European Commission

Combined Draft (Renewal) Assessment Report prepared according to Regulation (EC) N° 1107/2009
and
Proposal for Harmonised Classification and Labelling (CLH Report) according to Regulation (EC) N° 1272/2008

THYMOL;
5-methyl-2-(propan-2-yl) phenol

Volume 3 – B.6 (AS)

Rapporteur Member State: Spain
Co-Rapporteur Member State: Greece

Version History

When	What
July 2022	Initial DRAR – RMS Spain
November 2022	DRAR after CoRMS and Applicant comments
February 2023	RAR updated after EFSA CoCh received on 24/01/2023

The RMS is the author of the Assessment Report. The Assessment Report is based on the validation by the RMS, and the verification during the EFSA peer-review process, of the information submitted by the Applicant in the dossier, including the Applicant's assessments provided in the summary dossier. As a consequence, data and information including assessments and conclusions, validated and verified by the RMS experts, may be taken from the applicant's (summary) dossier and included as such or adapted/modified by the RMS in the Assessment Report. For reasons of efficiency, the Assessment Report should include the information validated/verified by the RMS, without detailing which elements have been taken or modified from the Applicant's assessment. As the Applicant's summary dossier is published, the experts, interested parties, and the public may compare both documents for getting details on which elements of the Applicant's dossier have been validated/verified and which ones have been modified by the RMS. Nevertheless, the views and conclusions of the RMS should always be clearly and transparently reported; the conclusions from the applicant should be included as an Applicant's statement for every single study reported at study level; and the RMS should justify the final assessment for each endpoint in all cases, indicating in a clear way the Applicant's assessment and the RMS reasons for supporting or not the view of the Applicant.

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B.6. TOXICOLOGY AND METABOLISM DATA

B.6.1. ABSORPTION, DISTRIBUTION, METABOLISM AND EXCRETION IN MAMMALS

A total of seven studies have been submitted to address this point for the renewal process, five of which were presented in the original DAR (2011) in support of the inclusion of thymol in Annex I of Directive 91/414/EEC. These studies were deemed acceptable following evaluation and peer review at EU level. For the renewal process, pharmacokinetic studies in human (oral route) and mouse (intraperitoneal and inhalation routes) has been provided. A comparative in vitro metabolism study has not been submitted to meet the data requirements of the Commission Regulation (EU) 283/2013.

All available information has been evaluated (or re-assessed) according to the current guidelines.

B.6.1.1. Absorption, distribution, metabolism and excretion by oral route

B.6.1.1.1. Rat: Metabolism and excretion of thymol

Data point	CA 5.1.1 (EU data requirement)
Report author	Austgulen, L.T., Solheim, E. and Scheline, R.R.
Report year	1987
Report title	Metabolism in rats of p-Cymene derivatives: carvacrol and thymol
Report No	Not applicable, published study (*Pharmacology & Toxicology*, 61, 98-102)
Document No	KCA 5.1.1/01
Guidelines followed in study	Not stated
Deviations from current test guideline OECD TG 417 (2010)	-Test material not characterised (batch no. reported). -The selection of the dose was not justified and only one dose was tested in the study. -Method and results poorly described. No individual data were provided (overall values only).
Previous evaluation	Yes, evaluated and accepted in DAR (2011)
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Supporting only

Materials and Methods

Test chemical	Thymol, Purity > 99%, Batch no.: not stated
Vehicle	Propylene glycol
Test system	Male albino rats (Wistar)
Test method	A single dose of thymol dissolved in propylene glycol (1mmol/kg bw, equivalent to 150 mg/kg bw) was given orally (by stomach tube) to six male rats. Similar animals receiving solvent only served as controls. Urine samples were collected at -10ºC at 24h intervals over a period of 72h. Analyses were carried out on the urine samples following conjugate hydrolysis using a glucuronidase and sulphatase preparation. Metabolites were semi-quantified as major or minor components by comparison of integrated peak areas from the gas chromatograms relative to the major metabolite.

Results

Thymol was rapidly absorbed and metabolised after oral administration and was almost completely excreted in the urine within 24 h (Table B.6.1.1.1). Only much smaller amounts of the most prominent metabolites were found in the 24-48 hour samples and no metabolites were found in the 48-72 hour samples.

Table B.6.1.1.1. Urinary excretion of thymol and its metabolites after single oral dose

Compound	Metabolite number	Relative amount detected	Occurrence
Thymol	**Parent**	**Major compound**	**6/6**
2,5-Dihydroxy-*p*-cymene	**T1**	**+**	**6/6**

Compound	Metabolite number	Relative amount detected	Occurrence
2-(2-Hydroxy-4-methylphenyl)propan-1-ol	**T2**	**+++**	**6/6**
5-Hydroxymethyl-2-(1-methylethyl)phenol	**T3**	**++**	**6/6**
2-(4-Hydroxymethyl-2-hydroxyphenyl)propan-1-ol	**T4**	**Trace**	**1/6**
2-(4-Hydroxymethyl-2-hydroxyphenyl)propionic acid	**T5**	**++**	**6/6**
3-Hydroxy-4-(1-methylethyl)benzoic acid	**T6**	**+**	**6/6**

Major metabolite assigned 100%
+++ >50% of the major metabolite
++ 10-50% of the major metabolite
+ 1-10 % of the major metabolite

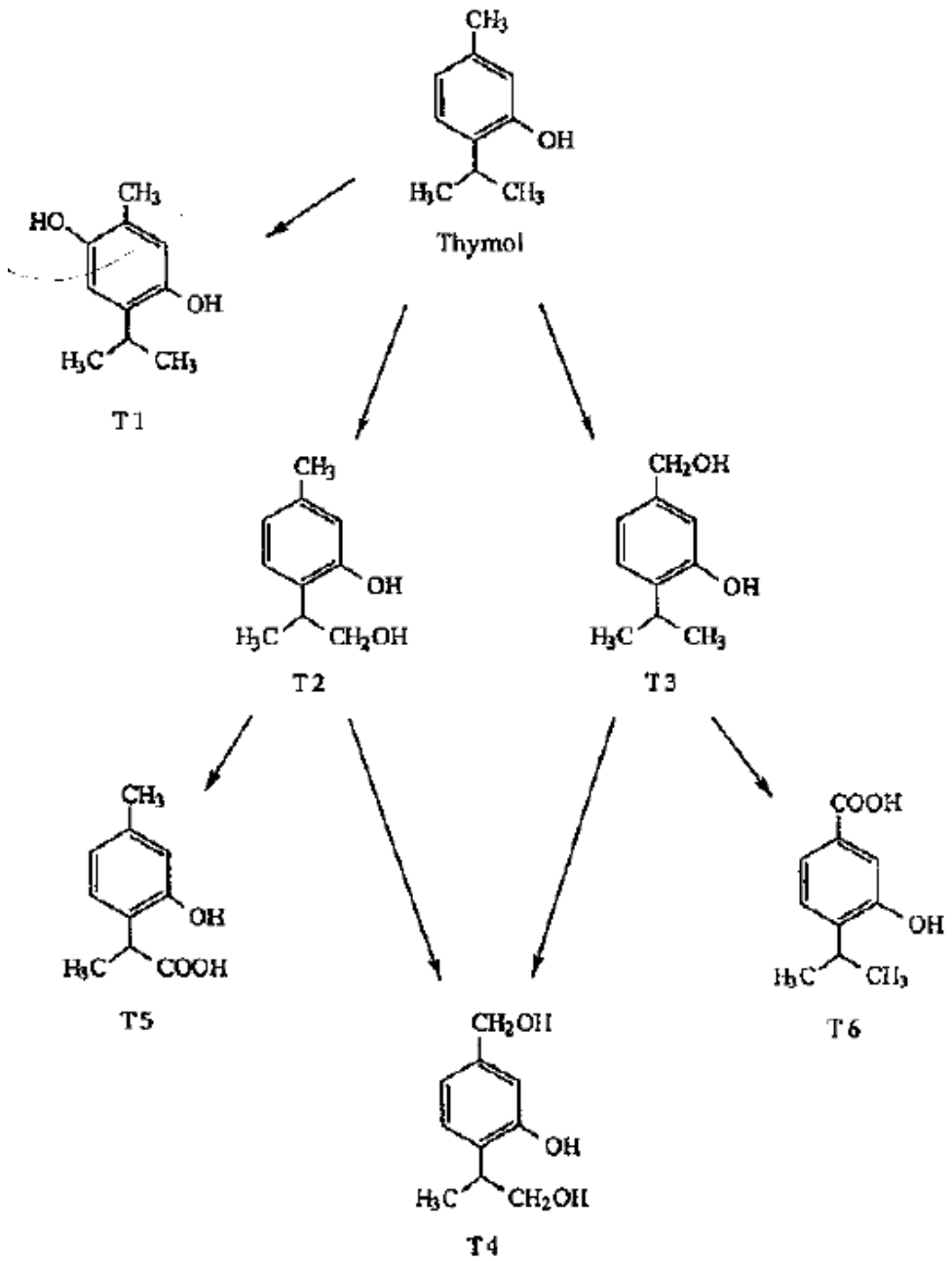


Figure B.6.1.1.1. Proposed structures of metabolites and metabolic pathways of thymol in rat

Figure B.6.1.1.1 displays the chemical structures of identified metabolites of thymol in urine and the proposed metabolic pathway of thymol. Based on the abundance in urine of these metabolites (Table B.6.1.1.1) the

primary metabolic route involves an oxidative pathway whereby hydroxylation of the isopropyl group leads to the alcohol (T2) and further oxidised propionic acid derivative (T5). Minor metabolic routes involve aromatic ring oxidation (T1) and methyl group oxidation to lead to the phenol (T3) and benzoic acid (T6) metabolites.

Assessment and conclusion by applicant: The study has been previously reviewed at EU level and is considered to be acceptable for renewal of the active substance. EFSA (EFSA Journal 2012;10(11):2916) agreed endpoint: Moderately rapid excretion *via* urine (at least 50% within 24 hours) – based on limited studies with thymol and information from structurally-related chemicals. Predominantly conjugation with glucuronic acid or sulfate; hydroxylation and other oxidative reactions occur to a lesser extent.
Assessment and conclusion by RMS: **Outcome and conclusion of the study:** The RMS agrees with the notifier's assessment and conclusion. This study is a scientific publication from a peer-reviewed journal. The study is pre-guideline and a compliance check with the current OECD TG 417 (2010) has identified the following deviations: no characterisation of the test item, the selection of the dose was not justified and only one dose was tested in the study, limited level of reporting (no individual results reported). Based on these deficiencies in the study, the RMS deems the study as supporting information to evaluate the metabolism and excretion of thymol in rats.

B.6.1.1.2. Rabbit: Metabolism and urinary excretion of thymol

Data point	CA 5.1.1 (EU data requirement)
Report author	Takada, M., Agata, I., Sakamoto, M., Yagi, N. and Hayashi, N.
Report year	1979
Report title	On the metabolic detoxication of thymol in rabbit and man
Report No	Not applicable, published study (*J. Toxicol. Sci.*, Vol 4, 341-350)
Document No	KCA 5.1.1/02
Guidelines followed in study	Not stated
Deviations from current test guideline OECD TG 417 (2010)	-Test material not characterised (batch and purity not reported). -Species not fully characterised (strain/sex) -The selection of the dose was not justified -Poorly description of the method and results
Previous evaluation	Yes, evaluated and accepted in DAR (2011)
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Supporting information (see deviations)

Materials and Methods

Test chemical	Thymol, Purity: not specified; Lot/Batch No.: not specified
Test system	Rabbit (strain/sex not provided)
Test method	Thymol filled in a capsule at a dose of 500 mg/kg bw was administered orally to three rabbits. Urine samples were collected and analysed 24, 48 and 72h post dosing. Total amount of glucuronic and sulphate acids were determined from urine at acidic pH following extraction with ether and subsequently purified. Total glucuronic acid was measured by the naphthoresorcinol picrate method (Ishidate and Nambara, 1957) and the ethereal sulphuric acid was determined by Fiscke's modification of benzidine method. Urinary metabolites were separated by gas chromatography and analysed by thin layer chromatography. Further determination of thymol glucuronide conjugate in urine was carried out by methylation and successive acetylation by acetic anhydride and pyridine of the glucuronide fraction to yield a purified crystalline substance. A comparison by infrared spectroscopy and melting point with the reference standard compound was carried out.

Results

Urinary excretion of glucuronic acid and ethereal sulphuric acid markedly increased at 24h compared to control values following a single oral dose of thymol (Table B.6.1.1.2). At 48h only glucuronic acid was slightly increased and both conjugates returned to control levels at 72h. The extent of glucuronic acid excretion is considerably higher than the ethereal sulphate, which suggests glucuronic conjugation is the primary metabolic route.

Table B.6.1.1.2. Glucuronic and ethereal sulphuric acid in urine following single oral dose of thymol

Conjugate	Rabbit	Urine				
		Control	Control	24 h	48 h	72 h
Glucuronic acid	A	42.0	61.5	181.6	93.3	64.5
	B	21.2	64.8	186.3	75.2	35.6
	C	61.6	39.4	183.0	45.8	29.4
	Average ± SD	41.6 ± 15.4	55.2 ± 11.2	183.6 ± 2.0	71.4 ± 19.5	43.2 ± 15.2
Ethereal sulphuric acid	A	8.0	22.4	40.0	26.8	12.8
	B	8.0	28.0	81.6	35.2	16.0
	C	8.0	9.6	73.6	6.4	3.2
	Average ± SD	8.0	20.0 ± 7.7	65.1 ± 18.0	22.8 ± 12.0	10.7 ± 5.9

Results are expressed as mg excreted for 24h; SD Standard deviation

The isolation of thymol glucuronide conjugate from urine was confirmed by comparison with the reference thymol conjugate.

Assessment and conclusion by applicant:

The study has been previously reviewed at EU level and is considered to be acceptable for renewal of the active substance.

EFSA (EFSA Journal 2012;10(11):2916) agreed endpoint: Moderately rapid excretion *via* urine (at least 50% within 24 hours) – based on limited studies with thymol and information from structurally-related chemicals. Predominantly conjugation with glucuronic acid or sulfate; hydroxylation and other oxidative reactions occur to a lesser extent.

Assessment and conclusion by RMS:

Outcome and conclusion of the study: This study is a scientific publication from a peer-reviewed journal. The study is pre-guideline and compliance with the current OECD TG 417 (2010) has been assessed. A few deviations from guidance have been identified: no characterisation of the test item, species not fully characterised (strain/sex), the selection of the dose is not justified and overall very limited detail given in the study, both method and results. Based on these method deficiencies of the study, the RMS deems the study as supporting information to evaluate the metabolism and excretion of thymol in rabbits.

B.6.1.1.3. Human: Metabolism and urinary excretion of thymol

Data point	CA 5.1.1 (EU data requirement)
Report author	Takada, M., Agata, I., Sakamoto, M., Yagi, N. and Hayashi, N.
Report year	1979
Report title	On the metabolic detoxication of thymol in rabbit and man
Report No	Not applicable, published study (*J. Toxicol. Sci.*, Vol 4, 341-350)
Document No	KCA 5.1.1/02
Guidelines followed in study	Not stated
Deviations from current test guideline	Not applicable
Previous evaluation	Yes, evaluated and accepted in DAR (2011)
GLP/Officially recognised	No

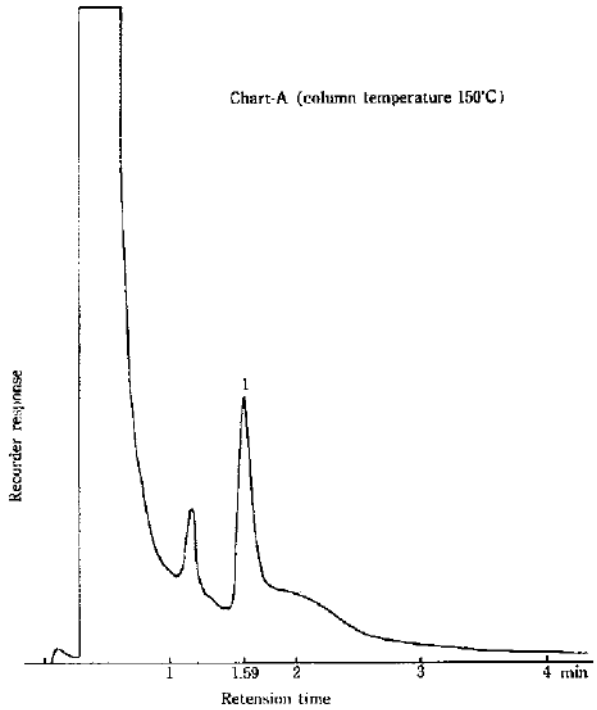
testing facilities	
Acceptability/Reliability	Supporting information (see deviations)

Materials and Methods

Test chemical	Thymol, Purity: not specified; Lot/Batch No.: not specified
Test system	Human male volunteers
Test method	Two male volunteers were administered a single oral dose of thymol (600 mg) and their 24h urine was collected. The urine was filtered through cotton and a series of extraction procedures with ether at various pH were carried out: Fraction [M1]: pH 5.7 and extraction for 24h with ether. Fraction [M2]: pH 2 and extraction for 30 or 60h Fraction [M3]: precipitate resulted from purification of [M2] with ether/methanol The fraction M3 was applied to enzymatic hydrolysis with beta-glucuronidase incubation at 37ºC and extracted with dichloromethane to yield fraction M5. All fractions were submitted for analysis using thin layer chromatography and gas chromatography.

Results

The analysis of 24h urine samples of two male volunteers resulted in the identification of small amounts of free thymol (Figure B.6.1.1.3/1) from fraction M1 (ether extraction at pH 5.7) and a more significant amount of thymol and thymolhydroquinone were detected in fraction M2 (ether extraction at pH 2).

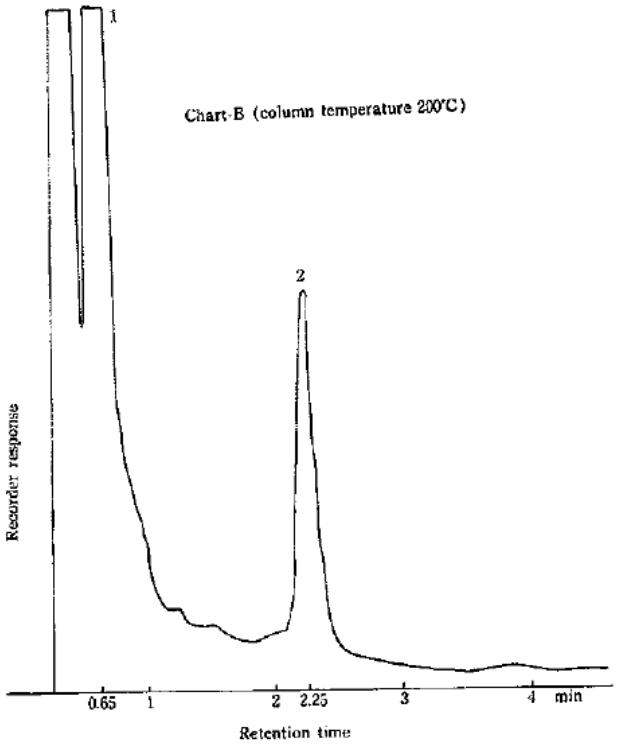


*Peak No.1: Thymol

Figure B.6.1.1.3/1. Gas chromatogram of urinary [M1] fraction

Fraction M1 is postulated as the unconjugated thymol based on the assumed stability at pH 5.2 of thymol glucuronic and sulphate conjugates. Fraction M2 corresponds to the urine extracted at acidic pH 2 that it is assumed to hydrolyse the sulphate conjugates but not glucuronides. Based on this assumption, both thymol and thymohydroquinone identified in M2 relate to the sulphate conjugate of these compounds.

The analysis of fraction M3 resulted in the detection of various compounds glucuronide conjugates (unidentified structures).



*Peak No 1: Thymol: Peak No.2: Thymohydroquinone

Figure B.6.1.1.3/2. Gas chromatogram of urinary [M2] fraction

Assessment and conclusion by applicant: The study has been previously reviewed at EU level and is considered to be acceptable for renewal of the active substance. EFSA (EFSA Journal 2012;10(11):2916) agreed endpoint: Moderately rapid excretion *via* urine (at least 50% within 24 hours) – based on limited studies with thymol and information from structurally-related chemicals. Predominantly conjugation with glucuronic acid or sulfate; hydroxylation and other oxidative reactions occur to a lesser extent.
Assessment and conclusion by RMS: **Outcome and conclusion of the study:** The outcome of the study shows that thymol (free and conjugated) and thymohydroquinone are detected in urine after oral administration. No quantification has been given in the study. This study is a scientific publication from a peer-reviewed journal. No guidance is available for this type of study. The RMS deems the study as supporting information to evaluate the metabolism and excretion of thymol in humans based on poorly described method and results.

B.6.1.1.4. Pharmacokinetics: Summary report

Data point	CA 5.1.1 (EU data requirement)
Report author	The European Agency for the Evaluation of Medicinal Products – Committee for Veterinary Medicinal Products
Report year	1996
Report title	Thymol
Report No	Not applicable, published study (EMEA/MRL/075/96-FINAL)
Document No	KCA 5.1.1/03

Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
Previous evaluation	Yes, evaluated and accepted in DAR (2011)
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Supporting information

Materials and Methods

Test chemical	Thymol (Purity: not stated; batch no.: not stated)
Test method	Pharmacokinetics

Results

Thymol is readily absorbed in the gastrointestinal tract. A small amount is oxidised to thymohydroquinone. About 50% of the absorbed substance is excreted *via* the kidney, either unconjugated or as a glucuronide or sulphate conjugate in 24h. The metabolite thymohydroquinone is also excreted *via* the kidney.

Assessment and conclusion by applicant:

The study has been previously reviewed at EU level and is considered to be acceptable for renewal of the active substance.

EFSA (EFSA Journal 2012;10(11):2916) agreed endpoint: Moderately rapid excretion *via* urine (at least 50% within 24 hours) – based on limited studies with thymol and information from structurally-related chemicals. Predominantly conjugation with glucuronic acid or sulfate; hydroxylation and other oxidative reactions occur to a lesser extent

Assessment and conclusion by RMS:

Outcome and conclusion of the study: This publication is a summary report on thymol pharmacokinetics. The RMS deems the study as supporting information only based on the limited information provided to evaluate the ADME properties of thymol (quantification and identification of metabolites, further information on absorption /distribution and excretion).

B.6.1.1.5. Metabolism: Summary Report

Data point	CA 5.1.1 (EU data requirement)
Report author	Mattia, A. and Sipes, G.I.
Report year	2005
Report title	WHO Food Additives Series 46: Phenol and Phenol derivatives
Report No	Not applicable, published study (www.inchem.org)
Document No	KCA 5.1.1/04
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
Previous evaluation	Yes, evaluated and accepted in DAR (2011)
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Supporting information

Materials and Methods

Test chemical	Thymol (Purity: not stated; batch no.: not stated)
Test method	ADME

Results

Phenol derivatives such as thymol are rapidly absorbed from the GI tract and share the same metabolic pathways. Thymol is reported to be excreted after conjugation with sulphate and glucuronic acid. Thymoquinol has been detected as a minor oxidation product of thymol (ring hydroxylation).

Assessment and conclusion by applicant: The study has been previously reviewed at EU level and is considered to be acceptable for renewal of the active substance. EFSA (EFSA Journal 2012;10(11):2916) agreed endpoint: Moderately rapid excretion *via* urine (at least 50% within 24 hours) – based on limited studies with thymol and information from structurally-related chemicals. Predominantly conjugation with glucuronic acid or sulfate; hydroxylation and other oxidative reactions occur to a lesser extent.
Assessment and conclusion by RMS: **Outcome and conclusion of the study:** This publication is a summary report that includes absorption, distribution and excretion information of phenol and derivatives (including thymol). The RMS deems the study as supporting information only based on the limited information provided to evaluate the ADME properties specific to thymol (quantification and identification of metabolites, further information on absorption /distribution and excretion).

B.6.1.1.6. Mouse: Pharmacokinetics

Data point	CA 5.2.1 (EU data requirement)
Report author	[redacted]
Report year	2009
Report title	Thymol: Oral (Gavage) Administration Pharmacokinetic Study in the Mouse
Report No	8202028
Document No	KCA 5.1.1/05
Guidelines followed in study	Not stated
Deviations from current test guideline OECD TG 417 (2010)	None
Previous evaluation	Yes, evaluated and accepted in DAR (2011)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	Yes

Materials and Methods

Test chemical	Thymol (Purity 99.7%; Batch no. 103366)
Test system	Hsd:ICR (CD.1®) mice (♂,♀)
Vehicle	Olive oil
Test method	The objective of the study is to investigate the pharmacokinetics of thymol in mice following a single oral (gavage) administration and to support a previously conducted mouse bone marrow micronucleus test (Vol.3, AS, B.6.4.2.1-01). Preliminary studies: Two preliminary studies were conducted prior to the main study. Groups of two mice per sex were administered a single oral (gavage) dose of 1250 mg/kg bw or 1000 mg/kg bw of thymol suspended in olive oil at a volume of 10 mL/kg. The higher dose was selected as it was used in the micronucleus study (Vol.3, AS, B.6.4.2.1-01) and it was deemed the maximum tolerated dose. Animals were observed once within half an hour of dosing, four times within four hours of dosing, twice daily on Days 2, 3 and 4, and once daily on Days 5, 6, 7 and 8. Body weights were recorded pre-treatment on Day -3

	and on Days 1, 4 and 8. Main study: Mice (15/sex) were administered a single oral (gavage) dose of 1000 mg/kg bw thymol suspended in olive oil at a volume of 10 mL/kg. Animals were observed on Day 1 at 1, 2, 4, 8, 12 and 24 hours post dose. Body weights were recorded pre-treatment on Day -7 and before dosing. Blood samples for pharmacokinetics (0.5 mL nominal) were taken from all main study animals during Day 1 at 1, 2, 4, 8, 12 and 24 hours after dosing. Terminal samples (1, 2, 4, 12 and 24h samples) were collected by cardiac puncture. In life samples (8h sample) were collected by orbital sinus puncture under isofluorane anaesthesia. Animals from 8h samples were used for the 24h samples. Samples were mixed gently by hand and then continuously for at least 2 min on an automatic mixer and placed in a Kryorack until centrifugation at 4ºC. The plasma was separated and frozen at <-50ºC. Plasma samples were analysed for thymol using GS-MS. The limit of quantification was 20mg/mL for olive oil. Pharmacokinetics analysis of the plasma was performed using WinNonlin Enterprise v.4.0.1.

Results

Preliminary studies

No mortality occurred in any of the two preliminary studies.

Clinical signs (1250 mg/kg bw dose) observed in both male and female animals were ataxia, bradypnoea, prone posture, decreased activity, ptosis and piloerection, ranging from 15min to 4 h post-dose. All animals appeared normal on Day 2.

Clinical signs (1000 mg/kg bw dose) observed in both male and female animals were ataxia, decreased activity, bradypnoea, ptosis and piloerection ranging from 5min post dose to 4 hours post dose. Prone posture, pallor extremities, loss of righting reflex and ptosis were observed in one male from 2 to 4h after dose. Prone and pallor extremities were also observed in one female from 15min to 4h after doses.

Body weight was unaffected by treatment with thymol.

Main study

No mortality occurred in this study.

Clinical signs included increased activity, piloerection, ataxia and decreased activity ranging from 1 to 8h post-dose in both male and female animals. Additional symptoms included bradypnoea (1male at 8h), ataxia (4 males up to 2h post dose and 6 females up to 4h post dose) and both hunched posture and lethargy (1 male at 2h post dose, 1 female at 4h post dose).

The mean plasma concentration of thymol over a 24h period are displayed in Table B.6.1.1.6/1. Figure B.6.1.1.6 displays the plot of concentration over time of thymol in mice.

Table B.6.1.1.6/1: Mean plasma concentration values of thymol in male and female mice after administration of thymol

Time point (h)	Mean concentration (ng/mL)	Standard deviation
1	4891	2779
2	5899	8163
4	6305	12704
8	2798	5255
12	227	120
24	379	525

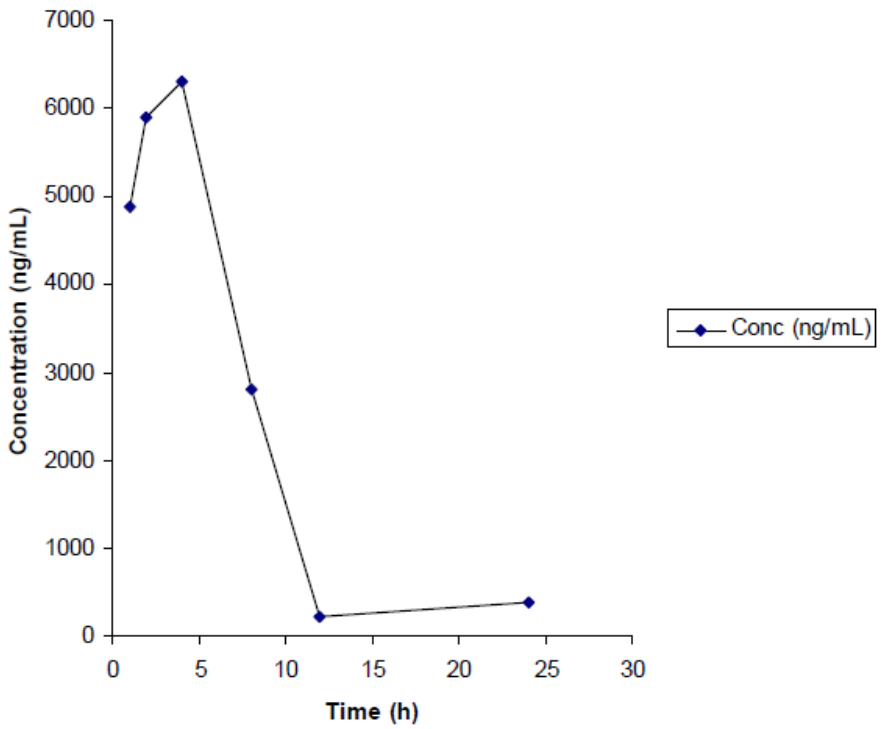


Figure B.6.1.1.6: Mean concentration of thymol over time (male and female mice)

The pharmacokinetic parameters of thymol are displayed in Table B.6.1.1.6/2.

Table B.6.1.1.6/2: Pharmacokinetic parameters of thymol after oral administration

Dose (mg/kg bw)	$AUC_{0\text{-}24h}$ (hours*ng/mL)	$AUC_{0\text{-}infinity}$ (hours*ng/mL)	Cmax (ng/mL)	Tmax (hours)	Half life (hours)
1000	44964	Not calculated*	6305	4	Not calculated*

**some animals had higher concentration at 24h after dosing than previous sampling time (8h)*

Assessment and conclusion by applicant:

The study has been previously reviewed at EU level and is considered to be acceptable for renewal of the active substance.

EFSA (EFSA Journal 2012;10(11):2916) agreed endpoint: Moderately rapid excretion *via* urine (at least 50% within 24 hours) – based on limited studies with thymol and information from structurally-related chemicals. Predominantly conjugation with glucuronic acid or sulfate; hydroxylation and other oxidative reactions occur to a lesser extent.

Assessment and conclusion by RMS:

Outcome and conclusion of the study: Pharmacokinetic parameters of thymol have been calculated following a single oral dose in mice (male and female). The study supports an *in vivo* micronucleus study of thymol in mice (Vol.3, AS, B.6.4.2.1-01). Plasma concentration peaked at 4h and a concentration of 6305 ng/mL.

The RMS deems the study as acceptable to evaluate the pharmacokinetic parameters of thymol in mice.

B.6.1.1.7. Human: Pharmacokinetics

Data point	CA 5.2.1 (EU data requirement)
Report author	Kohlert, C., Schindler, G., Marz, R.W., Abel, G., Brinkhaus, B., Derendorf, H., Grafe, E.U. and Veit, M.

Report year	2002
Report title	Systemic availability and pharmacokinetics of thymol in humans
Report No	Not applicable, published study (*J. Clin. Pharmacol.*, 42, 731-737)
Document No	KCA 5.1.1/06
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Yes

Materials and Methods

Test chemical	Thymol, Lot/Batch No.: not stated
Test systems	Male human volunteers (mean age 29.5 ± 6.74)
Test method	Twelve healthy male volunteers received a single dose of a Bronchipret® TP tablet, which contains the equivalent amount of 1.08 mg of thymol. Venous blood samples were collected once before subjects were administered the medication and 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 24, 31, 38, 48, 55, 62, and 72 hours after administration. Urine was collected at 0 to 3, 3 to 6, 6 to 9, 9 to 14, 14 to 24, 24 to 31, 38 to 48, 48 to 55, 55 to 62, and 62 to 72 hours after administration. For the determination of total concentration of thymol, plasma and urine samples were analysed by headspace solid-phase microextraction (HS-SPME) prior to gas chromatographic analysis. The identification of phase II metabolites and the determination of free thymol in both plasma and urine were carried out exemplarily in 2 subjects each. Pharmacokinetics data were determined by non-compartmental analysis using MS Excel based on the equations by Gibaldi and Perrier. C_{max} and T_{max} were determined directly from the data. The AUC0-clast was calculated using the linear trapezoidal rule. The terminal elimination half life was determined by linear regression of the terminal phase of the semi-logarithmic plasma concentration vs time profiles.

Results

Thymol showed a rapid absorption with maximum plasma levels of 93.1 ± 24.5 ng/mL (mean ± SD) that were reached after 1.97 ± 0.77 hours (mean ± SD) (Table B.6.1.1.7). Full pharmacokinetic parameters have been calculated in eleven individuals (Table B.6.1.1.7).

Table B.6.1.1.7: Pharmacokinetic parameters of thymol after single oral administration

	Mean	SD	Minimum value	Median	Maximum Value	Geometric Mean	Number
Dose (mg)	1.08						
C_{max} (ng/mL)	93.11	24.47	55.90	99.01	125.82	90.04	11
t_{max} (h)	1.97	0.77	0.80	2.03	3.13	1.81	11
$t_{1/2}$ (h)	10.2	1.4	8.3	9.9	12.9	10.1	11
$AUC_{0\text{-}Clast}$ (ng*h/mL)	837.3	278.5	456.7	835.8	1281.6	793.6	11
MRT_{abs} (h)	12.6	2.1	8.1	12.5	15.2	12.4	11
MAT (h)	0.53	0.04	0.46	0.54	0.59	0.53	11
CL_{tot}/f (L/h)	1.2	0.3	0.8	1.1	1.8	1.2	11
Vdss/f (L)	14.7	5.1	6.1	13.8	23.3	13.9	11
Vdarea/f (L)	17.7	5.6	10.8	17.2	29.5	16.9	11

C_{max} peak plasma concentration; t_{max} time to reach C_{max}; $t_{1/2}$ elimination half-life; $AUC_{0\text{-}clast}$ area under the concentration time-curve from time 0 to Clast; MRT_{abs} mean residence time after extravascular administration; MAT mean absorption time; $CL_{tot/f}$ total body clearance with respect to unknown bioavailability f; Vdss/f volume of distribution at steady state with respect to unknown bioavailability f; Vdarea/f volume of distribution during the elimination phase with respect to unknown bioavailability.

Free thymol could not be detected in human plasma or urine. Only thymol sulphate but not the glucuronide conjugate was identified in human plasma and it could be detected 20min after administration. Plasma concentration of thymol (as sulphate conjugate) over time in eleven volunteers is displayed in Figure B.6.1.1.7/1.

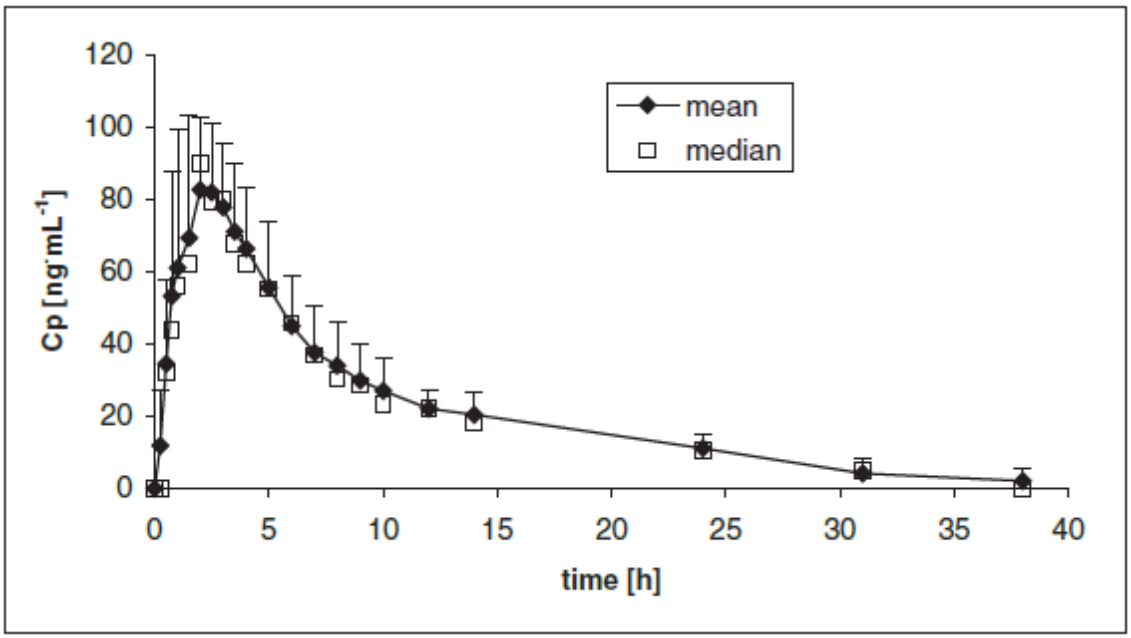


Figure B.6.1.1.7/1: Plasma thymol (as thymol sulphate) concentration over time in eleven volunteers

In urine, however, phase II conjugates of thymol were both identified (sulphate and glucuronide) and the ratio was constant over sampling times (Figure B.6.1.1.7/2).

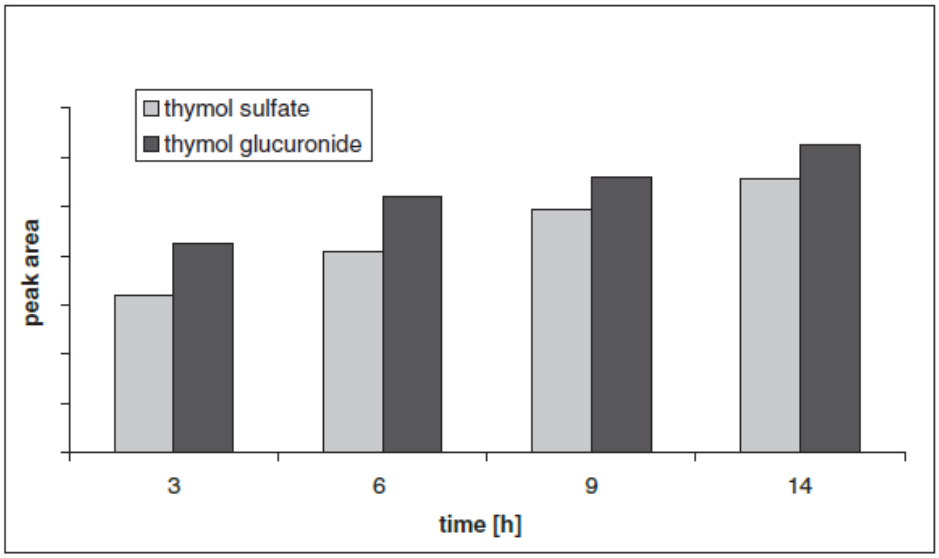


Figure B.6.1.1.7/2: Cumulative renal excretion of thymol sulphate and thymol glucuronide in one subject.

Elimination of thymol conjugates was detectable for the first 24h interval, with most being eliminated after 6h. The combined amount of both thymol sulphate and glucuronide excreted in 24h urine was 16.2 % ± 4.5%.

<u>Assessment and conclusion by applicant</u>:

This new study is considered to be acceptable for the renewal of thymol; the results are consistent with available data showing rapid absorption, moderately rapid excretion *via* urine, and predominantly conjugation with glucuronic acid or sulfate (reliable with restrictions; not GLP, only blood and urine collected).

Assessment and conclusion by RMS:

Outcome and conclusion of the study: This study is a scientific publication from a peer-reviewed journal. The RMS deems the study as acceptable to evaluate the pharmacokinetics of thymol in human. Based on the results of the study, thymol is rapidly absorbed and both thymol glucuronide and sulphate conjugates were identified in urine within 24h. Thymol sulphate only is detected in plasma and the maximum concentration was 93.1 ± 24.5 ng/mL at 1.97 ± 0.77 hours.

B.6.1.2. Absorption, distribution, metabolism and excretion by other routes

One study has been submitted to cover the ADME by other routes of thymol for the renewal process. This study has not been previously submitted and it has been evaluated as part of the renewal process.

There is no concern for toxicity following dermal exposure to thymol as acute dermal toxicity is expected low compared to acute toxicity following oral exposure. Studies by dermal route are not required.

B.6.1.2.1. Mouse: Pharmacokinetics intravenous dose

Data point	CA 5.2.1 (EU data requirement)
Report author	Xie, K., Tashkin, D.P., Luo, M.Z. and Zhang, J.Y.
Report year	2019
Report title	Pharmacokinetic study of thymol after intravenous injection and high-dose inhalation in mouse model
Report No	Not applicable, published study (*Pharmacol. Res. Perspect.*, 7 (5), 1-8)
Document No	KCA 5.1.1/07
Guidelines followed in study	Not stated
Deviations from current test guideline OECD TG 417 (2010)	-Test material not characterised (batch no. reported). -The selection of the dose was not justified and only one dose was tested in the study. -No individual data were provided (overall values only)
Previous evaluation	No, not previously evaluated
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Supporting information

Materials and Methods

Test chemical	Thymol, Purity: not stated; Batch no.: not stated
Vehicle	Saline solution
Test system	Male CD-1 mice
Test method	A single dose of 0.1 mg/mL of thymol in saline solution was directly injected as a bolus into 16 mice's tail vein at a volume of 0.16 mL within 2-3 seconds. The total amount of thymol injected was equivalent to 16 µg or 0.48 mg/kg bw. The mice were divided into two groups of 8 animals each for blood sampling. Mouse blood was collected from retro-orbital sinus under anesthesia (isoflurane) at baseline, 2, 10 and 30 min in one group and baseline, 5, 20 and 60 min post-dosing. The determination of thymol in mouse serum was carried out using LC/MS methodology. Pharmacokinetic parameters were calculated using Standard Phoenix/WinNonlin software.

Results

Thymol serum concentration over time is displayed in Table B.6.1.2.1/1. The maximum concentration of thymol occurred at 2min and it was 76.6 ng/mL. Thymol PK curve is displayed in Figure B.6.1.2.1. Thymol pharmacokinetics fit one compartmental model with first-order output. The resulting PK parameters are displayed in Table B.6.1.2.1/2.

Table B.6.1.2.1/1 : Thymol serum concentrations at various time points

Time (min)	Thymol concentration (ng/mL)*
0	0.0 ± 0.0
2	76.6 ± 6.0
5	41.4 ± 5.3
10	13.6 ± 1.0
20	3.7 ± 1.1
30	2.6 ± 0.8
60	0.2 ± 0.1
*mean ± SE (standard error)	

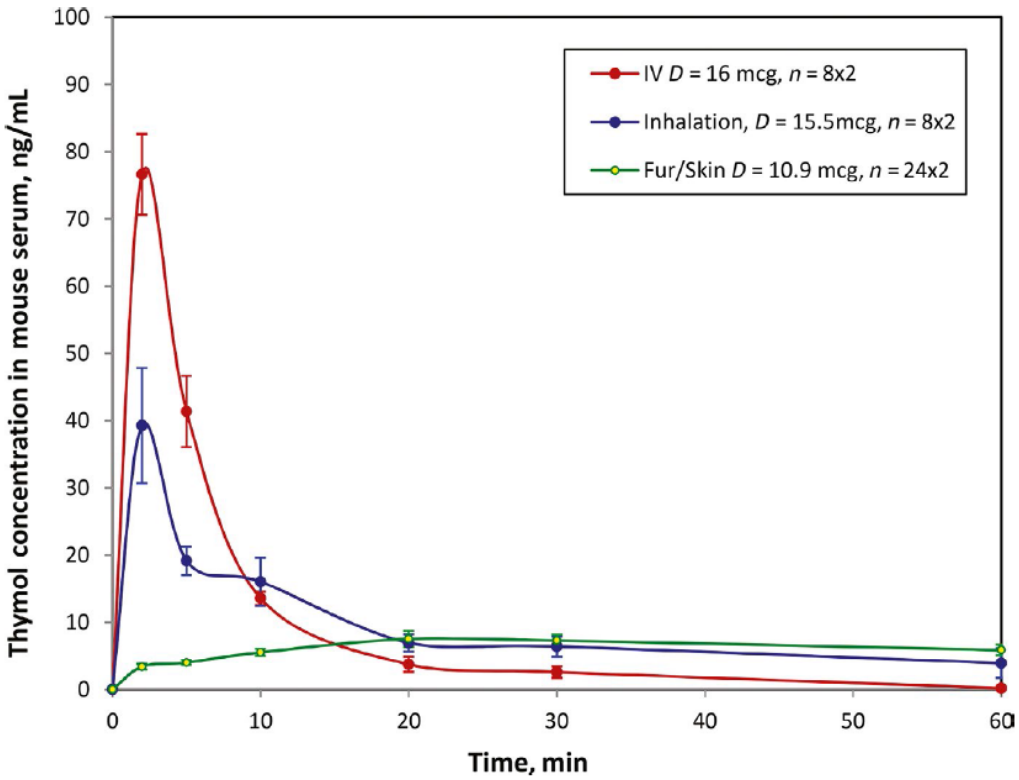


Figure B.6.1.2.1: Thymol serum concentration curve vs time in mouse model (experiment vs PK model)

Table B.6.1.2.1/2: Thymol pharmacokinetic parameters

Cmax (ng/mL)	$AUC_{0\text{-}60min}$ (ng/mL*min)	$AUC_{0\text{-}infinity}$ (ng/mL*min)	t_{max} (min)	$t_{1/2}$ (min)	Vd (mL)	Clt (mL/min)
78.3	626.7	626.7	2.00	3.43	136.4	27.6

Assessment and conclusion by applicant:

This new study is considered to be acceptable for the renewal of thymol as supportive information; the results are broadly consistent with available data showing rapid absorption (reliable with restrictions; study is similar to current OECD guideline but is not GLP and there are some deficiencies in the methodology)

Assessment and conclusion by RMS:

Outcome and conclusion of the study: This study is a scientific publication from a peer-reviewed journal. The RMS deems the study as supporting information to evaluate the pharmacokinetics of thymol in mice following intravenous injection. Based on the results of the study, thymol is rapidly detected in plasma and the maximum concentration was 78.3 ng/mL at 2 min. Deviations from the current OECD TG 417 (2010) include

no characterisation of the test item, no justification of the dose and very poor reporting of results (no individual data provided). Based on these deficiencies, the RMS deems the study as supporting information only.

