerative cellular proliferation after high dose-dependent overt tissue toxicity. Thus it is plausible that at lower exposure levels cellular protective mechanisms, trapping by glutathione and other scavengers of reactive metabolites, would constitute a threshold below which no overt toxicity 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 negative. The mouse strain used in the NTP carcinogenicity study, B6C3F1, is known to have a high spontaneous background incidence of liver tumours (Carmichael *et al*., 1997). It has been proposed that findings of liver tumours in mice are of little relevance to humans or, alternatively, they may be relevant only at very high (unrealistic) levels of exposure (Carmichael *et al*., 1997). This has also been reflected in the regulatory guideline ICH S1B, which notes that liver tumours in mice may not always have relevance to carcinogenic risk in humans and can potentially be misleading. NTP and IARC state that the most likely explanation for the mouse liver tumours is the formation of reactive metabolites, resulting in cytotoxicity and regenerative cell proliferation. There is also some experimental support for a cytotoxic mode of action in a study by McMillian *et al*. (2004), who performed a gene expression analysis on livers from male Sprague Dawley rats treated with pulegone. There was an increase in transcripts related to oxidative stress, probably linked to cytotoxicity.

Most probably, cytotoxicity needs to be present for prolonged periods for carcinogenesis to occur. This mechanism behind hepatocarcinogenesis has rarely been documented in humans, since discontinuation of exposure occurs if cytotoxicity is identified (Cohen, 2010). In the NTP rat carcinogenicity study, where pulegone-induced hepatocellular cytotoxicity was sustained through the lifetime of the animals, this was not sufficient to trigger carcinogenesis. The highest dose in the rat carcinogenicity study was 150 mg/kg/day. After conversion to human equivalent dose (HED) this corresponds to 24 mg/kg/day

or a total daily intake of 1200 mg pulegone in a 50 kg person. Presuming a comparable sensitivity to pulegone in humans as in rats (based on human case reports of pennyroyal poisoning it seems that at least acute hepatotoxicity occurs at broadly the same doses as in rats) this would mean that a daily intake of >1200 mg pulegone for several years would be required to cause (theoretical) hepatocarcinogenesis. Such high doses of pulegone are not clinically relevant, especially not in a chronic administration setting.

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. However, based upon the lack of predisposing factors (e.g. urinary tract infection, urolithiasis, epithelial hyperplasia) in the rat repeat-dose toxicity and carcinogenicity studies, the NTP considered the urinary bladder tumours to be related to mutagenic activity of pulegone. However, as described above in 2.6., the Comet assay performed on liver, kidney and bladder cells did not demonstrate any genotoxicity of pulegone. Menthofuran was weakly positive at high doses, but this was most probably due to high-dose cytotoxicity. It may be of importance to note, that in the NTP study a few rats at 75 and 150 mg/kg/day actually showed transitional epithelial hyperplasia in the urinary bladder. Two rats at 150 mg/kg/day also showed inflammation in the urinary bladder. Moreover, the results of the mechanistic study by Da Rocha *et al*. (2012) support that cytotoxicity followed by regenerative cell proliferation is the mode of action for pulegone-induced urothelial tumours in female rats.

The cytotoxicity to the bladder epithelium is linked to the excretion of pulegone and some of its metabolites in the urine at high concentrations (Da Rocha *et al*., 2012). Similar to the situation in the liver, cytotoxicity to the urinary bladder epithelium needs to be sustained for a long time period in order for carcinogenicity to develop. Dose levels required to produce urinary concentrations that could induce cytotoxicity in humans are likely within the range of $\geq$12 mg/kg/day (HED of 75 mg/kg in rats), corresponding to 600 mg daily intake in a 50 kg person. It is not likely that humans would be exposed to such high doses for the prolonged periods required to produce sustained cytotoxicity.

The authors of the NTP report speculate that there may be a link between the unusual finding of renal hyaline glomerulopathy and the urinary bladder tumours. This is based on an epidemiological study in human patients with chronic kidney disease (CKD), which found an increased risk of urinary tract and lung cancers in men but not women (Wong *et al*., 2009). As far as it is known, despite the fact that chronic nephropathy is commonly seen in the aging rat (in the NTP carcinogenicity study, the incidence of spontaneous chronic nephropathy was 84-100% in all groups, including controls), no similar association has been established in rats. Furthermore, in the investigative 4-week study by Da Rocha *et al*. (2012) no histopathological changes in the kidney were observed, indicating that the urinary bladder cytotoxicity was not associated with hyaline glomerulopathy.

Considerations on **the mode of action for tumour formation** presented above suggest strongly that pulegone is 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.

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

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 are no studies on DNA adducts of pulegone or menthofuran. 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 is 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 and more definitive in-vivo studies in humans are needed.