B.6.1.2.2. Mouse: Pharmacokinetics inhalation

Data point	CA 5.2.1 (EU data requirement)
Report author	Xie, K., Tashkin, D.P., Luo, M.Z. and Zhang, J.Y.
Report year	2019
Report title	Pharmacokinetic study of thymol after intravenous injection and high-dose inhalation in mouse model
Report No	Not applicable, published study (*Pharmacol. Res. Perspect.*, 7 (5), 1-8)
Document No	KCA 5.1.1/07
Guidelines followed in study	Not stated
Deviations from current test guideline OECD TG 417 (2010)	-Test material not characterised (batch no. reported). -The selection of the dose was not justified and only one dose was tested in the study. -No individual data were provided (overall values only)
Previous evaluation	No, not previously evaluated
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Supporting information

Materials and Methods

Test chemical	Thymol, Purity: not stated; Batch no.: not stated
Vehicle	HFA
Test system	Male CD-1 mice
Test method	A total of 16 mice were mounted into a specially-designed 21.5 L breathing tank. Fifteen sprays of the test article containing 0.5% thymol in HFA were delivered into the tank. Thirty seconds after the last spray the mice were allowed to breathe from inside the chamber for 10min. The total amount of thymol inhaled was calculated to be 15.5 µg or 0.47 mg/kg bw. The mice were divided into two groups of 8 animals each for blood sampling. Mouse blood was collected from retro-orbital sinus under anesthesia (isoflurane) at baseline, 2, 10 and 30 min in one group and baseline, 5, 20 and 60 min post-dosing. The determination of thymol in mouse serum was carried out using LC/MS methodology. Pharmacokinetic parameters were calculated using Standard Phoenix/WinNonlin software.

Results

Thymol serum concentration over time is displayed in Table B.6.1.2.2/1. The maximum concentration of thymol occurred at 2min and it was 39.2 ng/mL. Thymol PK curve is displayed in Figure B.6.1.2.2. Thymol pharmacokinetics fit two-compartmental model with first-order output. The resulting PK parameters are displayed in Table B.6.1.2.2/2.

Table B.6.1.2.2/1: Thymol serum concentrations at various time points

Time (min)	Thymol concentration (ng/mL)*
0	0.0 ± 0.0
2	39.2 ± 8.6
5	19.2 ± 2.1
10	16.0 ± 3.6
20	6.9 ± 1.3
30	6.4 ± 1.5

60	3.9 ± 2.2
*mean ± SE (standard error)	

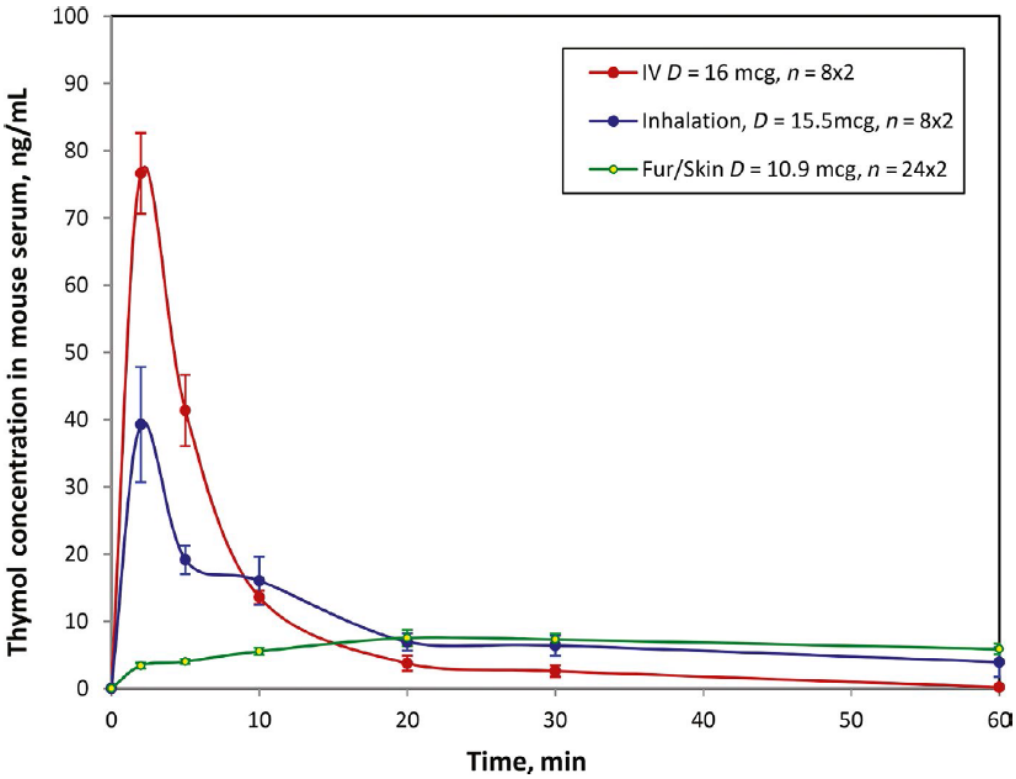


Figure B.6.1.2.2: Thymol serum concentration curve vs time in mouse model (experiment vs PK model)

Table B.6.1.2.2/2: Thymol pharmacokinetic parameters

Cmax (ng/mL)	$AUC_{0\text{-}60min}$ (ng/mL*min)	$AUC_{0\text{-}infinity}$ (ng/mL*min)	t_{max} (min)	$t_{1/2}$ (min)	Vd (mL)	Clt (mL/min)
42.3	464.3	484.2	2.00	4.73	64.7[a] 75.4[b]	9.48[a] 14.6[b]
[a] First compartment [b] Second compartment						

Assessment and conclusion by applicant:

This new study is considered to be acceptable for the renewal of thymol as supportive information; the results are broadly consistent with available data showing rapid absorption (reliable with restrictions; study is similar to current OECD guideline but is not GLP and there are some deficiencies in the methodology).

Assessment and conclusion by RMS:

Outcome and conclusion of the study: This study is a scientific publication from a peer-reviewed journal. The RMS deems the study as supporting information to evaluate the pharmacokinetics of thymol in mice *via* the inhalation route. Based on the results of the study, thymol is rapidly detected in plasma and the maximum concentration was 39.2 ng/mL at 2 min. Deviations from the current OECD TG 417 (2010) include no characterisation of the test item, no justification of the dose and very poor reporting of results (no individual data provided). Based on these deficiencies, the RMS deems the study as supporting information only.

B.6.2. ACUTE TOXICITY

No new data on acute toxicity have been submitted for the renewal process of thymol.

Acute toxicity studies presented in the original DAR (2011) are re-assessed according to current test guidelines and requirements.

B.6.2.1. Oral

B.6.2.1.1. Acute oral toxicity study in rats and guinea pigs with thymol

Data point	CA 5.2.1 (EU data requirement)
Report author	Jenner, P.M., Hagan, E.C., Taylor, J.M., Cook, E.L. and Fitzhugh, O.G.
Report year	1964
Report title	Food Flavourings and Compounds of Related Structure I. Acute Oral Toxicity
Report No	Not applicable, published study (*Fd Cosmet. Toxicol*., **2**, 327-343)
Document No	KCA 5.2.1/01
Guidelines followed in study	This study is pre-guideline. Similar to OECD TG 401 (1987, deleted in 2002)
Deviations from current test guideline OECD TG 420 (2001)	Very limited information provided in the study (method, dosing and results poorly described; no necropsy).
Previous evaluation	Yes, evaluated and accepted in DAR (2011)
GLP/Officially recognised testing facilities	No (prior to the enforcement of GLP)
Acceptability/Reliability	Supportive only (methodological deficits)

Materials and Methods

Test chemical	Thymol (purity unknown). 20% dilution in propylene glycol. According to the publication: *The commercially available material was used. No attempt was made to secure chemically pure compounds*. Therefore, a 20% dilution of an unknown thymol in propylene glycol was tested, instead of the technical active substance.
Vehicle	Propylene glycol.
Test system	Male and female young adult Osborne-Mendel rats Male and female guinea pigs
Test method	Thymol (20% w/v in propylene glycol) was administered orally by intubation to groups of 10 young adult rats evenly divided by sex and guinea pigs (male and female) (mice were also used for other compounds but not for thymol). Rats and guinea pigs were fasted 18 h prior to treatment. Mice were treated on full stomachs. Animals were observed usually for 2 weeks during which time the development of toxic signs was followed and time of death recorded. Such observation was continued until animals appeared normal and showed weight gain. In a few cases, where no acute toxic signs were seen, the animals were observed for only one week. The LD_{50} was calculated according to Litchfield & Wilcoxon.

Results

The LD_{50} slope function and their confidence limits, together with toxic signs and time of death are recorded for thymol in Table B.6.2.1.1.

Table B.6.2.1.1: Results of acute oral studies with thymol

Compound	Species	LD_{50} (mg/kg) with 95% confidence limits	Slope function with 95% confidence limits	Toxic signs and time of death
Thymol (20% w/v in propylene glycol)	Rat	980 (817-1180)	1.6 (1.3-2.0)	Time of death: 4 h – 5 days. Depression, ataxia, coma on high doses
	Guinea pig	880 (740-1050)	1.6 (1.2-2.1)	Time of death: 4 h – 5 days. Irritated gastro-intestinal tract, tremors, coma, respiratory failure

Assessment and conclusion by applicant: The study has been previously reviewed at EU level and is acceptable for renewal of the active substance. The EU-agreed acute oral LD_{50} in rats is 980 mg/kg bw and 880 mg/kg bw in guinea pigs (EFSA Journal 2012;10(11):2916).
Assessment and conclusion by RMS: This study was carried out prior to OECD Test Guidelines on acute oral toxicity, and several relevant differences with internationally accepted guidelines have been detected (see deviations point). Therefore, RMS deems this study as supportive, but not acceptable for classification purposes. It should be noted that LD_{50} values (980 mg/kg bw in rats and 880 mg/kg bw in guinea pigs) were obtained with the commercially available thymol that was tested, instead of the technical active substance.

B.6.2.1.2. Acute oral toxicity study in mice with thymol

Data point	CA 5.2.1 (EU data requirement)
Report author	Hasegawa R. Nakaji Y., Kurokawa Y. and Tobe M.
Report year	1989
Report title	Acute toxicity tests on 113 environmental chemicals
Report No	Not applicable, published study (*Science Reports of the Research Institutes, Tohoku University*, 36(1-4):10-16)
Document No	KCA 5.2.1/02
Guidelines followed in study	No claim made for Guideline compliance. Study performed according to the requests from the Ministry of health and welfare and other Japanese agencies.
Deviations from current test guideline OECD TG 420 (2001)	Publication on a review of acute toxicity studies performed since 1966. Squalene was used as vehicle but the doses of thymol assayed were not indicated. Very limited information provided in the study (method, dosing and results poorly described).
Previous evaluation	Yes, evaluated and accepted in DAR (2011)
GLP/Officially recognised testing facilities	Not stated (studies from 1966 pre-GLP. Data of the study on thymol not provided)
Acceptability/Reliability	Supportive only (methodological deficits)

Materials and Methods

Test chemical	Thymol (CAS 89-83-8; chemicals used were of the highest purity available) in squalene (dose not indicated).
Vehicle	Squalene
Test system	Male and female ddY mice
Test method	Thymol diluted in squalene (concentration not provided) was administered orally after 15 h of food deprivation. Ten animals were used for each dosing and at least 6 increasing dose levels were selected and tested to confirm both LD_0 and LD_{100} values. No data on the used dose levels is provided (only the resulting LD_{50} values). Clinical signs and symptoms were monitored during the experimental period (14 days). Anatomical examination was performed after death or at terminal sacrifice on day 14. LD_{50} values were calculated using the Litchfield & Wilcoxon method. No other details were reported.

Results

The observed clinical signs were hypoactivity and ataxic gait. Small intestine congestion was found at necropsy. The LD_{50} values were calculated to be 1200 mg/kg bw in males and 1050 mg/kg bw in females.

Assessment and conclusion by applicant: The study has been previously reviewed at EU level and is acceptable for renewal of the active substance. The EU-agreed acute oral LD_{50} in mice is 1200 mg/kg bw in males and 1050 mg/kg bw in females (EFSA Journal 2012;10(11):2916).
Assessment and conclusion by RMS: The publication includes data of acute toxicity studies performed since 1966 (pre-guideline), and no indication is given about the conditions thymol was assayed. Several relevant differences with internationally accepted guidelines have been detected (see deviations point). Therefore, RMS deems this study as supportive, but not acceptable for classification purposes.

B.6.2.1.3. Acute oral toxicity studies in several species with thymol

Data point	CA 5.2.1 (EU data requirement)
Report author	Escobar A.
Report year	2006
Report title	Final Report on the Safety Assessment of Sodium p-Chloro-m-Cresol, p-Chloro-m-Cresol, Chlorothymol, Mixed Cresols, m-Cresol, o-Cresol, p-Cresol, Isopropyl Cresols, Thymol, o-Cymen-5-ol, and Carvacrol
Report No	Not applicable, published study (*International Journal of Toxicology*, 25 (Suppl. 1): 29–127).
Document No	KCA 5.2.1/03
Guidelines followed in study	Not applicable. The publication is a review of published literature on several related substances (included thymol) used as cosmetic biocides/preservatives and/or fragrance ingredients. No data on the test guidelines followed in each study was provided.
Deviations from current test guideline	Not applicable (review of published literature). Test substances not characterised. Poorly described methods and results.
Previous evaluation	Yes, evaluated and accepted in DAR (2011)
GLP/Officially recognised testing facilities	Not stated for any study (review of published literature).
Acceptability/Reliability	Supportive only (review of published literature)

Executive summary

Escobar (2006) reviewed a number of publications related to the acute oral toxicity of thymol (among other related compounds). Several studies, including the publications of Jenner and Hasegawa summarised above (B.6.2.1.1 and B.6.2.1.2), are cited. The data provided in the publication for each study is presented in Table B.6.2.1.3.

Table B.6.2.1.3: Results of acute oral toxicity studies with thymol

Species	Concentration and vehicle	Mode of administration	Doses (mg/kg bw)	Clinical signs/ Mortality	LD_{50} (mg/kg bw)	Reference
Rabbit	Undiluted	Gelatine capsule Single dose	500, 750, 2000 and 3000	Only deaths occurring in the first 16 days were considered related to the treatment: -500 mg/kg bw: 6/6 animals survived 16 days or more. -750 mg/kg bw: 1/1 animals survived less than 16 days. -2000 mg/kg bw: 4/5 animals survived less than 16 days. -3000 mg/kg bw: 2/2 animals survived less than 16 days.	**LD_{50} between 500 and 750 mg/kg bw**	Livingston (1921)

Species	Concentration and vehicle	Mode of administration	Doses (mg/kg bw)	Clinical signs/ Mortality	LD_{50} (mg/kg bw)	Reference
Rabbit	50% in Olive oil	Gelatine capsule Single dose	250, 500, 750, 1000, 1500, 2000 and 3000	Only deaths occurring in the first 16 days were considered related to the treatment: -250 mg/kg bw: 1/5 animal survived less than 16 days. -500 mg/kg bw: 0/10 animals survived less than 16 days. -750 mg/kg bw: 1/5 animals survived less than 16 days. -1000 mg/kg bw: 3/6 animals survived less than 16 days. -1500 mg/kg bw: 2/5 animals survived less than 16 days. -2000 mg/kg bw: 3/5 survived less than 16 days. -3000 mg/kg bw: 2/2 survived less than 16 days.	**LD_{50} between 750 and 2000 mg/kg bw**	
Mouse (male)	Cottonseed oil	Gavage	620, 940, 1400 and 2100	All deaths occurred within 24 h. Only depression at lower doses. Depression and prostration at higher doses.	**LD_{50} = 1800 mg/kg bw**	McOmie *et al.* (1949)
Rat (Osborne -Mendel)	20% in propylene glycol.	Intubation	Not stated	Rats died within 4h to 5d Depression and ataxia in most dose groups, and coma at greater doses.	**LD_{50} rat = 980 mg/kg bw**	Jenner *et al.* (1964) B.6.2.1.1
Guinea pig	20% in propylene glycol.	Intubation	Not stated	Guinea pigs showed irritated gastrointestinal tract, tremors, coma and respiratory failure.	**LD_{50} guinea pig =880 mg/kg bw**	
Mouse (ddY males and females)	Squalene (concentration not stated)	Not stated	Not stated	Hypoactivity and ataxic gait. Small intestinal congestion.	**LD_{50} = 1050 mg/kg bw (f)** LD_{50} = 1200 mg/kg bw (m)	Hasegawa *et al.* (1989) B.6.2.1.2
Mouse	Not stated	Not stated	Not stated	Not stated	**LD_{50} mouse = 640 mg/kg bw**	Instituto Superiore di Sanità (1999)
Cat	Not stated	Not stated	Not stated	Not stated	**LD_{50} cat = 250 mg/kg bw**	
Rabbit	Not stated	Not stated	Not stated	Not stated	**LD_{50} rabbit = 750 mg/kg bw**	
Mouse (male)	Alcohol/propylene glycol/water	Gavage	Not stated	Decrease in spontaneous movemets, piloerection and paralysis of anterior limbs within 1h. Death occurred within 5 to 6 h.	**LD_{50} = 640 mg/kg bw**	RIFM (2001e)
Mouse (white)	10% in peanut oil	Gavage	500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1100, 1200,	Effects were observed from 1100 mg/kg bw and above: 1/8 mice died with 1100 3/8 mice died with 1200	**LD_{50} between 1200 and 1300 mg/kg bw**	

Species	Concentration and vehicle	Mode of administration	Doses (mg/kg bw)	Clinical signs/ Mortality	LD_{50} (mg/kg bw)	Reference
			1300, 1400 and 1500	4/8 mice died with 1300 6/8 mice died with 1400 8/8 mice died with 1500		
Mouse (white)	10% in aqueous emulsion	Gavage	500, 550, 600, 650, 700, 750 and 800	Effects were observed from 600 mg/kg bw and above: 1/8 mice died with 600 4/4 mice died with 650 6/8 mice died with 700 8/8 mice died with 750 8/8 mice died with 800	**LD_{50} between 600 and 650 mg/kg bw**	

Conclusion:

The cited LD_{50} values in mice, rabbits, guinea pigs and cats ranged from 250 mg/kg bw (in cats) to 1800 mg/kg bw (in male mice).

Assessment and conclusion by applicant:

The study has been previously reviewed at EU level and was accepted as supporting information.

The EU-agreed acute oral LD_{50} in rats is 980 mg/kg bw and 880 mg/kg bw in guinea pigs (EFSA Journal 2012;10(11):2916).

Assessment and conclusion by RMS:

The publication is a review of published literature on several related substances (included thymol) used as cosmetic biocides/preservatives and/or fragrance ingredients, including thymol. Provided data on the reported studies is very limited. Therefore, RMS deems this information as supportive, but not acceptable for classification purposes.

The lowest value of LD_{50} reported in this publication is 250 mg/kg bw in cats. However, for this study not even the concentration, vehicle, mode of administration used or number of treated animals is provided. With such data lacking, RMS deems this result cannot define the LD_{50} value for thymol and rather an overall range of doses should be considered.

The results obtained in the rest of the publications reviewed in this paper present LD_{50} values that lie between 300 and 2000 mg/kg bw, confirming the classification of thymol as acute oral toxicity, category 4 (H302) as it is already included in Annex VI of Regulation (EC) No 1272/2008.

B.6.2.1.4. Acute oral toxicity in mice

Data point	CA 5.2.1 (EU data requirement)
Report author	BG Chemie
Report year	2000
Report title	Thymol
Report No	None, published report. (Toxicological evaluation, No. 259)
Document No	KCA 5.9.2/01
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Not applicable (secondary source review)
Acceptability/Reliability	Supportive only (secondary source review)

Executive summary

BG Chemie (2000) is a toxicological evaluation of thymol that includes a review of several publications related to the acute oral toxicity of thymol. Table B.6.2.1.4 includes information on acute oral toxicity in animals with

thymol, that was reported in BG Chemie (2000) but was different from the data recoiled in the previous points (B.6.2.1.1 to B.6.2.1.3).

Table B.6.2.1.4: Data on acute oral toxicity of thymol

Species	Concentration and vehicle	Mode of administration	Doses (mg/kg bw)	Clinical signs/ Mortality	LD_{50} (mg/kg bw)	Reference
Mouse (male)	1 to 4% aqueous solutions in cottonseed oil	Orally by gavage	620 to 2100	Depressed general condition. Death occurred within 48 h. Gross pathology revealed haemorrhages in the small intestines and severe oedema and congestion of the lungs.	**LD_{50} = 1800 mg/kg bw**	McOmie *et al.* (1949)
Mouse (groups of 8)	thymol as a 10% peanut oil solution	Oral	Not indicated	Drowsiness and paralysis occurred after 2 to 5 minutes. At the later stages: muscle twitching and spasms. Deaths occurred after 6 to 12 hours. The survivors recovered slowly.	**LD_{50} = 1300 mg/kg bw**	Oelkers, (1940)
	thymol as a 10% aqueous emulsion				**LD_{50} = 650 mg/kg bw**	

Assessment and conclusion by applicant:

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Assessment and conclusion by RMS:

BG Chemie (2000) is a toxicological evaluation of thymol that includes a review of several publications related to the acute oral toxicity of thymol. The publication was provided by the applicant to support the Medical data point (B.6.9.2 and B.6.9.3). However, this toxicological report also includes information on acute oral toxicity in animals, different from the data recoiled in the previous points (B.6.2.1.1 to B.6.2.1.3).

Therefore, RMS has added this point B.6.2.1.4 to include just the new (or more explained) data reported in BG Chemie (2000) that was not reported in the previous points. However, the data is considered supporting information, since it is provided by a secondary source review.

B.6.2.2. Dermal

B.6.2.2.1. Acute dermal toxicity study with thymol

Data point	CA 5.2.2 (EU data requirement)
Report author	US-Environmental Protection Agency
Report year	2006
Report title	Thymol, Exemption for a requirement of a tolerance
Report No	Not applicable, published paper (Federal register, 71(11) 18th January, 2006)
Document No	KCA 5.2.2/01
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
Previous evaluation	Yes, evaluated and accepted in DAR (2011)
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Supportive only (secondary source review)

Executive summary

The US EPA publication, among other data, states that the waiver submitted to support the acute dermal toxicity data requirement was based upon information collected from a report by the Environmental Risk Management Agency (ERMA, 2005) of New Zealand and Anonymous (2000) which found dermal LD_{50} value of >2000 mg/kg bw.

Assessment and conclusion by applicant:

The information has been previously reviewed at EU level and was accepted as supporting information.

The EU-agreed endpoint: based on published information in the rabbit and other quoted values in the literature, LD_{50} likely to be >2000 mg/kg bw.

Assessment and conclusion by RMS:

The publication includes data provided to EPA as a waiver of the requirements of a tolerance for residues of the thymol on honey, honeycomb, and honeycomb with honey in a very specific use (when applied as treatment to decrease de incidence of *Varroa* mite infestation in the honeybee).

Only the LD_{50} value was provided and, therefore, this publication is considered as supportive information.

B.6.2.2.2. Acute dermal toxicity study with thymol

Data point	CA 5.2.2 (EU data requirement)
Report author	Escobar A.
Report year	2006
Report title	Final Report on the Safety Assessment of Sodium p-Chloro-m-Cresol, p-Chloro-m-Cresol, Chlorothymol, Mixed Cresols, m-Cresol, o-Cresol, p-Cresol, Isopropyl Cresols, Thymol, o-Cymen-5-ol, and Carvacrol
Report No	Not applicable, published study (*International Journal of Toxicology*, 25(Suppl. 1):29–127)
Document No	KCA 5.2.1/03
Guidelines followed in study	Not applicable. The publication is a review of published literature on several related substances (included thymol) used as cosmetic biocides/preservatives and/or fragrance ingredients. No data on the test guidelines followed in each study was provided.
Deviations from current test guideline	Not applicable. Test substance not characterised. Poorly described method and results.
Previous evaluation	Yes, evaluated and accepted in DAR (2011)
GLP/Officially recognised testing facilities	Not stated for any study (review of published literature).
Acceptability/Reliability	Supportive only (review of published literature)

Executive summary

Escobar (2006) reviewed a number of publications related to the acute dermal toxicity of thymol (among other related compounds). Two studies are cited. The data provided in the publication for each study is presented in Table B.6.2.2.2.

Table B.6.2.2.2: Results of acute dermal toxicity studies with thymol

Species	Concentration and vehicle	Mode of administration	Doses (mg/kg bw)	Clinical signs/ Mortality	LD_{50} (mg/kg bw)	Reference
Rabbit	Not stated	Application to shaved back 24 h exposition	420 mg/kg bw	No apparent systemic effect. No deaths occurred. The skin turned parchment-like within 24 h. Complete necrosis of superficial skin layers occurred after 10 days.	**LD_{50} > 420 mg/kg bw**	McOmie *et al.* (1949)
Rabbit New Zealand	Not stated	Application to the clipped abraded	2000 mg/kg bw	No deaths occurred. At 24 h: 10/10 had	**LD_{50} > 2000 mg/kg bw**	RIFM (1972b as cited

Species	Concentration and vehicle	Mode of administration	Doses (mg/kg bw)	Clinical signs/ Mortality	LD_{50} (mg/kg bw)	Reference
(10 animals)		abdominal skin 24 hours exposition 7 d observation Necropsy was performed but no results were reported		moderate erythema, 8/10 moderate oedema and 2/10 slight oedema On day 7: 3/10 showed moderate erythema and 4/10 slight erythema.		in RIFM 2001e)

Conclusion:

Acute dermal LD_{50} value stated to be >2000 mg/kg bw.

Assessment and conclusion by applicant:

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance as supporting information.

The EU-agreed endpoint: based on published information in the rabbit and other quoted values in the literature, LD_{50} likely to be >2000 mg/kg bw.

Assessment and conclusion by RMS:

The publication is a review of published literature on several related substances (included thymol) used as cosmetic biocides/preservatives and/or fragrance ingredients, including thymol. Provided data on the reported studies is very limited. Therefore, RMS deems this information as supportive, but not acceptable for classification purposes.

B.6.2.2.3. Acute dermal toxicity in rats

Data point	CA 5.2.2 (EU data requirement)
Report author	BG Chemie
Report year	2000
Report title	Thymol
Report No	None, published report. (Toxicological evaluation, No.259)
Document No	KCA 5.9.2/01
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Not applicable (secondary source review)
Acceptability/Reliability	Supportive only (secondary source review)

Executive summary

BG Chemie (2000) is a toxicological evaluation of thymol that includes a review of several publications related to the acute dermal toxicity of thymol. Table B.6.2.2.3 includes information on acute dermal toxicity in animals with thymol, that was reported in BG Chemie (2000) but was different from the data recoiled in the previous points (B.6.2.2.1 to B.6.2.2.2).

Table B.6.2.2.3: Data on acute dermal toxicity of thymol

Method/ Species	Concentration and vehicle	Mode of administration	Doses (mg/kg bw)	Clinical signs/ Mortality	LD_{50} (mg/kg bw)	Reference
EEC/B.3 (1984) Rats SPF-Wistar (5 male and 5	thymol (99.5 to 99.6% pure) mixed into a paste with Cremophor	Dermal 24-h occlusive exposure in the shorn dorsal and flank skin (10% of body surface)	2000	No deaths and no signs of toxicity (14-day observation period) 3/5 male and 2/5 females showed brownish discoloration	**LD_{50} > 2000 mg/kg bw**	Bayer, (1986 a)

Method/ Species	Concentration and vehicle	Mode of administration	Doses (mg/kg bw)	Clinical signs/ Mortality	LD_{50} (mg/kg bw)	Reference
female)				at application site which lasted for up to 4 days. No substance-related changes observed at terminal necropsy.		

Assessment and conclusion by applicant:

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Assessment and conclusion by RMS:

BG Chemie (2000) is a toxicological evaluation of thymol that includes a review of several publications related to the acute oral toxicity of thymol. The publication was provided by the applicant to support the Medical data point (B.6.9.2 and B.6.9.3). However, this toxicological report also includes information on acute oral toxicity in animals, different from the data recoiled in the previous points (B.6.2.2.1 to B.6.2.2.2).

Therefore, RMS has added this point B.6.2.2.3 to include just the new (or more explained) data reported in BG Chemie (2000) that was not reported in the previous points. However, the data is considered supporting information, since it is provided by a secondary source review.

B.6.2.3. Inhalation

Data point	CA 5.2.3 (EU data requirement)
Report author	US-Environmental Protection Agency
Report year	2006
Report title	Thymol, Exemption for a requirement of a tolerance
Report No	Not applicable, published paper (Federal register, 71(11) 18th January, 2006)
Document No	KCA 5.2.2/01
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
Previous evaluation	Yes, evaluated and accepted in DAR (2011)
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Supportive only (secondary source review)

Materials and Methods

Test chemical	Thymol as preservative (0.01%) in the anaesthetic halothane
Vehicle	Not indicated
Test system	Not applicable
Test method	Not applicable

Executive summary

The US EPA publication, among other data, states that the waiver submitted to support the acute inhalation toxicity data requirement was based upon information from the U.S. Food and Drug Administration Center for Drug Evaluation and Research (Ref. 19). According to the report, thymol is added to the anaesthetic halothane as a preservative (0.01%), and may be used to anaesthetise dogs, cats and other non-food animals for periods exceeding 4 hours using concentrations typically around 5%. Thymol concentration from these conditions has been calculated to be ≈5 mg/L. Since this is the limit concentration for an acute inhalation study and since this exposure concentration is without adverse effects in various animals it is considered unnecessary to conduct a study.

Assessment and conclusion by applicant:

Thymol is used in veterinary anaesthetics without adverse effects, at a concentration equivalent to the limit concentration specified in OECD guidelines.

The report has been previously reviewed at EU level and was accepted as supporting information.

Assessment and conclusion by RMS:

The publication includes data provided to EPA as a waiver of the requirements of a tolerance for residues of the thymol on honey, honeycomb, and honeycomb with honey in a very specific use (when applied as treatment to decrease de incidence of *Varroa* mite infestation in the honeybee).

This publication is considered as supportive information.

B.6.2.4. Skin irritation

Data point	CA 5.2.4 (EU data requirement)
Report author	BG Chemie
Report year	2000
Report title	Thymol
Report No	None, published report. (Toxicological evaluation, No. 259)
Document No	KCA 5.9.2/01
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Not applicable (secondary source review)
Acceptability/Reliability	Supportive only (secondary source review)

Executive summary

BG Chemie (2000) is a toxicological evaluation of thymol that includes a review of several publications related to the toxicity of thymol. Table B.6.2.4 includes information on the skin irritation study in animals that was reported in BG Chemie (2000).

Table B.6.2.4: Data on skin irritation/corrosion of thymol

Method, Species, strain, sex, no/group	Test substance	Dose levels, conditions and duration of exposure	Results	Reference
Primary skin irritancy OECD 404 6 adult albino rabbits (both sexes)	Thymol (99.5% pure) Made to a paste with water	500 mg of test substance (paste) Applied to the mechanically depilated flank skin for a 4-hour semi-occlusive exposure. Findings were scored 1, 24, 48 and 72 h, and after 7 and 14 days.	Thymol proved corrosive in all of the animals. The necrotic changes were irreversible in all of the animals up to the end of the observation period.	Bayer (1986 b)

Assessment and conclusion by applicant:

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Assessment and conclusion by RMS:

BG Chemie (2000) is a toxicological evaluation of thymol that includes a review of several publications related to the toxicity of thymol. The publication was provided by the applicant to support the Medical data point (B.6.9.2 and B.6.9.3). However, this toxicological report also includes information on a skin irritancy study with thymol and, therefore, the RMS has added this point to include it as supportive information.

B.6.2.5. Eye irritation

B.6.2.5.1. Eye irritation test

Data point	CA 5.2.5 (EU data requirement)
Report author	McOmie W.A., Anderson H.H. and Estess F.M.
Report year	1949
Report title	Comparative toxicity of certain t-Butyl substituted cresols and xylenols
Report No	Not applicable, published paper (Journal of the American Pharmaceutical Association, 38:366-369)
Document No	KCA 5.2.5/01
Guidelines followed in study	This study is pre-guideline.
Deviations from current test guideline OECD TG 405 (2021)	Only a brief description of the method used and the effect produced by thymol (together with other alkyl phenols) on the eyes of rabbits is provided in this publication. No data is provided on the strain, sex or number of animals used, the duration of the exposure, results after 48 hours or the individual data on the different parameters (conjunctival redness and chemosis, and iris and corneal lesions).
Previous evaluation	Yes, evaluated and accepted in DAR (2011)
GLP/Officially recognised testing facilities	No (prior to the enforcement of GLP)
Acceptability/Reliability	Supportive only (methodological deficits)

Materials and Methods

Test chemical	Thymol containing less than 1% of impurities.
Vehicle	Ethylene glycol
Test system	Rabbits (no further details)
Test method	0.03 mL of a solution of thymol diluted at 40% in ethylene glycol was applied directly on the cornea of rabbits. Corneas were observed after 1, 24 and 72 hours, and fluorescein test was performed after 24h. The damage to the eyes was scored using the Draize method.

Results

Draize scores are summarised in the table below. At 1 hour the corneal score was 32 out of a maximum of 100. After 24 hours the entire surface of the cornea was damaged and after 24 and 72 hours the treated eye was closed so completely that eye injury could not be evaluated by the scoring method. An estimate of 80/100 was made for irritation at 24 and 72 hours.

Table B.6.2.5.1: Draize scores in rabbit eyes after application of a 40% thymol solution

Observation time	1 h	24 h	72 h	Fluorescein test at 24 h
Score	32	80*	80*	100

**Treated eye was closed so completely that eye injury could not be evaluated by the scoring method*

Conclusion

Thymol was found to be extremely irritant to the eyes of rabbits.

Assessment and conclusion by applicant:

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

EU-agreed endpoint: Extremely irritant.

Assessment and conclusion by RMS:

The study is prior to any test guidelines and, therefore, presents many deficiencies. However, the results obtained for thymol as extremely irritant are in line with the actual classification of thymol as corrosive (Skin Corr. 1, H314), included in Annex VI of Regulation (EC) No. 1272/2008.

Therefore, classification as serious eye damage, category 1 (Eye Dam.1) is also required (although the hazard statement H318 is already included in the hazard statement H314 for skin corrosion: Causes severe skin burns and eye damage).

B.6.2.5.2. Eye irritation study

Data point	CA 5.2.5 (EU data requirement)
Report author	BG Chemie
Report year	2000
Report title	Thymol
Report No	None, published report. (Toxicological evaluation, No. 259)
Document No	KCA 5.9.2/01
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Not applicable (secondary source review)
Acceptability/Reliability	Supportive only (secondary source review)

Executive summary

BG Chemie (2000) is a toxicological evaluation of thymol that includes a review of several publications related to the toxicity of thymol. The previous publication of McOmie *et al.*, 1949 (B.6.2.5.1) was also mentioned in BG Chemie (2000) but no new data was provided for this study. Therefore, table B.6.2.5.2 includes only information on the eye irritation study in animals that was reported in BG Chemie (2000) and not included in the previous data point.

Table B.6.2.5.2: Data on eye irritation of thymol

Method, Species, strain, sex, no/group	Test substance	Dose levels, conditions of exposure	Results	Reference
Eye irritancy of thymol OECD 405 3 adult female albino rabbits	Thymol (99.5% pure)	100 µl (bulk weight approx.. 60 mg) instilled into the conjunctival sac of one eye. Rinsed out with physiological saline solution after 24h. Findings were scored according with Draize: 1h after rinsing, and again 24, 48 and 72 hours, and 7, 14 and 21 days.	Irritation scores: - Conjunctivae: 2 to 2.3 - Iris: 1.0 - Cornea: 1.3 to 2.7. Up to day 21 the animals had positive fluorescein test results and, as of the day 14 of the observation period, 2 rabbits developed pannus while the third showed swelling in the lower part of the eyeball. Thymol was evaluated as strong irritant on the basis of these irreversible findings.	Bayer (1986 b)

<u>Assessment and conclusion by applicant</u>:

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<u>Assessment and conclusion by RMS</u>:

BG Chemie (2000) is a toxicological evaluation of thymol that includes a review of several publications related to the toxicity of thymol. The publication was provided by the applicant to support the Medical data point (B.6.9.2 and B.6.9.3). However, this toxicological report also includes information on eye irritation with thymol and, therefore, the RMS has added this point to include it as supporting information.

B.6.2.6. Skin sensitization

B.6.2.6.1. Skin sensitization study in guinea pig

Data point	CA 5.2.6 (EU data requirement)
Report author	Klecak G., Gelbick H. and Frey J.R.
Report year	1977
Report title	Screening of fragrance materials for allergenicity in the guinea pig
Report No	Not applicable, published study (*Journal of the Society of Cosmetic Chemists*, 28:53-64)
Document No	KCA 5.2.6/01
Guidelines followed in study	This study is pre-guideline.
Deviations from current test guideline OECD TG 406 (2021)	The study investigated, in a battery of tests, 32 chemicals that had previously been described in the literature as being allergenic for man. No positive controls were used. Limited reporting on the methodology followed with thymol: a general methodology is described for all the substances. However, 10% was the higher concentration tested for thymol due to systemic toxicity (which was also not described). Therefore, in the case of thymol, method and results were poorly described (dermal responses after application in preliminary and main study, clinical signs, body weights...).
Previous evaluation	Yes, evaluated and accepted in DAR (2011)
GLP/Officially recognised testing facilities	No (prior to the enforcement of GLP)
Acceptability/Reliability	Supportive only (methodological deficits)

Materials and Methods

Test chemical	Thymol (purity not stated)
Vehicle	DT: isotonic saline Not specified for the other 3 techniques
Test system	Himalayan white-spotted guinea pigs (male and female)
Test method	32 chemicals that had previously been described in the literature as being allergenic for man, where investigated in a battery of tests in guinea pigs by these four techniques: <u>Open Epicutaneous Test (OET)</u>: *Preliminary test*: Test substances were tested undiluted and in a range of solvents (including acetone, ethanol, diethyl phthalate) at concentrations of 30, 10, 3, 1, 0.3, 0.1 and 0.03% to determine the minimal irritating and the maximum tolerated concentration. A single application of 0.025 mL was applied to an area measuring 2 cm² on the clipped flank skin (6-8 animals per group). Application sites were left uncovered. Reactions were scored after 24 h using an “all or none” criterion to determine a dose-response curve. The minimal irritating concentration was defined as the lowest concentration causing mild erythema in at least 25% of the animals in the group, and the maximal non-irritant concentration was the highest concentration causing no macroscopically discernible reactions in any animal in the group. *Induction*: On day 0, 0.1 mL of test substances at 4 to 6 concentrations between 100, 30, 10, 3, 1, 0.3, 0.1 and 0.03% were applied to an area measuring 8 cm² on the clipped flank skin of the animals (6-8 animals per group). No data is provided on the concentrations used for each component, however it can be concluded from the publication that thymol was used at a 3% concentration and up to 10%. Applications were repeated daily for 21 days on the same skin site. Application sites were left uncovered and the reactions scored 24 h after each application. The maximum non-irritant and the minimal non-irritant concentrations were determined. The following scale was used to evaluate irritation: 0 = no reaction; 0.5 = red spots; 1 = confluent redness; 2 = redness plus swelling; 3 = redness, swelling, crusts; 4 = necrosis. *Challenge*: Animals previously treated in the induction phase, together with 6 to 8 untreated controls for each compound, were challenged on days 21 and 35 on the contralateral flank with the same compound at the minimal irritating concentration (3% in the case of thymol) and some lower nonirritant concentrations; 0.025 mL of the test substance was applied to an

	area of skin measuring 2 cm² and the reactions scored after 24, 48 and/or 72 h. A concentration was considered sensitising when at least 2 out of 8 animals showed positive reactions to non-irritating challenge concentrations. A range of solvents were used for the various test chemicals and included water, ethanol, acetone, vaseline, diethyl phthalate, polyethylene glycol. The vehicle chosen for thymol was not specified. Draize Test (DT): *Induction:* A dose of 0.05 mL of a 0.1% solution of thymol in isotonic saline was injected intradermally on day 0. Further 0.1 mL doses were injected on 9 alternate days. *Challenge:* The treated animals and untreated controls were challenged intradermally with the 0.1% solution on days 35 and 49. The evaluation criterion was the mean diameter of the papular reactions. Maximization Test (MT): *Induction:* On day 0 animals were administered paired intradermal injections of 0.1 mL of 5% test substance (vehicle not specified), 0.1 mL of 5% test substance in Freund's complete adjuvant (FCA) and 0.1 mL of FCA alone. In addition, 250 mg of 25% test substance in petrolatum (chosen because this substance always causes mild to moderate skin irritation under occlusion) were applied on day 8 to the clipped skin on the neck and covered with an occlusive dressing for 2 days. *Challenge:* On day 21 an occlusive patch test with the test substance at a subirritant concentration in petrolatum was applied to the flank for 24 h. Reactions were read 24 and 48 h after patch removal. Test with Freund's Complete Adjuvant (FCAT): *Inducction:* Doses of 0.05 mL undiluted test substance mixed with the same volume of FCA were injected intradermally into the neck on days 0, 2, 4, 7, and 9. Controls were similarly treated with 0.05 mL of FCA alone. *Challenge:* Animals were tested epicutaneously on days 21 and 35 as described in the section above (MT).

Results

Thymol was not found to be sensitising at a concentration of 10% with any of the four techniques used. Higher concentrations of thymol were not tested because of systemic toxicity.

Open epicutaneous test (OET): The minimum non-irritating concentration after 1 and 21 applications was 3%.

Negative results were reported for the four tests: OET, DT, MT and FCAT.

Table B.6.2.6.1: Skin sensitisation results with thymol

Test substance	OET				Allergenicity in guinea pigs			
	Min. irritating conc. %		Min. sensitising conc. %	Min. eliciting conc. %	OET	DT	MT	FCAT
	After 1 application	After 21 applications						
Thymol*	3	3	-	-	-	-	-	-

**Thymol was not sensitising at 10%. Higher testing concentrations were not used because of systemic toxicity*

Regarding the allergenicity to humans, the paper also describes a literature survey for sensitisation in humans. On the basis of this survey thymol was classified as "compound with confirmed sensitizing capacity for man" and "compound causing positive patch tests in eczematous patients with no history of sensitization either to this particular compound or to the complex allergen known to contain it".

Assessment and conclusion by applicant:

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

Thymol was not sensitising to guinea pig skin in the OET, the Draize test, the maximisation test and in the FCA test. The overall EU-agreed endpoint is: inconsistent results in animal tests, reports of skin sensitisation in humans.

Assessment and conclusion by RMS:

This published paper is prior to OECD test guidelines and GLP. The study investigated, in a battery of tests, 32 chemicals that had previously been described in the literature as being allergenic for man. Negative results in guinea pig are reported. However, no data on the concentrations used with thymol is provided, but that it was tested at concentrations up to 10% (the description of the method is the general technique used for the 32 chemicals, but thymol was not tested at concentrations higher than 10% because of systemic toxicity). Also skin reactions were not reported at any point. Altogether, RMS deems this study as supportive, but not acceptable for classification purposes.

B.6.2.6.2. Skin sensitization study with thymol

Data point	CA 5.2.6 (EU data requirement)
Report author	Escobar A.
Report year	2006
Report title	Final Report on the Safety Assessment of Sodium p-Chloro-m-Cresol, p-Chloro-m-Cresol, Chlorothymol, Mixed Cresols, m-Cresol, o-Cresol, p-Cresol, Isopropyl Cresols, Thymol, o-Cymen-5-ol, and Carvacrol
Report No	Not applicable, published study (*International Journal of Toxicology*, 25(Suppl. 1):29–127)
Document No	KCA 5.2.1/03
Guidelines followed in study	Not applicable. The publication is a review of published literature on several related substances (included thymol) used as cosmetic biocides/preservatives and/or fragrance ingredients. No data on the test guidelines followed in each study was provided.
Deviations from current test guideline	Not applicable. Test substance not characterised. Poorly described method and results.
Previous evaluation	Yes, evaluated and accepted in DAR (2011)
GLP/Officially recognised testing facilities	Not stated for any study (review of published literature).
Acceptability/Reliability	Supportive only (review of published literature)

Executive summary

Escobar (2006) reviewed a number of publications related to the skin sensitisation potential of thymol (among other related compounds). Three studies, including the publication of Klecak G. summarised above (B.6.2.6.1), are cited. The data provided in the publication for each study is presented in Table B.6.2.6.2.

Table B.6.2.6.2: Skin sensitisation results with thymol

Species/number/test	Induction/Challenge doses	Results and comments	Reference
Guinea pig 20 females GPMT	*Induction*: Intradermal injections of FCA plus distilled water, 10% thymol in FCA and 10% Thymol in FCA plus distilled water. 7d later, after pretreatment with 10% sodium lauryl sulfate, 0.2 ml of 10% thymol in FCA was applied topically for 48 h under occlusion. *Challenge*: 2w after topical application, 0.02 ml of 5, 10 or 20% thymol in acetone was applied topically. Reactions were graded at 24, 48 and 72 h after application. Reactions were graded per Draize.	The mean response was 0.4 using 20% thymol and 0.2 at 10% thymol 48 h later. Weak sensitization effects with 20% thymol were observed. Thymol was considered negative at 10% concentration under the conditions of this test.	CTFA (1997b)
Himalayan guinea pigs 6 – 8 male and female/group -Open Epicutaneous Test (OET) -Draize Test (DT) -Maximization test (GPMT) -Freud's Complete Adjuvant Test (FCAT)	OET: *Induction*: 3% by 21 daily open applications. *Challenge*: days 21 and 35 by open application to the contralateral flank. Reactions evaluated at 24, 48 and 72 h.	Negative results reported	Klecak *et al.* (1977) B.6.2.6.1
	DT: *Induction*: intradermal injection (day 0) of 0.05 ml thymol in a 0.1% solution (in saline). On 9 alternate days, 0.1 ml of thymol was injected. *Challenge*: intradermal on days 35 and 49. The evaluation criterion was the mean diameter of the papular reactions.	Negative results reported	
	GPMT: *Induction*: on day 0, 2 intradermal injections (0.1 ml at 5% concentration) with and	Negative results reported **Thymol was tested up to*	

	without FCA. On day 8, 250 mg of 25% thymol in petrolatum was applied under occlusion for 48 h. *Challenge*: 24-h patch test using thymol in petrolatum on day 21. Reactions were read at 24 and 48 h after removing the patch.	*10%*	
	FCAT: *Induction*: injection of 0.1 ml of a 50:50 mixture of thymol and FCA on days 0, 2, 3, 7 and 9. Control animals were treated similarly with 0.05 ml of FCA alone. *Challenge*: Days 21 and 35, a 24-h patch test using thymol in petrolatum.	Negative results reported	
Albino Dunkin-Hartley guinea pigs Cross-reactivity	Guinea pigs sensitized to *p*-methoxyphenol were tested for cross-reactivity to thymol. Vehicle was methylethyl ketone and arachis oil.	No effects were reported	Van Der Walle *et al.* (1982)

<u>Assessment and conclusion by applicant</u>:

The report has been previously reviewed at EU level and was accepted as supporting information.

The overall EU-agreed endpoint is: inconsistent results in animal tests, reports of skin sensitisation in humans.

<u>Assessment and conclusion by RMS</u>:

The publication is a review of published literature on several related substances (included thymol) used as cosmetic biocides/preservatives and/or fragrance ingredients. Provided data on the reported studies is very limited. Therefore, RMS deems this information as supportive, but not acceptable for classification purposes.

Weak positive results were observed in one of the studies with 20% thymol in the first publication reviewed. The other sensitization test of thymol presented negative results, but it was tested up to 10% thymol. As for the last publication included in this review, the negative results were referred to cross-reactivity of thymol with animals previously sensitized with *p*-methoxyphenol, and therefore, this result is not valid for the assessment of the sensitization potential of thymol.

B.6.2.7. Phototoxicity

Phototoxicity testing is required by Regulation (EU) No 283/2013 where the active substance absorbs electromagnetic radiation in the range 290-700 nm and is liable to reach the eyes or light-exposed areas of skin, either by direct contact or through systemic distribution. If the Ultraviolet/visible molar extinction/absorption coefficient of the active substance is less than 10 L × mol–1 × cm–1, no toxicity testing is required.

<u>Assessment and conclusion by applicant</u>:

Thymol does not absorb at >290 nm (EFSA Journal 2012; 10(11):2916 and Vol. 3 CA section 2 of this dRAR). Therefore, phototoxicity testing is not triggered for this active substance and no study has been performed.

<u>Assessment and conclusion by RMS</u>:

RMS agrees with the applicant. According to Regulation (EU) No 283/2013, the *in vitro* study is not required since the results of UV/Visible spectroscopy performed with the active substance thymol (99.7% pure) showed no absorption > 290 nm (White G.A., 2007).

B.6.3. SHORT-TERM TOXICITY

B.6.3.1. Oral 43-day study

Data point	CA 5.3.1 (EU data requirement)
Report author	[redacted]
Report year	1996
Report title	Combined repeat dose and reproductive/developmental toxicity screening test of thymol by oral administration in rats
Report No	4L421
Document No	KCA 5.3.2/01
Guidelines followed in study	OECD TG (1990) (no TG number specified in the report). OECD TG 422 (1996) is the nearest applicable guideline.
Deviations from current test guideline OECD TG 422 (2016)	- Duration: Females were dosed up to 3 days of lactation instead of 13 days after delivery. - Historical Control Data not provided. - Sensory reactivity to stimuli, functional observations not performed. - Ano genital distance (AGD) was not measured in pups. - Haematology and clinical chemistry was performed only in males. - Spleen, ovaries, uterus, brain, thyroid and heart not weighted. - Histopathological examination not including thyroids, lungs, lower GI tract, uterus, spinal cord, urinary bladder, lymph nodes, nerves or bone marrow. - Thyroid activity was not addressed (chemistry, histopathological examinations or organ weight assessment, not performed).
Previous evaluation	Yes, evaluated and accepted in DAR (2011) and DAR Addendum (2016)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	Supportive only (methodological deficits)

Materials and Methods

Test chemical	Thymol (Lot No. CAN1119, purity 99.6%)
Vehicle	3% gum arabic aqueous solution
Test system	SD rats (Crj:CD, SPF) male and female
Test method	Preliminary range-finding test: doses of 30, 100 and 300 mg/kg bw/day were administered to both male and female SD rats for 13 days before mating and 4 days during mating (17 days for male rats), and females were also dosed for 7-9 days after mating was confirmed. Based on the results of this preliminary test, the dose levels used for the main study were 0, 8, 40 and 200 mg/kg bw/day in 3% gum arabic aqueous solution. Main study: doses of 0, 8, 40 and 200 mg/kg bw/d of thymol were daily administered by a gastric tube to groups of 10 male and 10 female SD rats for 14 days prior to mating. Males continued to be dosed during mating and up to the day prior to necropsy (total of 43 days). Females continued to be dosed through mating, gestation and up to Day 3 of lactation (with the day of birth classed as Day 0). *Observations related to repeated dose toxicity:* Mortality, external appearance, and activity were observed daily before and after administration. Dead animals were quickly necropsied after discovery. Body weight was measured in males on the starting day of administration and once per week thereafter, and in females on the starting day of administration, once per week until mating, on days 0, 7, 14 and 20 of pregnancy following mating, and on days 0 and 4 of nursing (the day when mating was confirmed being day 0 of pregnancy, and the day when delivery was confirmed being day 0 of nursing). Males' food consumption was measured once per week from the administration start day, except for the mating period; for females it was measured once per week before mating, after mating was confirmed, on days 0, 7, 14 and 20 of gestation and on days 0 and 4 of lactation. Haematology and clinical chemistry were performed on males only, on the day of necropsy following an overnight fast. Gross necropsy, organ weights (pituitary, thymus, spleen, liver, kidneys, adrenal gland, testes, epididymis and prostate) and

	histopathology (brain, pituitary, thymus, heart, spleen, liver, kidneys, adrenal gland, stomach, testes, epididymis, prostate and ovaries) were performed for the control and the highest dose groups only, except for adrenals (which were examined also in intermediate doses of female groups), stomach (for which investigations were performed for all male groups, 2 females from control group, and 5 females from each of the 8 and 40 mg/kg bw/day groups, and 6 females from the highest dose group) and thymus (which was only examined in female groups). *Observations related to reproduction/development toxicity: see section B.6.6.1.1.* *Statistical analysis:* Measured data was tested by Bartlett's equal variance tests and, when the variance was consistent, one-way analysis of variance and when it was not consistent, the Kruskal-Wallis test was performed. When there was a significant difference between groups, and the number of animals in the groups was fixed, the method of Dunnett was used, and if it was not consistent, the method of Scheffé was used for the multiple comparison tests for mean values and order. Enumeration data were tested using the Fisher's exact probability test. The significance level was 5%.

Results on repeated dose toxicity

For results on reproduction/developmental toxicity, see section B.6.6.1.1.

Preliminary range-finding test

A range-finding study was performed using dose levels of 30, 100 and 300 mg/kg bw/day.

No obvious changes were observed after administration of 100 mg/kg bw/d, but salivation. However, rats receiving 300 mg/kg bw/day showed reduction in spontaneous motor activity, ambulatory ataxia, abdominal recumbency, bradypnea, ptosis and hypersalivation, as well as reduction in body-weight. Based on these results dose levels of 0, 8, 40 and 200 mg/kg bw/day were selected for the main study.

Main study

Mortality

One high dose male died on the final day of dosing (day 43 after start of administration). This male did not show any clinical signs prior to death. However, gross necropsy revealed thickening of the proventricular wall (stomach), atrial dilation and congestion of the liver and lung, which were confirmed histopathologically as slight augmentation of the proventricular mucosal epithelium (hyperplasia), slight congestion of the liver and moderate congestive oedema and slight infiltration of inflammatory cells in the lung.

In addition, one female in the high dose group died during gestation (day 33 after start of administration). However, its death was judged due to a dosing error (the necropsy showed perforation of the oesophagus, haemorrhage in thoracic cavity and hematoma in pericardium).

Clinical signs

Changes were noted only in the 200 mg/kg bw/day group: transient salivation was observed immediately following dose administration in the high dose group, starting from approximately 13-15 days after the start of the study and increasing in incidence such that almost all males and half the females were affected by the end of the study.

Females of the 200 mg/kg bw/day group also showed transient decrease in spontaneous motor activity, which was observed just after administration. In the period from day 1 to day 13 of the study, this sign was recorded in 9 of the 10 high dose females. However, 5 of these animals only showed the sign one day; in 2 animals it was recorded on 3 days of this period, and for the last 2 females, this effect was noted on 10 days. In addition, one of these last females showed this sign on the 19th day of gestation and on day 1 of lactation, showing also emaciation on days 1 and 2 of lactation.

The described decrease in spontaneous motor activity was accompanied by ataxic gait in 6 of the females.

Also, one high dose female (non-delivering) showed discharge of black mucous from the vagina on Day 24 of gestation.

No clinical signs were observed in any of the animals in the control, 8 and 40 mg/kg bw/day groups.

Table B.6.3.1/01: Clinical signs observed in males of the 200 mg/kg bw/day group

	Grade	DAYS after commencement of treatment																					
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Number of animals		10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
No Abnormality		10	10	10	10	10	10	10	10	10	10	10	10	10	9	10	9	9	6	7	5	6	4
Salivation	+1	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1	1	4	3	5	4	6
	+2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		DAYS after commencement of treatment (cont.)																					
		22	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**
Number of animals		10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	9
No Abnormality		2	4	5	7	4	3	4	3	3	4	5	8	3	5	5	3	4	7	4	4	5	9
Salivation	+1	8	6	5	3	5	7	6	7	7	6	5	2	7	5	5	7	6	3	5	4	5	-
	+2	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1	2	-	-

+1 slight; +2 moderate

Table B.6.3.1/02: Clinical signs observed in females of the 200 mg/kg bw/day group

	Grade	DAYS after commencement of treatment															DAYS of gestation						
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**0**	**1**	**2**	**3**	**4**	**5**	**6**
Number of animals		10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
No Abnormality		10	6	7	8	7	7	10	7	9	8	7	8	7	8	10	9	9	10	9	9	10	9
Emaciation		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Decrease in locomotor activity	+1	-	3	3	2	3	3	-	3	1	2	3	2	3	2	-	-	-	-	-	-	-	-
	+2	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ataxic gait		-	3	3	2	3	1	-	2	1	1	3	2	3	1	-	-	-	-	-	-	-	-
Salivation	+1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1	-	1	1	-	1
	+2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		DAYS of gestation (cont.)															DAYS of lactation				
		7	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**		**0**	**1**	**2**	**3**	**4**
Number of animals		10	10	10	10	10	10	10	10	10	10	10	9	9	9		8	8	8	8	8
No Abnormality		10	8	9	9	10	10	9	8	10	9	10	9	8	9		7	6	6	5	8
Emaciation		-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	1	1	-	-
Decrease in locomotor activity	+1	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-
	+2	-	-	-	-	-	-	-	-	-	-	-	-	1	-		-	-	1	-	-
Ataxic gait		-	-	-	-	-	-	-	-	-	-	-	-	1	-		-	-	1	-	-
Salivation	+1	-	2	1	1	-	-	1	2	-	1	-	-	-	-		-	1	2	1	-
	+2	-	-	-	-	-	-	-	-	-	-	-	-	-	-		1	-	-	2	-

+1 slight; +2 moderate

Body weight

In males of the 200 mg/kg bw/day group, a decrease in bodyweight gain was noted from day 28 after the start of administration to study termination (10.8-13.3%), however, these differences were not statistically significant. On the other hand, no differences in bodyweight or bodyweight gain were observed in females groups.

Table B.6.3.1/03: Group mean body weights (BW) and body weight gain (BWG) in male rats

Dose level (mg/kg bw/d)	BW - Days after start of treatment; group mean ± SD (g)

	0	7	14	21	28	35	42
0	348 ± 10.1	402 ± 11.9	443 ± 15.1	475 ± 15.9	506 ± 17.7	535 ± 21.4	551 ± 28.8
8[#]	349 ± 8.8	407 ± 12.0	445 ± 16.6	471 ± 18.8	499 ± 20.1	523 ± 25.0	534 ± 26.5
40	348 ± 9.4	402 ± 17.0	442 ± 21.1	467 ± 24.7	495 ± 33.1	522 ± 40.9	545 ± 50.5
200 (% variation vs control)	349 ± 10.4 (↑0.3)	399 ± 16.4 (↓0.7%)	438 ± 16.1 (↓1.1%)	463 ± 17.3 (↓2.5%)	486 ± 25.3 (↓4.0%)	512 ± 28.0 (↓4.3%)	529 ± 33.0 (↓4.0%)

	BWG - Days after start of treatment; group mean ± SD (g)					
	7	14	21	28	35	42
0	54 ± 8.05	95 ± 13.5	127 ± 16.2	158 ± 17.9	187 ± 20.7	203 ± 28.3
8[#]	58 ± 6.3	96 ± 11.6	123 ± 15.6	150 ± 17.7	175 ± 21.7	185 ± 21.4
40	54 ± 13.3	94 ± 16.5	119 ± 20.0	147 ± 28.8	174 ± 36.1	197 ± 46.0
200 (% variation vs control)	51 ± 7.9 (↓5.6)	90 ± 9.6 (↓5.3)	115 ± 10.6 (↓9.4)	137 ± 18.1 (↓**13.3**)	164 ± 26.5 (↓**12.3**)	181 ± 26.5 (↓**10.8**)

[#] *n=9. Data for one animal were excluded from assessment, since it was discovered that there had been an administration error (non-fatal).*

Table B.6.3.1/04: Group mean body weights (BW) and body weight gain (BWG) in female rats

Dose level (mg/kg bw/d)	BW group mean ± SD (g)								
	Days after start of treatment			Days (gestation)				Days (lactation)	
	0	7	14	0	7	14	20	0	4
0	212 ± 5.9	224 ± 12.4	235 ± 14.7	247 ± 18.6	285 ± 19.4	322 ± 22.0	404 ± 26.6	292 ± 20.8	313 ±12.2
8	212 ± 7.4	225 ± 5.8	238 ± 9.7	251 ± 11.7	291 ± 14.0	331 ± 18.0	411 ± 22.5	298 ± 18.1	320 ± 13.8
40	210 ± 7.6	226 ± 6.8	237 ± 9.9	250 ± 10.5	289 ± 13.7	329 ± 16.2	412 ± 22.0	392 ± 14.4	324 ±11.5
200	211 ± 5.4	223 ± 7.4	234 ± 9.1	251 ± 12.5	287 ± 10.3	324 ± 9.3	[¥]400 ± 22.9	[¥¥]293±24.0	[¥¥]314±23.3

	BWG group mean ± SD (g)					
	Days after start of treatment		Days (gestation)			Days (lactation)
	7	14	7	14	20	4
0	11 ± 8.2	23 ± 9.9	38 ± 8.6	75 ± 10.2	157 ±15.7	20 ± 12.9
8	13 ± 3.0	26 ± 4.7	40 ± 7.9	80 ±12.9	159 ±18.0	22 ± 11.4
40	16 ± 5.1	27 ± 8.6	39 ± 8.1	79 ± 12.4	162 ± 18.5	31 ± 10.2
200	13 ± 5.9	24 ± 8.5	36 ± 9.2	73 ± 9.4	[¥]148 ± 14.4	[¥¥]21 ± 11.4

[¥] *n=9. Data for one animal are not included, since this animal died on gestation day 18 (day 33 of administration) due to an error during administration.*
[¥¥] *n=8. Data for one female are not included, since this animal died on gestation day 18 due to an error during administration. In addition, data for another female are not included since this animal did not deliver.*

Food consumption

No significant differences were observed between the control group and all test substance groups throughout the whole period.

Haematology (males only)

Within the differential leukocyte counts, slightly increased lymphocytes were observed (statistically significant as percentage values for all treated groups, but not statistically significant as absolute values). Also slightly reduced monocytes were observed in all treated groups (statistically significant as percentage values for mid and high dose groups but, as absolute values, it was only statistically significant the 40 mg/kg bw/day group); data are shown in the table below. The differences were described by the study authors as being within the laboratory background data (not provided). No other haematological changes were observed.

Table B.6.3.1/05: Differential leukocyte counts in male rats

	Thymol dose (mg/kg bw/d)			
	0	8	40	200
Number of animals	10	9[#]	10	9[@]
% values; group mean ± SD				

	Thymol dose (mg/kg bw/d)			
	0	**8**	**40**	**200**
Lymphocytes (% variation vs control)	76 ± 3.2	**82 ± 3.4*** **(↑7.9%)**	**84 ± 5.2**** **(↑10.5%)**	**85 ± 4.2**** **(↑11.8%)**
Neutrophils				
Segmented	14 ± 2.8	11 ± 3.4	10 ± 4.6	10 ± 3.4
Band	0 ± 0.4	0 ± 0.0	1 ± 0.5	0 ± 0.0
Eosinophils	1 ± 0.7	1 ± 1.4	1 ± 0.7	1 ± 1.3
Basophils	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
Monocytes (% variation *vs* control)	9 ± 3.9	6 ± 1.5	**4 ± 1.4**** **(↓55.6%)**	**4 ± 1.1*** **(↓55.6%)**
Absolute values (x 10^2/µL); group mean ± SD				
Lymphocytes	79 ± 21.2	91 ± 24.9	94 ± 25.2	108 ± 19.5
Neutrophils				
Segmented	15 ± 4.4	12 ± 3.7	12 ± 6.5	13 ± 4.8
Band	0 ± 0.4	0 ± 0.0	1 ± 0.7	0 ± 0.0
Eosinophils	1 ± 0.9	1 ± 1.6	1 ± 0.8	1 ± 1.9
Basophils	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
Monocytes (% variation *vs* control)	9 ± 4.6	7 ± 2.3	**5 ± 2.2*** **(↓44.4%)**	6 ± 2.3

[#] *Data for one animal were excluded from assessment, since it was discovered that there had been an administration error (non-fatal).*
[@] *One male died on Day 43 of treatment.*
[*] *Significantly different from the control group value at P<0.05*
[**] *Significantly different from the control group value at P<0.01*

Clinical chemistry (males only)

Triglycerides were significantly lower in the 8 mg/kg bw/day group but, since there was no significant change in the 40 and 200 mg/kg bw/day groups, this was not considered to be treatment related. No other significant differences were noted between the control group and any of the test substance groups.

Organ weights

An increased trend in thymus weights was observed in high dose male group, reaching a 9.8% increment for absolute weight and a 15.3% increase for the relative weight, however, these increases did not display statistically significant differences.

On the other hand, no significant differences were noted between control group and tested groups for either absolute organ weights or relative (to body weight) organ weights for both males and females.

Table B.6.3.1/06: Absolute and relative organ weights of male rats

Dose level (mg/kg bw/d)	Absolute organ weight of males								
	Final BW (g)	**Pituitary (mg)**	**Thymus (mg)**	**Liver (g)**	**Kidneys (g)**	**Adrenals (mg)**	**Testes (g)**	**Prostate (g)**	**Epididymis (g)**
0	528	14.3	356	14.48	3.14	70.2	3.54	0.71	1.28
8[#]	510	14.4	353	13.37	3.07	63.5	3.45	0.63	1.28
40	519	14.2	361	14.55	3.34	64.9	3.48	0.65	1.29
200[@] (% variation *vs* control)	506	14.7 (↑2.6%)	391 **(↑9.8%)**	14.12 (↓2.5%)	3.20 (↑2.0%)	65.7 (↓6.4%)	3.55 (↑0.3%)	0.73 (↑2.7%)	1.27 (↓0.5%)
Dose level (mg/kg bw/d)	**Relative organ weights of males**								
	Final BW (g)	**Pituitary ($x10^{-3}$)**	**Thymus ($x10^{-3}$)**	**Liver**	**Kidneys**	**Adrenals ($x10^{-3}$)**	**Testes**	**Prostate**	**Epididymis**
0	528	2.7	67	2.74	0.59	13.3	0.67	0.13	0.24
8[#]	510	2.8	69	2.62	0.60	12.4	0.68	0.12	0.25

40	519	2.8	69	2.79	0.64	12.6	0.67	0.13	0.25
200[@] (% variation *vs* control)	506	2.9 (↑7%)	78 (↑**15.3**%)	2.79 (↑1.7%)	0.64 (↑6.8%)	13.0 (↓2.7%)	0.70 (↑4.6%)	0.14 (↑6.6%)	0.25 (↑3.7%)

[#] *n=9. Data for one animal were excluded from assessment, since it was discovered that there had been an administration error (non-fatal).*
[@] *n=9. Data for the animal that died on Day 43 of treatment was not included.*

Table B.6.3.1/07: Absolute and relative organ weights of female rats

Dose level (mg/kg bw/d)	Absolute organ weight of females					
	Final BW (g)	Pituitary (mg)	Thymus (mg)	Liver (g)	Kidneys (g)	Adrenals (mg)
0	313	21.3	212	13.78	1.98	71.7
8	320	21.3	232	13.5	1.98	71.9
40	324	21.9	216	15.09	2.02	77.7
200[¥]	341	20.3	210	14.91	2.05	70.1
Dose level (mg/kg bw/d)	**Relative organ weights of females**					
	Final BW (g)	**Pituitary ($x10^{-3}$)**	**Thymus ($x10^{-3}$)**	**Liver**	**Kidneys**	**Adrenals ($x10^{-3}$)**
0	313	6.8	68	4.41	0.63	22.9
8	320	6.7	73	4.22	0.62	22.5
40	324	6.8	67	4.66	0.63	24
200[¥]	341	6.5	65	4.75	0.65	22.3

[¥] *n=8. Data from 2 females that did not bear offspring were excluded from assessment.*

Gross necropsy

In the animals scheduled for necropsy, thickening of the forestomach wall (thickening of the proventricular wall) was observed in 9 males and 1 female in the 200 mg/kg bw/day group. The surface of the thickened proventricular mucosa was whitened and rough.

Reduction in the size of the thymus was observed in 1 female from each of the 40 and 200 mg/kg bw/day groups and whitening of the adrenal was observed in 2 females in the 200 mg/kg bw/day group.

Table B.6.3.1/08: Gross necropsy findings in rats

Sex	**Male**					**Female**			
Fate	**Scheduled sacrifice**				**Dead**	**Scheduled sacrifice**			
Dose (mg/kg bw/d)	**0**	**8**	**40**	**200**	**200**	**0**	**8**	**40**	**200**
Number of animals	**10**	**9[#]**	**10**	**9[@]**	**1[@]**	**10**	**10**	**10**	**9[¥]**
Thymus									
small	0	0	0	0	0	0	0	1	1
Stomach									
thickening of forestomach wall	0	0	0	9	1	0	0	0	1
Heart									
Dilatation of atrium	0	0	0	0	1	0	0	0	0
Lung									
Congestion	0	0	0	0	1	0	0	0	0
Liver									
Congestion	0	0	0	0	1	0	0	0	0
Adrenal									
whitish	0	0	0	0	0	0	0	0	2

Sex	Male					Female			
Fate	Scheduled sacrifice				Dead	Scheduled sacrifice			
Dose (mg/kg bw/d)	0	8	40	200	200	0	8	40	200
Number of animals	**10**	**9[#]**	**10**	**9[@]**	**1[@]**	**10**	**10**	**10**	**9[¥]**
Brain									
dilatation of cerebral ventricle	0	0	0	1	0	0	0	0	0

[#] *Data for one animal were excluded from assessment, since it was discovered that there had been an administration error (non-fatal).*
[@] *One male died on Day 43 of treatment.*
[¥] *Data for one female are not included, since this animal died on gestation day 18 (day 33 of administration) due to an error during administration.*

Histopathology

The gross effects in the forestomach, adrenals and thymus were confirmed at histopathological examination and these effects were considered to be due to the test substance.

The forestomach thickening was confirmed as hyperplasia of the mucosal epithelium and was observed in males and females at 40 and 200 mg/kg bw/day. Thickening of the preventricular membrane was described as being due to hyperplasia of the stratified squamous epithelium and accelerated keratinisation. In some cases, the hyperplasia was accompanied by infiltration of inflammatory cells and oedema.

Bilateral increase of fatty droplets in the fascicular zone of the adrenal was observed in a high dose female. This case was one of the two in which whitening of the adrenal gland could be observed by eye. No abnormality was observed in the other female or in any of the other females in the 8 and 40 mg/kg bw/day groups.

Thymic involution (slight) was confirmed in the two females showing shrinkage of the thymus at necropsy.

Findings in other organs and tissues were considered to be incidental spontaneous lesions (except for the decedent animal), since they were also observed in control animals.

Table B.6.3.1/09: Histopathological findings in rats

Sex	Grade	Male					Female			
Fate		Scheduled sacrifice				Dead	Scheduled sacrifice			
Dose (mg/kg bw/d)		0	8	40	200	200	0	8	40	200
Number of animals		**10**	**9[#]**	**10**	**9[@]**	**1[@]**	**10**	**10**	**10**	**9[¥]**
Thymus										
Involution	+	*	*	*	*	*	0	0	1	1
Stomach										
Oedema, forestomach	+	0	0	4	5	0	0/2[&]	0/5[&]	0/5[&]	0/5[&]
Erosion, forestomach	+	0	0	0	0	0	0/2[&]	0/5[&]	1/5[&]	0/5[&]
Hyperplasia, mucosa, forestomach	+ ++	0 0	0 0	9 0	4 5	1 0	0/2[&] 0/2[&]	0/5[&] 0/5[&]	2/5[&] 0/5[&]	3/5[&] 1/5[&]
Inflammatory cell infiltration, forest.	+	0	0	6	9	0	0/2[&]	0/5[&]	0/5[&]	2/5[&]
Heart										
Inflammatory cell infiltration, focal	+	6	*	*	1	0	0	*	*	1
Spleen										
Extramedullary haematopoiesis	+	0	*	*	0	0	0	*	*	1
Lung										
Congestive oedema	++	*	*	*	*	1	*	*	*	*
Inflammatory cell infiltration	+	*	*	*	*	1	*	*	*	*
Liver										
Congestion	+	0	*	*	0	1	0	*	*	0
Extramedullary haematopoiesis	+	0	*	*	0	0	0	*	*	1
Haemorrhage, focal	+	2	*	*	0	0	0	*	*	0
Microgranuloma	+	9	*	*	6	0	1	*	*	0
Kidney										
Basophylic change, tubular epithelium	+	1[U]	*	*	0	0	3[U]	*	*	2
Calcification, corticomedullary junction	+	2[U]	*	*	1	0	1[U]	*	*	1[U]
Cyst	+	1[U]	*	*	0	0	1[U]	*	*	0

Sex	Grade	Male					Female			
Fate		Scheduled sacrifice				Dead	Scheduled sacrifice			
Dose (mg/kg bw/d)		0	8	40	200	200	0	8	40	200
Number of animals		**10**	**9#**	**10**	**9@**	**1@**	**10**	**10**	**10**	**9¥**
Fibrosis, focal	+	0	*	*	0	0	1U	*	*	1U
Hyaline droplet, tubular epithelium	+	2B	*	*	1B	0	0	*	*	0
Testis										
Atrophy, seminiferous tubule	+	1	*	*	0	0				
Epididymis										
Inflammatory cell infiltration	+	1	*	*	1	0				
Prostate										
Inflammatory cell infiltration	+	10	*	*	4	0				
Pituitary										
Cyst	+	0	*	*	0	0	0	*	*	1
Hyperplasia, rathke's pouch	+	1	*	*	0	0	0	*	*	0
Adrenal										
Increased fatty droplets, fascicular zone	+	0	*	*	0	0	0	0	0	1B
Brain										
Dilatation of cerebral ventricle	+	0	*	*	1	0	0	*	*	0

Grade + Slight, ++ Moderate
** Not examined; U Unilateral; B Bilateral*
& No. of animals showing lesion / no. of animals examined
 # Data for one animal were excluded from assessment, since it was discovered that there had been an administration error (non-fatal).
@ One male died on Day 43 of treatment.
¥ Data for one female are not included, since this animal died on gestation day 18 (day 33 of administration) due to an error during administration.

Conclusion on repeated dose toxicity

For conclusions on reproduction/developmental toxicity, see section B.6.6.1.1.

Histopathological changes in the forestomach of male and female parent animals were observed in groups given 40 mg/kg bw/day and above. These effects are considered consistent with the irritant properties of thymol (local site of contact irritation). The transient hypersalivation observed following dosing in the high dose group may have been related to this irritant effect in the upper GI tract.

The study authors attributed the reduced motor activity and ataxia following dosing to pharmacological effects of thymol (suppressive action on the central nervous system or muscular contraction), and the thymus effects were attributed to stress.

The local NOAEL for repeated dose toxicity was 8 mg/kg bw/day in this study, based on effects on the forestomach at 40 and 200 mg/kg bw/day.

The systemic NOAEL for repeated dose toxicity was 40 mg/kg bw/day in this study, based on reduced motor activity and ataxia, and increased thymus (absolute and relative) weight observed at 200 mg/kg bw/day.

Assessment and conclusion by applicant:

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is: Target/critical effect; gastric irritation in rats. The NOAEL is 8 mg/kg bw/d. The EU-agreed ARfD (0.08 mg/kg bw/d) is derived from this value.

Assessment and conclusion by RMS:

According to the report (1996), the study follows the OECD test guidelines of 1990, without specifying any guide number. However, the method followed is very close to the guideline OECD TG 422 of 1996 (replaced on 28th July 2015). Although the study provides the most extended data on thymol repeated-dose and reproductive toxicity of thymol available, it is considered supportive due to methodological deficits observed (see deviations point).

No Historical Control Data (HCD) is provided, which is especially relevant since the haematological findings were discarded in the study report, as being within the laboratory background data. These findings are included in Table B.6.3.1/05 and consist in statistically significant reduction in the number of monocytes (percentage and absolute values) and an increase in lymphocyte counts (only percentage values are statistically significant) in mid and high dose males groups.

The decrease in the percentage of monocytes shows a dose-related trend, reaching a 55.6% variation at mid and high dose males groups (statistically significant for both dose groups). Absolute monocyte values do not show dose-related trend, and only the mid dose group shows statistically significant differences compared with control group.

Regarding the statistically significant increase of the percentage of lymphocytes, it should be noted that the variation observed was dose-related, statistically significant in all treated groups and reached an 11.8% variation at the high dose male group. Absolute lymphocyte counts showed a dose-related trend, but with no statistical significance.

The quality of the available data on thymol does not allow a proper assessment of these findings. This is the only available study that performs determination of leukocytes, preventing the comparison of the prevalence of these effects with other toxicological studies. Additionally, the haematological analysis was performed only at the end of the study, not allowing a comparison of the data within the groups (throughout the study and/or before the administration of the test substance). Moreover, although the study report considered these effects as not toxicologically relevant, with no HCD to compare the results of the study, RMS cannot confirm such affirmation.

On the other hand, RMS deems available data is not strong enough to consider these findings an adverse effect of thymol: absolute monocytes reduction is not dose-related and, as for the percentage value, the dose-effect relationship stops at 40 mg/kg bw/day (the variation keeps the same at 200 mg/kg bw/day), reducing the weight of the dose-relationship effect in the assessment. Regarding lymphocyte counts, only relative values were statistically significant, reaching a variation of 11.8% at 200 mg/kg bw/day, which is also not considered enough evidence to consider that the administration of thymol caused the effect.

Under the conditions of the study, RMS deems the gastric irritative signs as LOCAL effects, given the corrosive properties of the test substance and the gavage administration. Therefore, based on the histopathological changes in the forestomach of male and female rats at 40 mg/kg bw/day, the Local NOAEL is 8 mg/kg bw/day.

As for systemic values, RMS considers the systemic NOAEL is 40 mg/kg bw/day, based on the reduced motor activity and ataxia in females and the increased thymus weight (absolute and relative) observed at 200 mg/kg bw/day.

NOAEL values of this study, for reproductive toxicity, are included in section B.6.6.1.1 (Fertility NOAEL is 200 mg/kg bw/day and Developmental NOAEL is 40 mg/kg bw/day).

B.6.3.2. Oral 19-week study

Data point	CA 5.3.2 (EU data requirement)
Report author	Hagan E.C., Hansen W.H., Fitzhugh O.G., Jenner P.M. and Jones W.I.
Report year	1967
Report title	Food flavouring and compounds of related structure II. Subacute and chronic toxicity
Report No	Not applicable, published study (*Food and Cosmetics Toxicology*, 5:141-157)
Document No	KCA 5.3.2/02
Guidelines followed in study	Not indicated. Not applicable.
Deviations from current test guideline OECD TG 408 (2018)	Poorly described method and the reporting of the results was very limited with results for each substance summarised only as a single entry in a summary table. Only the effects believed to be due to the administration of the compound are listed. The test was not performed with the technical active substance but with the commercially available thymol used as food additive.
Previous evaluation	Yes, evaluated and accepted in the DAR

GLP/Officially recognised testing facilities	No (prior to the enforcement of GLP)
Acceptability/Reliability	Supportive only (methodological deficits)

Materials and Methods

Test chemical	In the case of thymol (which was included in the diet) neither purity nor batch were indicated. According to the publication: *Commercially available materials, rather than pure chemicals, were used since the purpose of these studies was to evaluate the toxicity of these materials in relation to their use as food additives*.
Vehicle	Diet (prepared and distributed weekly)
Test system	Weanling Osborne-Mendel rats (male and female)
Test method	Thymol was administered to groups of 5 male and 5 female Osborne-Mendel rats in feed at the dose levels of 1000 ppm or 10000 ppm for 19 weeks (equivalent to 90 or 900 mg/kg bw/day in diet). A common control group of 5 males and 5 females was used for 3 compounds (benzyl cinnamate, eugenol and thymol). The weight, food intake and general condition of all animals were recorded weekly. Haematological examinations were made at the end of the study. At the termination of the study, the rats were sacrificed and exsanguinated. Tissues of all rats were examined macroscopically. Liver, kidneys, spleen, heart and testes were weighed. Histopathological examination was performed on these organs and also on the remaining abdominal and thoracic viscera, and on one hind leg for bone, bone marrow and muscle on 3 or 4 rats from both sexes, from the high dose group and the control group. If changes attributable to the test item were found in the high dose group, additional examination on lower dosage levels were performed.

Results

The reporting of the results was very limited with results for each substance summarised only as a single entry in a summary table. Only the effects believed to be due to the administration of the compound are listed.

According to the authors, no effect on growth or haematology and no macroscopic changes in the tissue were observed in the high and low dose level (10000 and 1000 ppm, respectively).

No microscopic changes were observed in the high dose group and, therefore, no microscopic examination was performed in the low dose group.

Assessment and conclusion by applicant:

The study has been previously reviewed at EU level and was accepted as supporting information.

There were no effects up to the highest dose tested (~500 mg/kg bw/d; dietary exposure).

Assessment and conclusion by RMS:

This publication reports the study of the subacute and/or chronic toxicity of 48 food flavourings in rats. The study was performed previous to test guidelines or the enforcement of international GLP. The reporting of methodology followed and the results obtained for thymol is very limited. Therefore, RMS deems this study as supportive information.

For this renewal assessment, the conversion of concentrations in feed into daily doses has been performed following the Guidance on selected default values to be used by the EFSA Scientific Committee, Scientific Panels and Units in the absence of actual measured data, 2012 (EFSA Journal 2012;10(3):2579).

Following this guideline, a default factor of 0.09 for rats should be used for subchronic studies starting usually at week 5 to 7 (in this case, weanling rats were used). Therefore, administered doses in this study have been calculated as 90 and 900 mg/kg bw/day in diet.

According to the guideline, the converting factor of 0.05 that was used in the previous DAR (calculated by the Notifier using default conversion factors), and by the applicant in this renewal assessment should be applied for chronic studies (or subacute or subchronic studies starting in a later age).

It should be noted that the results obtained in this study were obtained with the commercially available thymol used as food additive, instead of the technical active substance.

B.6.3.3. Other routes

Studies by other routes have not been submitted. The RMS deems not necessary to conduct these studies since:

According to Regulation 283/2013 "*for human risk assessment additional dermal studies shall be considered on a case by case basis, unless the active substance is a severe irritant*." Since thymol is currently classified as Skin Corr. 1, H314 this study is not required.

Additionally, "*for volatile active substances (vapour pressure >10^{-2} Pascal) expert judgement (for example based on route-specific kinetic data) shall be required to decide whether the short term studies have to be performed by inhalation exposure*." Vapour pressure of thymol is 3.4 Pa at 20 ºC. However, regarding the lack of adverse effects in animals at doses greater than 5 mg/L (acute exposure) according to the US EPA report summarised in section B.6.2.3 the study is deemed not necessary.

B.6.4. GENOTOXICITY

Genotoxicity potential of thymol has been assessed by i*n vitro* studies (gene mutations in bacteria and mammalian cells, chromosomal aberrations in mammalian cells and DNA damage in mammalian cells) and *in vivo* studies in somatic cells (chromosomal aberrations). Thirteen studies were already considered in the previous DAR (2011) and were provided by the applicant for the renewal, and six studies were newly provided for this renewal.

B.6.4.1. In vitro studies

B.6.4.1.1. Bacterial gene mutation

B.6.4.1.1-01. First study

Data point	CA 5.4.1 (EU data requirement)
Report author	Stammati, A., Bonsi, P., Zucco, F., Moezelaar, R., Alakomi, H.L. and von Wright, A.
Report year	1999
Report title	Toxicity of selected plant volatiles in microbial and mammalian short-term assays
Report No.	Not applicable, published study (Food Chem. Toxic. 37, 813-823).
Document No.	KCA 5.4.1/01
Guidelines followed in study	Not specified in the study report.
Deviations from OECD TG 471 (2020)	- Batch No. of test chemical not reported. No stability determination of the test substance. - Only two strains were tested, none of them with AT base pair at the primary reversion site. - No justification for the choice of solvent was included. - No details of preliminary test were reported. - Individual plate counts were not available. - 4-Nitroquinoline-N-oxide is indicated as control positive (-S9) for WP2 strain. - 2-Aminoanthracene should not be used as the sole indicator of the efficacy of the S9-mix. - No statistics were provided. - No historical control data were reported.
Previous evaluation	Yes, evaluated and considered as supporting information in DAR (2011)
GLP	No
Reliability	Supportive only

Materials and Methods

Test chemical	Thymol; purity: >99 %; batch No.: not stated. Source: Fluka, Buchs, Switzerland.
Solvent	Ethanol (85%)
Negative control	Solvent (10 µL/plate)
Positive control (-S9)	4-Nitroquinoline-N-oxide (0.5 µg/plate) for TA98. Sodium azide (0.5 µg/plate) for TA100.
Positive control (+S9)	2-Aminoanthracene (0.5 µg/plate)
Test system	*S. typhimurium* TA98 and TA100.
Metabolic activation	Rat liver S9 induced by phenobarbitone, obtained from Robens Institute of Health and Safety, UK.
Test method	A standard Ames plate incorporation assay (Maron and Ames, 1983) was applied without pre-incubation. A preliminary plate incorporation assay was carried out to determine non-toxic dose levels. 6.25, 12.50 and 25 µL of 0.1 M thymol per plate (equivalent to 94, 188 and 375 µg/plate) were tested by triplicate in *S. typhimurium* strains.

Criteria for assessment of results	The result was considered positive if the number of colonies found was at least twice the background number, within the non-toxic dose range.
HCD	Not reported
Statistical analysis	No statistical analysis was stated.

Results

Thymol produced an increase in revertants with strain TA100, both with and without S9. However, the responses did not show any dose-related trend, and the magnitude of the increase was less than two-fold, which was not considered to be biologically significant.
The study authors stated that several attempts were made to find a dose inducing at least a twice the spontaneous background number of revertants, but this was not possible within the non-toxic dose range (data not shown).

Table B.6.4.1.1-01: Results of Ames test performed on thymol

Test chemical	Concentration (µL/plate)	Revertants/plate (mean ± SD)			
		TA98		TA100	
		-S9	+S9	-S9	+S9
Ethanol (85%)	10	32.0 ± 3.0	26.3 ± 3.2	94.7 ± 9.6	106.7 ± 1.5
Thymol (0.1M)	6.25	38.3 ± 3.2	23.7 ± 3.2	147.0 ± 6.1	134.0 ± 7.0
Thymol (0.1M)	12.5	37.0 ± 2.6	24.0 ± 5.6	176.0 ± 12.5	150.3 ± 11.0
Thymol (0.1M)	25.0	41.7 ± 4.5	28.0 ± 2.0	152.0 ± 12.0	135.0 ± 16.6
Positive control		1096.5 ± 67.2	284.0 ± 56.6	591.0 ± 56.6	1003.5 ± 84.1

Assessment and conclusion

Assessment and conclusion by applicant:

The study has been previously reviewed at EU level and was accepted as supporting information.
The EU-agreed endpoint is: Overall no genotoxic potential *in vivo via* the oral route.

Assessment and conclusion by RMS:

RMS agrees with the applicant's assessment: a negative result was observed in this test.
The study is only considered as supporting information to evaluate gene mutation in bacteria due to the deviations explained above.

B.6.4.1.1-02. Second study

Data point	CA 5.4.1 (EU data requirement)
Report author	Azizan, A. and Blevins, R.D.
Report year	1995
Report title	Mutagenicity and antimutagenicity testing of six chemicals associated with the pungent properties of specific spices as revealed by the Ames *Salmonella*/microsomal assay.
Report No.	Not applicable, published study (Archives of Environmental Contamination and Toxicology, 28, 248-258).
Document No.	KCA 5.4.1/02
Guidelines followed in study	Not specified in the study report.
Deviations from OECD TG 471 (2020)	- Batch of test chemical not reported. No stability determination of the test substance. - Only three strains tested, instead of five. None of the tested strains had AT base pair at the primary reversion site. - Tested concentrations were not reported. - No individual or mean data were provided. - S9 was not described. - No positive controls and historical control data were provided. - No statistics were provided.

Previous evaluation	Yes, evaluated and considered as supporting information in DAR (2011)
GLP	No
Reliability	Not acceptable

Materials and Methods

Test chemical	Thymol, in the crystalline form. Batch No.: not stated. Obtained from commercial sources.
Solvent	Dimethylsulphoxide (DMSO)
Negative control	DMSO
Positive control	Not stated
Test system	*S. typhimurium* strains TA97, TA98 and TA100.
Metabolic activation	S9 (no further information)
Test method	Thymol was diluted in DMSO to give a final concentration of 1 mg/mL and was tested (at concentrations not reported but indicated to be non-toxic) for mutagenicity with or without S9, in triplicate plates by the pre-incubation method. The pre-incubation time was 20 min. A second assay was performed with TA100 in the presence of metabolic activation and incubation time of 60 min.
Criteria for assessment of results	A mutagenic ratio greater than two inferred a positive mutagenic response.
HCD	Not reported
Statistical analysis	No statistical analysis was stated.

Results

Thymol gave a mutagenic ratio lower than 2 in both assays (with pre-incubation time of 20 or 60 min) and no dose-related response was observed (data not shown in the study).

Assessment and conclusion

Assessment and conclusion by applicant: The study has been previously reviewed at EU level and was accepted as supporting information. The EU-agreed endpoint is: Overall no genotoxic potential *in vivo* via the oral route.
Assessment and conclusion by RMS: RMS considers this study as non-acceptable, given the significant lack of information in the reported data. See the deviations above, for more details.