According to a human relevance framework concept developed by IPCS and ILSI, liver tumours in rodents formed as the result of sustained cytotoxicity and regenerative proliferation are in principle considered relevant for evaluating human cancer risk, provided that similar metabolism occurs in the animal model and humans (Holsapple *et al*., 2006). Dose-, species-, and sex-dependent differences in elimination of pulegone-derived ^{14}C have been observed (see above 2.4.) and differences in metabolite patterns reported in the literature seem to indicate that dose- and/or species-dependent differences in the metabolism and formation of reactive metabolites might also exist. However, available data is not sufficient to make any definite conclusions in this regard.

Is there evidence for matrix effect vis-à-vis pulegone and menthofuran as isolated substances versus herbal medicinal products containing pulegone and menthofuran?

Escobar *et al*. (2015) examined the toxicity of the essential oil (peperina oil) of *Minthostachys verticillata*, a plant related to peppermint featuring 64.7% pulegone in the essential oil. Groups of 10 Wistar rats were treated for 90 days by 0, 70, 260 and 460 mg/kg of the essential oil in feed, corresponding to the pulegone doses of 45.2, 168 and 297 mg/kg bw per day. No toxicity was reported at any dose level and the authors claim a NOAEL of 460 mg/kg bw per day of *Minthostachys verticillata* essential oil containing about 300 mg/kg bw of pulegone. For pure pulegone, the LOEL in the NTP 3-month study was 37.5 mg/kg, in contrast to about 300 mg/kg in the study of Escobar *et al*. (2015), who explain this discrepancy with the potential protective effect of other constituents, e.g. menthone. This latter constituent is also present in mint and peppermint oils. However, several important differences were found when comparing the study of Escobar *et al*. (2015) and the NTP 3-month study: (i) dietary administration instead of oral gavage, which results in a more uncertain exposure; (ii) lack of complete histopathological evaluation (only three tissues examined); (iii) a different rat strain (Wistar) was used in the Escobar *et al*. study as compared with the NTP studies, which used F344 rats. Consequently, no firm conclusion on potential matrix effect can be made.

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 (which NTP considers significant) the IARC working Group regards pulegone as non-genotoxic current conclusion: genotoxic potential of pulegone and menthofuran is unlikely, but cannot yet be convincingly refuted
Genotoxicity in-vivo	Comet assay negative in liver, kidney and bladder cells (menthofuran slightly positive in liver cells most probably due to high-dose cytotoxicity) micronucleus tests consistently negative
Carcinogenicity in rodents	NTP and IARC: 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 quantitative differences are likely, but their significance is not known
Other information	some evidence of non-linearity of metabolic activation and adduct formation
WoE conclusion	IARC: class 2B (possibly carcinogenic to humans) cell cytotoxicity and regenerative proliferation driven by reactive metabolites and GSH depletion as a probable mechanism of action overall conclusion: toxicity and carcinogenicity of pulegone have a 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, raised concerns about the implications for public health of intake of preparations containing pulegone and menthofuran. The uncertainties about the genotoxic potential have been sufficiently addressed by more appropriate Comet assay in liver, kidney and bladder cells and micronucleus assay in bone marrow which both gave essentially negative results. Also 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. It is concluded that efficient scavenging or mitigating of reactive metabolites at lower pulegone exposures is likely to create **a threshold**. Consequently, the risk assessment scenario adopted below is based on the above considerations.

Selection of a study for setting a NOAEL

On the basis of the following considerations, the NTP 3-month repeat-dose toxicity study in rats is concluded to be the most relevant study for establishing a NOAEL:

- Rodent carcinogenicity studies are aimed at determining the carcinogenic potential of a drug or chemical substance. Although non-neoplastic findings are recorded in a lifetime rodent

bioassay their incidence and character may be influenced by the age of the animals and increasing spontaneous background pathology. Therefore it is not appropriate to use a carcinogenicity study to set a NOAEL for a non-neoplastic toxicological finding.

- The rat toxicity study used by the CEFS to establish the ADI value of 0.1 mg/kg bw was of short duration (28 days) and no detailed information was published (Thorup *et al*., 1983). Thus this study is considered to be inferior to the NTP 3–month study for the present purpose.
- A recently published 3-month study in rats (Escobar *et al*., 2015) using peperina oil containing a high concentration of pulegone is considered inferior to the NTP 3-month study based on (i) a mixture is used; (ii) dietary administration instead of oral gavage (more uncertain exposure); (iii) lack of complete histopathological evaluation (only three tissues examined). In addition, a different rat strain (Wistar) was used in the Escobar *et al*. study as compared with the NTP studies, which used F344 rats.

There were no histopathological findings in the 3-month NTP mouse study; thus the rat was the most sensitive species and the 3-month rat study should be used in a conservative approach. The value of 37.5 mg/kg bw per day, based on liver and kidney toxicity in the NTP 3-month repeat-dose toxicity study, is taken as a NOAEL value.