B.6.4.1.1-03. Third study

Data point	CA 5.4.1 (EU data requirement)
Report author	Florin, I., Rutberg, L., Curvall, M. and Enzell, C.R.
Report year	1980
Report title	Screening of tobacco smoke constituents for mutagenicity using the Ames test
Report No.	Not applicable, published study (Toxicology, 15(3), 219-232).
Document No.	KCA 5.4.1/03
Guidelines followed in study	Not specified in the study report.
Deviations from current test guideline **OECD TG 471 (2020)**	- Batch of test chemical not reported. No stability determination of the test substance. - Only four tested strains instead of five. None of the tested strains with AT base pair at the primary reversion site. - One concentration used instead of a range of concentrations. - No specific control positive (-S9) for each strain was used. - 2-Aminoanthracene should not be used as the sole indicator of the efficacy of the S9-mix. - No individual or mean data were provided.

	- No repeat assay was performed. - No statistics were provided. - No historical control data.
Previous evaluation	Yes, evaluated and considered as supporting information in DAR (2011).
GLP	No (pre-GLP requirement)
Reliability	Only supportive

Materials and Methods

Test chemical	Thymol; purity ≥ 97%; batch No.: not stated.
Solvent	Ethanol
Negative control	Not stated
Positive control (-S9)	N-methyl-N´-nitro-N-nitrosoguanidine
Positive control (+S9)	2-aminoanthracene
Test system	*S. typhimurium* strains TA98, TA100, TA1535 and TA1537
Metabolic activation	Rat liver S9 derived from male Sprague-Dawley rats induced with Aroclor 1254 or 3-methylcholanthrene.
Test method	The test substance was tested in spot tests with and without metabolic activation. A single concentration was tested (3 µmol/plate, equivalent to 450 µg/plate).
Criteria for assessment of results	Not stated
HCD	Not reported
Statistical analysis	No statistical analysis was stated.

Results

Neither precipitation, nor cytotoxicity were observed at the only dose of thymol tested.
Thymol was not found to be mutagenic.

Assessment and conclusion

Assessment and conclusion by applicant: The study has been previously reviewed at EU level and was accepted as supporting information. The EU-agreed endpoint is: Overall no genotoxic potential *in vivo via* the oral route.
Assessment and conclusion by RMS: RMS considers this study as supporting information to evaluate gene mutation in bacteria due to the deviations explained above.

B.6.4.1.1-04. Fourth study

Data point	CA 5.4.1 (EU data requirement)
Report author	Shibuya, T., Sakamoto, K., Kawakami, K., Hara, T.., Shimizu, Y., Matsuki, Y., Nakagomi, M. and Iida, S.
Report year	1996
Report title	Reverse mutation test of thymol on bacteria.
Report No.	None
Document No.	KCA 5.4.1/04
Guidelines followed in study	Not specified in the study report.
Deviations from OECD TG 471 (2020)	- Limited level of reporting. - No information about precipitation was reported. - No statistics were provided. - No historical control data. - 2-Aminoanthracene should not be used as the sole indicator of the efficacy of the S9-mix.
Previous evaluation	Yes, evaluated and considered as acceptable in DAR (2011).
GLP	No

Acceptability	Yes

Materials and Methods

Test chemical	Thymol, purity >98 %; batch/Lot No.: CAN1119.
Solvent	Dimethylsulphoxide (DMSO)
Negative control	DMSO
Positive control (-S9)	2-(2-furyl)3-(5-nitro 2-furyl)-acrylamide (0.1 µg/plate for TA98, 0.01 µg/plate for TA100 and 0.01 µg/plate for WP2 *uvrA*) Sodium azide (0.5 µg/plate for strain TA1535) 9-Aminoacridine (80 µg/plate for strain TA1537)
Positive control (+S9)	2-aminoanthracene (0.5 µg/plate for TA98, 1 µg/plate for TA100 strain, 2 µg/plate for TA1535 and TA1537 strains, and 10 µg/plate for WP2 *uvrA* strain).
Test system	*S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *E. coli* WP2 *uvrA.*
Metabolic activation	Rat liver S9 derived from 7-week male Sprague-Dawley rats induced with phenobarbital and 5,6-benzoflavone.
Test method	Thymol was tested for mutagenicity, with or without S9, using the plate incorporation method. Dose-finding study: The test was conducted with a common ratio of approximately 3 in a range of 50-5000 µg/plate. Without S9 mix, antibacterial properties were observed at 500 µg/plate or higher for all strains. With S9 mix, TA1535 and TA1537 were found to have antibacterial properties at 500 µg/plate or higher. Otherwise, antibacterial properties were observed at 1500 µg/plate or higher. Main study: The thymol doses were 15.6-500 µg/plate in the test without S9 mix, and 62.5-2000 µg/plate for the test with S9 mix (31.3-1000 µg/plate for TA1535 and TA1537), with the common ratio set at 2. Each concentration was tested in triplicate. The study was conducted twice to confirm the reproducibility of the results.
Criteria for assessment of results	In both tests, the number of mutant colonies with or without S9 mix did not increase by a factor of 2 or more compared to the solvent control value for the five test bacteria used. Based on these results, thymol was deemed to be non-mutagenic (negative) for the test system used.
HCD	Not reported
Statistical analysis	No statistical analysis was stated.

Results

In experiment I and II, the number of mutant colonies with or without S9 mix did not increase 2-fold or more compared to the solvent control value for any of the five test bacteria used (see tables B.6.4.1.1-04/1 and B.6.4.1.1-04/2). Positive controls gave acceptable results.

Based on these results, thymol was deemed to be non-mutagenic (negative) for the test system used.

Table B.6.4.1.1-04/1: Number of revertants in the Ames test performed with thymol. Experiment I

Concentration (µg/plate)	S9	Number of colonies/plate (mean ± SD)				
		Base-pair substitution type			Frameshift type	
		TA100	TA1535	WP2 *uvr*A	TA98	TA1537
0	**-S9**	130 ± 8.2	18 ± 6.6	23 ± 2.5	33 ± 4.4	10 ± 2.6
15.6		133 ± 6.1	15 ± 3.5	19 ± 6.2	34 ± 5.9	8 ± 1.5
31.3		134 ± 15.0	19 ± 3.1	20 ± 6.0	32 ± 2.9	11 ± 4.0
62.5		126 ± 2.3	16 ± 1.7	14 ± 4.4	28 ± 9.5	9 ± 2.3
125		111 ± 10.6	17 ± 1.5	15 ± 6.1	27 ± 1.5	9 ± 4.4
250		108 ± 9.2	10 ± 4.4	11 ± 4.7	30 ± 3.2	6 ± 1.7*
500		86 ± 7.0*	2 ± 2.0*	10 ± 4.9*	16 ± 2.0*	0 ± 0.00*
0	**+S9**	130 ± 16.9	14 ± 4.2	24 ± 6.0	37 ± 2.5	12 ± 5.0
31.3		ND	17 ±1.5	ND	ND	9 ± 4.5
62.5		131 ± 13.9	19 ± 6.0	30 ± 0.6	42 ± 6.0	13 ± 1.5
125		128 ± 5.5	17 ± 6.2	25 ± 7.8	38 ± 0.6	9 ± 3.5
250		146 ± 9.0	11 ± 4.0	20 ± 4.0	42 ± 3.0	9 ± 1.0

500		118 ± 8.5	9 ± 1.5*	21 ± 8.9	32 ± 1.5	9 ± 1.7*
1000		0 ± 0.00*	0 ± 0.00*	7 ± 6.5*	0 ± 0.00*	0 ± 0.00*
2000		0 ± 0.00*	ND	0 ± 0.00*	0 ± 0.00*	ND
Positive control	**-S9**	603 ± 7.5	137 ± 15	110 ± 10.1	774 ± 37.2	736 ± 91.8
	+S9	1361 ± 134	254 ± 21.5	1259 ± 85.9	465 ± 10.3	236 ± 44.8

** Inhibition was observed against growth of the bacteria.*
ND not done.

Table B.6.4.1.1-04/2: Number of revertants in the Ames test performed with thymol. Experiment II

Concentration (µg/plate)	S9	Number of colonies/plate (mean ± SD)				
		Base-pair substitution type			Frameshift type	
		TA100	TA1535	WP2 *uvr*A	TA98	TA1537
0	**-S9**	141 ± 17.2	12 ± 3.2	20 ± 3.5	23 ± 9.1	12 ± 3.8
15.6		111 ± 3.1	9 ± 3.2	24 ± 4.6	27 ± 2.3	9 ± 3.5
31.3		117 ± 4.6	12 ± 2.6	20 ± 2.1	23 ± 2.6	9 ± 4.0
62.5		121 ± 7.5	11 ± 3.1	20 ± 5.0	28 ± 1.7	9 ± 4.0
125		128 ± 20.1	14 ± 2.3	17 ± 8.7	21 ± 1.5	8 ± 1.0
250		104 ± 3.5	11 ± 1.5	16 ± 1.7	24 ± 8.2	5 ± 0.6
500		0 ± 0.00*	0 ± 0.00*	1 ± 2.3*	6 ± 7.2*	0 ± 0.00*
0	**+S9**	138 ± 10.2	13 ± 2.5	22 ± 1.5	30 ± 1.2	13 ± 0.00
31.3		ND	16 ± 4.0	ND	ND	19 ± 1.2
62.5		151 ± 14.7	19 ± 4.0	23 ± 4.0	38 ± 3.2	14 ± 1.0
125		127 ± 17.7	15 ± 6.4	25 ± 3.1	42 ± 12.7	15 ± 2.5
250		141 ± 15.0	13 ± 2.5	23 ± 5.5	48 ± 6.5	14 ± 5.1
500		111 ± 25.6	0 ± 0.00*	24 ± 4.2	16 ± 4.5	2 ± 3.5*
1000		0 ± 0.00*	0 ± 0.00*	14 ± 2.1	0 ± 0.00*	0 ± 0.00*
2000		0 ± 0.00*	-	0 ± 0.00*	0 ± 0.00*	-
Positive control	**-S9**	678 ± 13.0	310 ± 13.8	147 ± 5.6	893 ± 37.2	1456 ± 164
	+S9	1012 ± 143	282 ± 26.9	1360 ± 73.1	299 ± 27.2	253 ± 12.0

** Inhibition was observed against growth of the bacteria*
ND not done.

Assessment and conclusion

Assessment and conclusion by applicant:

The study has been previously reviewed at EU level and was accepted.

The EU-agreed endpoint is: Overall no genotoxic potential *in vivo via* the oral route.

Assessment and conclusion by RMS:

A negative result was obtained in this test.

The study was considered as acceptable to evaluate gene mutation in bacteria. Although there were several deviations from OECD TG 471 (2020) and is a no-GLP study, the deviations are not considered to have influenced the outcome of the test. Moreover, the information reported was rather complete: among others, controls were included, and individual data were reported together with means and standard deviations.

B.6.4.1.1-05. Fifth study

Data point	CA 5.4.1 (EU data requirement)
Report author	Llana-Ruiz-Cabello, M., Maisanaba, S., Puerto, M., Prieto, A.I., Pichardo, S., Jos, A. and Cameán, A.M.
Report year	2014
Report title	Evaluation of the mutagenicity and genotoxic potential of carvacrol and thymol using the Ames *Salmonella* test and alkaline, Endo III and FPG-modified comet assays with the human cell line Caco-2.
Report No.	Not applicable, published study (Food and Chemical Toxicology, 72: 122–128).
Document No.	KCA 5.4.1/10
Guidelines followed in	OECD TG 471 (1997)

study	
Deviations from OECD TG 471 (2020)	- Batch of test chemical not reported. - TA104 strain was used instead of TA1535. - In the TG, 2-NF is recommended as positive control for TA98 without metabolic activation, but here it was used to check studies also performed with TA97A, TA102 and TA104. 2-AF was not included in the TG. Given the positive response in all the positive controls, this deviation was considered of minimal relevance. - Limited level of reporting. - No information about precipitation was reported. - No historical control data.
Previous evaluation	No, not previously submitted
GLP	No
Reliability	Supportive only

Materials and Methods

Test chemical	Thymol. Purity: 99.5%. Batch/Lot No.: Unknown. Source: Sigma-Aldrich, Spain.
Solvent	DMSO
Negative control	Untreated and DMSO
Positive control (-S9)	2-Nitrofluorene (2-NF, 0.1 µg/plate) for TA97A, TA98, TA102 and TA104. Sodium azide (NaN_3, 1 µg/plate) for TA100.
Positive control (+S9)	2-Aminofluorene (2-AF, 20 µg/plate) for all the strains.
Test system	*Salmonella typhimurium* TA97A, TA98, TA100, TA102 and TA104.
Metabolic activation	S9 derived from rats livers induced with Aroclor 1254.
Test method	This test was performed by the plate incorporation method. The highest dose was selected by a cytotoxic pre-test with human cell line. Thymol was tested at 15.6, 31.3, 62.5, 125 and 250 µM, in three independent experiments, and in the presence and absence of S9 activation. All concentrations were tested in triplicate. Revertant colonies were counted, and the background lawn was inspected for signs of either toxicity or compound precipitation.
Criteria for assessment of results	None reported
HCD	Not reported
Statistical analysis	Statistical analysis was performed using the analysis of variance (ANOVA) followed by Dunnett's multiple comparison. The normality of the distribution and the homogeneity of variances were confirmed using Kolmogorov and Smirnov's test, and Bartlett's test, respectively. The differences were considered significant at $p \leq 0.05$.

Results

Thymol did not produce any increase in the number of revertants in any of the treated strains up to the highest concentration (250 µM), either in the presence or in the absence of S9. Consequently, thymol was not mutagenic in this bacterial assay at the concentrations tested.

The toxicity of thymol, measured as a decrease in the background lawn and in revertant frequency, was not observed at any concentration tested. The positive controls produced statistically significant increases ($p < 0.01$) in the number of revertant colonies, confirming the sensitivity of the test system and the activity of the S9 mix.

These results are shown in table B.6.4.1.1-05.

Table B.6.4.1.1-05: Number of revertants per plate in the Ames test performed with thymol

Concentration (µM)	Number of colonies (mean ± SD)									
	TA97A		TA98		TA100		TA102		TA104	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Untreated control	288±11	261±9	17±3	25±2	118±6	87±3	238±33	252±25	273±7	310±22
15.6	299±41	287±7	17±4	33±4	105±20	112±13	297±40	362±6	330±6	429±43
31.3	314±14	340±35	18±1	31±3	139±17	123±16	364±24	382±28	418±28	498±55
62.5	267±14	306±12	24±8	28±4	80±3	83±13	256±45	311±32	281±18	394±23
125	177±36	206±27	21±4	24±3	70±12	75±7	252±15	192±11	320±13	502±43
250	261±48	273±24	21±3	33±4	105±8	111±11	307±10	346±29	464±17	423±19
Positive control	**647±13****	**553±23****	**1081±250****	**1124±248****	**1076±112****	**1026±110****	**620±173****	**542±47****	**791±62****	**861±122****
Solvent control	196±11	200±13	20±6	26±10	98±16	105±8	268±22	273±17	296±22	309±15

*** $p < 0.01$ very significant differences from controls*

Assessment and conclusion

Assessment and conclusion by applicant: This new study is considered to be acceptable for the renewal of thymol (reliable with restrictions; study not GLP). The EU-agreed endpoint is: Overall no genotoxic potential *in vivo via* the oral route.
Assessment and conclusion by RMS: A negative result was obtained in this test. The study was considered as supporting information to evaluate gene mutation in bacteria. This study was not performed under GLPs and it presented several deviations from OECD TG 471 (2020) as can be seen above.

B.6.4.1.2. Mammalian cell gene mutation

B.6.4.1.2-01. First study

Data point	CA 5.4.1 (EU data requirement)
Report author	Lloyd, M.
Report year	2011
Report title	Mutation at the *hprt* locus of mouse lymphoma L5178Y cells (MLA) using the Microtitre^{R} fluctuation technique.
Report No.	8226097
Document No.	KCA 5.4.1/08
Guidelines followed in study	OECD TG 476 (1997)
Deviations from OECD TG 476 (2016)	- Maximum concentration in Experiment 1 in the absence of metabolic activation did not achieve between 20 and 10% the RS. This deviation was of minimal relevance given that the value was closed to 20 and that in Experiment 2 this deficiency was corrected by increasing the highest dose. - Historical control data (HCD) range and distribution were not available. Only the HCD means were reported.
Previous evaluation	Yes, evaluated and considered as acceptable in DAR (2011).
GLP	Yes
Acceptability	Yes

Materials and Methods

Test chemical	Thymol. Purity: 99.7 %. Batch/Lot No.: 103366.
Solvent	Dimethylsulphoxide (DMSO)
Negative control	DMSO
Positive control (-S9)	4-nitroquinoline 1-oxide (NQO)
Positive control (+S9)	Benzo[a]pyrene (B[a]P)
Test system	L5178Y *tk*$^{+/-}$ (3.7.2C) mouse lymphoma cells
Metabolic activation	Liver S9 derived from male Sprague-Dawley rats induced with Aroclor 1254
Test method	Cells were grown in RPMI 1640 media. Concentrations selected for the mutation experiments were based on the results of the cytotoxicity range-finder experiment. Range-finder test: 50, 100, 200, 400, 800, 1600 µg/mL were tested ± S9 Experiment 1: without S9: 20, 40, 60, 80, 100, 120, 140, 160, 180, 200 µg/mL with S9: 10, 20, 30, 40, 50, 60, 75, 90, 105, 120, 140 µg/mL Experiment 2: without S9: 10, 20, 40, 50, 60, 70, 80, 90, 100, 125 µg/mL with S9: 10, 20, 40, 50, 60, 70, 80, 90, 100, 110, 125, 140 µg/mL Duplicate suspension cultures were used in each mutation assay ±S9. Exposure duration was 3 h and cells were incubated for 7 days before measurement of viability (as relative survival, RS) and mutation frequency (MF). MF was calculated as the 6-thioguanine (6TG) resistant mutants/10^6 viable cells, 7 days after treatment was calculated.

Criteria for assessment of results	The test chemical was considered to be mutagenic in this assay if: 1. The MF at one or more concentrations was significantly greater than that of the negative control ($p \leq 0.05$). 2. There was a significant concentration-relationship as indicated by the linear trend analysis ($p \leq 0.05$). 3. The effects described above were reproducible. Results that only partially satisfied the assessment criteria described above were considered on a case-by-case basis.
HCD	Mean MF in the historical negative control = 2.36 (-S9) 2.73 (+S9). Normal range is considered not more than three times the historical negative control mean. Mean MF in the historical positive control with 0.15 µg/mL NQO = 25.35 Mean MF in the historical positive control with 0.2 µg/mL NQO = 35.84 Mean MF in the historical positive control with 2 µg/mL B[a]P = 18.89 Mean MF in the historical positive control with 3 µg/mL B[a]P = 38.10
Statistical analysis	Statistical significance of mutant frequencies was carried out according to the UKEMS guidelines. The control log mutant frequency (LMF) was compared with the LMF from each treatment concentration and the data were checked for a linear trend in MF with test article treatment. These tests require the calculation of the heterogeneity factor to obtain a modified estimate of variance.

Results

In the cytotoxicity range-finder experiment, precipitation was observed from 400 µg/mL. The highest concentration that survived the treatment was 100 µg/mL in the absence and presence of S9 (see table B.6.4.1.2-01/1). No marked changes in osmolality or pH were observed at the highest concentration analysed (800 µg/mL) compared to the vehicle controls (data not reported).

Table B.6.4.1.2-01/1: Percentage RS in the range-finder experiment

Treatment (µg/mL)	-S9 % RS	+S9 % RS
0	100	100
50	68	16
100	53	15
200	0	0

In Experiment 1, one culture at 100 µg/mL in the absence of S9 was lost due to excessive toxicity during the expression period; the remaining culture was plated for viability and 6TG resistance but was lost due to contamination of the mutant plates. Concentrations from 120 µg/mL in the absence of S9 and from 140 µg/mL in the presence of S9 were considered too toxic. The highest concentrations analysed were 80 µg/mL in the absence of S9 and 105 µg/mL in the presence of S9, which gave 24% and 15% RS, respectively (see table B.6.4.1.2-01/2).

In Experiment 2, the highest concentrations analysed were 90 µg/mL in the absence of S-9 and 110 µg/mL in the presence of S-9, which gave 16% and 20% RS, respectively (see table B.6.4.1.2-01/3).

In Experiments 1 and 2, in the absence of S9 no significant increases in MF at any concentration or statistically significant linear trends were observed following treatment with thymol. In the presence of S9 in Experiment 1, no significant increases in MF were observed at any concentration. A weak but statistically significant linear trend was observed, but without marked increases in MF at any concentration tested, this observation was not considered biologically relevant. In the presence of S9 in Experiment 2, a statistically significant increase in MF was observed at 80 µg/mL, but there were no statistically significant findings at any other dose level. Negative control data and positive control data were consistent with the laboratory historical data. See tables B.6.4.1.2-01/02 and B.6.4.1.2-01/03 for further details.

Table B.6.4.1.2-01/2: Mean RS and MF after 3-hour treatment with thymol - Experiment 1

Concentration (µg/mL)	Without S9		Concentration (µg/mL)	With S9	
	% RS	MF		% RS	MF
0	100	1.34	**0**	100	1.39#
20	82	1.69	**10**	81	2.61
40	58	3.12	**20**	65	0.30
60	37	2.42	**30**	54	2.46
80	24	2.77	**40**	46	0.89
			50	48	0.75
			60	47	1.90
			75	38	1.07
			90	21	2.53
			105	15	2.88
Linear trend: ns			**Linear trend ***		
NQO (0.15 µg/mL)	72	27.75	**B[a]P (2 µg/mL)**	80	10.43
NQO (0.2 µg/mL)	ne		**B[a]P (3 µg/mL)**	45	28.34

MF 6-TG resistant mutants/10^6 viable cells 7 days after treatment
ne not evaluated due to contamination
ns not significant
** Test for linear trend $\chi 2$ (one-sided), significant at 5% level*
 # Based on one replicate only due to contamination

Table B.6.4.1.2-01/3: Mean RS and MF after 3-hour treatment with thymol - Experiment 2

Concentration (µg/mL)	Without S9		Concentration (µg/mL)	With S9	
	% RS	MF		% RS	MF
0	100	1.20	**0**	100	1.69
10	87	1.55	**10**	78	3.53
20	76	1.93	**20**	56	4.49
40	56	0.81	**40**	52	2.19
50	59	0.37	**50**	46	2.07
60	46	1.84	**60**	39	1.29
70	35	0.89	**70**	34	2.13
80	24	1.19	**80**	32	**5.32***
90	16	1.70	**90**	29	3.51
			100	25	3.22
			110	20	2.61
Linear trend: ns			Linear trend: ns		
NQO (0.15 µg/mL)	37	13.45	**B[a]P (2 µg/mL)**	99	10.59
NQO (0.2 µg/mL)	45	29.09	**B[a]P (3 µg/mL)**	52	42.46

MF 6-TG resistant mutants/10^6 viable cells 7 days after treatment.
** Test for linear trend $\chi 2$ (one-sided), significant at 5%.*

Assessment and conclusion

<u>Assessment and conclusion by applicant</u>:

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance. The EU-agreed endpoint is: Overall no genotoxic potential in vivo via the oral route.

<u>Assessment and conclusion by RMS</u>:

In this *in vitro* gene mutation test performed in mouse lymphoma cells, thymol was found negative both in the absence and presence of metabolic activation.

The study has been considered as acceptable, no major deviations were found.

B.6.4.1.2-02. Second study

Data point	CA 5.4.1 (EU data requirement)
Report author	Maisanaba, S., Prieto, A.I., Puerto, M., Gutiérrez-Praena, D., Demir, E., Marcos, R. and Cameán, A.M.
Report year	2015
Report title	*In vitro* genotoxicity testing of carvacrol and thymol using the micronucleus and mouse lymphoma assays.
Report No.	Not applicable, published study (Mutation Research, 784-785: 37–44).
Document No.	KCA 5.4.1/11
Guidelines followed in study	Not stated.
Deviations from OECD TG 490 (2016)	- A short-term treatment assay with metabolic activation was not carried out. - It is stated that the highest concentration was one with a RTG of 20%, but RTG for the highest concentration in the tabulated data were 93 and 101% in experiment 1 and 2, respectively. - Batch/Lot no. was unknown. - There were not measurements of pH. - There was no information about signs of precipitation. - HCD was not reported. - It was unclear in the article which was the concentration of the positive control (MMS), several concentrations were reported.
Previous evaluation	No, not previously submitted
GLP	No
Reliability	Supportive only

Materials and Methods

Test chemical	Thymol. Purity: 99.5 %. Batch/Lot No.: unknown. Source: Sigma Aldrich, Spain.
Solvent	DMSO
Negative control	RPMI 1640 medium
Positive control	Methanesulfonate (MMS, 1 µg/mL)
Test system	L5178Y/*Tk*$^{\pm}$ mouse lymphoma cells
Test method	Cytotoxicity was determined by the relative total growth (RTG) after a treatment of 4 h of thymol. The recommended highest concentration was one with an RTG of 20%. Thereby, the selected ranges for thymol, taking into account the obtained RTG values were 0–250 µM. Thymol was applied to L5178Y/*Tk*$^{\pm}$ mouse lymphoma cells at 7.8, 15.65, 31.25, 62.5, 125 and 250 µM during 4 h without S9, in duplicate. Replicates were exposed to 4 µg/mL trifluorothymidine (TFT) for the mutation analysis. Plates were also incubated for 12 days. To assist the scoring of TFT mutation colonies, 2.5 mg/mL MTT was added to each well and the plates were incubated for 4 h. After this time, the mutant colonies of each plate were counted. Colony size was estimated. The induced mutant frequency (IMF) was determined according to the formula IMF =MF−SMF, where MF is the test culture mutant frequency and SMF is the spontaneous mutant frequency.
Criteria for assessment of results	Positive responses are determined as those that for any treatment meet or exceed the global evaluation factor (GEF, 126 for the microwell assay) and also when a positive trend test is obtained.
HCD	Not reported
Statistical analysis	The statistical approach included the one-way ANOVA followed by Dunnett's test, which was used to evaluate the significance of the differences in MF between the control and treated cultures. Results were considered statistically significant when the *p-value* was <0.05.

Results

Thymol did not present a mutagenic response, and the cytotoxicity test did not show any bioavailability reduction (taking into account the RTG values) after exposure to this substance (up 250 µM). For further data see table B.6.4.1.2-02).

Table B.6.4.1.2-02: Summary of mutagenicity in mouse lymphoma cells treated with thymol for 4 h (-S9)

Concentration (µM)	% plating efficacy	MF ($x10^{-6}$)	RTG	MF (S/L)	IMF (MF-SMF)
Experiment 1					
0	95	138	100	81/57	-
7.8	95	152	110	55/97	14
15.65	80	176	160	68/108	38
31.25	74	177	137	80/97	39
62.5	74	168	140	87/80	30
125	95	182	90	98/84	44
250	104	177	93	81/96	39
MMS (10 µg/mL)	104	**773***	87	648/125	635
Experiment 2					
0	100	137	100	77/60	-
7.8	82	162	97	97/65	25
15.65	76	171	118	105/66	34
31.25	75	174	99	108/66	37
62.5	106	110	86	78/32	-27
125	96	146	106	99/47	9
250	76	158	101	103/55	21
MMS (10 µg/mL)	47	**986***	77	765/221	849

Small/large colonies (S/L) colony mutant frequencies); SMF, spontaneous mutant frequency; IMF, induced mutant frequency.
** $p \leq 0.001$*

Assessment and conclusion

<u>Assessment and conclusion by applicant</u>:

This new study is considered to be acceptable for the renewal of thymol (reliable with restrictions; study not GLP).
The EU-agreed endpoint is: Overall no genotoxic potential *in vivo* via the oral route.

<u>Assessment and conclusion by RMS</u>:

In this test performed in mouse lymphoma cells, thymol was negative for gene mutation analysed *in vitro* without metabolic activation. A short-term study with metabolic activation was not performed and given the negative result of the short-term study without metabolic activation, this study was necessary in order to comply with acceptability criteria of the OECD TG 490 (2016). Dose selection was also doubtful (see deviations above).
Taking all together, this test was regarded as supplemental only by the RMS.

B.6.4.1.3. Mammalian chromosome aberration

B.6.4.1.3-01. First study (Chromosomal aberration assay)

Data point	CA 5.4.1 (EU data requirement)
Report author	Hikiba, H., Watanabe, E., Barrett, J.C. and Tsutsui, T.
Report year	2005
Report title	Ability of fourteen chemical agents used in dental practice to induce chromosome aberrations in Syrian Hamster Embryo cells.
Report No	Not applicable, published study (Journal of Pharmaceutical Sciences, 97: 146-152).
Document No	KCA 5.4.1/05
Guidelines followed in	Not specified in the study report.

study	
Deviations from OECD TG 473 (2016)	- Batch no. was unknown. - The source of SHE cells was unknown. - The method for preparation of the metabolic activation system was not reported. - There is not information about the methods of slide preparation and staining technique used. - There is no information about the precipitation. - The number of harvested cells is not indicated. - The method for estimating cytotoxicity is not the recommended in the TG. Furthermore, cytotoxicity was not measured in the assay carried out with metabolic activation. - Only 100 metaphase cells per dose were examined rather than 300 specified in the current guideline. - It is not stated if the negative control includes the solvent. - Positive controls were not included in the assay. - Historical negative and positive control data is not reported.
Previous evaluation	Yes, evaluated and considered as acceptable in DAR (2011).
GLP	No
Acceptability	Supportive only

Materials and Methods

Test chemical	Thymol. Purity: > 98 %. Batch/Lot No.: unknown. Source: Iwaki Seiyaku, Tokyo.
Solvent	DMSO at 500 mM
Negative control	Unknown
Positive control	None
Test system	Syrian hamster embryo (SHE) cells. Doubling time: 16 h.
Metabolic activation	Rat liver post-mitochondrial supernatant (PMS)
Test method	*Cytotoxicity assay* SHE cells were treated with 0, 130, 260, 390, 520 µM of thymol for 24 h. After harvesting with 0.1% trypsin, the cells were incubated in triplicate for 7 days for colony formation. The relative colony-forming efficiency was expressed as the no. of colonies in the treated dishes divided by the no. in the control dishes x 100. Actual colony-forming efficiency of control cells was 13 ± 0.7 %. *Chromosome aberration assay* The chromosome aberration test was performed by a long treatment method in which SHE cells (n= 5 x 10^5) were plated into 75 cm^2 flasks, incubated overnight, and treated for 24 h with 0, 130, 260, 390, 520 µM of thymol without metabolic activation system. Other cells were treated for 3 h with 5 % PMS, with 0, 130, 260 and 390 µM of thymol. Colcemid (0.2 µg/mL) was added to the cultures 3 h before cell harvesting. Then, the cells were harvested with 0.1% trypsin. For determination of chromosome aberrations, 100 metaphases were scored per experimental group. Achromatic lesions greater than the width of the chromatid were scored as gaps unless there was displacement of the broken piece of chromatid. If there was displacement, they were scored as breaks.
Criteria for assessment of results	Not stated
HCD	Not reported
Statistical analysis	A χ^2 test was performed.

Results

Cytotoxicity assay

Relative colony forming efficiency of thymol in the absence of metabolic activation can be seen in table B.6.4.1.3-01 and it was excessive at the highest dose tested (520 µM).

Chromosome aberration assay

Thymol did not induce statistically significant increases in the levels of chromosome aberrations in SHE cells in absence of metabolic activation (see table B.6.4.1.3-01).
With metabolic activation, thymol induced statistically significant increases in the levels of chromosome aberrations at all concentrations tested.

Table B.6.4.1.3-01: Chromosome aberrations in SHE cells

Thymol concentration (µM)	Relative colony forming efficiency (%)	Type of aberrations (%)								Aberrant metaphases (%)
		CG	ICG	CB	ICB	E	O	D	F	
24-hour exposure, without metabolic activation										
0	100	0	0	0	0	0	0	0	0	0
130	94	1	1	1	1	0	0	0	0	4.0
260	91	1	1	1	0	0	0	0	0	3.0
390	83	2	0	3	0	0	0	0	0	5.0
520	8	Few metaphases								
3-hour exposure, with metabolic activation										
0	nr	0	0	0	0	0	0	0	0	0
130	nr	2	2	1	0	2	0	0	0	**6.0***
260	nr	4	1	10	0	8	0	0	0	**19.0****
390	nr	5	0	13	1	8	0	0	0	**17.0****

nr No recorded, CG chromatid gaps, ICG isochromatid gaps, CB chromatid breaks, ICB isochromatid breaks, E exchanges, O ring chromosomes, D dicentric chromosomes, F fragmentations,
** P<0.05 (χ^2 test), **P<0.01 (χ^2 test)*

Assessment and conclusion

<u>Assessment and conclusion by applicant</u>:

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.
The EU-agreed endpoint is: Overall no genotoxic potential *in vivo* via the oral route.

<u>Assessment and conclusion by RMS</u>:

In this test performed in Syrian Hamster Embryo cells, thymol was positive for structural chromosomal aberrations analysed *in vitro*, but only with metabolic activation. Cytotoxicity data are not available in these conditions.
The study has been considered as supportive only due to the deviations described above.

B.6.4.1.3-02. Second study (Chromosomal aberration assay)

Data point	CA 5.4.1 (EU data requirement)
Report author	Tanaka N., Yamakage, K., Kusakabe, H., Hashimoto, K., Nagao, T. and Ohta, R.
Report year	1996
Report title	*In vitro* chromosomal aberration test of thymol on cultured Chinese hamster cells.
Report No.	None
Document No.	KCA 5.4.1/06
Guidelines followed in study	Methods Concerning Tests Using New Chemical Substances (March 31, 1984, private company #237, medical dept. #306, 62 bureau #303) and OECD Toxicity Guidelines 473.
Deviations from OECD TG 473 (2016)	- The original report in Japanese was not provided. Two documents were submitted: one of them was not readable and the other one seems to be a translation in which not all the information is included and some information is doubtful (for example, the use of colestimide in the test). Therefore, information from this study is considered as not adequately presented. - Batch No. was unknown.

	- There was no information about doubling time of the cell line. - Neither the metaphase-arresting substance, nor the time at which it was added to the culture, were adequately reported. - There was no information about the solubility. There were no measurements of pH. - Cell density at seeding was not reported. - None cytotoxicity parameter was reported. - Only 200 metaphase cells per dose were examined for chromosome aberrations rather than 300 specified in the current guideline. - Historical negative and positive control data were not reported.
Previous evaluation	Yes, evaluated and considered as acceptable in DAR (2011)
GLP	It is stated that the final test was implemented based on the compliance of the Chemical Substance GLP Standards. Nevertheless, the document is not signed.
Acceptability	Supportive only

Materials and Methods

Test chemical	Thymol. Purity: > 98 %. Batch/Lot No.: unknown.
Solvent	DMSO
Negative control	DMSO
Positive control (-S9)	Mitomycin C
Positive control (+S9)	Cyclophosphamide
Test system	CHL/IU cells of Chinese hamster origin from the Research Resource Bank (JCRB)
Metabolic activation	Rat liver S9 derived from Sprague-Dawley rats induced with phenobarbital and benzoflavone. S9 mix composition ml/10 mL S9 3 20 mM HEPES (pH 7.2) 2 50 mM $MgCl_2$ 1 330 mM KCl 1 50 mM G-6-P 1 40 mM NADP 1 Distilled water 1
Test method	*Preliminary assay* Dose selection for the main test was based on a preliminary growth inhibition test in which a 50% growth inhibitory concentration was determined. Very few details are provided in the paper and no data are reported. *Chromosome aberration assay* Cells were incubated with controls at the above-mentioned concentrations and with thymol at concentrations of 0.02, 0.04 and 0.08 mg/mL: - For 24 or 48 h, in the absence of S9 (*continuous processing*). - For 6 h, followed by 18-hour culture in fresh medium, in the presence and absence of S9 (*short-term processing*). Each group was tested in duplicate. Neither the metaphase-arresting substance nor the time at which it was added, were adequately reported. Then, the cells were harvested, fixed and stained with Giemsa. For determination of chromosome aberrations and polyploid cells, 200 and 300 metaphase cells were analysed, respectively.
Criteria for assessment of results	In the case that two tests confirmed a significant decrease, it was positive. In the case that the trend test did not confirm a significant decrease, it was negative. In the case that the structural aberrations were less than 100, and the polyploid cells were less than 400 in the observed cells, the results were named impossible to be judged due to the cell toxicity.
HCD	Not reported
Statistical analysis	Regarding the appearance frequency of cells containing chromosome aberrations,

	the Lin Method was considered, and the Fisher Direct Probability Method 3 was applied to background data and test substance processing group times, multiplicity was considered, and the family-wise significant level was verified to be 5%. In the case that the Fisher Direct Probability method confirmed a significant decrease, the Cochrane Armitage Trend Test was carried out regarding dose dependency.

Results

Continuous processing

There was no increase in the incidence of cells with chromosome aberrations following 24 or 48 h of incubation with 0.02 or 0.04 mg/mL of thymol in the absence of S9.

The high dose group (0.080 mg/mL) could not be analysed due to the significant cell toxicity.

Results are summarised in table B.6.4.1.3-02/1.

Short-term processing

In the absence of S9, there was marked cytotoxicity at 0.08 mg/m after 6 h of incubation with thymol followed by 18 h of culture, but it was still possible to score 77 cells. Of these cells, three (3.9%) showed chromosome aberrations (apart from gaps), which indicated a higher rate than the negative control rate. However, clear conclusions could not be drawn in the presence of marked cytotoxicity.

At 0.08 mg/mL in the presence of S9, there was a statistically significant increase in the incidence of cells with chromosome aberrations with gaps included and, when gaps where excluded from the analysis, an increase in the incidence of chromosome aberrations without statistical significance was observed.

Results are summarised in table B.6.4.1.3-02/2.

Table B.6.4.1.3-02/1: Chromosome aberrations in the continuous processing (without S9)

Group	Conc (mg/mL)	No. of cells analysed	No. of structural aberrations							Others	No. of cells with aberrations		Polyploid (%)
			gap	ctb	cte	csb	cse	mul	total		TAG (%)	TA (%)	
24-hour exposure period													
Control	**0**	200	0	0	1	0	0	0	1	0	1 (0.5)	1 (0.5)	0.50
DMSO	**0**	200	0	0	0	0	0	0	0	0	0 (0.0)	0 (0.0)	0.13
Thymol	**0.02**	200	1	0	0	0	0	0	1	0	1 (0.5)	0 (0.0)	0.38
	0.04	200	2	0	1	0	0	0	3	0	3 (1.5)	1 (0.5)	0.00
	0.08	15 (T)	0	0	0	0	0	0	0	0	0 (0.0)	0 (0.0)	0.00 (T)
MC	**0.00005**	200	3	29	61	0	4	0	97	0	72 (36)	70 (35)	0.00
48-hour exposure period													
DMSO	**0**	200	0	0	0	0	1	0	1	0	1 (0.5)	1 (0.5)	0.38
Thymol	**0.02**	200	0	1	0	0	0	0	1	0	1 (0.5)	1 (0.5)	0.13
	0.04	200	0	0	0	0	0	0	0	1	0 (0.0)	0 (0.0)	0.63
	0.08	0 (T)	-	-	-	-	-	-	-	-	-	-	- (T)
MC	**0.00005**	200	4	31	59	0	1	20	115	5	60 (30)	57 (28.5)	0.75

Abbreviations gap chromatid gap and chromosome gap, ctb chromatid break, cte chromatid exchange, csb chromosome break, cse chromosome exchange (dicentric and ring etc), mul multiple aberrations, TAG total number of cells with aberrations, TA total number of cells with aberrations except gap, T group excluded from analysis, impossible to be judged due to the cell toxicity, MC mitomycin.

Table B.6.4.1.3-02/2: Chromosome aberrations in the short-term processing

Group	Conc (mg/mL)	No of cells analysed	No. of structural aberrations							Others	No. of cells with aberrations		Polyploid (%)
			gap	ctb	cte	csb	cse	mul	total		TAG (%)	TA (%)	
Without S9 mix – 6-hour exposure period													
Control	**0**	200	0	0	0	0	0	0	0	0	0 (0.0)	0 (0.0)	0.00
DMSO	**0**	200	1	0	0	0	1	0	2	0	2 (1.0)	1 (0.5)	0.13
Thymol	**0.02**	200	1	0	0	0	0	0	1	0	1 (0.5)	0 (0.0)	0.38
	0.04	200	1	0	0	0	0	10	11	0	2 (1.0)	1 (0.5)	0.00
	0.08	77 (T)	1	1	2	0	0	0	4	0	4 (5.2)	3 (3.9)	0.58 (T)
CPA	**0.00005**	200	0	0	1	0	0	0	1	0	1 (0.5)	1 (0.5)	0.13
With S9 mix – 6-hour exposure period													
DMSO	**0**	200	1	1	0	1	0	0	3	0	3 (1.5)	2 (1.0)	0.25
Thymol	**0.02**	200	1	1	0	0	0	0	2	1	2 (1.0)	1 (0.5)	0.13
	0.04	200	0	0	1	0	0	0	1	0	1 (0.5)	1 (0.5)	0.13
	0.08	200	1	5	7	0	2	0	15	0	**11* (5.5)**	10 (5.0)	0.75
CPA	**0.00005**	200	10	193	272	1	1	360	837	1	176 (88)	175 (87.5)	0.00

Abbreviations gap chromatid gap and chromosome gap, ctb chromatid break, cte chromatid exchange, csb chromosome break, cse chromosome exchange (dicentric and ring etc), mul multiple aberrations, TAG total number of cells with aberrations, TA total number of cells with aberrations except gap, CPA cyclophosphamide.

** Significantly different from historical control with respect to TAG and polyploidy by Fisher s exact test (p<0.05) using a Bonferroni correction for multiple comparison.*

Assessment and conclusion

Assessment and conclusion by applicant: The study has been previously reviewed at EU level and is acceptable for renewal of the active substance. The EU-agreed endpoint is: Overall no genotoxic potential *in vivo* via the oral route.
Assessment and conclusion by RMS: In this test performed in CHL/IU cells from Chinese hamster lungs, thymol was negative for structural chromosomal aberrations analysed *in vitro*. Although a statistically significant increase in chromosome aberrations was observed in the cells treated with the higher dose tested of thymol (a concentration stated to be cytotoxic although cytotoxicity parameters are not reported) in the short-term test with metabolic activation, it was statistically significant only when the gaps were included in the analysis. In the short-term test, with and without metabolic activation, a non-statistically significant increase in chromosome aberrations was observed excluding the gaps. No statistically significant differences were observed in the continuous tests. Taking all together, the test was considered to yield a negative result and regarded by the RMS as supplemental only, due to the deviations described above.

B.6.4.1.3-03. Third study (Chromosomal aberration assay)

Data point	CA 5.4.1 (EU data requirement)
Report author	Buyukleyla, M. and Rencuzogullari, E.
Report year	2009
Report title	The effects of thymol on sister chromatid exchange, chromosome aberration and micronucleus in human lymphocytes.
Report No.	Not applicable, published study (Ecotoxicology and Environmental Safety, 72(3): 943-947).
Document No.	KCA 5.4.1/07
Guidelines followed in study	IPCS guidelines (Albertini, R.J., Anderson, D., Douglas, G.R., Hagmar, L., Hemminki, K., Merlo, F., Natarajan, A.T., Norppa, H., Shuker, D.E.G., Tice, R., Waters, M.D., Aitio, A., 2000. IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. Mutat. Res. 463, 111–172)
Deviations from OECD TG 473 (2016)	- Batch No. was unknown. - There was no information about the precipitation. - The number of harvested cells was not indicated. - According to the TG, separated lymphocytes should be cultured for 48 h in the presence of PHA in order to induce cell division prior to exposure to the test chemical. In the 48-hour treatment, cells were cultivated only for 24 h before adding the thymol. - The types of chromosomal aberrations were not detailed in the study table, although the most abundant were mentioned in the study text. - Historical negative and positive control data were not reported.
Previous evaluation	Yes, evaluated and considered as acceptable in DAR (2011).
GLP	No
Acceptability	Yes

Materials and Methods

Test chemical	Thymol. Purity: 99.6 %. Batch/Lot No.: unknown. Source: Sigma.
Solvent	DMSO
Negative control	Untreated and DMSO (4 µL/mL)
Positive control	Ethyl methanesulfonate (0.7 µg/mL)
Test system	Human peripheral lymphocytes from whole blood cells from four healthy donors (two male and two female, non-smokers, age: 23–26).
Metabolic activation	None
Test method	The method of Evans (1984) was followed with minor modifications (Evans, H.J.,

	1984. Human peripheral blood lymphocytes for the analysis of chromosome aberrations in mutagen tests, Handbook of Mutagenicity Test Procedures, second ed. Elsevier Sciences Publishers, Amsterdam, pp. 405–427). Lymphocytes were grown in MEM eagle culture liquid with 15% of foetal calf serum and 2.5 mg/L phytohaemagglutinin (PHA), and were treated with 25, 50, 75 and 100 µg/mL of thymol for 24 h (added 48 h after initiating the culture) and 48 h (added 24 h after initiating the culture), without metabolic activation. Colchicine (0.06 µg/mL) was added to the cultures 2 h before harvesting; cells were then harvested, fixed and stained using 5% Giemsa stain. 100 metaphases per donor (400 metaphases per concentration) were analysed for chromosome aberrations. Gaps were not evaluated as chromosome aberrations. In addition, 400 cells (100 cells from each donor) were scored for replication index (RI) and the mitotic index (MI) was also determined by scoring 3000 cells from each donor.
Criteria for assessment of results	Not reported
HCD	Not reported
Statistical analysis	The t-test was used for the statistical significance of all the parameters. Dose–response relationships were determined from the correlation and regression coefficients for the percentage of structural and total chromosome aberrations, RI and MI.

Results

Thymol induced structural chromosome aberrations at all concentrations and treatment periods when compared with controls, without dose-dependent manner. Structural abnormalities were more common in chromatids (chromatid breaks) than in chromosomes (chromosome breaks, sister union, dicentric chromosome) and thymol did not induce numerical abnormalities such as aneuploidy and euploidy. See table B.6.4.1.3-03. The mitotic index was below 50% at the highest dose level tested in the 48-hour treatment.

Table B.6.4.1.3-03: Structural chromosome aberrations in human lymphocytes treated with thymol

Test substance	Concentration (µg/mL)	Abnormalities		Structural CA ± SE (%)	MI ± SE (% compared to solvent control)
		Chromatid type	Chromosome type		
24-hour treatment					
Untreated	-	14	9	5.75 ± 0.62	6.04 ± 1.19
DMSO	4 µL/mL	22	12	8.50 ± 0.64	5.23 ± 0.84
EMS	0.7	64	54	29.50 ± 0.64	3.69 ± 0.68
Thymol	25	47	16	**15.75 ±2.78***	6.99 ± 0.41 (134)
	50	80	10	**22.50 ± 0.28*****	**3.45 ± 0.82* (66)**
	75	72	8	**20.00 ± 1.68****	4.58 ± 0.95 (88)
	100	58[1]	24[1]	**27.50 ± 2.95****	**2.89 ± 0.86* (55)**
48-hour treatment					
DMSO	4 µL/mL	27	5	8.00 ± 0.70	7.05 ± 0.79
EMS	0.7	93	30	30.75 ± 5.07	4.11 ± 1.08
Thymol	25	32	11	10.75 ± 1.25	6.18 ± 0.42 (88)
	50	55	9	**16.00 ± 2.94***	**4.27 ± 0.72* (61)**
	75	55	11	**16.50 ± 1.19****	4.74 ± 1.05 (67)
	100	64[1]	11[1]	**34.09 ± 0.94*****	**2.58 ± 0.30***** (37)**

[1] 298 and 220 cells were scored, respectively due to excessive toxicity
** Significantly different from solvent control p≤ 0.05, ** p≤ 0.01, *** p≤ 0.001*

Assessment and conclusion

<u>Assessment and conclusion by applicant</u>:

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.
The EU-agreed endpoint is: Overall no genotoxic potential *in vivo* via the oral route.

Assessment and conclusion by RMS:

In this test performed in human lymphocytes, thymol was positive for structural chromosomal aberrations analysed *in vitro* by a continuous exposure to the substance and without metabolic activation.

Although the short-term tests recommended in the OECD test guideline 473 (2016), with and without metabolic activation, were not performed in this study, they were not deemed necessary given the positive response obtained in the available test. It was stated in this study that numerical abnormalities, aneuploidy or euploidy were not induced by thymol, although no tabulated data was shown. In this context, the RMS interpreted the mentioned "euploidy" as polyploidy. Regarding aneugenicity, this kind of test is not usually considered as appropriate to investigate such parameters, and apparently no modification of the method was applied to cover this endpoint.

Taking all together, this test was considered to yield a positive result and was regarded by the RMS as acceptable, as the deviations described above were minimal.

B.6.4.1.3-04. Fourth study (Chromosomal aberration assay)

Data point	CA 5.4.1 (EU data requirement)
Report author	Someya, H., Higo, Y., Ohno, M., Tsutsui, T.W. and Tsutsui, T.
Report year	2008
Report title	Clastogenic activity of seven endodontic medications used in dental practice in human dental pulp cells
Report No.	Not applicable, published study (Mutation Research, 650: 39-47).
Document No.	KCA 5.4.1/09
Guidelines followed in study	None stated
Deviations from OECD TG 473 (2016)	- Batch No. was unknown. - There was no information about doubling time of the cell line. - There was no information about the solubility. There were no measurements of pH. - Recommended cytotoxicity parameters were not used and the one used was not described. - No appropriate positive control for no metabolic activation conditions was used. - Cell density at seeding was not reported. - Only 100 metaphase cells per dose were examined for chromosome aberrations rather than 300 specified in the current guideline. - Experimental condition of metabolic activation was not tested. - Historical negative and positive control data were not reported.
Previous evaluation	No, not previously submitted
GLP	No
Reliability	Supportive only

Materials and Methods

Test chemical	Thymol. Purity >98%. Batch/Lot No.: unknown. Source: Iwaki Seiyaku, Japan.
Solvent	DMSO
Negative control	DMSO
Positive control	Cyclophosphamide (50 µM) with or without metabolic activation (5%)
Test system	Human dental pulp cells (D824 cells) from a lower third molar extracted from a 23-year old woman.
Metabolic activation	Liver post-mitochondrial supernatant (PMS) from male Sprague-Dawley rats treated with sodium phenobarbital and 5,6-benzoflavone (added only to the positive control).
Test method	In a preliminary assay, cytotoxicity was measured to select concentrations for analysis of chromosome aberrations and it was determined as no. of cells treated with thymol relative to the no. of cells in the control cultures x 100. Triplicate cultures of D824 cells were treated with thymol for 3 or 30 h at various concentrations (not

	stated). After washings with fresh medium, cells were incubated for 27 h, and the no. of cells was counted after harvesting with trypsin. *Chromosome aberration assay* The concentrations that induced cytotoxicity greater than 50% the one of the control were used in principle in all experiments for assessing chromosome aberrations. No metabolic activation systems were applied. Triplicate cultures were treated with thymol for 3 or 30 h at 30, 100 and 300 µM, then washed and incubated for a further 27 h. 3 h before the end of incubation, 0.02 µg/mL colcemid was administered. Metaphase chromosomes were prepared, and 100 cells per dose were counted. The aberrations scored were chromatid gaps, chromosome gaps, chromatid breaks, chromosome breaks, chromatid exchanges, dicentric chromosomes, ring chromosomes, and fragmentations. Achromatic lesions greater than the width of the chromatid were scored as gaps unless there was displacement of the broken piece of chromatid; if there was displacement, these were scored as breaks.
Criteria for assessment of results	Not reported
HCD	Not reported
Statistical analysis	Statistical analysis was performed by χ^2-test to assess the significance of the difference in the incidences of chromosome aberrations between control cultures and cultures treated with test agents. The level of significance in the statistical analysis was determined at $p < 0.05$.

Results

No increases in the levels of chromosome aberrations were observed in cells treated for 3 h with thymol, which also failed to induce polyploidy and endoreduplication in D824 cells.
Thymol induced a statistically significant increase in the levels of chromosome aberrations that were observed in cells treated for 30 h. This increase occurred in a concentration-dependent manner. No statistically significant increases in the percentage of cells with polyploidy or endoreduplication were elicited by thymol after 30 h.
A concentration-dependent growth inhibition was observed. The positive control gave a positive response in the assay.

Table B.6.4.1.3-04: Chromosome aberrations in D824 cells induced by thymol

Concentration (µM)	Relative cell No. (%)	No. of metaphases scored	Type of structural aberration[a] (%)								Aberrant metaphases (%)	Polyploidy and endoreduplication (%)
			ctg	csg	ctb	csb	cte	D	O	F		
3-hour treatment without metabolic activation												
0	100	500	0.8	0	0	0	0	0	0	0	0.8	2.0
30	83	100	0	0	0	0	0	0	0	0	0	1.0
100	77	100	1	0	0	0	0	0	0	0	1.0	2.0
300	78	100	0	0	1	0	0	0	0	0	1.0	3.0
30-hour treatment without metabolic activation												
0	100	500	1	0	0	0	0	0	0	0	1.0	2.0
30	92	100	3	0	0	0	0	0	0	0	3.0	3.0
100	91	100	6	0	2	0	0	0	0	0	**6.0****	5.0
300	65	100	11	0	3	0	1	0	0	0	**14.0****	7.0
Cyclophosphamide, with or without metabolic activation												
50 (-PMS)	87	100	0	0	0	0	0	0	0	0	0	3.0
50 (+PMS)	47	100	20	0	10	0	3	0	0	0	**35.0****	0

[a] ctg, chromatid gaps; csg, chromosome gaps; ctb, chromatid breaks; csb, chromosome breaks; cte, chromatid exchanges; D, dicentric chromosomes; O, ring chromosomes; F, fragmentations.
*** Statistically significant difference from control ($p < 0.01$; $\chi 2$-test)*

Assessment and conclusion

<u>Assessment and conclusion by applicant</u>:

This new study is considered to be acceptable for the renewal of thymol (reliable with restrictions; study is similar to current OECD guideline but is not GLP and there are some deficiencies in the methodology). The EU-agreed endpoint is: Overall no genotoxic potential *in vivo via* the oral route.
Assessment and conclusion by RMS: In this test performed in human dental pulp cells (D824 cells), thymol was positive for structural chromosomal aberrations analysed *in vitro* after a treatment of 30 h without metabolic activation, and negative after a treatment of 3 h. However, the result was considered as equivocal, given that there was no cytotoxicity information. Taking all together, this test was considered to yield a equivocal result and regarded by the RMS as supplemental only, due to the deviations described above.

B.6.4.1.3-05. Fifth study (Micronucleus test)

Data point	CA 5.4.1 (EU data requirement)
Report author	Buyukleyla, M. and Rencuzogullari, E.
Report year	2009
Report title	The effects of thymol on sister chromatid exchange, chromosome aberration and micronucleus in human lymphocytes.
Report No.	Not applicable, published study (Ecotoxicology and Environmental Safety, 72(3): 943-947).
Document No.	KCA 5.4.1/07
Guidelines followed in study	IPCS guidelines (Albertini, R.J., Anderson, D., Douglas, G.R., Hagmar, L., Hemminki, K., Merlo, F., Natarajan, A.T., Norppa, H., Shuker, D.E.G., Tice, R., Waters, M.D., Aitio, A., 2000. IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. Mutat. Res. 463, 111–172).
Deviations from OECD TG 487 (2016)	- Batch No. was unknown. - There was no information about the precipitation and the pH. - To estimate cytotoxic and cytostatic activity the NDI was calculated (see test method below). It presents a very slight modification compared to the CBPI (cytokinesis-block proliferation index) recommended in the TG, but it is minimal and regarded valid by the RMS. Taking this opinion into consideration, the cytotoxicity did not reach the reduction in NDI to 45± 5% of the concurrent negative control, as it is recommended in the TG when maximum concentration is based on cytotoxicity. However, excessive toxicity was reported at the highest concentration used. - Historical negative and positive control data were not reported.
Previous evaluation	Yes, evaluated and considered as acceptable in DAR (2011).
GLP	No
Acceptability	Yes

Materials and Methods

Test chemical	Thymol. Purity: 99.6 %. Batch/Lot No.: unknown. Source: Sigma.
Solvent	DMSO
Negative control	Untreated and DMSO (4 µL/mL).
Positive control	Ethyl methanesulfonate (0.7 µg/mL)
Test system	Human peripheral lymphocytes from whole blood cells from four healthy donors (two male and two female, non-smokers, age: 23–26 years).
Metabolic activation	None
Test method	For the analysis of micronuclei in binucleated lymphocytes, 0.2 mL of fresh blood was used to establish cultures. The cells were treated with 25, 50, 75 and 100 µg/mL of thymol for 24 h and 48 h, without metabolic activation. Cytochalasin B (6 µg/mL) was added after 44 h of incubation to block cytokinesis. Cells were harvested after a further 24-hour incubation. In all subjects, 2000 binucleated lymphocytes were scored from each donor (8000 binucleated cells per concentration).

	1000 viable cells were examined to determine the frequency of cells with 1, 2, 3, or 4 nuclei and to calculate the nuclear division index (NDI), using the following formula: NDI=(M1)+(2xM2)+(3xM3)+(4xM4)/N where M1–M4 represent the number of cells with 1–4 nuclei and N is the total number of viable cells scored (Fenech, 2000).
Criteria for assessment of results	Not reported
HCD	Not reported
Statistical analysis	The t-test was used for the statistical significance of all the parameters. Dose–response relationships were determined from the correlation and regression coefficients for the mean MN and NDI.

Results

Thymol induced an increase in the frequency of micronucleus at all concentrations and treatment periods when compared with controls without a dose-dependent effect (see table B.6.4.1.3-05). Thymol dose-dependently decreased the NDI at 75 and 100 µg/mL for 24 h and at all concentrations, except 25 mg/mL, for 48-hour treatment periods.

Table B.6.4.1.3-05: Frequency of micronucleus and NDI in human lymphocytes treated with thymol

Test substance	Concentration (µg/mL)	Micronucleated binuclear cells (%) ± SE	NDI ± SE (% compared to solvent control)
24 h treatment without metabolic activation			
Untreated	-	0.405 ± 0.11	1.395 ± 0.063
DMSO	4 µL/mL	0.460 ± 0.17	1.380 ± 0.058
EMS	0.7	2.917 ± 0.41	1.272 ± 0.078
Thymol	25	**1.875 ± 0.43***	1.310 ± 0.058 (95)
	50	**1.877 ± 0.44***	1.222 ± 0.078 (89)
	75	**1.702 ± 0.49***	**1.147 ± 0.060* (83)**
	100	**2.530 ± 0.46*[1]**	**1.087 ± 0.031** (79)**
48 h treatment without metabolic activation			
DMSO	4 µL/mL	0.602 ± 0.11	1.335 ± 0.034
EMS	0.7	5.120 ± 1.70	1.190 ± 0.050
Thymol	25	**1.725 ± 0.10***	1.280 ± 0.051 (96)
	50	**1.992 ± 0.43***	**1.147 ± 0.041* (86)**
	75	**1.415 ± 0.16***	**1.112 ± 0.022** (83)**
	100	**2.237 ± 0.39*[1]**	**1.065 ± 0.013*** (80)**

[1] 5130 and 2868 cells were scored, respectively due to excessive toxicity
** Significantly different from solvent control p≤ 0.05, ** p≤ 0.01, *** p≤ 0.001*

Assessment and conclusion

<u>Assessment and conclusion by applicant</u>:

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.
The EU-agreed endpoint is: Overall no genotoxic potential *in vivo* via the oral route.

<u>Assessment and conclusion by RMS</u>:

In this test performed in human lymphocytes, thymol was positive for micronuclei analysed *in vitro* by a continuous exposure to thymol and without metabolic activation.

Although the short-term tests recommended in the OECD test guideline 487 (2016), with and without metabolic activation, were not performed, they were not deemed necessary given the positive response obtained in this test.

Taking all together, this test was considered to yield a positive result without metabolic activation, and acceptable as deviations described above were minor.

B.6.4.1.3-06. Sixth study (Micronucleus test)

Data point	CA 5.4.1 (EU data requirement)
Report author	Maisanaba, S., Prieto, A.I., Puerto, M., Gutiérrez-Praena, D., Demir, E., Marcos, R. and Cameán, A.M.

Report year	2015
Report title	*In vitro* genotoxicity testing of carvacrol and thymol using the micronucleus and mouse lymphoma assays.
Report No.	Not applicable, published study (Mutation Research, 784-785: 37–44).
Document No.	KCA 5.4.1/11
Guidelines followed in study	Based on OECD 487 (2010)
Deviations from OECD TG 487 (2016)	- A short-term treatment assay without metabolic activation was not carried out. - To estimate cytotoxic and cytostatic activity the NDI was calculated (see test method below). It presents a very slight modification compared to the CBPI (cytokinesis-block proliferation index) recommended in the TG, but it is minimal and regarded valid by the RMS. Taking this opinion into consideration, the cytotoxicity did not reach the reduction in NDI to 45 ± 5% of the concurrent negative control, as it is recommended in the TG when maximum concentration is based on cytotoxicity. - Batch/Lot no. was unknown. - There were not measurements of pH. - There were no information about the type and composition of metabolic activation system. - There was no information about signs of precipitation. - The number of cells with micronuclei given separately for each treated and control culture was not reported. - HCD were not reported.
Previous evaluation	No, not previously submitted
GLP	No
Reliability	Supportive only

Materials and Methods

Test chemical	Thymol. Purity: 99.5 %. Batch/Lot No.: unknown. Source: Sigma Aldrich, Spain.
Solvent	DMSO
Negative control	RPMI 1640 medium
Positive control (-S9)	Mitomycin C (MMC, 0625 µg/mL)
Positive control (+S9)	Cyclophosphamide (CP, 8 µg/mL)
Test system	L5178Y/*Tk*± mouse lymphoma cells
Metabolic activation	S9 mix
Test method	Five different concentrations of thymol were selected according to the EC50 (without or with S9) previously obtained in a cell viability test at a range of 0-250 µM. Cells were treated at 15.62, 31.25, 62.5, 125 and 250 µM of thymol diluted in DMSO (stock at 0.5 M) during 24 h without S9 and 4 h with S9. Two duplicate cultures/concentrations were done. After these periods, cells were exposed to cytochalasin B (6 µg/mL) for 20 h and then harvested, fixed and stained with Giemsa. Micronuclei frequencies were analysed in at least 2000 binucleated cells per concentration. In addition, cells were scored to evaluate the nuclear division index (NDI), using the following formula: NDI=(M1)+(2xM2)+(3xM3)+(4xM4)/N
Criteria for assessment of results	Not reported.
HCD	Not reported
Statistical analysis	The statistical approach included the one-way ANOVA followed by Dunnett's test, which was used to evaluate the significance of the differences in micronucleated cells and NDI between the control and treated cultures. Results were considered statistically significant when the *P-value* was < 0.05.

Results

Neither the frequency of binucleated cells presenting at least one micronucleus (BNMN), nor the frequency of total micronuclei (data not shown) scored per 2000 binucleated cells, showed a significant increase with respect to the control values. Also, no significant differences were found in NDI values between the experimental groups and the negative control. Although additional nuclear anomalies, such as bridges or buds, were checked, no significant presence was observed (data not shown). Positive controls gave a satisfactory response.

Table B.6.4.1.3-06: BNMN and NDI in mouse lymphoma cells treated with thymol

Test substance	Concentrations	BNMN (%) ± SD	NDI ± SD
Without S9 (24 h)			
Negative control	-	0.9 ± 0.1	1.3 ± 0.0
Mitomycin C	0.0625 µg/mL	**15.6 ± 3.0***	1.4 ± 0.1
Thymol	15.62 µM	0.7 ± 0.3	1.4 ± 0.0
	31.25 µM	0.6 ± 0.3	1.5 ± 0.1
	62.5 µM	1.0 ± 0.5	1.5 ± 0.0
	125 µM	0.8 ± 0.2	1.3 ± 0.0
	250 µM	1.2 ± 0.1	1.5 ± 0.1
With S9 (3-6 h)			
Negative control	-	0.9 ± 0.1	2.0 ± 0.1
Cyclophosphamide	8 µg/mL	**3.6 ± 0.4***	2.0 ± 0.0
Thymol	15.62 µM	0.7 ± 0.3	1.9 ± 0.0
	31.25 µM	1.0 ± 0.4	2.0 ± 0.1
	62.5 µM	0.8 ± 0.5	2.0 ± 0.1
	125 µM	1.1 ± 0.1	2.0 ± 0.1
	250 µM	1.1 ± 0.1	1.9 ± 0.1

** $p < 0.01$*

Assessment and conclusion

Assessment and conclusion by applicant:

This new study is considered to be acceptable for the renewal of thymol (reliable with restrictions; study not GLP).
The EU-agreed endpoint is: Overall no genotoxic potential *in vivo via* the oral route.

Assessment and conclusion by RMS:

In this test performed in mouse lymphoma cells, thymol was negative for micronuclei analysed *in vitro* by a continuous exposure to thymol without metabolic activation and by short-term exposure with metabolic activation. A short-term study without metabolic activation was not performed.
Taking all together, this test was regarded as supplemental only by the RMS, due to the deviations described above and given that it was non-GLP.

B.6.4.1.4. DNA damage

B.6.4.1.4-01. First study (Sister chromatid exchange analysis)

Data point	CA 5.4.1 (EU data requirement)
Report author	Buyukleyla, M. and Rencuzogullari, E.
Report year	2009
Report title	The effects of thymol on sister chromatid exchange, chromosome aberration and micronucleus in human lymphocytes.
Report No.	Not applicable, published study (Ecotoxicology and Environmental Safety, 72(3): 943-947).
Document No.	KCA 5.4.1/07
Guidelines followed in study	IPCS guidelines (Albertini, R.J., Anderson, D., Douglas, G.R., Hagmar, L., Hemminki, K., Merlo, F., Natarajan, A.T., Norppa, H., Shuker, D.E.G., Tice, R., Waters, M.D., Aitio, A., 2000. IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. Mutat. Res. 463, 111–172).
Deviations from OECD TG 479 (1986), deleted in 2014.	This TG was deleted in 2014. However, for this assessment the version of the TG from 1986 was compared as follows: - There was no information about the solubility.

	- It should be stated whether or not centromeric switch of label is scored as an SCE. - The number of SCEs, the number of chromosomes and the calculated mean number of SCEs per chromosome should be listed separately for each metaphase for all treated and control cultures. - Cells should be exposed to the test chemical also in the presence of metabolic activation. - Rationale for dose selection was not clearly explained.
Previous evaluation	Yes, evaluated and considered as acceptable in DAR (2011)
GLP	No
Reliability	Supportive only

Materials and Methods

Test chemical	Thymol. Purity: 99.6 %. Batch/Lot No.: unknown. Source: Sigma.
Solvent	DMSO
Negative control	Untreated and DMSO (4 µL/mL)
Positive control	Ethyl methanesulfonate (0.7 µg/mL)
Test system	Human peripheral lymphocytes from whole blood cells from four healthy donors (two male and two female, non-smokers, age: 23–26 years).
Metabolic activation	None
Test method	The method of Perry and Thompson (1984) was followed with minor modifications (Perry, P.E., Thompson, E.J., 1984. The methodology of sister chromatid exchanges. Handbook of Mutagenicity Test Procedures, second ed. Elsevier Sciences Publishers, Amsterdam, pp. 495–529). Lymphocytes were grown in MEM eagle culture liquid with 15% of foetal calf serum and 2.5 mg/L phytohaemagglutinin (PHA), and were treated with 25, 50, 75 and 100 µg/mL of thymol for 24 h (thymol added 48 h after initiating the culture) and 48 h (thymol added 24 h after initiating the culture), without metabolic activation. Colchicine (0.06 µg/mL) was added to the cultures 2 h before harvesting; cells were then harvested, fixed and stained using modified fluorescence plus Giemsa. 100 second-division metaphases (25 cells per sample) were analysed for sister chromatid exchange (SCE). In addition, 400 cells (100 cells from each donor) were scored for replication index (RI).
Criteria for assessment of results	Not reported
HCD	Not reported
Statistical analysis	The t-test was used for the statistical significance of all the parameters. Dose–response relationships were determined from the correlation and regression coefficients for the mean MN and NDI.

Results

Thymol significantly increased the mean SCE in human lymphocytes treated with all doses when compared with both untreated and DMSO controls, except at 100 µg/mL for 24 h and at 75 and 100 µg/mL for 48 h, at which excessive toxicity was shown (see table B.6.4.1.4-01).
The RI was statistically significantly decreased at the highest concentration for the 48-hour treatment time in a concentration dependent manner.

Table B.6.4.1.4-01: Mean SCE and RI in cultured human lymphocytes treated with thymol

Test substance	Concentration (µg/mL)	Min-Max SCE	SCE/cell ± SE	RI ± SE
24-hour treatment				
Untreated	-	2-15	8.52 ± 0.85	2.54 ± 0.08
DMSO	4 µL/mL	2-16	8.91 ± 0.23	2.48 ± 0.05
EMS	0.7	6-57	22.77 ± 7.49	2.31 ± 0.03
Thymol	25	2-25	**10.40 ± 0.46***	2.48 ± 0.02

Test substance	Concentration (µg/mL)	Min-Max SCE	SCE/cell ± SE	RI ± SE
	50	2-17	**11.31 ± 0.14*** **	2.40 ± 0.06
	75	2-24	**10.69 ± 0.45***	2.35 ± 0.09
	100	3-28	9.97 ± 1.32[1]	2.36 ± 0.17
48-hour treatment				
DMSO	4 µL/mL	2-16	9.73 ± 0.96	2.49 ± 0.08
EMS	0.7	4-66	25.07 ± 4.38	2.09 ± 0.10
Thymol	25	2-17	**10.78 ± 0.70***	2.43 ± 0.09
	50	2-21	**12.62 ± 1.09***	2.14 ± 0.16
	75	2-23	10.96 ± 0.97	2.08 ± 0.23
	100	3-23	12.49 ± 1.84[1]	**1.92 ± 0.17***

[1] 91 and 96 cells were scored, respectively due to excessive toxicity
** Significantly different from solvent control p≤ 0.05, ** p≤ 0.01, *** p≤ 0.001*

Assessment and conclusion

Assessment and conclusion by applicant: The study has been previously reviewed at EU level and is acceptable for renewal of the active substance. The EU-agreed endpoint is: Overall no genotoxic potential *in vivo* via the oral route.
Assessment and conclusion by RMS: In this test performed in human lymphocytes, thymol was positive for sister chromatid exchange analysed *in vitro* by a continuous exposure to thymol and without metabolic activation. The TG for the "*In vitro* Sister Chromatid Exchange Assay in Mammalian Cells" (OECD TG 479, 1986) was deleted in 2014. Taking all together, this test was considered to yield a positive result, but it was regarded by the RMS as supplemental only, due to the deviations described above.

B.6.4.1.4-02. Second study (Comet assay)

Data point	CA 5.4.1 (EU data requirement)
Report author	Llana-Ruiz-Cabello, M., Maisanaba, S., Puerto, M., Prieto, A.I., Pichardo, S., Jos, A. and Cameán, A.M.
Report year	2014
Report title	Evaluation of the mutagenicity and genotoxic potential of carvacrol and thymol using the Ames *Salmonella* test and alkaline, Endo III and FPG-modified comet assays with the human cell line Caco-2.
Report No.	Not applicable, published study (Food and Chemical Toxicology, 72: 122–128).
Document No.	KCA 5.4.1/10
Guidelines followed in study	Not stated
Deviations from current test guideline	No TG is currently available.
Previous evaluation	No, not previously submitted
GLP	No
Reliability	Supportive only

Materials and Methods

Test chemical	Thymol. Purity: 99.5%. Batch/Lot No.: Unknown. Source: Sigma-Aldrich, Spain.
Solvent	DMSO
Negative control	Medium without foetal calf serum
Positive control	H_2O_2 (100 µM)
Test system	Human colon intestinal cell line Caco-2
Test method	Caco-2 human cells were exposed during 24 and 48 h to 62.5, 125 and 250 µM of thymol. After extraction, DNA was stained with SYBR Gold nuclei acid gel, and more than

	100 nuclei per experimental point were analysed. To investigate oxidative DNA damage, the cells were treated with two different enzymes in addition to thymol: Endo III, the substrates of which are oxidised pyrimidines, and FPG, which recognises the common oxidised purine 8-oxoGua and ring-opened purines or formamidopyrimidines. After this treatment the samples were processed as in the standard comet assay. Three independent experiments were carried out.
Criteria for assessment of results	None reported
HCD	Not reported
Statistical analysis	The total scores of the different groups were compared using the non-parametric Kruskal–Wallis test, followed by the Mann–Whitney U-test when the first test showed differences. The differences were considered significant at $p \leq 0.05$.

Results

Thymol (0–250 µM) did not induce DNA strand-breaks in Caco-2 cells at any concentrations tested after 24 or 48 h of exposure, even following treatment with Endo III or FPG.
These results can be found in Figures B.6.4.1.4-02/1 and B.6.4.1.4-02/2 below.

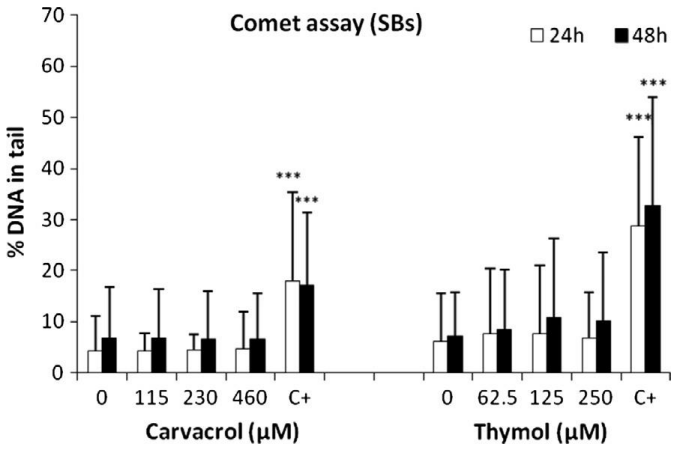

Figure B.6.4.1.4-02/1: The level of DNA damage was measured in Caco-2 cells after 24 and 48 h of exposure to 62.5, 125 or 250 µM thymol (right) as the formation of strand breaks by the standard comet assay. The levels of DNA strand-breaks (SBs) are expressed as % DNA in tail. All values are expressed as mean ± SD. ***Significantly different from control ($p < 0.001$).

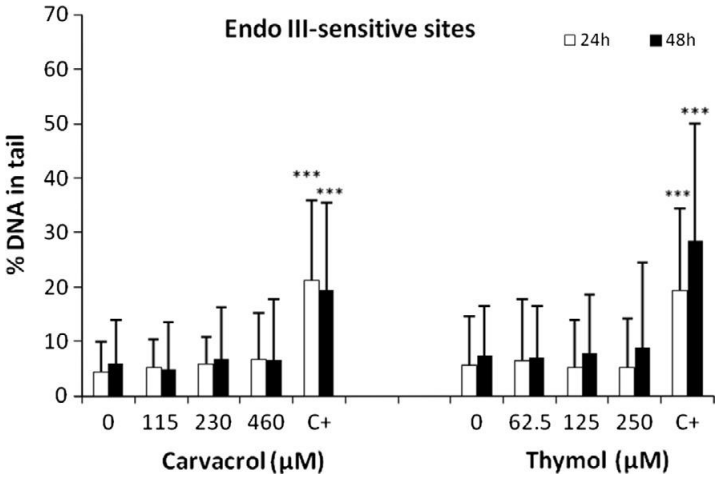

Figure B.6.4.1.4-02/2: The level of DNA damage was measured in Caco-2 cells after 24 and 48 h of exposure 62.5, 125 or 250 µM thymol (right) as End III-sensitive sites by the modified comet assay. The levels of oxidised

pyrimidines are expressed as % DNA in tail. All values are expressed as mean ± SD. ***Significantly different from control ($p < 0.001$).

Assessment and conclusion

Assessment and conclusion by applicant: This new study is considered to be acceptable for the renewal of thymol (reliable with restrictions; study not GLP). The EU-agreed endpoint is: Overall no genotoxic potential *in vivo via* the oral route.
Assessment and conclusion by RMS: A negative result was obtained in this test. In the results of these comet assays, mean values were not reported. The study was considered as supporting information to evaluate DNA damage *in vitro*.

B.6.4.1.4-03. Third study (Comet assay)

Data point	CA 5.4.1 (EU data requirement)
Report author	Aydin S., Başaran A.A. and Başaran N.
Report year	2005
Report title	The effects of thyme volatiles on the induction of DNA damage by the heterocyclic amine IQ and mitomycin C.
Report No.	Not applicable, published study (Mutation Research, 581: 43–53).
Document No.	KCA 5.4.1/12
Guidelines followed in study	Not stated
Deviations from current test guideline	No TG is currently available.
Previous evaluation	No, not previously submitted
GLP	No
Reliability	Supportive only

Materials and Methods

Test chemical	Thymol. Purity: not stated. Batch/Lot No.: Unknown. Source: Fluka AG, Switzerland.
Solvent	DMSO
Negative control	DMSO
Positive control	Mitomycin C (MMC) 2-amino-3-methylimidazo[4,5-f]-quinoline (IQ)
Test system	Human lymphocytes from a healthy 48-year-old female donor (who was non-smoker and not exposed to radiation or drugs).
Test method	Lymphocytes were exposed to thymol at 0.005, 0.01, 0.025, 0.05, 0.1, 0.2, 0.5, 1 and 2 mM for 0.5 h at 37°C. The replicate experiments were carried out with blood samples from the same donor collected at different time intervals. Three separate experiments were performed. Cells were checked for viability by trypan blue exclusion. 100 cells two replicate slides were analysed from each sample and the DNA damage was scored visually. The following grades were distinguished: grade 0 (no damage), grade 1 (low damage), grade 2 (medium damage), grade 3 (high damage) and grade 4 (very high damage). A total damage score for the slide was derived by multiplying the number of cells assigned to each level of damage by the numerical value of the grade and summing over all grades giving a maximum possible score of 400 (corresponding to 100 cells of grade 4). One slide reader performed the analyses, thus minimizing variability due to subjective scoring.
Criteria for	None reported

assessment of results	
HCD	Not reported
Statistical analysis	Statistical comparison of the results from controls, MMC and IQ groups were carried out by the one-way analysis of variance (ANOVA) test and post hoc analysis of group differences was performed by the LSD test.

Results

Cell viability was more than 90%.
No additional DNA strand breakage was observed at thymol concentrations of 0.005, 0.01, 0.025, 0.05 and 0.1 mM. However, at the higher concentrations of 0.2, 0.5, 1 and 2 mM, significant increases in DNA damage were seen compared with the negative control (see table B.6.4.1.4-03).

Table B.6.4.1.4-03: Effects of thymol on DNA damage in human lymphocytes in the Comet assay

Concentration (mM)	Mean No. of cells from each grade of damage					Damage score
	Undamaged (0)	Low (1)	Medium (2)	High (3)	Very high (4)	
Negative control	79	14	4	2	1	32
0.005	84	11	3	1	1	25
0.01	80	12	5	2	1	31
0.025	78	13	4	4	1	37
0.05	77	11	7	4	1	41
0.1	78	8	9	3	1	40
0.2	65	15	7	7	6	**73***
0.5	56	13	13	13	5	**97***
1	23	23	28	19	8	**165****
2	10	12	32	17	29	**243****

** p<0.01, ** p<0.001*

Assessment and conclusion

<u>Assessment and conclusion by applicant</u>:

This new study is considered to be acceptable for the renewal of thymol (reliable with restrictions; study not GLP).
The EU-agreed endpoint is: Overall no genotoxic potential *in vivo via* the oral route.

<u>Assessment and conclusion by RMS</u>:

A positive result was obtained in this Comet assay in human lymphocytes from 0.2 mM thymol treatment.
No numerical values were reported for the DNA damage. Instead of the percentage of DNA measured in the tail, a classification in grades was reported and although the analysis was performed by a reader, the parameters that defined the different grades of DNA damage were not specified.
Besides of this comet assay to analyse the effect of thymol in DNA damage, an experiment to test the effect of thymol in DNA damage induced by MMC and IQ was carried out. However, taking into account that this effect is not a requirement of EU Regulation No. 283/2013, this part of the paper was not included in this summary by the RMS. Both substances (MMC and IQ) were used as positive controls, but tabulated data from experiments with them were not provided and only graphs were shown to demonstrate their effects. Therefore, they were not included here.
The study was considered as supporting information only to evaluate DNA damage by thymol *in vitro*.

B.6.4.2. *In vivo* studies in somatic cells

B.6.4.2.1. Mammalian chromosome aberration

B.6.4.2.1-01. First study (Micronucleus test)

Data point	CA 5.4.2 (EU data requirement)
Report author	Shibuya, T., Horiya, N., Katoh, M., Hara, T., Sekino, S., Matsuki, Y., Iida, S. and Nakagomi, M.
Report year	1996
Report title	Micronucleus test of thymol using mice
Report No.	Not reported. According to the applicant: Hatano Research Institute, Food and Drug Safety Center, Kanagawa, Japan.
Document No.	KCA 5.4.2/01
Guidelines followed in study	No stated.
Deviations from OECD TG 474 (2016)	- Weight variations of the animals at the commencement of the study were not reported and animal conditions was not recorded in the main test. - Only a single sample time (24 h) was used in the main study, although this was justified based on the preliminary MN test. No numerical results from this preliminary test were reported. - The number of cells scored for micronuclei per animal was lower than recommended (2000 *vs* 4000), although more animals were used (5 mice per each sex, instead of each for one sex only).
Previous evaluation	Yes, evaluated and accepted in the DAR (2011)
GLP	No
Reliability	Yes

Materials and Methods

Test chemical	Thymol. Purity: > 98%. Batch/Lot No.: CAN1119. Source: Wako Pure Chemicals Industries.
Solvent	Pharmacopeia olive oil
Negative control	Pharmacopeia olive oil
Positive control	Cyclophosphamide (50 mg/kg bw)
Test system	Crj:BDF1 mice (males and females), Charles River Japan, 9 weeks at study initiation.
Test method	*Preliminary toxicity test* 5 mice/sex/group were treated by gavage with a single dose of thymol (500, 750, 1000, 1250, 1500, 1750 and 2000 mg/kg bw). General condition and mortality were observed daily for 4 days. *Preliminary micronucleus (MN) test* The same dose of 1250 mg/kg bw was set as maximum for both males and females. 5 males/group were treated by gavage with a single dose of thymol (1250 mg/kg bw) with sample times of 24, 48 and 72 h. *MN assay* 5/sex/group were treated by gavage with a single dose of thymol (156.3, 312.5, 625 and 1250 mg/kg bw, considering the lower dose to provide contingency of death being found at the higher dose). Bone marrow samples from femurs were prepared after 24 h and acridine orange was used as stain. Samples were coded to be ramdomised during the analysis. For each individual specimen, 2000 separate polychromatic erythrocytes (PCE) were observed, and the number of cells with MN was recorded. Also, the proportion of normochromatic erythrocytes (NCE) and PCE were determined in 500 separate erythrocytes.
Criteria for assessment of results	None reported
HCD	Not reported, although it was stated that the frequencies of appearance of

	micronuclei in the controls were within the relative ranges for the spread of background data from the past 5 years (mean ±3SD).
Statistical analysis	For the frequency of appearance of MN, significant differences were tested by 5% the level between the solvent group on the one hand and the treatment and positive control groups on the other hand, in Fisher´s test. Bonferroni correction was applied to take into account their multiplicity. Also, with regard to dose dependency of the frequency of appearance of MN, the Cochran-Armitage trend test was applied with 5% level. For the proportion of juveniles among erythrocytes, a t-test was carried out between the solvent group on the one hand and the treatment and positive control groups on the other hand. In testing, a Bonferroni correction was applied to take into account their multiplicity.

Results

Preliminary test
A decline in spontaneous activity was found in all administration groups. With increases in dose (no doses detailed), toxic signs such as staggering, abdominal position and respiratory stress were observed. No death occurred in males but in females 2 deaths occurred in each of the 1500 and 1750 mg/kg bw groups. The maximum tolerated doses of 2000 mg/kg bw in males and 1250 mg/kg bw in females were selected for the preliminary MN test.

Preliminary MN test
Mortality was initially recorded in the 1500, 1750 and 2000 mg/kg male groups in the preliminary micronucleus test and therefore, the same dose of 1250 mg/kg bw was set as maximum for both males and females. No MN induction or suppression of bone-marrow proliferation was observed at sampling times of 24, 48 and 72 h. Based on the results of the preliminary MN test, the sample time for the main test was set at 24 h.

Main MN test
No statistically significant increase in the frequency of appearance of MN compared with the solvent control group was found in any thymol dose group. Moreover, no dose-dependent increasing trend was observed (see tables B.6.4.2.1-01/1 and B6.4.2.1-01/2). For the positive control a significant increase was found in males and females. In none of the administration groups was the proportion of juveniles among all erythrocytes found to be significantly different from the solvent control groups, and no evidence was obtained that thymol had sufficiently exposed the target cells.

Table B.6.4.2.1-01/1: Summary of MN induction in PCE in male mice

Group	Animal No.	MNPCE / PCE[a]	PCE / ERY[b]
Solvent control Olive oil (10 mL/kg bw)	1	2 / 2000	261 / 500
	2	3 / 2000	261 / 500
	3	4 / 2000	174 / 500
	4	0 / 2000	232 / 500
	5	3 / 2000	286 / 500
	Total	12 / 10000	1214 / 2500
	% mean ±SD	0.12 ± 0.08	48.6 ± 8.6
Thymol 312.5 mg/kg bw	11	5 / 2000	238 / 500
	12	3 / 2000	293 / 500
	13	1 / 2000	272 / 500
	14	5 / 2000	311 / 500
	15	6 / 2000	279 / 500
	Total	20 / 10000	1393 / 2500
	% mean ±SD	0.20 ± 0.10	55.7 ± 5.4
Thymol 625 mg/kg bw	16	3 / 2000	152 / 500
	17	1 / 2000	263 / 500
	18	1 / 2000	207 / 500
	19	8 / 2000	306 / 500
	20	6 / 2000	276 / 500
	Total	19 / 10000	1204 / 2500
	% mean ±SD	0.19 ± 0.16	48.2 ± 12.3
Thymol 1250 mg/kg bw	21	1 / 2000	228 / 500
	22	3 / 2000	198 / 500

Group	Animal No.	MNPCE / PCE[a]	PCE / ERY[b]
	23	3 / 2000	308 / 500
	24	1 / 2000	319 / 500
	25	7 / 2000	287 / 500
	Total	15 / 10000	1340 / 2500
	% mean ±SD	0.15 ± 0.12	53.6 ± 10.5
Positive control Cyclophosphamide 50 mg/kg bw	26	27 / 2000	179/ 500
	27	17 / 2000	164/ 500
	28	22 / 2000	201 / 500
	29	51 / 2000	298 / 500
	30	40 / 2000	299 / 500
	Total	157 / 10000	1141 / 2500
	% mean ±SD	**1.57 ± 0.70*** **	45.6 ± 13.1

a No. of MN PCE/total no. of PCE observed
b No. of PCE/total no. of erythrocytes observed
*** Data significantly different from the solvent control at 0.1% level*

Table B.6.4.2.1-01/2: Summary of MN induction in PCE in female mice

Group	Animal No.	MNPCE / PCE[a]	PCE / ERY[b]
Solvent control Olive oil (10 mL/kg bw)	51	7 / 2000	293 / 500
	52	4 / 2000	297 / 500
	53	0 / 2000	347 / 500
	54	5 / 2000	337 / 500
	55	1 / 2000	322 / 500
	Total	17 / 10000	1596 / 2500
	% mean ±SD	0.17 ± 0.14	63.8 ± 4.8
Thymol 312.5 mg/kg bw	61	5 / 2000	242 / 500
	62	2 / 2000	302 / 500
	63	3 / 2000	302 / 500
	64	3 / 2000	352 / 500
	65	1 / 2000	318 / 500
	Total	14 / 10000	1516 / 2500
	% mean ±SD	0.14 ± 0.07	60.6 ± 8.0
Thymol 625 mg/kg bw	66	3 / 2000	341 / 500
	67	1 / 2000	304 / 500
	68	3 / 2000	306 / 500
	69	2 / 2000	335 / 500
	70	6 / 2000	283 / 500
	Total	15 / 10000	1569 / 2500
	% mean ±SD	0.15 ± 0.09	62.8 ± 4.8
Thymol 1250 mg/kg bw	71	2 / 2000	347 / 500
	72	3 / 2000	355 / 500
	73	1 / 2000	304 / 500
	74	3 / 2000	342 / 500
	75	2 / 2000	256 / 500
	Total	11 / 10000	1604 / 2500
	% mean ±SD	0.11 ± 0.04	64.2 ± 8.2
Positive control Cyclophosphamide 50 mg/kg bw	76	28 / 2000	306 / 500
	77	31 / 2000	272 / 500
	78	31 / 2000	225 / 500
	79	17 / 2000	275 / 500
	80	36 / 2000	294 / 500
	Total	143 / 10000	1372 / 2500
	% mean ±SD	**1.43 ± 0.35*** **	54.9 ± 6.2

a No. of MN PCE/total no. of PCE observed
b No. of PCE/total no. of erythrocytes observed
*** Data significantly different from the solvent control at 0.1% level*

Assessment and conclusion

Assessment and conclusion by applicant: The study authors note that "no evidence was obtained that the investigational substance had sufficiently exposed the target cells", however they considered the test results to be valid since dose levels only slightly higher than the high dose level had been shown to be lethal in the preliminary studies. An additional oral gavage toxicokinetic study (Vol.3, AS. B.6.1.1.6) was submitted to validate the findings of the mouse micronucleus study. Excessive systemic toxicity was observed at the dose of 1250 mg/kg bw so a lower dose of 1000 mg/kg bw was used. This study demonstrated that thymol at a dose of 1000 mg/kg bw was readily absorbed by mice of both sexes (plasma T_{max} = 4 h) and widely distributed around the body (C_{max} = 6305 ng/mL). This provides reassurance that in the negative mouse micronucleus study conducted at a dose of 1250 mg/kg bw, the bone marrow was exposed. The study has been previously reviewed at EU level and is acceptable for renewal of the active substance. The EU-agreed endpoint is: Positive results for clastogenicity in vitro. Negative results in a rat bone marrow in vivo chromosome aberration assay in rats. Overall, no genotoxic potential in vivo via the oral route.
Assessment and conclusion by RMS: The RMS agrees with the conclusion by the applicant. A negative result was obtained in this *in vivo* micronucleus test in mice erythrocytes extracted from bone marrow. The test substance was systemically detected in a toxicokinetic study (Vol.3, AS. B.6.1.1.6), which was performed in the same species and with the same administration route that this MN test. So, in the opinion of the RMS, there was sufficient evidence of target organ exposure. The study has been considered as acceptable to evaluate the clastogenicity and aneugenicity caused by thymol *in vivo*. Although some deviations were found, these are not considered to have influenced the outcome of the test.