Considering the setting of an acceptable uncertainty factor, the available data suggest a comparable pattern of response to high pulegone exposure of rats and humans regarding potential generation of reactive metabolites, acute liver toxicity and glutathione depletion as the basis for determining a threshold for toxicity. This justifies a reduction of the uncertainty factor from 100 to 50 for extrapolating the NOAEL from rats to a safe intake level in humans. Using an uncertainty factor of 50, the acceptable exposure would be 0.75 mg/kg bw per day. The daily dose for an adult of 50 kg body weight would thus be 37.5 mg/person/day. It has to be noted that this limit value is based on a 3-month repeat dose toxicity study, which may seem rather short regarding potentially long treatment durations by pulegone-containing herbal medicinal preparations. However, a comparison of the critical target organs in rats of the 3-month repeat-dose toxicity study and the 2-year (life-time) carcinogenicity study shows no significant difference suggesting that the 3-months study is sufficiently predictive for relevant long-term effects. In addition, a convincing threshold mechanism (glutathione depletion) is proposed below which no adverse effects are expected. Since the proposed acceptable intake level includes a safety factor of 50 (i.e. far below the assumed threshold) any accumulative effects resulting from long term repeated daily use are highly unlikely. Therefore, the above proposed acceptable intake level of 37.5 mg/day is considered to be applicable (safe) for chronic use although it is derived from a rat 3-month repeat-dose toxicity study.

3. Conclusions and recommendations of the HMPC

3.1. Toxicological conclusions

1. On the basis of recent rodent subchronic 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 in-vitro experimental data.
2. Liver and kidney toxicities are induced at relatively high pulegone doses only when protective cellular levels of glutathione are depleted demonstrating a clear threshold for toxicity.
3. Neoplasms were observed in female rats (bladder) and male and female mice (liver) in the NTP study. A majority of evidence points to a probable mode of action behind these neoplasms in the

form of sustained cytotoxicity and cell proliferation caused by bioactivation of pulegone (and menthofuran) to reactive cytotoxic metabolites, and not genotoxicity.

4. Non-relevance of rodent neoplasms to human carcinogenesis seems probable because of the above consideration of mode of action, which would require a long-term sustained exposure to pulegone and menthofuran at doses which are not relevant in human situations.

5. There are no new data on pharmacovigilance, but prevailing opinion is that no certain cases of liver toxicity in humans are associated with the use of peppermint oil or mint oil.

6. As a recommendation, the HMPC proposes that an acceptable exposure limit is 0.75 mg/kg bw per day, which is higher than the current TDI value of 0.1 mg/kg bw per day.

3.2. Recommended limit values for herbal medicinal products

Evaluation of herbal medicinal products containing pulegone and menthofuran should be based on the following thresholds. Companies marketing medicinal products containing pulegone and menthofuran should check, whether their medicinal products are complying with these exposure limits. If necessary, appropriate variations to ensure compliance should be finalized within two years after publication of this document.

Oral use

The value of 37.5 mg/kg bw per day, based on the NTP 3-month repeat dose toxicity study, is taken as a NOAEL value. With the use of an uncertainty factor of 50, the acceptable exposure would be 0.75 mg/kg bw per day. The daily dose for an adult of 50 kg body weight[5] would thus be 37.5 mg/person/day.
The intake (pulegone + menthofuran) of 37.5 mg/person/day (even if the limit presents the overall intake from all sources) can be accepted for herbal medicinal products. It should be understood that as the limit is set for a life-long exposure.
For treatment durations of less than 1 year an intake (pulegone + menthofuran) of 75.0 mg/day can be accepted. In the case of intermittent dosing, the acceptable daily intake should be based on the total number of dosing days instead of the time interval over which the doses are administered. For example, a drug administered once per week for 5 years (i.e., 260 dosing days) would have an acceptable intake per dose of 75.0 mg.

Dietary background

The main source of human exposure to pulegone and menthofuran different from medicinal products would be from foodstuff and beverages (e.g., mint/peppermint flavored alcoholic/non-alcoholic beverages, confectionery, chewing gum). According to JECFA, estimated per capita intake in Europe is reported to be 2 µg/day for pulegone and 25 µg/day for menthofuran (IPCS, 2001). In certain cases the maximum permitted levels of pulegone in food may lead sporadically to higher intakes in subjects consuming mint flavored beverages or confectionery. However, the overall intake is unlikely to result in exposure levels leading human adverse effects (the highest proposed daily dose of 75 mg pulegone in medicinal products for (up to 1-year) short-term treatment is 72-120-fold below the estimated dose leading to moderate to severe toxicity in the documented cases of human intoxication with pennyroyal oil; see section 2.4).

[5] For ~18% (average) of the European population the body weight is given with less than 60 kg [EUROPEAN COMMISSION 2006]. This 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).

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 15 mg/day (life–long exposure) or 30 mg/day for a short-term exposure (less than 1 year).

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 amount of pulegone + menthofuran within the daily dose is <37.5 mg for adults (life-long exposure) or <75 mg for short-term exposure (less than 1 year). The use 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 37.5 mg for adults.

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).

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 15 mg/day (life–long exposure) or 30 mg/day for a short-term exposure (less than 1 year).

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