B.6.4.2.1-02. Second study (Chromosome aberrations)

Data point	CA 5.4.2 (EU data requirement)
Report author	Azirak, S. and Rencuzogullari, E.
Report year	2008
Report title	The *in vivo* genotoxic effects of carvacrol and thymol in rat bone marrow cells
Report No.	Not applicable, published study (Environmental Toxicology, 23(6): 728-735).
Document No.	KCA 5.4.2/02
Guidelines followed in study	OECD test guideline 475 (1997)
Deviations from OECD TG 475 (2016)	- The batch No. was unknown. - The group size was small (2/sex *vs* 5/sex specified in the guideline). - Different sample times were used: a later sample time (24 h after the first sample time) would normally be used. - At least, 200 metaphases should be analysed for each animal and in this study 100 metaphases were examined. - Individual animal data were not reported. - No information on toxicity in the animals was reported. - No justification was given for the choice of dose levels used. - The rats were slightly old: 12-16 weeks old *vs* the 6-10 weeks old recommended in the guideline. - Intraperitoneal injection is generally not recommended in the guideline since it is not an intended route of human exposure. - The injected volume was not reported. - Randomisation of the samples was not mentioned in the study. - The results did not distinguish between males and females, they are reported as combined. - No HCD were reported. - The positive control used is not included in the list of possible positive controls.

Previous evaluation	Yes, evaluated and accepted in the DAR (2011).
GLP	No
Reliability	Supportive

Materials and Methods

Test chemical	Thymol. Purity: 99.6%. Batch/Lot No.: unknown. Source: Sigma
Solvent	DMSO
Negative control	DMSO (1000 µL/kg)
Positive control	Urethane (400 mg/kg bw)
Test system	Sprague-Dawley rats (males and females), 12-16 weeks old, weight 200.28 ± 2.65 g.
Test method	2 rats/sex/dose were intraperitoneally treated with thymol (40, 60, 80 and 100 mg/kg bw) for 6, 12 and 24 h. To arrest mitosis, colchicine (3 mg/kg bw) was injected intraperitoneally 2 h before the animals were sacrificed. Bone marrow cells from femur were fixed, spread on glass slides and air-dried. The slides were stained with Giemsa. 100 metaphases per animal (totally 400 metaphases per group) were examined for the occurrence of the structural and numerical chromosome aberrations (CA). The Mitotic Index (MI) was also determined by scoring 3000 cells from each animal.
Criteria for assessment of results	Not reported
HCD	Not reported
Statistical analysis	The t test was used for the statistical significance for the percentage of structural, numerical and total CA and MI. Dose response relationships were determined from the correlation and regression coefficients for the percentage of structural, numerical and total CA and MI.

Results

Thymol statistically significantly induced structural CA compared with controls, in all the concentrations and treatment times tested. There were dose-dependent effects on induction of structural and numerical CA at 6, 12 and 24 h treatment periods (see table B.6.4.2.1-02). Thymol was capable of inducing structural CA (especially chromatid type abnormalities) and numerical CA (especially the polyploidy).
Thymol decreased the MI at all concentrations and treatment times, with a more marked effect, up to 50% reduction, at the 24-hour sample time.

Table B.6.4.2.1-02: Structural and numerical CA and MI in rat bone marrow cells treated with thymol

Test substance dose (mg/kg bw)	Structural CA		Numerical CA (polyploidy)	% with structural aberrations ± SE	% with numerical aberrations ± SE	MI (%) ± SE (% compared to solvent control)
	Chromatid type break	Chromosome type break				
Sampling time: 6 h						
Control	-	-	-	-	-	5.61±0.21
DMSO	2	-	1	0.50±0.28	0.25 ± 0.25	4.91±0.06
40	8	8	8	**4.0±0.40****	2.00 ± 0.57	4.70±0.08 (96)
60	8	12	9	**5.0 ±0.40****	2.25 ± 0.75	**3.94±0.11** (80)**
80	7	14	9	**5.25±0.75****	**2.25±0.62***	**3.76±0.14** (77)**
100	15	10	10	**6.25±0.62****	**2.50±0.28****	**3.74±0.28* (76)**
Urethane	11	16	6	6.75±0.47	1.50 ± 0.28	3.61±0.29 (74)
Sampling time: 12 h						
DMSO	3	-	4	0.75 ± 0.25	1.00 ± 0.40	4.45±0.13
40	11	6	7	**4.25±0.47****	1.75 ± 0.47	3.99±0.22 (90)
60	9	12	10	**5.00±0.40****	2.50 ± 0.95	**3.58±0.16* (80)**
80	10	14	9	**6.00±0.40*****	2.25 ± 0.47	**3.58±0.12** (80)**
100	18	18	14	**9.00±1.29****	**3.50±0.64***	**3.42±0.09** (77)**
Urethane	23	17	11	10.00±1.58	2.75±0.25	2.60±0.18 (58)
Sampling time: 24 h						

Test substance dose (mg/kg bw)	Structural CA		Numerical CA (polyploidy)	% with structural aberrations ± SE	% with numerical aberrations ± SE	MI (%) ± SE (% compared to solvent control)
	Chromatid type break	Chromosome type break				
DMSO	6	-	5	1.50 ± 0.28	1.25 ± 0.25	4.35 ± 0.20
40	19	18	7	**9.25 ± 1.60***	1.75 ± 1.10	**2.86 ± 0.27* (66)**
60	41	20	11	**15.2±1.18*****	2.75 ± 1.18	**2.51±0.11*** (58)**
80	38	28	13	**16.5±1.04*****	**3.25 ± 0.47***	**2.54±0.11*** (58)**
100	53	35	17	**22.0±0.40*****	4.25 ± 1.25	**2.19±0.12*** (50)**
Urethane	60	36	22	24.0 ± 3.67	5.50 ±0.95	2.51±0.09 (58)

** Statistically significantly different from solvent control $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$*

Assessment and conclusion

Assessment and conclusion by applicant: The study has been previously reviewed at EU level and is acceptable for renewal of the active substance. The EU-agreed endpoint is: Overall no genotoxic potential *in vivo via* the oral route.
Assessment and conclusion by RMS: A positive result was obtained in this *in vivo* chromosome aberration test performed in rats treated with thymol. The study was considered only as supportive information to evaluate the clastogenicity caused by thymol *in vivo*, due to the above-mentioned deviations from the current test guideline and that it is a non-GLP study.

B.6.4.2.1-03. Third study (Chromosome aberrations)

Data point	CA 5.4.2 (EU data requirement)
Report author	[redacted]
Report year	2009
Report title	Induction of chromosome aberrations in the bone marrow of treated rats
Report No.	8201846
Document No.	KCA 5.4.2/03
Guidelines followed in study	OECD TG 475 (1997)
Deviations from OECD TG 475 (2016)	- Death should not be observed in the highest dose tested. - The later sample collection was 26 h after the first sampling time and not 24 h as it is recommended in the guideline.
Previous evaluation	Yes, evaluated and accepted in the DAR (2011).
GLP	Yes
Acceptability	Yes

Materials and Methods

Test chemical	Thymol. Purity: 99.7%. Batch/Lot No.: 103366.
Solvent	Methylcellulose (1%)
Negative control	Methylcellulose (1%)
Positive control	Cyclophosphamide (35 mg/kg), treated animals were terminated 16 h post dosing.
Test system	Sprague-Dawley Crl:CD ® (SD) rats (males and females), 7-8 weeks old, weight 185-338 g.
Test method	*Range finding test* 3 rats/sex/group received orally by gavage a single dose of 1000, 1400 and 2000 mg/kg bw of thymol (10 mL/kg) and were observed for 2 days. Clinical signs and body weights were recorded in all animals: observations were performed immediately after dose administration and at least 4 times in the 4 h following administration. Observations were also recorded at least once on each of the non-dose administration days.

	Main test 6 rats/sex/group received orally by gavage a single dose of 0, 500, 1000 and 2000 mg/kg bw of thymol (10 mL/kg) and positive control, and bone marrow samples were collected 16 h after administration. Animals were not fasted prior to dose administration. Clinical signs and body weights were recorded in all animals: observations were performed immediately after dose administration and at least 4 times in the 4 h following administration. Observations were also recorded at least once on each of the non-dose administration days. Body weights were recorded on the day of dosing and on each day of bone marrow sampling. About 2 h prior to bone marrow sampling, rats were intraperitoneally dosed with 2 mg/kg of colchicine (10 mL/kg) to arrest dividing cells in metaphase. Bone marrow was sampled from the femurs, fixed and stained with Giemsa. Where possible, 200 metaphases from each animal were analysed for chromosome aberrations. Only cells with 40-42 chromosomes were considered acceptable. Cells with more than this no. of chromosomes were recorded separately. Calculation of the mitotic index (MI) was based on an examination of 1000 cells per animal. *Satellite group* 6 rats/sex/group (satellite groups) were dosed with solvent control and 2000 mg/kg bw of thymol (20 mL/kg, due to concerns over the viscosity) and bone marrow samples were collected 42 h after administration. Blood was collected 0.5, 1, 2, 4, 8 and 24 h after dosing for analysis of thymol concentration. Bone marrow samples were processed as described above.
Acceptance criteria	This assay was considered valid if the following criteria are met: 1. The heterogeneity $\chi 2$ test demonstrates acceptable variability between animals within groups, particularly where no positive responses are seen, and 2. The proportion of cells with structural aberrations (excluding gaps) in negative control animals falls within the normal range, and 3. At least five animals out of each group at each kill time are available for analysis, and 4. The positive control chemical (CPA) induces statistically significant increases in the number of cells with structural aberrations.
Criteria for assessment of results	A test article was considered as clearly positive if: 1. A statistically significant increase in the proportion of cells with structural aberrations occurred at one or more concentration and/or sample time, and 2. The proportion of cells with structural aberrations at such data points exceeded the normal range. Increased incidence of cells with gaps or increased proportions of cells with structural aberrations not exceeding the normal range, or occurring only at very high or very toxic concentrations were concluded as "equivocal". Cells with exchange aberrations or cells with greater than one structural aberration occur very infrequently in vehicle control animals. Their appearance is therefore considered to be of particular significance. The test article was considered as negative in this assay if none of the above criteria were met. Biological relevance was taken into account, for example consistency of response within and between dose levels.
HCD	**Table B.6.4.2.1-03/1: Chromosome aberrations in rat bone marrow**

Parameters	No. of animals	Mean % frequency of aberrations	% frequency of aberrations			
			0	>0,≤1	>1,≤2	>2,≤3
Structural aberrations including gaps	117	0.11	85.5	13.7	0.9	

Structural aberrations excluding gaps	117	0.08	90.6	8.5	0.9	
Numerical aberrations	117	0.42	61.5	29.1	7.7	1.7
Polyploid cells	58	0.23	72.4	22.4	5.2	

+ 100 or 200 cells scored per animal for structural aberrations

Calculated in June 2008 by CLEH Statistics, from audited report data from 12 studies started between July 2003 and September 2007.

Statistical analysis	The proportion of cells with structural aberrations excluding gaps for each treatment group was compared with the proportions in vehicle controls by using a 2x2 $\chi 2$ contingency test. Probability values of $p \leq 0.05$ were accepted as significant. The proportions of cells with structural aberrations including and excluding gaps for each treated group were examined in relation to historical negative control ranges. A further statistical analysis (linear trend test) was used to evaluate possible dose-response relationships.

Results

Range finding test

In this range-finding test, animals were given a single dose of thymol at 1000, 1400 and 2000 mg/kg bw. Clinical signs were limited to mouth rubbing and increased salivation in animals dosed at 1000 mg/kg bw (immediately post dosing) and in one female at 2000 mg/kg bw. Signs observed in this female included piloerection, prone, ptosis, bradypnoea and hypothermia 2 h post dosing, and therefore this animal was killed *in extremis*.
Whilst lethality was observed 2 h post dosing on day 1, this was limited to this mentioned female at 2000 mg/kg bw and, according to the clinical signs, the death was considered as not related to the treatment. Therefore, a dose of 2000 mg/kg bw (the maximum recommended dose, in accordance with current regulatory guidelines) was deemed to be a suitable maximum dose for this assay.

Main test

Two males treated at 2000 mg/kg bw and sampled at 16 and 42 h either were found dead or killed *in extremis*. One female treated at 2000 mg/kg bw was also found dead (42 h sample time).
Several clinical signs of toxicity were reported. Ataxia was observed in males and females from 1000 mg/kg bw, 0.5 h after treatment. Decreased activity was observed in both sexes also from 1000 mg/kg bw, 1 h after treatment. Piloerection and lethargy was reported in males and females at 2000 mg/kg bw sampled at 42 h, 1 h after dosing. Most of the effects were reversible.

Table B.6.4.2.1-03/2: Clinical signs observed in rats treated with thymol

Treatment (mg/kg bw)	No. of animals with the detailed clinical signs found at each time point					
	Day 1 (hours after administration)				Day 2	Day 3
	0	0.5	1	2		
Range finding test – males						
1000	3 (MR, SA)	3 (√)	3 (√)	3 (√)	3 (√)	3 (√)
1400	3 (√)	3 (√)	3 (√)	3 (√)	3 (√)	3 (√)
2000	3 (√)	3 (√)	3 (√)	3 (√)	3 (√)	3 (√)
Range finding test – females						
1000	3 (MR, SA)	3 (√)	3 (√)	3 (√)	3 (√)	3 (√)
1400	3 (√)	3 (√)	3 (√)	3 (√)	3 (√)	3 (√)
2000	3 (√)	3 (√)	3 (√)	2 (√) 1 (PI, PN, PT, BB, HT*)	2 (√)	2 (√)
Main test, 16 h sample - males						
Vehicle	6 (√)	6 (√)	6 (√)	6 (√)	6 (√)	
500	6 (√)	6 (√)	6 (√)	6 (DA)	6 (√)	
1000	6 (√)	6 (AX)	6 (AX, DA)	6 (AX, DA)	6 (√)	

Treatment (mg/kg bw)	No. of animals with the detailed clinical signs found at each time point					
	Day 1 (hours after administration)				Day 2	Day 3
	0	0.5	1	2		
2000	5 (√) 1 (BG, #)	5 (AX)	5 (AX, DA)	5 (AX, DA)	5 (√)	
Main test, 42 h sample - males						
Vehicle	6 (√)	6 (√)	6 (√)	6 (√)	6 (√)	6 (√)
2000	6 (√)	6 (AX, DA)	6 (AX, LE, PI)	5 (AX, LE, PI) 1 (US, BD*)	3 (PI, DA, FF) 2 (PI, DA)	5 (√)
Main test, 16 h sample - females						
Vehicle	6 (√)	6 (√)	6 (√)	6 (√)	6 (√)	
500	6 (√)	6 (√)	6 (√)	6 (DA)	6 (√)	
1000	6 (√)	6 (AX)	6 (AX, DA)	6 (AX, DA)	6 (√)	
2000	6 (√)	6 (AX)	6 (AX, DA)	6 (AX, DA)	6 (√)	
Main test, 42 h sample - females						
Vehicle	6 (√)	6 (√)	6 (√)	6 (√)	6 (√)	6 (√)
2000	6 (√)	6 (AX, DA)	6 (AX, LE)	6 (AX, LE, PI, PT)	5 (PI, SN, DA) 1 (+)	5 (SN)

*√ no abnormality detected. * animal killed in extremis. # animal died immediately post dosing. + animal found dead. AX ataxia. BB bradypnoea. BD dyspnoea, BG gasping, DA decreased activity, FF soft faeces. HT hypothermia, LE lethargy. MR mouth rubbing. PI piloerection. PN prone. PT ptosis. SA increased salivation. SN areas of red/brown staining around the snout. US unconscious.*

A small decrease in bodyweight change between days 1 and 3 was observed in rats at 2000 mg/kg bw compared to control rats (see table B.6.4.2.1-03/3): in males sampled 42 h post dosing (vehicle +5.9%; 2000 mg/kg bw +1.7%) and females sampled both at 16 (vehicle +1.5%; 2000 mg/kg bw -1.5%) and 42 h (vehicle +4.1%; 2000 mg/kg bw +0.5%).

Table B.6.4.2.1-03/3: Bodyweight gain in rats treated with thymol

Treatment (mg/kg bw)	Bw day 1	Bw day 3	% Bw change (from day 1 to day 3)
Range finding test – males			
1000	283	271	-4.2
1400	294	293	-0.3
2000	293	290	-1
Range finding test – females			
1000	204	196	-3.9
1400	206	208	+1
2000	204	196	-3.9
Main test, 16 h sample - males			
Vehicle	271	281	+3.7
500	278	283	+1.8
1000	266	272	+2.3
2000	272	278	+2.2
Main test, 42 h sample - males			
Vehicle	290	307	+5.9
2000	295	300	+1.7
Main test, 16 h sample - females			
Vehicle	201	204	+1.5
500	200	202	+1
1000	201	206	+2.5
2000	203	200	-1.5
Main test, 42 h sample - females			
Vehicle	197	205	+4.1
2000	204	205	+0.5

The frequencies of cells with structural chromosome aberrations in males and females treated with thymol at all doses were similar to the concurrent vehicle controls. The frequency of cells with numerical aberrations in all thymol treated animals also fell within the expected distribution of the historical negative control range. Results are summarised in table B.6.4.2.1-03/4.

Table B.6.4.2.1-03/4: Structural aberrations in bone marrow cells from rats treated *in vivo* with thymol

Treatment (mg/kg bw)	No. of animals	Cell scored	Cells with aberrations including gaps (%)	Cells with aberrations excluding gaps (%)	Mean MI % (% compared to solvent control)
Male group data 16-hour sample					
Vehicle	6	1200	2 (0.2)	2 (0.2)	6.3
500	6	1200	2 (0.2)	2 (0.2)	7.5 (119)
1000	6	1178	1 (0.1)	1 (0.1)	6.9 (110)
2000	5	1000	1 (0.1)	1 (0.1)	7.2 (114)
CPA	6	467	241 (51.6)	**240 (51.4)*** **	1.2 (19)
Male group data 42-hour sample					
Vehicle	6	1200	1 (0.1)	1 (0.1)	6.5
2000	5	1000	1 (0.1)	1 (0.1)	6.9 (106)
Female group data 16-hour sample					
Vehicle	6	1143	1 (0.1)	1 (0.1)	6.6
500	6	1114	4 (0.4)	4 (0.4)	5.9 (89)
1000	6	1123	1 (0.1)	1 (0.1)	6.0 (91)
2000	6	1196	3 (0.3)	2 (0.2)	5.2 (79)
CPA	6	548	224 (40.9)	**221 (40.3)*** **	0.9 (14)
Female group data 42-hour sample					
Vehicle	6	1200	2 (0.2)	2 (0.2)	6.3
2000	5	1000	4 (0.4)	3 (0.3)	3.6 (57)

**** Statistically significant $p \leq 0.001$*

Results of bioanalysis confirmed that animals dosed at 2000 mg/kg bw were systemically exposed to thymol. The plasma concentration of thymol reached a maximum 2 and 8 h after administration of 2000 mg/kg bw to females and males, respectively (see B.6.4.2.1-03/5).

Table B.6.4.2.1-03/5: Mean concentration of thymol in plasma of rats at 2000 mg/kg bw

Time (h)	Sex	Mean concentration (ng/mL ± SD)
2	M	1083 ± 397
	F	5154 ± 2205
4	M	1201 ± 787
	F	1895 ± 313
8	M	3381 ± 2333
	F	4208 ± 2238
24	M	2145 ± 231
	F	2574 ± 915

Assessment and conclusion

Assessment and conclusion by applicant:

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance. The EU-agreed endpoint is: Overall no genotoxic potential *in vivo via* the oral route.

Assessment and conclusion by RMS:

A negative result was obtained in this *in vivo* oral chromosome aberration test performed in rats treated with thymol.

The study was considered as acceptable to evaluate the clastogenicity caused by thymol *in vivo*. The exposure of bone marrow was confirmed by the bioanalysis of thymol in plasma. The deviations observed from the current OECD TG 475 (2016) were considered of minimal relevance.

B.6.4.3. *In vivo* studies in germ cells

In vivo studies in germ cells are required by Regulation (EU) No. 283/2013 on a case-by-case basis, taking into account the information regarding toxicokinetics, the use and the anticipated exposure.
Further *in vivo* studies in germ cells are not considered necessary for thymol renewal, based on the available data.

B.6.4.4. Photomutagenicity

According to Commission Regulation (EU) No. 283/2013, if the Ultraviolet/visible molar extinction/absorption coefficient of the active substance and its major metabolites is less than 1000 L × mol^{-1} × cm^{-1}, photomutagenicity testing is not required. UV/Visible spectroscopy of thymol showed no absorption at >290 nm (White G.A., 2007), therefore the study is not required.

B.6.5. LONG-TERM TOXICITY AND CARCINOGENESIS

No long-term toxicity or carcinogenicity data are available for thymol.

Assessment and conclusion

Assessment and conclusion by applicant:

Thymol occurs naturally in a variety of fruits (including grapes), vegetables, herbs and spices. It is used in cosmetics as a fragrance, as a flavouring agent in food, in dentistry and dental preparations for its anti-microbial properties, as a medical disinfectant, in human medicine for the treatment of worms and parasites, topical treatments and respiratory disorders and is also a constituent of oils used in aromatherapy. Thymol is an approved food additive in the EU (Commission Implementing Regulation (EU) No. 872/2012) and has generally recognized as a safe (GRAS) status in the US. The JECFA review (2001) on Phenol and phenol derivatives concluded that there is no safety concern arising from the use of thymol as a flavouring agent in food.

As discussed in Document M-CA, Section 6; thymol is present in mouthwashes such as Listerine at a concentration of 0.064% (0.64 mg/mL); directions for use recommend rinsing with 20 mL of undiluted product i.e. containing almost 13 mg thymol. The concentration in some toothpastes is 0.51% (equivalent to 5.1 mg/g). In addition, thymol is used in ointment, such as Vicks Vaporub (0.09%), to relieve congestive symptoms associated with sinusitis, nasal catarrh, head cold and nasal congestion and in antiseptic cream where the concentration is 0.04%. Thymol is also added as a preservative to the anaesthetic halothane and at anaesthetic induction concentrations the atmospheric concentration of thymol has been calculated to be approximately 5%. Historically, it was given to healthy adults in large doses to treat intestinal parasites (doses of 2 g equivalent to 33 mg/kg bw for a 60 kg person). Thymol is naturally occurring in citrus fruit, which are popularly consumed and their essential oils are widely used in food and confectionary. Indeed, it has been demonstrated in Document M-CA, Section 6 that the use of thymol in the plant protection product Mevalone does not result in exposure to residues higher than natural background exposure.

Therefore, a large number of humans have been and continue to be regularly exposed to the active ingredient via physical contact and in their diet with no known reports of adverse effects; the available data and information do not show any concern regarding the long-term exposure of humans.

The generation of longer-term *in vivo* data is not justified for this substance and has not been performed. No classification is proposed for carcinogenicity.

Assessment and conclusion by RMS:

The RMS agrees with the justification given by the applicant to waive the data requirement regarding long-term toxicity and carcinogenesis of thymol.

B.6.6. REPRODUCTIVE TOXICITY

All the studies included in this section were already included in the previous DAR (2011) and DAR Addendum 2016, and have been provided by the applicant for the renewal of thymol: one repeated dose and reproductive/developmental toxicity study in rats, provided in Japanese acompained by its English translation (two word-documents), and also two published non-guideline studies in rats. Only the first study was GLP compliant but presented methodological deficits and, therefore, all the data was considered as supportive information only.

B.6.6.1. Generational studies

B.6.6.1.1. Repeated dose and reproductive/developmental study in rats

Data point	CA 5.6.1 (EU data requirement)
Report author	[redacted].
Report year	1996
Report title	Combined repeat dose and reproductive/developmental toxicity screening test of thymol by oral administration in rats
Report No	4L421
Document No	KCA 5.3.2/01
Guidelines followed in study	OECD TG (1990) according to the report, but no TG specified. OECD 422 (1996) is the nearest applicable guideline.
Deviations from current test guideline OECD TG 422 (2016)	- Duration: Females were dosed up to 3 days of lactation instead of 13 days after delivery. - Historical Control Data not provided. - Sensory reactivity to stimuli, functional observations not performed. - Ano genital distance (AGD) was not measured in pups. - Haematology and clinical chemistry was performed only in males. - Spleen, ovaries, uterus, brain, thyroid and heart not weighted. - Histopathological examination not including thyroids, lungs, lower GI tract, uterus, spinal cord, urinary bladder, lymph nodes, nerves or bone marrow.
Previous evaluation	Yes, evaluated and accepted in DAR (2011) and DAR Addendum (2016)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	Supportive only (methodological deficits)

Materials and Methods

Test chemical	Thymol (Lot No. CAN1119, purity 99.6%)
Vehicle	3% gum arabic aqueous solution
Test system	SD rats (Crj:CD, SPF) male and female
Test method	Preliminary range-finding test: doses of 30, 100 and 300 mg/kg bw/day were administered to both male and female SD rats for 13 days before mating and 4 days during mating (17 days for male rats) and females for 7-9 days after mating was confirmed. No obvious changes were observed after administration of 100 mg/kg bw/day, but salivation. However, rats receiving 300 mg/kg bw/day showed reduction in spontaneous motor activity, ambulatory ataxia, abdominal recumbency, bradypnea, ptosis and hypersalivation, as well as reduction in body-weight. Based on these results, the dose levels used for the study were 0, 8, 40 and 200 mg/kg bw/day in 3% gum arabic aqueous solution. Main study: doses of 0, 8, 40 and 200 mg/kg bw/day of thymol were daily administered by a gastric tube to groups of 10 male and 10 female SD rats for 14 days prior to mating. Males continued to be dosed during mating and up to the day prior to necropsy (total of 43 days). Females continued to be dosed through mating, gestation and up to Day 3 of lactation (with the day of birth classed as Day 0). *Observations related to repeated dose toxicity: see section B.6.3.1.1.*

	Observations related to reproduction/development toxicity: Oestrous cycle was monitored by daily vaginal smears from the start of the study (14-day pre-mating period) through to confirmation of mating. Mating was confirmed when vaginal plug had been formed or sperm was observed in the vaginal smear, and that date was taken as day 0 of gestation. From these observations, the following items were calculated: - days required for mating - number of oestrus cycles until confirmation - mating rate (%): (number of mated animals/number of cohabiting animals)*100 - conception rate (%): (number of pregnant animals/number of mated animals)*100 All females where mating was confirmed were allowed to deliver naturally and the delivery observed. Delivery day was taken as day 0 of lactation. Neonates were allowed to be suckled until day 4 of lactation and were observed daily for general condition, suckling, nest building and possible cannibalism. At necropsy (4th day of lactation) ovaries and uterus of each adult female were examined for *corpora lutea* and implantation sites. Females who had not delivered for 25 days after confirmation of mating were necropsied and the uteri of animals where no implantations were observed visually were immersed in 2% KOH aqueous solution to confirm any possible implantations. From these observations, the following items were calculated: - gestation period - birth rate (%): (number of live births/number of pregnant females)*100 - implantation rate (%): (number of implantations/number of *corpora lutea*)*100 - Delivery rate (%): (total number of births/number of implantations)*100 At birth the number of live and still births was recorded and pups were sexed and examined for external abnormalities. The general condition of the pups and maternal behaviour were observed daily and pups were weighed on Day 0 and Day 4. All pups were necropsied on Day 4 (except those that could not be examined due to cannibalism). From these observations, between days 0 and 4 of lactation, the following items were calculated: - birth rate (%): (number of confirmed live births/total number of births)*100 - neonatal survival rate: (number of live births at lactation day 4/number of confirmed births *100 *Statistical analysis:* Measured data was tested by Bartlett's equal variance tests and, when the variance was consistent, one-way analysis of variance and when it was not consistent, the Kruskal-Wallis test was performed. When there was a significant difference between groups, and the number of animals in the groups was fixed, the method of Dunnett was used, and if it was not consistent, the method of Scheffé was used for the multiple comparison tests for mean values and order. Enumeration data were tested using the Fisher's exact probability test. The significance level was 5%. Data for neonates used mean values calculated for each mother animal as the statistical units.

Results on reproduction/developmental toxicity

For results on repeated dose toxicity, see section B.6.3.1.1.

Oestrus cycle

There was no effect on oestrus cycle in any group: each group showed an approximately normal oestrus cycle of 4 or 5 days, with no significant difference noted between the control group and the administration groups.

Reproductive performance

All mating and fertility indices were similar between the groups. Pre-coital time (pairing days until mating) was longer in the treated groups showing a dose-related trend (not statistically significant), reaching an increase of 56% compared to controls.

Delivery and lactation

One female at 200 mg/kg bw/day failed to deliver. This female showed discharge of black mucous from the vagina on Day 24 of pregnancy, and 3 dead foetuses were found in the uterus at necropsy (only 3 implantation sites were present in the uterus of this animal). With the exception of this high dose female, all other females

delivered normally. There were no significant differences between the gestation period, number of *corpora lutea*, number of implantations, birth rate and delivery rate of the control and test groups.

In the 200 mg/kg bw/day group, the number of implantations, implantation rate and delivery rate were slightly lower than other groups, which is a reflection of the number of implantations in the non-delivery animal as no abnormalities were noted in the test results of the other animals in this group.

Also in the 200 mg/kg bw/day, the quantity of milk in the stomach of newborns on day 1 of nursing was small in the litter of the dam that showed symptoms of wasting on post-natal days 1-2. This was considered secondary to the emaciation showed by the female around delivery rather than any effect of the test substance on lactation. Otherwise, no abnormality was found in dams of any group.

Effects on neonates

Survival

During the lactation period at the high dose level, 5 of the 17 pups of one dam's litter died and showed small quantity of milk in the stomach on Day 1. This dam was the one that showed symptoms of wasting around delivery and, therefore, the effect on pups was considered secondary to a temporary deterioration of its general condition. Otherwise, no other abnormalities were seen in dams of any group.

Pup weights at birth and on Day 4 were lower at the high dose level (10.2% and 8.8% lower for males and females, respectively) and body weight gains decreased up to 14.6% for males and 10.5% for females. However, the differences were not statistically significant.

Table B.6.6.1.1: Summary of reproductive findings in rats

Finding	Dose group (mg/kg bw/d)[a]			
	0	**8**	**40**	**200**
Number of pairs mated	10	10	10	10
Oestrous cycle (days)	4.3±0.41	4.3±0.35	4.6±0.60	4.3±0.42
Number of pairs with evidence of mating	10	10	10	10
Pairing days until mating	2.3±1.06	2.7±1.16 (↑17%)	3.0±0.94 (↑30%)	3.6±3.89 (↑56%)
Number of pregnant females	10	10	10	10
Number of females with live pups	10	10	10	8[b]
Gestation length (days)	22.3±0.48	22.5±053	22.4±0.52	22.4±0.52
Number of *corpora lutea*	17.2±1.81	17.5±1.72	18.3±2.26	17.9±1.97
Number of implantation sites	15.9±1.37	16.5±1.96	17.2±2.57	15.6±4.58
Implantation index (%)[c]	92.9	94.4	93.9	86.9
Delivery index (%)[d]	96.7	90.7 (↓6.2%)	93.5 (↓3.3%)	84.9 (↓12%)
Number of pups delivered	15.4±1.78	15.0±2.16	16.0±2.05	16.4±1.77
Number of live pups on Day 0	15.3±1.70	14.8±2.10	15.8±1.81	16.1±1.73
Live birth index (%)[e]	99.4	98.7	99.0	98.5
Sex ratio at birth (male/female)	0.83 (70/84)	0.74 (64/86)	1.05 (82/78)	0.87 (61/70)
Number of live pups on Day 4	15.3±1.70	14.6±2.46	15.6±1.90	15.3±1.98
Viability index on Day 4 (%)[f]	100.0	98.3	98.7	94.9 (↓5.1%)
Pup weight on Day 0 (g)	6.8±0.66 (m) 6.4±0.62 (f)	6.7±0.53 (m) 6.3±0.52 (f)	6.5±0.85 (m) (↓4.4%) 6.1±0.78 (f) (↓4.7%)	6.1±0.56 (m) **(↓10.3%)** 5.8±0.46 (f) (↓9.4%)
Pup weight on Day 4 (g)	10.8±1.06 (m) 10.2±1.06 (f)	10.7±1.07 (m) 10.2±1.06 (f)	10.5±1.09 (m) (↓2.8%) 10.2±1.11 (f)	9.7±1.29 (m) **(↓10.2%)** 9.3±1.16 (f) (↓8.8%)
Weight gain of pups Days 0 to 4	4.1±0.61 (m) 3.8±0.63 (f)	4.0±0.59 (m) 3.9±0.57 (f)	4.0±0.48 (m) (↓2.4%) 4.1±0.53 (f) (↑7.9%)	3.5±0.79 (m) **(↓14.6%)** 3.4±0.78 (f) **(↓10.5%)**

[a] Values expressed as Mean ± S.D.
[b] one gravid female died due to dosing error, one female failed to deliver (3 dead foetuses were found in the uterus at necropsy)

[c] Implantation index (%) = (Number of implantation sites / number of *corpora lutea*) x 100
[d] Delivery index (%) = (Number of pups delivered / number of implantation sites) x 100
[e] Live birth index (%) = (Number of live pups on Day 0 / number of pups delivered) x 100
[f] Viability index on Day 4 (%) = (Number of live pups on Day 4 / number of live pups on Day 0) x 100

Externally visible abnormalities in neonates were restricted to the 40 mg/kg bw/day group and consisted of one pup with missing tail and one pup with protrusion of the naval region (confirmed at necropsy as naval hernia).
In animals which survived to Day 4 the only other abnormalities identified at necropsy were two cases of thymic remnant in the neck (at 8 mg/kg bw/day). In dead pups patent *foramen ovale* was found in 1, 2 and 1 pups in the 8, 40 and 200 mg/kg bw/day groups respectively. These patterns of abnormal findings do not suggest any treatment related effect.

Regarding the reproductive/developmental toxicity, the study report concludes that, in the conditions of the present trial, the no-observed effect level for repeat dose toxicity was 8mg/kg for both males and females and the no-observed effect level for reproduction/development toxicity was 200mg/kg for both male and female parent animals and 40mg/kg for infant animals.

Assessment and conclusion by applicant:

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

The EU-agreed endpoint is: target / critical effect: decreased weight gain of pups in early lactation, and no effects reproductive performance (EFSA Journal 2012;10(11):2916). The offspring NOAEL of 40 mg/kg bw/day was used to derive the AOEL.

Assessment and conclusion by RMS:

According to the report (1996), the study follows the OECD test guidelines of 1990, without specifying any guide number. However, the followed method is very close to the OECD test guideline 422 of 1996 (replaced on 28th July 2015). This study provides the most extended data available for the assessment of the repeated-dose toxicity of thymol. However, the study is deemed as supportive due to the methodological deficits observed (see deviations point).

All the fertility indices were similar between the groups. Although pre-coital time showed a dose-related increasing trend (not statistically significant) in treated groups compared to controls, this increase in the pairing days until mating was associated with the suppressive effect of thymol on activity at high dose levels.

Therefore, under the conditions of the study, since no adverse effects were observed in the reproductive performance, the Fertility NOAEL is 200 mg/kg bw/day.

The Developmental NOAEL is 40 mg/kg bw/day, based on the reduction in pup weight and weight gain observed at 200 mg/kg bw/day, under the conditions of the study.

NOAEL values of this study, for repeated-dose toxicity, are included in section B.6.3.1.1 (Local NOAEL is 8 mg/kg bw/day and systemic NOAEL is 40 mg/kg bw/day).

B.6.6.1.2. Effects on reproduction potency of female rats

Data point	CA 5.6.1 (EU data requirement)
Report author	US-Environmental Protection Agency
Report year	2006
Report title	Thymol, Exemption for a requirement of a tolerance
Report No	Not applicable, published paper (Federal register, 71(11) 18th January, 2006)
Document No	KCA 5.2.2/01
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
Previous evaluation	Yes, evaluated and accepted in DAR (2011)
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Supportive only (secondary source review)

Materials and Methods

Test chemical	Thymol. No data on the specifications provided.
Vehicle	Not indicated
Test system	Rats, chicken, mice, hamsters and rabbits
Test method	Not indicated (secondary source review)

Executive summary

The US EPA publication gives a brief indication about reproductive toxicity (developmental) data on thymol:

- Embryonic chickens have multiple malformations following thymol injection into the yolk or air sac. This was an argument given in a public comment to support the objection to "any tolerance exemption, or waiver allowing more than zero residue of thymol on food". However, the EPA report argued that the results from the chicken study are of questionable relevance to mammals, and added two references where no developmental effects were reported:
- Developmental malformations have not been found following thymol exposure to other mammalian especies such as mice, rats, hamsters and rabbits (Environmental Risk Management Agency of New Zealand, 2005).
- A publication (Mortazavi *et al*., 2003) reported no external tissue abnormalities in foetuses following dosing of female rats with an infusion of the plant *Satureja khuzestanica* (which has the components thymol and carvacrol).

This last study was described in the DAR (2011) as a published abstract with very few details (Mortazavi *et al.*, 2003. Effects of *Satureja khuzestanica* on reproduction potency of female rats. Neurotoxicology and Teratology, 25:392-393). The summary from the DAR (2011) is transcribed here:

«The study was conducted with an herbal infusion of a plant (grown in Iran), the oil from which contains 93.9% carvacrol, 1.0% eugenol, 0.8% *p*-cymene and 0.6% thymol. The details of preparation of the test material are not given so for this assessment it is assumed when calculating the thymol dose administered that the concentrations in essential oil apply to the material used to prepare the dosing solution (though it is not explicitly stated that oil was used (as opposed to other extracts which might be less concentrated) so this is a worst-case estimate for thymol dose). The test material was administered to rats via drinking water at 7.5 mg/mL and 15 mg/mL for 14 days prior to mating. Assuming 0.6% thymol, a daily water intake for young female rats of 25 mL/day and a body weight of 200 g, this equates to thymol intakes of 5.6 and 11.3 mg/kg bw/day.

It is not clear whether dosing stopped at mating or continued through gestation. Very few details are provided such as group size or strain of rats and no numerical data are presented. After mating females were maintained up to Day 18 of gestation when the uterus was excised, weighed and evaluated for live, dead and resorbed foetuses. Live foetuses were weighed and evaluated for skeletal abnormalities and soft tissue anomalies (methods not stated). It is stated that there was no difference in external abnormalities between treated and control groups. It is also indicated that the number of live offspring was increased at the low dose but there were no live offspring at the high dose. These latter findings are difficult to understand, but the absence of abnormal foetuses in this study gives some (limited) reassurance regarding developmental toxicity».

Assessment and conclusion by applicant:

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance as supporting information; secondary source with limited reporting.

Assessment and conclusion by applicant:

The publication includes data provided to EPA as a waiver of the requirements of a tolerance for residues of the thymol on honey, honeycomb, and honeycomb with honey in a very specific use (when applied as treatment to decrease de incidence of *Varroa* mite infestation in the honeybee).

This EPA report referred to the publication of Mortazavi *et al.*, 2003 to support the lack of teratogenicity effects of thymol, but results are only provided in a brief sentence (which has been included in the third point of the executive summary).

For the renewal of the inclusion of thymol, the applicant provided the US-EPA report, but not the abovementioned study. However, since the DAR (2011) summarised the abstract of Mortazavi et al., 2003, it

has been transcribed to this section of the draft Renewal Assessment Report.

RMS deems this study as supportive information.

No data on sexual function or fertility were included. The only reported results were that no difference in external abnormalities between treated and control groups were found.

According to the data available, an estimation on the content of thymol was included in the assessment of this study in the DAR (2011). The concentration of thymol was estimated using the content of thymol in the essential oil as a "worst-case", considering that it is more concentrated than other extracts. Therefore, the highest concentration of thymol expected in the tested material is a 0.6% of thymol (estimated to be a 5.6 and 11.3 mg/kg bw/day). It should be noted that the administered test material was an herbal infusion of the plant rather than the essential oil (which is a mixture of 93.9% carvacrol, 1.0% eugenol, 0.8% *p*-cymene and 0.6% thymol).

Thus, the lack of adverse effects in the treated groups might be attributable either to the lack of toxicity of the components of the infusion of the plant, or to the low concentration of thymol administered.

B.6.6.1.3. Effects on male rat fertility

Data point	CA 5.6.1 (EU data requirement)
Report author	Haeri S., Minaie B., Amin G., Nikfar S., Khorasani R., Esmaily H., Salehnia A. and Abdollahi M.
Report year	2006
Report title	Effect of *Satureja khuzestanica* essential oil on male rat fertility
Report No	Not applicable, published paper (Fitoterapia, 77:495-499)
Document No	KCA 5.6.1/03
Guidelines followed in study	None
Deviations from current test guideline	Not applicable. Limited level of reporting, no reference to thymol.
Previous evaluation	Yes, evaluated and accepted in DAR (2011)
GLP/Officially recognised testing facilities	Not stated
Acceptability/Reliability	Supportive only (methodological deficits)

Materials and Methods

Test chemical	*Satureja khuzestanica* essential oil (SKEO). Thymol was not defined as part of the composition of the SKEO. According to the publication: *There are evidences indicating that carvacrol and flavonoids are the main constituents of Satureja spp. The Iranian SKEO has been well analysed by GC-mass and found to be flavonoids, mainly p-cymene and carvacrol* (the report includes references to those affirmations).
Vehicle	Tap water
Test system	Adult male and virgin female Wistar rats
Test method	Six adult male rats per dose group were treated for 45 days with 0, 75, 150 and 225 mg/kg bw/day of *Satureja khuzestanica* essential oil via drinking water. On day 45 males were mated with female rats with normal estrus cycles (1 male x 2 females). Sperm in the vaginal smear was the proof of mating. Mated female rats were killed on day 21 of pregnancy. Mating success, litter size, number of implantation sites, resorbed foetuses and *corpora lutea* were recorded. Male rats were killed on day 45; serum was collected for LH, FSH, estradiol and testosterone levels detection by radioimmunoassay kits. Testes and accessory sex organs were weighed and a histopathological study was performed. Effects on fertility were determined by potency index, fertility index, fecundity and post implantation loss.

Results

It is stated that potency, fecundity, fertility index, litter size and FSH and testosterone concentrations were all significantly increased. Weights of testes, seminal vesicles and prostate were increased at the high dose and

histopathological effects were recorded at the mid and high dose (increased numbers of spermatogonium, spermatid cords, Leydig cells and spermatozoids, plus hypertrophic Sertoli cells). No further details are presented. It is not possible to differentiate whether these effects could be due to thymol or other components in this essential oil. No adverse effects on reproductive performance are identified in this limited study.

Assessment and conclusion by applicant:

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance. This study is considered to be acceptable as supporting information as it does not provide specific information on thymol for a full assessment.

Assessment and conclusion by RMS:

RMS deems this study as supportive information. The publication does not even mention the presence of thymol in the material tested (*Satureja khuzestanica* essential oil, SKEO). However, an estimation of the content of thymol in the administered doses might be performed using the composition of the essential oil of *Satureja khuzestanica* (included the summary of the study of Mortazavi *et al.* (2003), transcribed from the DAR (2011) in the previous point B.6.6.1.2). According to this data, the essential oil of *Satureja khuzestanica* is more concentrated than other extracts, containing 93.9% carvacrol, 1.0% eugenol, 0.8% *p*-cymene and 0.6% thymol. With this data, the estimated administered doses of thymol in this study are 0.45, 0.90 and 1.35 mg/kg bw/day.

No adverse effects on reproductive performance were identified, although several parameters were reported as statistically increased (potency, fecundity, fertility index, litter size and FSH and testosterone concentrations).

Considering that the estimated content of thymol in the test material assayed was 0.6%, the lack of adverse effects on reproductive performance in the treated groups might be attributable either to the lack of toxicity of the components of the essential oil, or to the low concentration of thymol administered.

B.6.6.2. Developmental toxicity studies

The main data on developmental toxicity provided by the applicant is a combined repeated-dose reproductive/developmental toxicity-screening test ([redacted]., 1996), summarised in points B.6.3.1 and B.6.6.1.1. RMS deems this study as supportive information due to methodological deficits.

Effects in offspring were addressed only up to post-natal day 4, where externally visible abnormalities in neonates showed low incidences and the abnormalities identified at necropsy did not suggest any treatment-related effect. Additionally, reduction in pup weight and weight gain was observed at 200 mg/kg bw/day, leading to the establishment of a developmental toxicity NOAEL of 40 mg/kg bw/day. See see B.6.6.1.1 for more details.

Additionally, a published non-guideline study with *Satureja khuzestanica* in rats has been presented. This publication, cited a study performed in rats, via drinking water, with an herbal infusion of the plant *Satureja khuzestanica* (Mortazavi *et al*., 2003, summarised also in point B.6.6.1.2). The only reported results were that no difference in external abnormalities between treated and control groups were found. The reported data of this study is very limited. Although no adverse effects on development were reported, the estimated content of thymol in the test material assayed (herbal infusion of the plant *Satureja khuzestanica*) was up to 0.6%, making it impossible to differentiate if the effects were due to the lack of reproductive toxicity of its components (included thymol), or to the low concentrations of thymol administered.

B.6.7. NEUROTOXICITY

According to Regulation (EU) No 283/2013, neurotoxicity studies shall be performed for active substances with structures that are similar or related to those capable of inducing neurotoxicity or delayed polyneuropathy, and for active substances which induce specific indications of potential neurotoxicity; neurological signs or neuropathological lesions in toxicity studies at dose levels not associated with marked general toxicity.

Thymol is not structurally-related to substances which are known to be neurotoxic.

No neurotoxicity studies were submitted in the original DAR (2011) in support of the inclusion of thymol in Annex I of Directive 91/414. No new data have been submitted for the renewal process.

The applicant identified in the literature search a published study investigating the effect of orally administered thymol on behavioural alterations, the blood brain barrier and brain neurotransmitters after oral administration (Baldissera *et al*., 2018). However, due to limitations in the study design the applicant did not considered it reliable nor relevant to the toxicological risk assessment for thymol.

RMS deems this study discarded by the applicant (Baldissera *et al*., 2018) should be considered as supporting information, since the results obtained in this new publication supports the evidence of a possible neurotoxic effect of thymol at doses not associated with marked general toxicity. Moreover, considering the transient neurological effects observed in several toxicological studies performed with thymol, RMS deems these new results should not be ruled out.

Accordingly, RMS has included this publication (Baldissera *et al*., 2018) in the assessment of the data on neurotoxicity of thymol.

B.6.7.1. Neurotoxicity studies in rodents

Data point	CA 5.7.1 (EU data requirement)
Report author	Baldissera M.D., Souza C.F., De Matos A.F.I.M., Doleski P.H., Baldisserotto B., Da Silva A.S. and Monteiro S.G.
Report year	2018
Report title	Blood-brain barrier breakdown, memory impairment and neurotoxicity caused in mice submitted to orally treatment with thymol
Report No	Not applicable, published paper (Environmental Toxicology and Pharmacology, 62 (2018):114–119)
Document No	KCA 9.6.5.1-01
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Supportive only (methodological deficits)

Materials and Methods

Test chemical	Thymol (purity > 98.5%. Batch data not provided)
Vehicle	Water
Test system	Male Swiss mice
Test method	Groups of 6 male mice received oral doses of 0, 10, 20 or 40 mg/kg bw thymol dissolved in water once a day for 30 days. Behavioural testing was conducted on day 30; pre-exposure tests were not performed and no detailed clinical observations were made. The following parameters were measured: - Behavioural tests: o Non-spatial long-term memory was evaluated using an inhibitory avoidance task. A step-down latency was assumed as a measure of acquisition considering a cut-off time of 300 s. o Open field: The open field (40 x 40 cm) was used to observe possible alterations on locomotor and exploratory activity. The floor was divided into 9 squares. The animals were gently placed in the centre and observed for 4 min. - Blood-brain barrier permeability: on day 30, the mice were injected intraperitoneally with

	1 mL of Evan's blue 1 h before euthanasia in order to determine the blood-brain barrier (BBB) integrity. After euthanasia, the brain tissue was collected and the left hemisphere was used to evaluate the BBB permeability by measuring Evan's blue dye extravasation (expressed as ng of Evan's blue/mg of tissue). The right hemisphere was used to determine the following variables: - Brain AChE and Na+, K+-ATPase activities were measured using the right hemisphere: AChE enzymatic activity was measured spectrophotometrically (expressed as µg AcSCh/h/mg of protein), and Na+, K+-ATPase activity was colorimetrically evaluated (expressed as nmol Pi released/min/mg of protein). - Brain Reactive Oxygen Species (ROS) levels and Xantine Oxidase (XO) activity were also measured using the right hemisphere: ROS levels were evaluated by the DCFH oxidation method using fluorescence (results expressed as U DCF/mg of protein). XO activity was expressed as UI/mg of protein. - Brain acetylcholine (ACh) levels were evaluated using a commercial assay kit (ng/mg of protein). - Brain protein content was evaluated by the method of Coomassie blue G dye, using serum bovine albumin as the standard. - Brain thymol quantification was performed by high performance liquid chromatography (HPLC-DAD).

Results

In the behaviour test, thymol at 20 and 40 mg/kg bw reduced the latency time compared to the control group (statistically significant difference). However, thymol exerted no effects on open-field test (data not showed).

Analyses showed an statistically significant increase of the BBB permeability in animals treated with thymol (20 and 40 mg/kg bw), indicating a breakdown of the BBB.

Also, thymol increased the AChE activity (40 mg/kg bw), the ROS levels and the cerebral xanthine oxidase (XO) activity (20 and 40 mg/kg bw) and decreased the Na+, K+-ATPase activity (20 and 40 mg/kg bw) when compared to mice of control group.

Additionally, an statistically significant increase in the content of thymol in brain tissue was detected in mice treated with 20 and 40 mg/kg bw thymol when compared to the control group.

Assessment and conclusion by applicant: This new study is not considered reliable and is therefore not used for the toxicological risk assessment of thymol (the study is not GLP, groups sizes are small (n=6, single sex), methodological deficiencies).
Assessment and conclusion by RMS: RMS considers this study as supportive information and agrees with the applicant regarding the methodological deficiencies observed (no GLP, no guideline method followed, only 6 male mice per group, etc.). However, considering the lack of neurotoxicity data on thymol, together with the neurological effects observed in different studies provided in the dossier of thymol (see Volume 1 section 2.6.7 for an overview of all the data), RMS deems these results should not be overlooked. Furthermore, the results reported in this publication show dose-related trend and statistically significant differences from the 20 mg/kg bw group, compared with the control group, for several neurological parameters. RMS deems these results suggest that thymol might elicit neurotoxicity at certain concentrations and the assessment of the potential neurotoxic effects of thymol should be adequately addressed.

B.6.7.2. Delayed polyneuropathy studies

No studies submitted.

Assessment and conclusion by RMS:

Outcome and conclusion of the study: The RMS agrees with the applicant and these studies are not deemed necessary. Thymol bears no structural similarity to organophosphates, carbamates or other known inducers of delayed neurotoxicity.

B.6.8. OTHER TOXICOLOGICAL STUDIES

B.6.8.1. Toxicity studies on metabolites and relevant impurities

There were not available toxicity studies on metabolites or relevant impurities.

B.6.8.2. Supplementary studies on the active substance

B.6.8.2.1. Acute intraperitoneal study in mice

Data point	CA 5.8.2 (EU data requirement)
Report author	Viana G.S.B., Matos F.F., Araujo W.L., Matos F.J.A. and Craveiro A.A.
Report year	1981
Report title	Essential oil of *Lippia grata*: pharmacological effects and main constituents
Report No	Not applicable, published study (*Quarterly Journal of Crude Drug Research*, 19(1):1-10)
Document No	KCA 5.8.2/01
Guidelines followed in study	None stated.
Deviations from current test guideline	Not applicable
Previous evaluation	Yes, evaluated and accepted in DAR (2011)
GLP/Officially recognised testing facilities	No (prior to the enforcement of GLP)
Acceptability/Reliability	Supportive only (methodological deficits)

Materials and Methods

Test chemical	Thymol (commercial grade. Purity not reported).
Vehicle	None
Test system	Swiss mice
Test method	Commercial thymol was purchased (together with carvacrol) for pharmacological comparisons with the *Lippia*-derived compounds (which were the actual materials investigated in this study). The pharmacological testing consisted in several *in vivo* and *in vitro* procedures, including an acute intraperitoneal toxicity test in mice with thymol. Intraperitoneal (i.p.) injection to 6 groups of 3 adults Swiss mice each (sex at random). Doses ranged from 33.3 – 233 mg/kg bw for thymol. The animals were observed for a period of 3 days.

Results

Mortality

Following administration, mortality were observed at doses from 73.3 mg/kg bw and above. A summary of mortalities is presented in Table B.6.8.2.1.

Clinical observations

Slight ataxia was observed at 50 mg/kg bw. From 73.3 mg/kg bw and above, ataxia was observed together with decreased spontaneous motor activity, and somnolence was added at the 110 mg/kg and higher doses.

Table B.6.8.2.1: Mortality and signs of toxicity in mice following single i.p. doses of thymol

Thymol dose (mg/kg bw)	No. of mice per group	No. of deaths	Signs of toxicity
33.3	3	0	No effect
50.0	3	0	Slight ataxia
73.3	3	1	Ataxia, decreased spontaneous motor activity
110.0	3	3	Ataxia, decreased spontaneous motor activity, somnolence
166.6	3	3	Ataxia, decreased spontaneous motor activity, somnolence

Thymol dose (mg/kg bw)	No. of mice per group	No. of deaths	Signs of toxicity
233.3	3	3	Ataxia, decreased spontaneous motor activity, somnolence

Conclusion

The report refers an aproximate 3 days LD_{50} of 110 mg/kg bw for commercial thymol administrated i.p. to mice.

Assessment and conclusion by applicant:

The study has been previously reviewed at EU level and was accepted as supporting information.

Assessment and conclusion by RMS:

The study investigates the pharmacological effects of the isolated active principles of several species of the genus *Lippia grata* Schauer. Commercial thymol and carvacrol were tested for pharmacological comparison with the *Lippia*-derived compounds. The pharmacological testing consisted in several *in vivo* and *in vitro* procedures, including acute toxicity in mice after intraperitoneal injection. Very limited report on the method and results was provided and, therefore, RMS deems this study as supportive.

B.6.8.2.2. Subcutaneous and intravenous toxicity studies with thymol

Data point	CA 5.8.2 (EU data requirement)
Report author	Escobar A.
Report year	2006
Report title	Final Report on the Safety Assessment of Sodium p-Chloro-m-Cresol, p-Chloro-m-Cresol, Chlorothymol, Mixed Cresols, m-Cresol, o-Cresol, p-Cresol, Isopropyl Cresols, Thymol, o-Cymen-5-ol, and Carvacrol
Report No	Not applicable, published study (*International Journal of Toxicology*, 25(Suppl. 1): 29–127)
Document No	KCA 5.2.1/03
Guidelines followed in study	Not applicable. The publication is a review of published literature on several related substances (included thymol) used as cosmetic biocides/preservatives and/or fragrance ingredients. No data on the test guidelines followed in each study was provided.
Deviations from current test guideline	Not applicable. Test substance not characterised. Poorly described method and results.
Previous evaluation	Yes, evaluated and accepted in DAR (2011)
GLP/Officially recognised testing facilities	Not stated for any study (review of published literature).
Acceptability/Reliability	Supportive only (review of published literature)

Materials and Methods

Test chemical	Thymol and other related compounds. Several studies described with no data on the specifications of the active substance.
Vehicle	Several studies described, with different vehicles (see table B.6.8.2.2)
Test system	Guinea pigs
Test method	-

Executive summary

Escobar (2006) reviewed a number of publications related to animal toxicology using thymol (among other related compounds). Several parenteral studies (subcutaneous, intraperitoneal and intravenous dosing), including the publication of Viana *et al.* summarised above (B.6.8.2.1), are cited. Data provided for each study is presented in Table B.6.8.2.2:

Table B.6.8.2.2: Acute parenteral toxicity with thymol

Test/species/number	Test substance/ vehicle	Dose/mode of administration/	Results and comments	Reference
Assessment of thymol on the thyroid gland Guinea pigs 4 young male	Thymol in olive oil	Doses: 106 or 233 mg/kg bw/day (20 or 40 mg/day, respectively) Subcutaneous (s.c.) dosing for 8 to 9 d Oxygen consumption was monitored and a histological examination of the thyroid was done	Clear thyroid activation was seen in 2 animals and weakly in a third animal. No effect was seen in the oxygen consumption in any animal.	Möller (1939)
		Dose: 243, 313 and 375 mg/kg bw/day (60, 80 and 100 mg/day, respectively) 9 injections over 16 days (s.c.)	Thyroid activation: after 2 injections at 243 mg/kg bw/day; after 3 injections at 313 mg/kg bw/day and after 4 injections at 375 mg/kg bw/day No effect was seen in the oxygen consumption in any animal.	
6 dogs	Thymol in oil	Doses not indicated. Perfusion (i.v.) for 20 to 25 min Monitoring: blood pressure, respiration and survival	Clinical signs at the dose of 60 mg/kg bw, were convulsions and respiratory arrest. Thymol LD_{50} in dog (i.v.) = 150 mg/kg bw	Caujolle and Franck (1944)
Rats	Thymol in ethylene glycol	1 to 3 ml/100 g bw Single subcutaneous (s.c.) injection	No convulsions were caused by the administration of thymol. No additional information available.	Matsumoto *et al.* (1963)
Mice (A/He5) Groups of males and females	Thymol Vehicle not specified	Serial two-fold dilutions of thymol (*via* not specified) 6 injections over 2 weeks	Maximum tolerated dose (MTD) of all five mice = 0.25 g/kg	Stoner *et al.* (1973)
Mice (SPF) 5 males	Thymol Vehicle not specified	6 to 8 doses of thymol intravenously (i.v.)	Thymol LD_{50} in mice (i.v.) = 100 mg/kg bw	James and Glen (1980)
Mice (Swiss) 3 mice/test group	Thymol	Doses from 33.3 to 233.3 mg/kg bw Intraperiteonal (i.p.) injections Observation up to 3 days post-adm.	Effects: 33.3 mg/kg bw. No effects. 50 mg/kg bw: nonspecific effects and slight ataxia. 73.3 mg/kg bw: 1/3 died; ataxia, decreased spontaneous motor activity 110 mg/kg pc: 3/3 died; ataxia, decreased spontaneous motor activity and somnolence. The same was observed in the 166.6 and 233.3 mg/kg bw groups Calculated Thymol LD_{50} in mice (i.p.) = 110 mg/kg bw. Actual testing of this dose resulted in the death of 3/3 animals in the first 3 days.	Viana *et al*. (1981) B.6.8.2.1
Mice Rats Rabbits	Thymol Vehicle not specified	Subcutaneous (s.c.) or intravenous (i.v.) injections	Thymol LD_{50} in mouse (s.c.) = 800 mg/kg bw Thymol LD_{50} in rat (s.c.) =1600	Instituto Superiore di Sanità

Test/species/number	Test substance/ vehicle	Dose/mode of administration/	Results and comments	Reference
Dogs (no more data provided)			mg/kg bw Thymol LD_{50} in rabbit (i.v.) = 60 mg/kg bw Thymol LD_{50} in mice (i.v.) = 100 mg/kg bw Thymol LD_{50} in dog (i.v.) = 150 mg/kg bw	(1999)
Mice Male	Thymol in an alcohol/propylene glycol/water vehicle	Doses not reported Subcutaneous (s.c.)	Calculated Thymol LD_{50} in male mice (s.c.) = 243 mg/kg bw.	RIFM (2001e)

Assessment and conclusion by applicant:

The study has been previously reviewed at EU level and was accepted as supporting information.

Assessment and conclusion by RMS:

The publication is a review of published literature on several related substances (included thymol) used as cosmetic biocides/preservatives and/or fragrance ingredients, including thymol. Provided data on the reported studies is very limited. Therefore, RMS deems this information as supportive.

B.6.8.2.3. Subcutaneous toxicity

Data point	CA 5.8.2 (EU data requirement)
Report author	BG Chemie
Report year	2000
Report title	Thymol
Report No	None, published report. (Toxicological evaluation, No. 259)
Document No	KCA 5.9.2/01
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Not applicable (secondary source review)
Acceptability/Reliability	Supportive only (secondary source review)

Materials and Methods

Test chemical	Thymol. No data on the specifications of the active substance.
Vehicle	No data
Test system	Guinea Pig
Test method	-

Executive summary

BG Chemie (2000) is a toxicological evaluation of thymol that includes a publication related to the subcutaneous toxicity of thymol (Möller, 1939). Table B.6.8.2.3 includes information of this publication, that was slightly more explained in BG Chemie (2000) than the provided in B.6.8.2.2 (Escobar, 2006) for the same study.

Table B.6.8.2.3: Data on subcutaneous toxicity of thymol

Test/species/number	Test substance/ vehicle	Dose/mode of administration/	Results and comments	Reference
Male guinea pigs	Thymol	Doses: 20 to 100 mg/animal/day Subcutaneous (s.c.) dosing for 8 to 9 d	Animals showed histological marked "activation" of the thyroid gland without any increase in oxygen consumption. According to the investigator, signs of activation included proliferation of the interstitial tissue, increase in follicular epithelium, abundance of blood and endonuclear cellular changes	Möller (1939)

Assessment and conclusion by applicant:

-

Assessment and conclusion by RMS:

BG Chemie (2000) is a toxicological evaluation of thymol that includes a review of several publications related to the toxicity of thymol. The publication was provided by the applicant to support the Medical data point (B.6.9.2 and B.6.9.3). However, this toxicological report also provides information on the subcutaneous toxicity study of Möller (1939), slightly more explained than the data already included in point B.6.8.2.2 (Escobar, 2006).

Therefore, RMS has added this point B.6.8.2.3 to include the information reported in BG Chemie (2000): which includes the description of the signs of the thyroid activation, within the range of doses (20 to 100 mg/animal/day) used in both Möller experiments, described in Escobar (2006). However, the data is considered supporting information, since it is provided by a secondary source review.

B.6.8.2.4. *In vitro* effect of thymol on human sperm motility and function

Data point	CA 5.8.2 (EU data requirement)
Report author	Chikhoune, A., Stouvenel, L., Iguer-Ouada, M., Hazzit, M., Schmitt, A., Lorès, P., Wolf, J.P., Aissat, K., Auger, J., Vaiman, D. and Touré, A.
Report year	2015
Report title	*In-vitro* effects of *Thymus munbyanus* essential oil and thymol on human sperm motility and function
Report No	None, published report (Reproductive BioMedicine Online, 31 (3): 411-420).
Document No	KCA 5.8.2/02
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Supportive only

Materials and Methods

Test chemical	Synthetic thymol, unknown batch No. and purity. Biochem Chemopharma, Canada.
Vehicle	Ethanol
Test system	Human semen samples obtained by masturbation, after 2-5 days abstinence, collected from five individuals with normal sperm parameters according to WHO values.
Test method	Samples were allowed to liquefy for 30 minutes before testing.

	Sperm vitality and motility Solutions of thymol at concentrations of 100, 200, 300, 400 and 500 µg/mL were prepared in ethanol; control samples were simultaneously prepared and tested in parallel by incubating semen samples with solutions of 2, 4, 6, 8 and 10% ethanol (corresponding to the concentrations of pure ethanol present in each dilution of thymol). The percentages of motile and viable spermatozoa were assessed for each dilution, immediately after the addition of the substance and 30 min later. All the experiments were conducted in duplicate. Sperm vitality was assessed according to WHO values by eosin-nigrosin staining (Vita Eosine, RAL Instruments) on sperm smears (100 spermatozoa were analysed for each individual, each time point and each dilution). Sperm motility was assessed by computer-aided sperm analysis with Hamilton Thorne version 10 HTM IVOS Analyzer (Hamilton-Thorne Biosciences, Beverly, MA USA). The percentage of motile and progressive spermatozoa was recorded together with the average path velocity (VAP), straight line velocity (VSL) and curvilinear velocity (VCL). The effective concentrations of thymol causing 50% of the spermatozoa to become immobilized, EC_{50}, were determined from the motility assay. *Transmission electron microscopy (TEM)* 2×10^7 spermatozoa were incubated from fresh ejaculate at 37°C for 30 min, with an equal volume of 500 µg/mL thymol. The spermatozoa were then washed with M2 medium, centrifuged at 300 g for 10 min and fixed by incubation in 0.1 M phosphate buffer pH 7 supplemented with 3% glutaraldehyde for 1.5 h at room temperature. The samples were then washed twice in phosphate-buffered saline and resuspended in 0.2 M sodium cacodylate buffer. The samples were then post-fixed by incubation with 1% osmium tetroxide, after which they were dehydrated by immersion in a graded series of alcohol solutions and embedded in Epon resin. Semi-thin sections were cut and stained with toluidine blue-Azur II. Ultra-thin sections (90 nm) were cut with a Reichert Ultracut S ultramicrotome, and were then stained with uranyl acetate and lead citrate. Sections were analysed with a JEOL 1011 microscope.
Statistical analysis	Unpaired Student's t-tests were carried out on the global population of spermatozoa. The significance of the effect of the essential oil on sperm vitality and motility, taking the individual into account, was also determined by analysis of variance. $p < 0.05$ was considered statistically significant.

Results

Sperm vitality and motility

The percentage of total progressive spermatozoa was statistically significantly decreased by concentrations of 200 µg/mL ($p < 0.001$) and above (see figure B.6.8.2.4).
This effect was dose-dependent and independent of exposure time. The percentage of viable spermatozoa decreased similarly, in a dose-dependent manner, beginning at a concentration of 200 µg/mL ($p < 0.01$).

The EC_{50} value (the effective concentration causing the immobilization of 50% of the spermatozoa in the population) was 223 µg/mL for thymol.

Transmission electron microscopy (TEM)

Ultrastructural analysis by TEM revealed abnormalities of the acrosome at a thymol concentration of 500 µg/mL (data not shown and no other concentrations of thymol tested). The plasma membrane of the flagellum did not appear to be altered (data not shown).

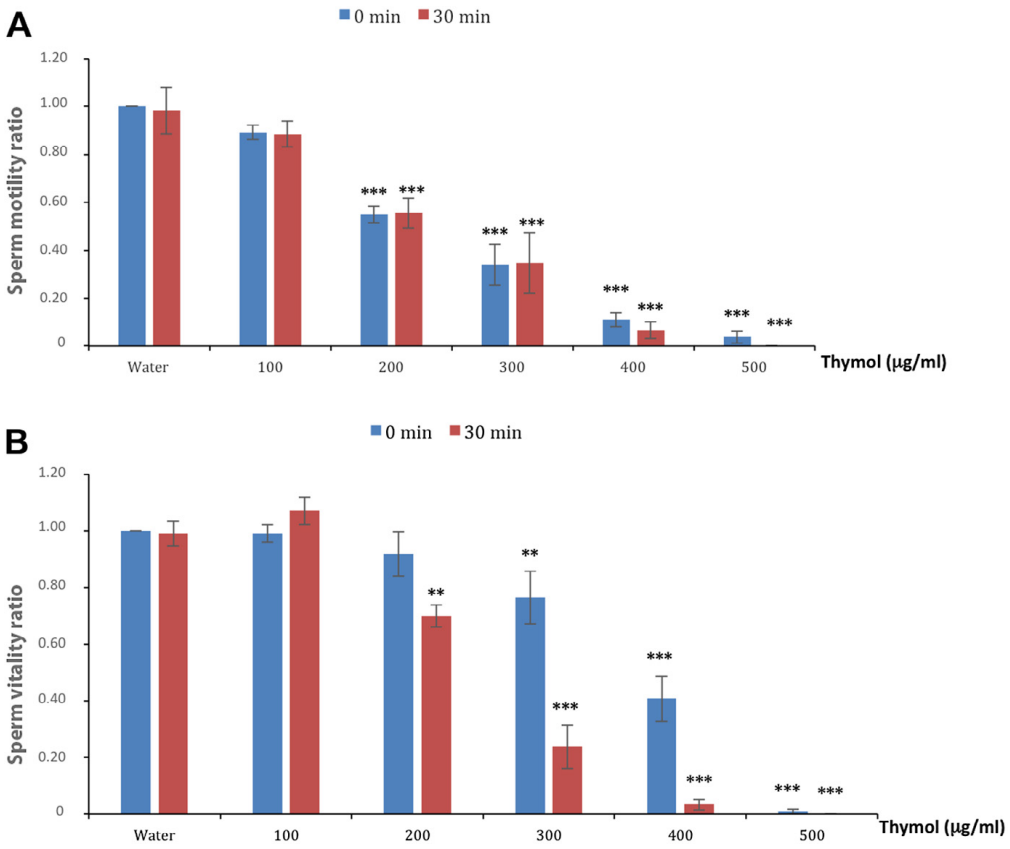

Figure B.6.8.2.4: Effect of thymol on human sperm motility and vitality. Sperm motility and vitality were recorded immediately (blue) or 30 min after addition of the thymol (red). All the percentages are reported as ratio with respect to control sample diluted in pure water. Data are expressed as means ± SD (n = 5). ** $p < 0.01$, *** $p < 0.001$.

Assessment and conclusion

<u>Assessment and conclusion by applicant</u>: This new study is not considered reliable and is therefore not used for the toxicological risk assessment of thymol (the study is not GLP, methodological deficiencies).
<u>Assessment and conclusion by RMS</u>: This study was considered as supportive information by the RMS. It provides information about the effect of thymol in the sperm motility and vitality, which are parameters that were not addressed in the information submitted in Reproduction section. It is noticed that the results of this published study should be carefully considered as they come from an *in vitro* assay.

B.6.8.2.5. Oral 21-day study in rabbits

Data point	CA 5.8.2 (EU data requirement)
Report author	Bacova, K., Zitterl-Eglseer, K., Chrastinova, L., Laukova, A., Madarova, M., Gancarcikova, S., Sopkova, D., Andrejcakova, Z. and Placha, I.
Report year	2020
Report title	Effect of thymol addition and withdrawal on some blood parameters, antioxidative defence system and fatty acid profile in rabbit muscle.
Report No	None, published report (Animals, Vol 10, Issue 8, 1248).
Document No	KCA 5.8.2/03
Guidelines followed in study	None
Deviations from current	Not applicable

test guideline	
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Supportive only

Materials and Methods

Test chemical	Thymol, unknown batch No. Purity ≥ 99.9%. Sigma Aldrich, St. Louis, MO, USA
Vehicle	Dietary admixture
Test system	M9 rabbits, 35 days initiation, 1006 ± 98 g and 1035 ± 107 g in control and thymol groups, respectively.
Test method	48 rabbits of both sexes were divided in control and treatment groups. Control animals received basal diet. The treatment group received a diet with thymol (250 mg/kg feed) for 21 days and thymol was withdrawn for the 7 next days. 8 starved rabbits (6 male and 2 female) in each group were slaughtered – stunned then quickly bled: at 56 (thymol addition group) or 63 days of age (thymol withdrawn). Bodyweight and feed intake were recorded individually once a week. Diets were analysed to determine their composition and thymol content in feed amounted to 148.9 ± 16.7 µg/g dry matter (DM). Samples of blood, plasma muscle, small intestinal wall, liver and hard faeces were collected for biochemical analyses and detection of thymol. Biochemical parameters measured were: aspartate aminotransferase (AST, µkat/L), alanine aminotransferase (ALT, µkat/L), alkaline phosphatase (ALP, µkat/L), total proteins (TP, g/L), urea (mmol/L), creatinine (µmol/L), triglycerides (mmol/L) and cholesterol (mmol/L).

Results

No effects of thymol addition in growth were observed during the study. 5 non-treatment-related deaths occurred, 3 in controls and 2 in thymol-treated rabbits.

Thymol concentrations were 0.05 ± 0.02 µg/L in plasma and 0.04 ± 0.03 µg/g of dry matter in intestinal wall after the 21-day treatment. But, after the 7-day withdrawal, it was not detected.
Thymol concentration in faeces was 0.89 ± 0.45 µg/g after 21 days of treatment, and 0.08 ± 0.04 µg/g after withdrawal.

Table B.6.8.2.5/1: Thymol content in feed, plasma, intestinal wall and faeces

Substance	TA	TW
Feed	148.90 ± 16.65	-
Plasma	0.05 ± 0.02	ND
Intestinal wall	0.04 ± 0.03	ND
Faeces	0.89 ± 0.45	0.08 ± 0.04

ND—not detected, TA—thymol addition and TW—thymol withdrawal.

Table B.6.8.2.5/2: Effect of thymol on biochemical parameters in rabbit blood

Parameter	TA			*p*-Value	TW			*p*-Value
	C	T	SD		C	T	SD	
AST (µkat/L)	0.45	0.34	0.12	0.1199	0.49	0.34	0.20	0.2948
ALT (µkat/L)	0.77	0.62	0.14	0.2677	0.76	0.69	0.32	1.0000
ALP (µkat/L)	2.00 [a]	1.52 [b]	0.42	0.0183	2.42	2.19	0.83	0.5476
TP (g/L)	36.20 [a]	61.86 [b]	13.88	0.0025	36.84 [a]	55.90 [b]	11.79	0.0079
Urea	5.18	5.31	0.50	1.0000	5.03 [a]	6.54 [b]	0.95	0.0079
Creatinine	79.67 [a]	115.40 [b]	19.55	0.0357	64.25 [a]	115.60 [b]	28.52	0.0159
Triglycerides	1.15	1.45	0.29	0.1508	1.53 [a]	0.84 [b]	0.49	0.0317
Cholesterol	2.20 [a]	1.17 [b]	0.80	0.0228	1.76	0.95	0.60	0.0556

[a,b] Values within a row with different superscript letters differed significantly ($p < 0.05$). Data are presented as mean ± standard deviation (SD). C—control diet, TA—thymol addition and TW—thymol withdrawal.

ALP and cholesterol levels were decreased thymol treatment, but not after thymol withdrawal. Urea significantly increased, and triglycerides decreased after thymol withdrawal. TP and creatinine significantly increased after thymol treatment and withdrawal.

Assessment and conclusion

Assessment and conclusion by applicant: The study is not acceptable (not reliable). Proposed category: 3 not reliable - Stability not determined - Only 1 dietary concentration level was investigated - Individual doses/consumption not reported - Limited parameters investigated therefore biological relevance of results obtained unclear
Assessment and conclusion by RMS: The RMS considered this study as supportive information. It provides information about the presence of thymol in plasma, intestinal wall and faeces after oral administration to rabbits for 21 days, and about the effect of thymol in the biochemical parameters.

B.6.8.3. Studies on endocrine disruption

The ED criteria according to Points 3.6.5 and 3.8.2 of Annex II of Regulation (EC) No 1107/2009, as amended by Commission Regulation (EU) 2018/605, and subsequently the ECHA/EFSA guidance document (2018), should be applied for all substances which have a pending decision on approval or renewal of approval.

The available dataset of in vivo mammalian toxicology studies for thymol consists mainly in a combined repeated dose and reproduction/developmental toxicity study conducted in rats (43 and 40 days for males and females, respectively), and a 19-week sub-chronic toxicity study conducted in rats .no guideline, checked for compliance with OECD TG 408). On the other hand, very short summaries of non-guideline published studies were also found within the documentation provided in the dossier (e.g. 8/9 and 16 days repeated subcutaneous injection study in guinea pigs, and a 14-days repeated toxicity study in rats) No 28 and 90 days short term toxicity studies, long term-carcinogenicity or generational studies were provided.

In vitro mechanistic data available from the US EPA CompTox Chemicals Dashboard (v4) for the thyroid, estrogenic, androgenic and steroidogenic pathways has been evaluated as part of the ED assessment (sections B.6.8.3.1, B.6.8.3.2, B.6.8.3.3 and B.6.8.3.4, respectively). In silico QSAR predictions from Danish database were also included (B.6.8.3.8).

Additionally, the data set also includes 2 studies provided by the applicant (B.6.8.3.5, and B.6.8.3.6).

B.6.8.3.1. Thyroid activity in ToxCast model

Data point	CA 5.8.3 (EU data requirement)
Report author	US EPA ToxCast: https://comptox.epa.gov/dashboard
Report year	Data extracted on January 2022
Report title	Thyroid activity in ToxCast model
Report No	Not applicable

Document No	Not applicable
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
Previous evaluation	No, not previously submitted
GLP	Not applicable
Acceptability	Yes

Materials and Methods

Test chemical	Thymol
Test system	US EPA ToxCast program
Test method	In the ToxCast program, thousands of chemicals, including thymol, were screened a panel of *in vitro* assays that measure receptor binding, enzyme activity, and transactivation, among numerous other activities. These assays are aimed at identification of potential endocrine activity for estrogen, androgen, thyroid, or steroidogenesis modalities. The US EPA ToxCast program has been consulted for the thymol https://comptox.epa.gov/dashboard/dsstoxdb/results?search=DTXSID9020617#invitrodb-bioassays-toxcast-data in relation to the thyroid pathway.

Results

The EDSP21 tab in the CompTox Chemicals Dashboard version 4 includes 12 thyroid receptor bioassays for thymol (table B.6.8.3.1, Study ID Matrix No: 1-12). Negative results were obtained in all of them (table B.6.8.3.1).

Table B.6.8.3.1: Summary of US EPA ToxCast EDSP21- thyroid bioactivity assays for thymol

Assay endpoint	Study ID Matrix	Assay type	Organism	Result	AC50	Flag
ATG_THRa1_TRANS_up	1	mRNA induction	human	Inactive	-	-
ATG_THRa1_TRANS_dn	2	mRNA induction	human	Inactive	-	-
LTEA_HepaRG_THRSP_up	3	mRNA induction	human	inactive	-	-
LTEA_HepaRG_THRSP_dn	4	mRNA induction	human	inactive	-	-
TOX21_TRHR_HEK293_Agonist	5	intracellular calcium	human	inactive		-
TOX21_TRHR_HEK293_Antagonist	6	intracellular calcium	human	Inactive	-	-
TOX21_TR_LUC_GH3_Agonist	7	Luciferase induction	Rat	Inactive	-	-
TOX21_TR_LUC_GH3_Antagonist	8	Luciferase induction	Rat	Inactive		
TOX21_TR_LUC_GH3_Antagonist_viability	9	cell viability	Rat	Inactive	-	-
TOX21_TSHR_HTRF_Agonist_ratio	10	cAMP measurement	human	Inactive	-	-
TOX21_TSHR_HTRF_Antagonist_ratio	11	cAMP measurement	human	Inactive	-	-
TOX21_TSHR_HTRF_wt_ratio	12	cAMP measurement	human	Inactive	-	-

Assessment and conclusion by Applicant: Three *in vitro* ToxCast™ thyroid assays (ATG_THRa1_TRANS_up, Tox21_TR_LUC_GH3_Agonist and Tox21_TR_LUC_GH3_antagonist) were listed for thymol under the endocrine disrupter screening programme assays (EDSP21) on the CompTox™ Dashboard. Data were available for the thyroid receptor in human liver cell lines and in rat pituitary gland cell lines. Thymol was inactive in these assays; there is no evidence for T-mediated activity.
Assessment and conclusion by RMS: **Outcome and conclusion of the study**: As of the date of this report, there were 12 assays on thyroid activity associated with the EDSP21 tab in the CompTox Chemicals Dashboard. Based on this battery of *in vitro* assays, thymol was inactive in all of them.

B.6.8.3.2. Estrogenic activity in ToxCast model

Data point	CA 5.8.3 (EU data requirement)
Report author	US EPA ToxCast: https://comptox.epa.gov/dashboard
Report year	Data extracted on January 2022.
Report title	Estrogenic activity in ToxCast model
Report No	Not applicable
Document No	Not applicable
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
Previous evaluation	No, not previously submitted
GLP	Not applicable
Acceptability	Yes

Materials and Methods

Test chemical	Thymol
Test system	US EPA ToxCast program
Test method	In the ToxCast program, thousands of chemicals, including Thymol, were screened a panel of *in vitro* assays that measure receptor binding, enzyme activity, and transactivation, among numerous other activities. These assays are aimed at identification of potential endocrine activity for estrogen, androgen, thyroid, or steroidogenesis modalities. The US EPA ToxCast program has been consulted for the thymol (https://comptox.epa.gov/dashboard/dsstoxdb/results?search=DTXSID9020617#invitrodb-bioassays-toxcast-data) in relation to the estrogenic pathway.

Results

Thymol was tested in 19 assays included in the ToxCast ER Bioactivity Model component. (Table B.6.8.3.2/1). The results were positive/active for only 2 assays (ATG_ERa_TRANS_up and OT_ER_ERbERb_0480) and negative for 17 assays.

Table B.6.8.3.2/1: Summary of US EPA ToxCast EDSP21- estrogenic bioactivity assays for thymol

Assay endpoint	Study ID Matrix	Assay type	Organism	Result	AC50 #	Flag
ACEA_ER_80hr	13	real-time cell-growth kinetics	human	Inactive	-	-
ACEA_ER_AUC_viability	14	real-time cell-growth kinetics	human	Inactive	-	-
ATG_ERa_TRANS_up	**15**	**mRNA induction**	**human**	**active**	**46.18**	**-**
ATG_ERE_CIS_up	16	mRNA induction	human	inactive	-	-
NVS_NR_hER	17	radioligand binding	human	inactive		-
OT_ERa_EREGFP_0120	18	fluorescent protein induction	human	Inactive	-	-

OT_ERa_EREGFP_0480	19	fluorescent protein induction	human	Inactive	-	-
OT_ER_ERaERa_0480	20	protein fragment complementation assay	human	Inactive		
OT_ER_ERaERa_1440	21	protein fragment complementation assay	human	Inactive	-	-
OT_ER_ERaERb_0480	22	protein fragment complementation assay	human	Inactive	-	-
OT_ER_ERaERb_1440	23	protein fragment complementation assay	human	Inactive	-	-
OT_ER_ERbERb_0480	**24**	**protein fragment complementation assay**	**human**	**Active**	**34.88**	**-**
OT_ER_ERbERb_1440	25	protein fragment complementation assay	human			
TOX21_ERa_BLA_Agonist_ratio	26	beta lactamase induction	human			
TOX21_ERa_BLA_Antagonist_ratio	27	beta lactamase induction	human			
TOX21_ERa_BLA_Antagonist_viability	28	ATP content	human			
TOX21_ERa_LUC_VM7_Agonist	29	luciferase induction	human			
TOX21_ERa_LUC_VM7_Antagonist_0.5nM_E2	30	luciferase induction	human			
TOX21_ERa_LUC_VM7_Antagonist_0.5nM_E2_viability	31	ATP content	human			

[#] *Chemical concentration (in µM) at which 50% of the maximum response is achieved.*

Both these positives/actives results are cell-based, multiplexed-readout assays that use human cell lines. ATG_ERa_TRANS_up uses HepG2 liver cell line with measurements taken at 24 hours after chemical dosing in a 24-well plate. On the other hand, the OT_ER_ERbERb_0480 assay uses HEK293T kidney cell line with measurements taken at 8 hours after chemical dosing in a 384-well plate. None of the positive assays presented flags. However, the cytotoxicity limit was 10 µM, so the AC50 value displayed in the assays was higher than the cytotoxicity limit, and therefore, the results were not reliable.

The CERAPP (from consensus) model indicates that thymol is inactive as an ER agonist, ER antagonist and as receptor binding. On the other hand, the scores from the ER ToxCast model predictions displayed a weak activity as an ER agonist with AUC (area under the curve) value of 0.00465; whereas was inactive as antagonist with AUC value of 0.0 (Table B.6.8.3.2/2).

Table B.6.8.3.2/2: ER ToxCast model predictions

Model	Agonist	Antagonist	Binding
ToxCast Pathway Model (AUC)	0.00465	0.00	-
CERAPP* Potency Level (From Literature)	-	Inactive (Inactive)	-
CERAPP* Potency Level (Consensus)	Inactive (Inactive)	Inactive (Inactive)	Inactive (Inactive)

**CERAPP Collaborative Estrogen Receptor Activity Prediction Project (CERAPP; Mansouri et al., CERAPP Collaborative Estrogen Receptor Activity Prediction Project. Environ Health Perspect. 2016 Jul;124 (7) 1023-33). The CERAPP is a large-scale modelling project which has investigated the efficacy of using predictive computational models trained on high-throughput screening data (e.g. from the EDSP21 initiative) to evaluate the ER-related activity of thousands of chemicals, and identify priorities for further testing.*

The ToxCast Model Dashboard includes predictions of the estrogen receptor activity of thymol, based on the Collaborative Estrogen Receptor Activity Prediction Project.

The 19 assays associated with the ToxCast ER prediction model are collectively included in the ED Excel spreadsheet/lines of evidence under Study ID Matrix No. 13-31.

Assessment and conclusion by Applicant:

Two of the ToxCast ER Model assays proposed in the excel file sheet gave positive results however the reported AC50 values are well above the limit of cytotoxicity for these assays and therefore the results are not considered relevant:

- OT_ER_ERbERb_0480: the AC50 was determined to be 34.88 µM with a limit of cytotoxicity reported to be 10 µM.

- ATG_ERa_TRANS_up: the AC50 was determined to be 46.18 µM with a limit of cytotoxicity reported to be 10 µM. Overall, negative results are reported for estrogen receptor agonist and antagonist activity. Therefore, thymol is not predicted to be an estrogen receptor agonist or antagonist.
Assessment and conclusion by RMS: **Outcome and conclusion of the study**: As of the date of this report, there were 19 assays associated with the ToxCast ER Bioactivity prediction model. Based on this battery of *in vitro* assays, thymol is considered to have weak bioactivity in the ER pathway. Only 2/19 bioactivity assays displayed positive results (ATG_ERE_TRANS_up and OT_ER_ERbERb_0480); and these results were not supported by CERAPP (literature and consensus) predictions. The cytotoxicity limit was 10 µM, so the AC50 value displayed in the assays was higher than the cytotoxicity limit. Therefore, these positive results should be considered carefully.

B.6.8.3.3. Androgenic activity in ToxCast model

Data point	CA 5.8.3 (EU data requirement)
Report author	US EPA ToxCast: https://comptox.epa.gov/dashboard
Report year	Data extracted on January 2022.
Report title	Androgenic activity in ToxCast model
Report No	Not applicable
Document No	Not applicable
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
Previous evaluation	No, not previously submitted
GLP	Not applicable
Acceptability	Yes

Materials and Methods

Test chemical	Thymol
Test system	US EPA ToxCast program
Test method	In the ToxCast program, thousands of chemicals, including thymol, were screened a panel of *in vitro* assays that measure receptor binding, enzyme activity, and transactivation, among numerous other activities. These assays are aimed at identification of potential endocrine activity for estrogen, androgen, thyroid, or steroidogenesis modalities. The US EPA ToxCast program has been consulted for the thymol (https://comptox.epa.gov/dashboard/dsstoxdb/results?search=DTXSID9020617#invitrodb-bioassays-toxcast-data) in relation to the androgenic pathway.

Results

Thymol was tested in 14 assays included in the ToxCast AR Bioactivity Model (Table B.6.8.3.3/1). Negative results were obtained in all of them (table B.6.8.3.3/1).

Table B.6.8.3.3/1: Summary of US EPA ToxCast EDSP21-androgenic bioactivity assays for thymol

Assay endpoint	Study ID Matrix	Assay type	Organism	Result	AC50	Flag
ATG_AR_TRANS_up	32	mRNA induction	human	Inactive	-	-
OT_AR_ARELUC_AG_1440	33	Luciferase induction	hamster	Inactive	-	-
OT_AR_ARSRC1_0480	34	protein fragment complementation assay	human	inactive	-	-
OT_AR_ARSRC1_0960	35	protein fragment complementation assay	human	inactive	-	-

TOX21_AR_BLA_Agonist_ratio	36	beta lactamase induction	human	inactive		-
TOX21_AR_BLA_Antagonist_ratio	37	beta lactamase induction	human	Inactive	-	-
TOX21_AR_BLA_Antagonist_viability	38	ATP-content	human	Inactive	-	-
TOX21_AR_LUC_MDAKB2_Agonist	39	Luciferase induction	human	Inactive		
TOX21_AR_LUC_MDAKB2_Antagonist_0.5nM_R1881	40	Luciferase induction	human	Inactive	-	-
TOX21_AR_LUC_MDAKB2_Antagonist_0.5nM_R1881_viability	41	ATP-content	human	Inactive	-	-
TOX21_AR_LUC_MDAKB2_Antagonist_10nM_R1881	42	Luciferase induction	human	Inactive	-	-
TOX21_AR_LUC_MDAKB2_Antagonist_10nM_R1881_viability	43	ATP-content	human	Inactive	-	-
UPITT_HCI_U2OS_AR_TIF2_Nucleoli_Agonist	44	Protein-protein binding	human	Inactive		
UPITT_HCI_U2OS_AR_TIF2_Nucleoli_Antagonist	45	Protein-protein binding	human	Inactive		

The scores from the AR ToxCast model predictions show that thymol is inactive as an AR agonist or antagonist with AUC (area under the curve) values of 0. The ToxCast Models Dashboard includes predictions of the androgen receptor activity of thymol based on the COMPARA. COMPARA (Consensus) prediction model shows that thymol is inactive as an AR agonist, AR antagonist and as AR binding (Table B.6.8.3.3/2).

Table B.6.8.3.3/2: AR ToxCast model predictions

Model	Agonist	Antagonist	Binding
ToxCast Pathway Model (AUC)	0.00	0.00	-
COMPARA (Consensus) #	Inactive	Inactive	Inactive

#COMPARA is a larger scale collaboration between 35 international groups, using QSAR models to predict AR activity using a common training set of 1746 compounds provided by U.S. EPA. A key result is a consensus model of AR agonist and antagonist activity that is run against the DSSTox chemical library.

The 14 assays associated with the ToxCast AR prediction model are collectively included in the ED Excel spread sheet/lines of evidence under Study ID Matrix No. 32-45.

Assessment and conclusion by Applicant:

None of the ToxCast AR Model assays proposed in the excel file sheet gave positive results.

Overall, negative results are reported for androgen receptor agonist and antagonist activity. Therefore, thymol is not predicted to be an androgen receptor agonist or antagonist.

Assessment and conclusion by RMS:

Outcome and conclusion of the study: As of the date of this report, there were 14 assays associated with the ToxCast AR Bioactivity prediction model. All assays in the battery were negative for AR activity. Moreover, according to the AR ToxCast model predictions, thymol was inactive for AR agonism, antagonism, and AR binding.

B.6.8.3.4. Steroidogenic activity in ToxCast model

Data point	CA 5.8.3 (EU data requirement)
Report author	US EPA ToxCast: https://comptox.epa.gov/dashboard
Report year	Data extracted on March 2021.
Report title	Steroidogenic activity in ToxCast model
Report No	Not applicable

Document No	Not applicable
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
Previous evaluation	No, not previously submitted
GLP	Not applicable
Acceptability	Yes

Materials and Methods

Test chemical	Thymol
Test system	US EPA ToxCast program
Test method	In the ToxCast program, thousands of chemicals, including thymol, were screened a panel of *in vitro* assays that measure receptor binding, enzyme activity, and transactivation, among numerous other activities. These assays are aimed at identification of potential endocrine activity for estrogen, androgen, thyroid, or steroidogenesis modalities. The US EPA ToxCast program has been consulted for the thymol (https://comptox.epa.gov/dashboard/dsstoxdb/results?search=DTXSID9020617#invitrodb-bioassays-toxcast-data) in relation to the steroidogenic pathway.

Results:

Thymol was tested in 2 assays included in the ToxCast Steroidogenesis Bioactivity Model (Table B.6.8.3.4, Study ID Matrix No.: 46-47). Negative results were displayed in these two assays (table B.6.8.4).

Table B.6.8.3.4: Summary of US EPA ToxCast EDSP21- steroidogenesis bioactivity assays for thymol

Assay endpoint	Study ID Matrix	Assay type	Organism	Result	AC50	Flag
TOX21_Aromatase_Inhibition	46	Luciferase induction	human	Inactive	-	-
TOX21_Aromatase_Inhibition_viability	47	ATP-content	human	Inactive	-	-

Assessment and conclusion by Applicant:

One *in vitro* ToxCast™ steroidogenesis assay (TOX21_Aromatase_Inhibition) was listed for thymol under the endocrine disrupter screening programme assays (EDSP21) on the CompTox™ Dashboard. A negative result is reported for this assay. Therefore, thymol is not predicted to have steroidogenesis activity.

Overall, there was no evidence for EAS-mediated activity in a range of *in vitro* assays. Following assessment of the integrated lines of evidence for the EAS-modalities, it is concluded that there is no evidence for EAS-mediated adversity in the available data.

Assessment and conclusion by RMS:

Outcome and conclusion of the study: As of the date of this report, there were 2 assays on steroidogenesis activity associated with the EDSP21 tab in the CompTox Chemicals Dashboard. Negative results were obtained in these two studies.

B.6.8.3.5: Androgenic/anti-androgenic activity assessment

Data point	CA 5.8.3 (No EU data requirement)
Report author(s)	Chen, J., Ahn, K.C., Gee, N.A., Gee, S.J., Hammock, B.D. and Lasley, B.L.
Report year	2007
Report title	Anti-androgenic properties of parabens and other phenolic containing small molecules in personal care products.
Report No	Not applicable, published study (*Toxicol. Appl.Pharmacol., 221,3:278–284*)
Document No	KCA 5.8.3/02
Guidelines followed in study	No test method available

Deviations from current test guideline	Not applicable
Previous evaluation	No, not previously submitted
GLP/ Officially recognised testing facilities	Not applicable, published study
Reliability	Supportive only

Materials and Methods

Test chemical	Thymol (≥99% purity). 17(beta)-hydroxy-4-androsten-3-one.
Controls	Negative: DMSO Positive: Known antiandrogens: Flutamide and Vinclozolin
Test system	*Cell-based human AR-mediated bioassay* The bioassay system employs human embryonic kidney HEK293 cells that lack critical steroid metabolizing enzymes. The cells are stably transfected with PCDNA6-hAR and an MMTVLuc. neo plasmid containing a luciferase reporting gene. The cells (designated as 2933Y) are highly responsive to endogenous steroids as well as synthetic compounds.
Test method	*Cell culture* 2933Y cells were cultured in DMEM with 10% FBS. Cells placed in a 96-well plate were incubated for 16 hours with 10% v/v of thymol alone, testosterone alone at a physiologically relevant dose, or a combination of testosterone and test compound. Cells were then lysed and cell lysates were exposed to Luciferin substrate. The luciferase activity induced by thymol or the testosterone was measured. Luciferase catalyses luciferin oxidation and the chemical energy of this reaction produces a light flash which is measured by the luminometer. The intensity of the light is expressed as relative light units (RLU) and is directly proportional to the induced luciferase activity. A relatively low concentration of Testosterone (T) (0.125 nM) was selected to test interactions of compounds in this report. The dominant circulating androgen in most mammalian systems is testosterone. The mean circulating concentration of T in humans ranges from less than 3.5 to 35 nM (Williams and Larsen, 2003, *Williams Textbook of Endocrinology. Saunders, Philadelphia*). Approximately 2–3% of circulating T is free, and non-sex hormone binding globulin (SHBG)-bound is considered bioactive (Siiteri *et al*., 1982, *Recent Prog. Horm.Res. 38, 457–510*.; Pardridge *et al*., 1988, *Oxf. Rev. Reprod. Biol. 10, 237–292*.). Thus, the selected dose of 0.125 nM T is physiologically relevant and enhances the likelihood of antagonist recognition at lower concentrations of the natural ligand since the stronger the effect of the androgen control, the greater the competition (higher concentration) of the antiandrogen required. In addition, the selected dose of 0.125 nM T can induce significant luciferase activity above the vehicle controls (20-fold induction) with a relatively small variance (coefficient of variance of 5–6% of relative light units). Use of a low dose of T also avoids the large variance and potential "hook effects" associated with the use of higher concentrations of T in the assay. Furthermore, the lower concentration allows for the observation of higher responses when testing compounds that augment or enhance the effects of T. *MTT assay* In the MTT assay, after 16 h of treatment as performed in the AR-mediated assay, 20 µL of 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) was added to each well and the plate was incubated at 37°C for 4 h. The absorbance was then measured for assessment of cell viability.
Data analysis	The values shown are mean±SD from three independent experiments for each dose tested. Data were analysed by one-way analysis of variance (ANOVA), followed by multiple comparisons test when appropriate, using Sigmstat Software. The level of significance was set at $p<0.05$. To test for agonist properties, treatments were compared to the negative control group containing vehicle only. For androgen antagonist properties, treatments were compared to the testosterone-positive control group.

Results

Assessment of androgenic and anti-androgenic activity in the cell-culture assay

Thymol did not exhibit cytotoxicity when tested alone at 10 µM or in combination with 0.125 nM of testosterone (T) as observed in MTT measurements. Moreover, no cytotoxicity was observed when testosterone was tested alone. Vehicle-treated groups and chemical-treated groups did not demonstrate statistically significant differences with respect to cell proliferation.

Thymol statistically significantly inhibited the transcriptional activity induced by 0.125 nM testosterone by 34% at 10 µM and by 11% at a concentration of 1.0 µM. Thymol did not exhibit androgenic activities at concentrations up to 10 µM when evaluated without testosterone. The two positive controls significantly inhibited the luciferase activity induced by 1.0 nM testosterone.

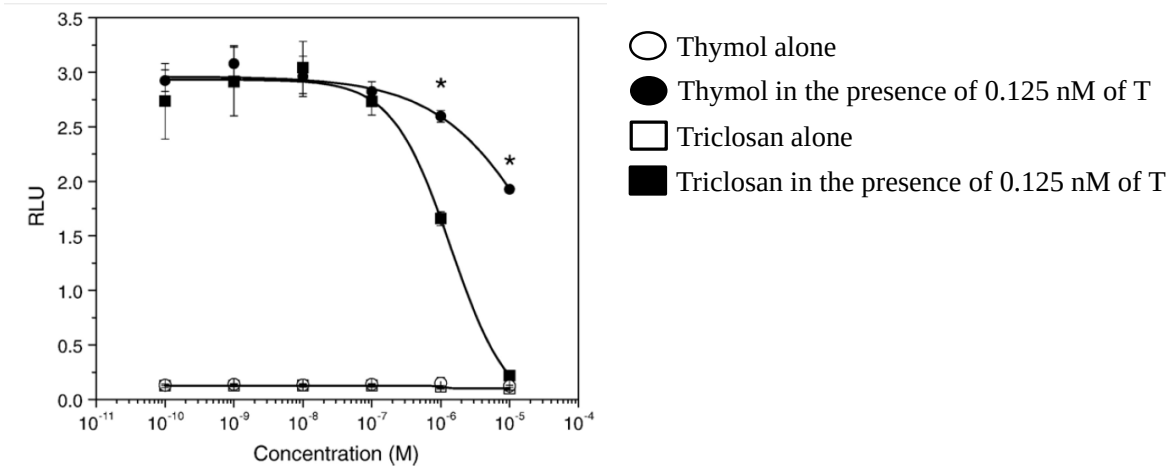

Figure B.6.8.3.5: The effect of thymol and triclosan on AR-mediated transcriptional activity induced by 0.125 nM T: *p<0.05 of T-induced transcriptional activity by thymol and triclosan of 1 µM and 10µM.

Assessment and conclusion by Applicant: This new study is considered to be acceptable for the renewal of thymol as supporting information (reliable with restrictions; study not GLP, does not follow validated methodology for this assay type). Under the conditions of the study, thymol did not appear to be androgenic *in vitro* when tested without testosterone although some inhibition of testosterone-induced transcriptional activity was observed. The ECHA/EFSA guidance notes that *in vitro* mechanistic test methods are proposed for screening and prioritisation purposes and should not be directly extrapolated to the intact endocrine system *in vivo*.
Assessment and conclusion by RMS: **Outcome and conclusion of the study:** This published study used a modified version of the current AR-STTA (Stably transfected Human androgen receptor transactivation assay for detection of androgenic/anti-androgenic activity of chemicals) guideline (OCED TG 458). There was no evidence that thymol possessed hAR agonist activity. However, thymol showed dose-dependent anti-androgenic activity in the mammalian stably-transfected cell line, in which thymol inhibited in a dose-response pattern the hAR transcriptional activity induced by testosterone, without any evidence of cytotoxicity. The IC_{50} was not calculated. The highest concentration of 10 µM tested was approximately 100000-fold in excess of the T concentration used, so exceed several orders of magnitude a natural scenario. However, the test methodology is well conducted and RMS deems that the potential anti-androgenic activity of thymol might be further investigated.

B.6.8.3.6. Estrogenic/anti-estrogenic/androgenic/anti-androgenic activity assessment

Data point	CA 5.8.3 (No EU data requirement)
Report author(s)	Michalíkova, K., Linhartova, L., Ezechias, M. and Cajthaml, T.
Report year	2019
Report title	Assessment of agonistic and antagonistic properties of widely used oral care antimicrobial substances toward steroid estrogenic and androgenic receptors
Report No	Not applicable, published study (*Chemosphere, 217:534-541*)

Document No	KCA 5.8.3/03
Guidelines followed in study	No detailed test guidelines are currently available to conduct this type of study.
Deviations from current test guideline	Not applicable
Previous evaluation	No, not previously submitted
GLP/ Officially recognised testing facilities	No
Reliability	Supportive only

Materials and Methods

Test chemical	Thymol (≥98% purity), 17β-estradiol (≥98% purity), 4,5a-dihydrotestosterone (≥99% purity).
Test system	*Recombinant yeast assay* → Yeast cells (*Saccharomyces cerevisiae* BMAEREluc/ERa, *S. cerevisiae* BMAERE/AR and *S. cerevisiae* BMA64luc). *Transcriptional activity of estrogen receptor* → human T47D breast carcinoma cell line. *Transcriptional activity of androgen receptor* →Transgenic reporter gene cell line AIZ-AR.
Test method	*Yeast assay* The strains *S. cerevisiae* BMAEREluc/ERa and BMAERE/AR produce luciferase extracellularly after stimulation of the estrogen and androgen receptors, respectively. These strains were used to assess the estrogen- and androgen-like activities of thymol (among 9 other substances). During the exponential phase, the yeast strains were incubated for 2.5 h with thymol in the presence (antagonist mode) or in the absence (agonistic mode) of either 17ß-estradiol or dihydrotestosterone. After the incubation, luciferin was added to the cultures and the samples were immediately measured by a luminometer. *T47D-CXCL12 assay* The T47D breast carcinoma cell line, using an altered secretion of cytokine CXCL12/SDF1 allowed the determination of estrogen receptor binding activities. T47D cells were exposed to thymol in ethanol at concentrations ranging from 1.6 to 500 µM. Each concentration was assessed in triplicate. 17ß-estradiol was used as positive control. The secretion of stroma cell-derived factor 1 (SDF1 also referred as CXCL12) was determined by an ELISA test as a response of the cells to the presence of the estrogen receptor agonist. *AIZ-AR reporter gene assay* Cells were exposed to thymol dissolved in ethanol either in the presence or absence of dihydrotestosterone as an antagonistic or agonistic mode of the assay, respectively. After 24h of incubation, the medium was removed, and the cells were lysed. The luciferase activity was measured on a luminometer.
Data analysis	In order to distinguish whether or not antagonism of thymol is competitive in nature, the Schild analysis was applied. This method involves the construction of two or more dose-response curves for agonist in the absence and presence of a fixed concentration of the antagonist. When the antagonists compete for the binding site of the receptor, we should observe: (1) parallel shifts to the right in the dose response curves and (2) no decrease in the maximum response that can be achieved. However, clear deviation from parallel shifts in the dose-response curves and significant depression of the maximum response indicate that the antagonism is not competitive. All results are expressed as the mean ± standard deviation. The dose-response data were fitted using four-parameter logistic curves and the inflection points of the curves represent the IC50 concentrations that induce half of the maximum effects.

Results

<u>Estrogenic/anti-estrogenic activity</u>

<u>Recombinant yeast assay</u>
Thymol did not trigger estrogenic effects even at the highest concentration levels used in the *S. cerevisiae* BMAEREluc/ERα yeast assay (data not shown). However, thymol exhibited significant anti-estrogenic effect in a concentration-dependent manner (figure B.6.8.3.6/1). The IC_{50} of the anti-estrogenic effect was 237±16 µM.

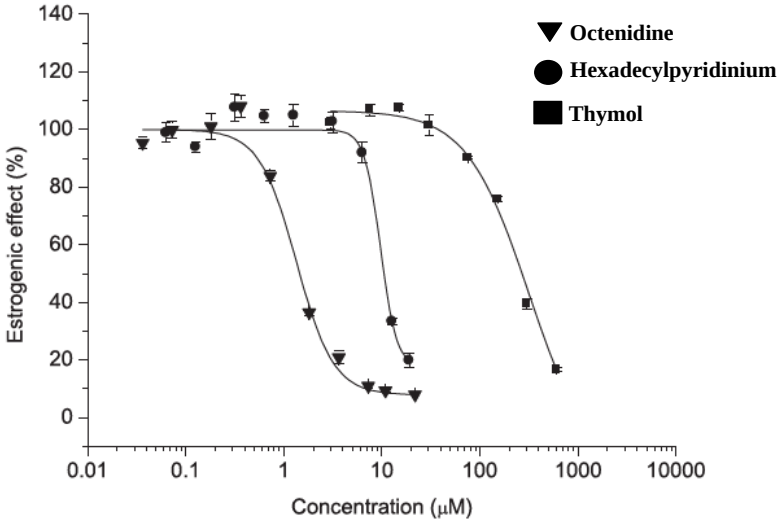

Figure B.6.8.3.6/1: Estrogenic/anti-estrogenic activity of thymol in presence of 17β-estradiol in recombinant yeast assay system

T47D-CXCL12 test

Thymol did not trigger estrogenic effects even at the highest concentration levels used in the *T47D-CXCL12 test*. However, thymol exhibited significant anti-estrogenic effect in a concentration-dependent manner (figure B.6.8.3.6/2). The IC_{50} of the anti-estrogenic effect was 177±39 µM.

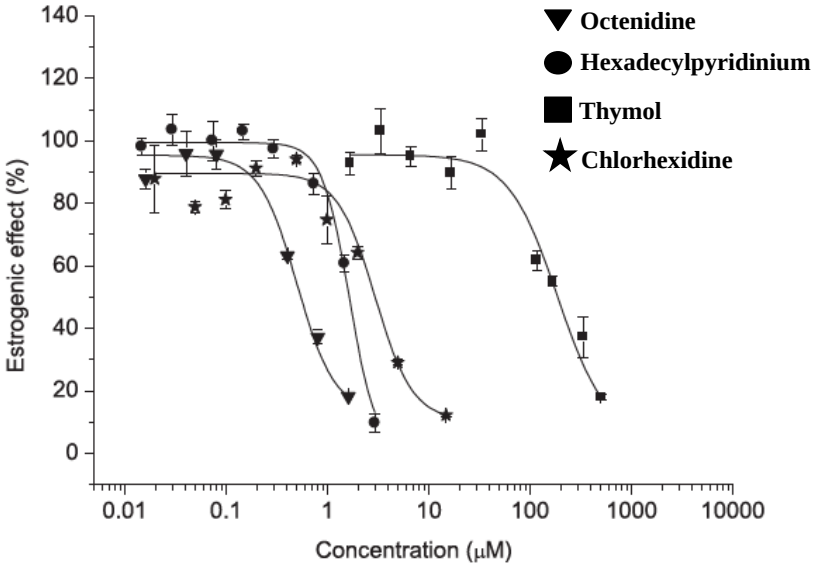

Figure B.6.8.3.6/2: Estrogenic/anti-estrogenic activity of thymol in presence of 17β-estradiol in T47D-CXCL12 test system

Androgenic/anti-androgenic activity

Recombinant yeast assay

No androgenic effect was observed in the recombinant yeast assay system. However, thymol exhibited significant anti-androgenic effect in a concentration-dependent manner (figure B.6.8.3.6/3). The IC_{50} of the anti-androgenic effect was 73±9 µM.

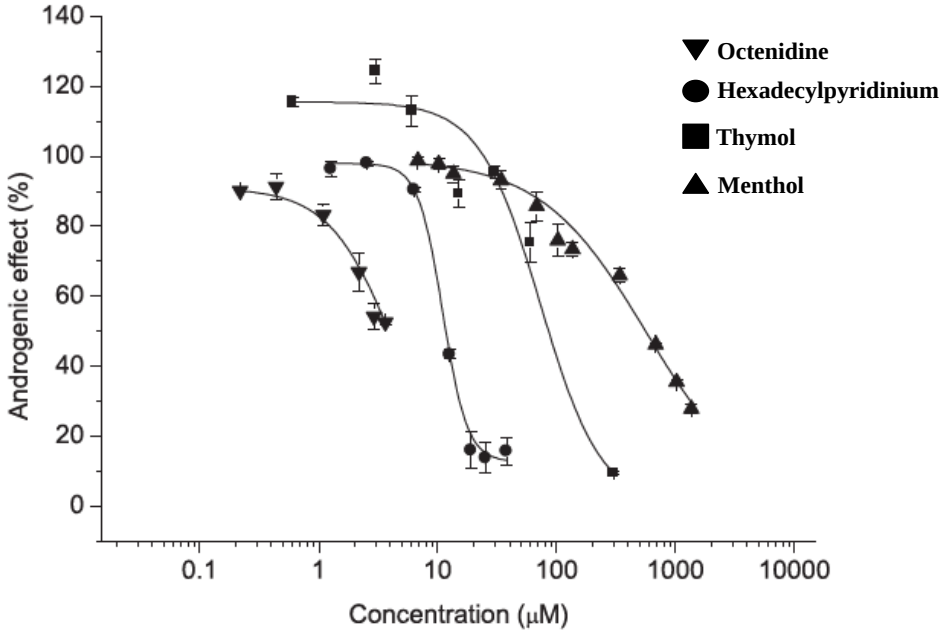

Figure B.6.8.3.6/3: Androgenic/anti-androgenic activity of thymol in presence of dihydrotestosterone in recombinant yeast assay system.

AIZ-AR test
Thymol antagonistic effect was not observed in the case of the AIZ-AR human cell line, which is probably due to sensitivity of the assay when higher applied concentrations were toxic to the cell line.

Schild analysis

The results of the Schild analysis indicated that thymol competed with the natural ligands for the binding sites of the receptor and therefore the antagonism is competitive in nature. Thymol shifted the dose-response curve of the agonist and did not decrease the maxima in both estrogenic and androgenic yeast assays.

Assessment and conclusion by applicant:

This new study is considered to be acceptable for the renewal of thymol as supporting information (reliable with restrictions; study not GLP, does not follow validated methodology for this assay type). Under the conditions of the study, thymol appeared to be a competitive antagonist of the estrogen and androgen receptors. The ECHA/EFSA guidance notes that *in vitro* mechanistic test methods are proposed for screening and prioritisation purposes and should not be directly extrapolated to the intact endocrine system *in vivo*.

Assessment and conclusion by RMS:

Outcome and conclusion of the study: There was no evidence that thymol possessed ER or AR agonist activity in the three different system assays performed. However, thymol showed dose-dependent anti-estrogenic and anti-androgenic activity. The concentrations of 17β-estradiol and testosterone used in the competitive assays were not specified, so it is not possible to determine the order of magnitude of the thymol inhibiting concentrations of estradiol and testosterone responses.

As noted, the thymol IC_{50} value displayed in the androgen receptor inhibition assay (73µM) was low than the value in the estrogen inhibition tests systems (237 and 177, for recombinant yeast and TT47D-CXCL12 assays, respectively). These values showed that thymol was a weak competitive inhibitor of estrogen receptor compared with its potential role to inhibit androgen receptor.

The anti-androgenic activity finding is in agreement with previous Chen *et al*. (B.6.8.3.5) study.

B.6.8.3.7. Thyroid activation in guinea pigs

Data point	CA 5.8.3 (No EU data requirement)
Report author	BG Chemie
Report year	2000
Report title	Thymol
Report No	None, published report. (Toxicological evaluation, No.259). This report includes a summary performed from the published study "Effects of thymol on thyroid gland". Möller, H. *et al., Arch. Exp.Pathol. Pharmak*.191: 615–620 (article in German).
Document No	KCA 5.9.2/01
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Not applicable (secondary source review)
Acceptability/Reliability	Supportive only (secondary source review)

Materials and Methods

Test chemical	Thymol. No data on the specifications of the active substance.
Vehicle	No data
Test system	Guinea Pig
Test method	*Experiment 1* Four young male guinea pigs received daily subcutaneous injections of thymol at 106 or 233 mg/kg bw/day (20 or 40 mg/day, respectively, for 8-9 days. *Experiment 2* Four young male guinea pigs received 9 subcutaneous injections over 16 days of thymol at 243, 313 and 375 mg/kg bw/day (60, 80 and 100 mg/day, respectively).

Results

Experiment 1

Clear thyroid activation was seen in 2 animals and weakly in a third animal. No effect was seen in the oxygen consumption in any animal.

Experiment 2

There was thyroid activation in the 60 mg/day (243 mg/kg/day) dose group after two injections, in the 80 mg/day (313 mg/kg/day) dose group after three injections, and in the 100 mg/day (375 mg/kg/day) dose group after four injections. No effect was seen in the oxygen consumption in any animal.

Assessment and conclusion by RMS:

Outcome and conclusion of the study: The report provided by BG Chemie includes a brief summary of a study published by Möller *et al*. in 1939 regarding thyroid effects after subcutaneous administration of thymol in guinea pigs.

The original study conducted by Möller (in German) has not been presented nor translated by the applicant to support the renewal dossier of the active substance thymol. Therefore, methodology, schedule administration and the findings were not properly ascertained by RMS. More details regarding the effects found in thyroids were needed, so an appropriate assessment cannot be performed.

B.6.8.3.8. *In silico* QSAR predictions

Data point	CA 5.8.3 (EU data requirement)
Report author	Danish QSA. https://qsar food.dtu.dk/
Report year	Data extracted on January 2022
Report title	Not applicable
Report No	Not applicable

Document No	Not applicable
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
Previous evaluation	No, not previously submitted
GLP	Not applicable
Acceptability	Yes

Materials and Methods

Test chemical	Thymol
Test system	Danish QSAR database
Test method	Qualitative structural activity relationship (QSAR) data was obtained for thymol from the Danish QSAR database and the OECD QSAR Toolbox v.4.5. The Danish (Q)SAR Database (https://qsar food.dtu.dk/) was released in November 2015 and has since then been expanded and updated a number of times. It currently holds many predictions for around 650000 substances and is a tool that allows industry, research, authorities and others to search for hazard information on chemical substances, especially those with little or no testing data. While the Danish (Q)SAR Database contains around 650000 substances, there may still be substances of interest to some users which are not contained in the database. Or even when contained, there may in some cases be interest to obtain more detailed predictions in the QSAR Prediction Reporting Format, which includes information on prediction probability, possible alerts, nearest analogs in the training set etc. The Danish (Q)SAR Models website offers users to make on-the-fly predictions for user-defined chemical structures by use of >30 models developed by DTU in the Leadscope software. The use of the commercial Leadscope® Enterprise Server software as a back-end to this website was made possible based on a collaboration agreement between Leadscope Inc. and the National Food Institute at the Technical University of Denmark.

Results

T-modality

In accordance with the OECD Conceptual Framework and the ECHA/EFSA GD on ED, Level 1, T-related non-test information was gathered for thymol. Qualitative structural activity relationship (QSAR) data was obtained for thymol from the Danish QSAR database.

The results of the Danish QSAR database for thymol in respect of T-mediated endocrine endpoints are summarised below.

Table B.6.8.3.8/01: The results of Danish QSAR database for thymol regarding T-modality

	Exp	Battery	CASE Ultra	Leadscope	SciQSAR
Thyroperoxidase (TPO) inhibition QSAR1 (Rat in vitro)	NEG	NA	NA	POS_IN	NA
Thyroperoxidase (TPO) inhibition QSAR2 (Rat in vitro)	NEG	NA	NA	POS_OUT	NA
Thyroid Receptor α Binding (Human in vitro)					
mg/L		12083.43	24028.56	83.39842	138.3017
µM		80438.23	159955.8	555.1752	920.6613
Positive for IC50 ≤ 10 µM					
Positive for IC50 ≤ 100 µM					
Domain		IN	IN	OUT	OUT
Thyroid Receptor β Binding (Human in vitro)					
mg/L		2895.454	4861.026	8.20234	929.8816

µM		19274.76	32359.38	54.60218	6190.132
Positive for IC50 ≤ 10 µM					
Positive for IC50 ≤ 100 µM					
Domain		IN	IN	OUT	OUT
Key: POS = Positive; NEG = Negative; IN = within the applicability domain of the model; OUT = outside of the applicability domain of the model					

The Danish QSAR Database reports that thymol is within the applicability domain of the model for thyroid receptor binding when the "battery" prediction is made. The battery approach can give more reliable predictions and reduces "noise" from the individual models estimates and thereby improve accuracy and/or broaden the applicability domain. However, when predictions were analysed individually through the three available models (CASE Ultra, Leadscope Predictive Data Miner and SciQSAR), only Case Ultra displayed that thymol was within the applicability domain.

On the other hand, the Danish QSAR Database reports that thymol gave positive results in respect of thyroid peroxidase (TPO) inhibition in two respective Leadscope global, binary composite QSAR models: QSAR1 and QSAR2 (but only QSAR1 was within the applicability domain of these models). These models were developed to predict the potential of substances to inhibit thyroperoxidase using the US EPA ToxCast data sets (training set for QSAR1 = 877 chemicals; training set for QSAR2 = 1519 chemicals) and the commercial software Leadscope® Predictive Data Miner. The highest ranking structural features associated with activity were versions of phenols, anisole and aniline whereas the most frequent structural features associated with inactivity included ethers, esters, aryl halides and a tertiary amine [1]. The structure of thymol only includes a phenol group, so the substance has one feature associated with TPO inhibition activity.

EAS-modality

In accordance with the OECD Conceptual Framework and the ECHA/EFSA GD on ED, Level 1, EAS-related non-test information was gathered for thymol. Qualitative structural activity relationship (QSAR) data was obtained for thymol from the Danish QSAR database, and results are summarised below.

Table B.6.8.3.8/02: Results of Danish QSAR database for thymol regarding EAS-modality

	Exp.	Battery	CASE Ultra	Leadscope	SciQSAR
Estrogen Receptor α Binding, Full training set (Human *in vitro*)	NEG	NEG IN	NEG IN	NEG IN	NEG IN
Estrogen Receptor α Binding, Balanced Training Set (Human *in vitro*)	NEG	NEG IN	NEG IN	NEG IN	INC OUT
Estrogen Receptor α Activation (Human		NEG IN	NEG IN	NEG IN	NEG OUT
Estrogen Receptor Activation, CERAPP data (*in vitro*)		NA	NA	INC_OUT	NA
Androgen Receptor Inhibition (Human *in vitro*)		NEG IN	NEG IN	NEG OUT	NEG IN
Androgen Receptor Binding, CoMPARA data (*in vitro*)	NEG	NA	NA	NEG IN	NA
Androgen Receptor Inhibition, CoMPARA data (*in vitro*)	NEG	NA	NA	INC OUT	NA
Androgen Receptor Activation, CoMPARA data (*in vitro*)	NEG	NA	NA	NEG_IN	NA

Key POS = Positive; NEG = Negative; IN = within the applicability domain of the model; OUT = outside of the applicability domain of the model

The Danish QSAR Database predictions report that thymol lacks the potential to interact with estrogen and androgen receptors.

[1] Rosenberg SA, Watt ED, Judson RS, Simmons SO, Paul Friedman K, Dybdahl M, Nikolov NG, Wedebye EB. QSAR models for thyroperoxidase inhibition and screening of U.S. and EU chemical inventories. *Computational Toxicology*. 2017, Vol 4,pp 11-21. https://doi.org/10.1016/j.comtox.2017.07.006.

OECD ToolBox

The results of the OECD QSAR Toolbox v.4.2 profilers for thymol in respect of E-related endpoints are shown below:

Table B.6.8.3.8/03: Results of the OECD QSAR Toolbox v.4.2 for thymol

[1] Estrogen Receptor Binding, alerts in	
parent only	Weak binder, OH group
metabolites from in vivo Rat metabolism simulator only	Moderate binder, OH group; Weak binder, OH group
metabolites from Rat liver S9 metabolism simulator only	Moderate binder, OH group; Weak binder, OH group
[2] rtER Expert System - USEPA, alerts in	
parent only	Alkoxyphenols
metabolites from in vivo Rat metabolism simulator only	No alert found
metabolites from Rat liver S9 metabolism simulator only	No alert found
OECD QSAR Toolbox v.4.2 profilers	
Profiler predictions are supporting information to be used together with the relevant QSAR predictions	

[1] Estrogen receptor binding: Weak binder, OH group: The OECD Toolbox v.4.2 predicts that thymol is not a potential ER binder (moderate/weak binder).

[2] rtER Expert System USEPA: Alkoxyphenols: USEPA profiler predicts that thymol meets the criteria of chemical classes that are potential ER binders, on the basis that is an alkoxyphenol substance.

Assessment and conclusion by RMS:

Outcome and conclusion of the study: The *in silico* QSAR predictions displayed that thymol is not a potential ER or AR binder.

B.6.9. MEDICAL DATA AND INFORMATION

B.6.9.1. Medical surveillance on manufacturing plant personnel and monitoring studies

Data point	CA 5.9 (EU data requirement)
Report author	Volpe, M.J.
Report year	2021
Report title	Manufacturer Medical Report
Report No	Not applicable
Document No	KCA 5.9.1/01
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No
Acceptability/ reliability	Yes

The manufacturing plant conducts regular medical monitoring of workers involved in the production, packaging or handling of thymol. There have been no reports of reactions or ill health in any workers.

B.6.9.2. Data collected on humans

Data point	CA 5.9.2 (EU data requirement)
Report author	BG Chemie
Report year	2000
Report title	Thymol
Report No	None, published report (Toxicological evaluation, No. 259).
Document No	KCA 5.9.2/01
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	Yes, evaluated and accepted in DAR (2011)
GLP/Officially recognised testing facilities	Not applicable (secondary source review)
Acceptability/Reliability	Supportive only (secondary source review)

Materials and Methods

Test chemical	Thymol
Vehicle	Mouthwash. Full composition not indicated.
Test system	Male patients
Test method	Not applicable.

Executive summary

In this publication on the toxicological evaluation of thymol, a review of experience in humans was performed. Several publications with information on the experience in humans are cited. Data provided for each study is presented in Table B.6.9.2:

Table B.6.9.2: Data on experience in humans with thymol

Type of data/report	Test substance/ vehicle	Relevant information about the study	Observations	Reference
Oral route				
Studies on the metabolism of thymol in 2 volunteers	0.6 g thymol/person	Single oral administration of thymol and the 24-h urine was analysed (thin-layer chromatography).	Thymol glucuronide, thymol sulfate were detected, to a lesser extent thymolhydroquinone sulfate and small amounts of unchanged thymol. No further quantitative details	Takada *et al.* (1979)
Dermal route (skin sensitization)				
Reported data	Thymol. No more data provided	No data	Thymol reportedly caused dermatitis in dentists	Schwartz *et al.* (1957)
Maximisation test in 25 volunteers	Thymol 4% in petrolatum	48-h occlusive exposure	No primary skin irritation nor allergenic reactions	Kligman (1972)
Patch-test in patients with contact dermatitis (38 dentist, 18 dental nurses and 28 dental technicians)	Thymol 1% in petrolatum		Only 1 dental nurse (1.2% of patients) developed a positive reaction to thymol	Berova *et al.* (1990)
43-year-old patient with chronic paronychia	Ethanolic antiseptic solution (“Listerine”) containing 0.6% thymol Patch-test: 1% thymol in petrolatum	3-week occlusive application of “Listerine” (0.6% thymol, 0.9% eucalyptus oil, 0.6% methyl salicylate, 0.4% menthol and traces of benzoic acid) Also 48-h patch test with the antiseptic and with the ingredients was performed to the patient and 3 controls	The 3-w application resulted in spreading pruritic dermatitis in the patient. 48-h patch test with the antiseptic produced a positive reaction in the patients and none in the 3 controls. 48-h patch test with thymol (1% in petrolatum) led to a reaction (2+). 48-h patch test with eucalyptus oil (1% in alcohol), methyl salicylate (2% in olive oil), menthol (1% in petrolatum) and benzoic acid (5% in petrolatum) proved negative in the patient and in the 3 controls. However, the patient used “Listerine” as mouthwash several times without developing any noticeable reactions. According to the author, thymol appears to be a weak sensitiser which needs prolonged contact, occlusive application and inflamed skin to produce sensitisation and dermatitis.	Fisher (1989)
Patch test in 365 patients at a dermatology clinic (1981 to 1986)	Thymol 1% formulation (composition not specified)	No data	2 cases (0.5%) of positive reactions	Itoh *et al.* (1988)
Test in 131 patients at a dermatology clinic (1979 - 1982)	Thymol 1% formulation (composition not specified)	This publication is previous to Itoh *et al.* (1988), but same investigators and similar group of patients	None of the individual reacted	Nishimura *et al.* (1984)
Patch test in 221 patients treated at a dermatology department	“spir. Dilute” solution of thymol (only data available)	No data	One female patient (0.45%) showed a positive reaction	Dohn (1980)
31-year-old woman with skin allergy to menthol	Thymol (no more data)	No data	It was possible to exclude a simultaneous allergy to thymol	Papa and Shelley (1964)

Type of data/report	Test substance/ vehicle	Relevant information about the study	Observations	Reference
Patch-test in 300 workers (217 women and 83 men, 20-27 years) of stomatology offices	Thymol (5% in glycerine)	12 different substances used in dental practice were patch-tested	39 individuals reacted to thymol, in a total of 213 who tested positive. 87 were negative	Djerassi and Berowa (1966)
Patch-test in 79 patients with eyelid dermatitis	Thymol (1% in petrolatum)	No data	All results were negative	Nethercott *et al.* (1989)
Patch-tests in 290 patients	Thymol (1% in petrolatum)	Thymol was assayed in the context of a study on allergising effect of topical medicaments	None of the 290 patients developed a positive reaction to thymol	Meneghini *et al.* (1971)
Patch-test in 100 eczematous patients of a dermatology clinic	Thymol (1% in petrolatum)	Numerous substances were patch-tested	None of the patients showed a positive reaction to thymol	Rantuccio and Meneghini (1970)
Patch-test in a 51-year-old sawmill worker	Thymol (0.01 to 0.015 ml, equivalent to 0.1 to 0.15 mg)	Within 2 years working, he developed itching vesicular dermatitis on the face, dorsa of his hands and the flexures of his forearms. Patch-test was performed with various extracts of wood and pure constituents.	Negative results for thymol in the patch-test. Strongly positive reactions to western red cedar, in particular	Bleumink *et al.* (1973)
Patch-test in 23 patients with contact dermatitis following the use of Hirudoid cream	Hirudoid cream, containing 0.1% of thymol Patch-test: thymol (not specified)	These patients developed allergic contact dermatitis over a period of 10 years. Individual components of the cream were patch-tested.	The individual components of the cream proved negative in the patch-test The cause of the allergies was identified as a reaction product of thymol, ethanolamine and formaldehyde. The last two substances are degradation products of 1,3,5-trihydroxyethylexahydrotriazine, of which Hirudoid cream contains 0.15%	Smeenk *et al.* (1987)
Other routes				
Postoperative "halothane hepatitis"	Halotane (inhalation anaesthetic) containing 0.01% thymol	No more data	It's been suggested that thymol might play a causative role in very rarely occurring instances of postoperative "halothane hepatitis"	Hutter and Laing (1993)
Olfactory threshold test in 9 to 12 subjects	Thymol in water	No more data	The olfactory threshold of thymol in water was found to be 500 µg/L	Dietz and Traud (1978)

Assessment and conclusion by applicant:

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance. The overall EU-agreed endpoint is: inconsistent results in animal tests, reports of skin sensitisation in humans. Thymol is classified as Skin Sens. 1 (H317) (EFSA Journal 2012;10(11):2916).

Assessment and conclusion by RMS:

This report is a toxicological evaluation of thymol that reviews the experience in humans and includes information of other publications, mainly about skin sensitization and two more publications about inhalation. The data is considered supporting information, since it is provided by a secondary source review.

Regarding skin sensitization data, conflicting evidence was provided, but since positive reactions were

observed in humans, RMS deems thymol should be considered a weak skin sensitizer, in agreement with the applicant.

Regarding the suggestion that thymol might play a causative role in postoperative "halothane hepatitis", considering subsequent papers, this effect is not considered provoked by thymol (see volume 1 section 2.6.2.3.1 for more details).

B.6.9.3. Direct observation

B.6.9.3.1. Review

Data point	CA 5.9.3 (EU data requirement)
Report author	BG Chemie
Report year	2000
Report title	Thymol
Report No	None, published report. (Toxicological evaluation, No.259)
Document No	KCA 5.9.3/01
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	Yes, evaluated and accepted in DAR (2011)
GLP/Officially recognised testing facilities	Not applicable (secondary source review)
Acceptability/Reliability	Supportive only (secondary source review)

Executive summary

Thyroid intoxication was diagnosed in three male patients following the use of a mouthwash containing thymol for periods of from 6 months to 3 years. Symptoms included weight loss, tremors, restlessness, sleeplessness and diarrhoea. Upon cessation of the use of the mouthwash the patients showed recovery and weight gain. There have been no other reports of systemic effects attributed to thymol. No further details (Edens, 1937).

A 3 week-old infant suffered a respiratory collapse when inhaling a cold remedy, which in addition to menthol and other volatile oils also contained thymol. The authors considered it very unlikely that the collapse was attributable to the remedy. No further details (Davis and Livingstone, 1986).

Assessment and conclusion by applicant:

The study has been previously reviewed at EU level and is acceptable for renewal of the active substance.

Assessment and conclusion by RMS:

This report is a toxicological evaluation of thymol that reviews the experience in humans, gathering information previously published. Reported intoxication cases have been summarised in this point.

B.6.9.3.2. Fatal large-volume mouthwash ingestion

Data point	CA 5.9.3 (EU data requirement)
Report author	Soo Hoo G.W., Hinds R.L., Dinovo E. and Renner S.W.
Report year	2003
Report title	Fatal large-volume mouthwash ingestion in an adult: a review and the possible role of phenolic compound toxicity
Report No	None, published report. (J. Intensive Care Med. May-Jun; 18(3): 150-5)
Document No	KCA 5.9.3/02
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	Yes, evaluated and accepted in DAR (2011)
GLP/Officially recognised	Not applicable (secondary source review)

testing facilities	
Acceptability/Reliability	Supportive only (secondary source review)

Materials and Methods

Test chemical	Poisoning case with mouthwash containing thymol.
Vehicle	Not applicable. Poisoning case.
Test system	Not applicable. Poisoning case.
Test method	Not applicable. Poisoning case.

Summary

A single patient developed cardiovascular collapse and multiorgan system failure following ingestion of almost 3 litres of mouthwash. He presented with a profound anion gap metabolic acidosis and a significant osmolar gap. He died despite full supportive care including dialysis and mechanical ventilation.

According to the paper, the ethanol in Listerine is insufficient to explain all of the metabolic disturbances of the patient before his death. It was concluded that phenolic compound toxicity (eucalyptol, menthol and thymol) likely contributed to this patient's presentation and constellation of symptoms.

The estimated amount of non-alcoholic ingredients ingested was almost of 7.74 g (1.92 g of thymol (0.064%); 2.76 g of eucalyptol (0.092%); 1.26 g of menthol (0.042%); and 1.8 g of methyl salicylate (0.060%)).

Assessment and conclusion by applicant: -
Assessment and conclusion by RMS: The first paragraph of this summary was included in the previous DAR, just before the summary of the poison case extracted from BG Chemie report (2000), but with no reference cited. The applicant included the information as part of the report of BG Chemie (2000). However, since this data could not be found in the report, RMS has searched for the publication to add the reference for this case, adding information on the estimated amount of thymol and the other non-alcoholic ingredients ingested by this person.

B.6.9.4. Epidemiological studies

There are no epidemiological studies available for thymol.

B.6.9.5. Diagnosis of poisoning (determination of active substance, metabolites), specific signs of poisoning, clinical test

Information on signs of poisoning is available in the accidental cases noted under B.6.9.3.

B.6.9.6. Proposed treatment: first aid measures, antidotes, medical treatment

According to the applicant, the following treatment is proposed. First aid measures include removal of patient to fresh air if inhalation exposure is suspected. It is recommended any contaminated clothing is immediately removed and that any exposed skin is washed with soap and water. In the case of ocular exposure, severe or lasting irritation may occur, affected eyes should be rinsed with water for at least 15 minutes. Following oral exposure, induce vomiting in patient and contact medical services. A doctor should be called if any symptoms persist or the patient feels unwell.

B.6.10. REFERENCES RELIED ON

LITERATURE SEARCH

B.6.10.1. Summary

In order to select the updated literature data relating to the active substance thymol, as required by Article 8(5) of Regulation (EC) No. 1107/2009 on the placing of plant protection products on the market, the applicant performed a review of the literature according to:

- EFSA (2011). Guidance of EFSA, Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009, EFSA Journal 2011;9(2):2092.

- AGES (2013). External scientific report, Case studies for the application of the Guidance of EFSA on Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009, using substances for which dossiers are submitted under Regulation (EU) No 1141/2010, EFSA supporting publication 2013: EN-511.

In this literature search, the aim was to find scientific peer-reviewed open literature on thymol dealing with toxicological and toxicokinetic studies, operator exposure, residues, fate and behaviour in the environment and ecotoxicological studies, which were published between 1/1/2005 and 16/02/2021 for thymol.

To achieve this goal, a wide range of reference collections was consulted utilising the online various databases.

The following initial search strategy was used:

- The search terms were defined initially using terms related to the active substance, its metabolites and plant protection products containing the active substance, including synonyms.

- Specific search terms were then used relating to the specific data requirements in question (i.e. for toxicology, human exposure, residues, environmental fate and behaviour and ecotoxicology) to refine the search.

- The search of publications was conducted, using the two-stage approach outlined above. A list of titles, authors and dates were extracted. Any duplicate summary records were removed, before proceeding.

The remaining summary records were then assessed for relevance in a two-step process:

- Firstly, a rapid assessment for relevance was conducted based on the titles and abstracts, to exclude summary records which were obviously irrelevant to the specific data requirements in question.

- Secondly, a detailed assessment of full-text documents was conducted to identify potentially relevant, irrelevant or relevant and reliable records that were not excluded in the first step.

The results of this search were as follows:

Study selection process	Toxicology (including human exposure)	Residues	Environmental fate and behaviour	Ecotoxicology
Total number of documents retrieved (with duplicates) (global search results related to the active substance, its metabolites and plant protection products containing the active substance)	35229			
Total number of documents retrieved (without duplicates) after focused search strategy (combining terms for	494	303	510	1120

Study selection process	Toxicology (including human exposure)	Residues	Environmental fate and behaviour	Ecotoxicology
active substance and specific data requirements)				
Number of publications excluded after rapid assessment for relevance (non-relevant, excluded literature)	481	284	507	983
Number of publications for which detailed assessment of full-text required after rapid assessment for relevance	13	19	3	137
Number of publications excluded from further consideration after detailed assessment for relevance (non-relevant, excluded literature)	3	9	0	93
Number of publications considered relevant following the two-step process (i.e. after detailed assessment of full-text)	**10**	**10**	**3**	**44**

For toxicology: 10 publications were considered relevant following the two-step process.

B.6.10.2. Search strategy

The following search strategy was used:

- The search terms were defined using terms related to the active substance, its metabolites and plant protection products containing the active substance, including synonyms.

- Specific search terms relating to the data requirements in question (i.e. for toxicology, human exposure, residues, environmental fate and behaviour and ecotoxicology) were then used to refine the search.

- These search terms are listed in Section B.6.10.2.4 below.

B.6.10.2.1. Date of the literature search

The literature search was conducted on 2nd July 2020 and 16th/17th February 2021.

B.6.10.2.2. Time period covered in the literature search

The search identified scientific peer-reviewed open literature published in the last 15 years (from 1/1/2005 to 16/02/2021) for thymol.

B.6.10.2.3. Bibliographic databases used in the literature review

A number of different sources were used in the literature search with the aim to locate all relevant scientific peer-reviewed open literature. The reference collections searched were AGRICOLA, AGRIS (FAO), PASCAL, PubMed (MEDLINE) inc. TOXNET, Science Direct, Springer and Wiley. All sources and the rationale for their inclusion in the search are presented in the table below.

Table B.6.10.2.3: Databases used in the literature search by the applicant

Database	Description/Rationale for Inclusion
1. AGRICOLA	Coverage of the database includes agricultural economics and rural sociology, agricultural production, animal sciences, chemistry, entomology, food and human nutrition, forestry, natural resources, pesticides, plant science, soils and fertilizers, and water resources. More than 6.7 million records (09/2019). 1970-present.
2. AGRIS (FAO)	AGRIS is one of the most comprehensive search engines in food and agricultural

Database	Description/Rationale for Inclusion
	scientific literature providing free access to millions of bibliographic records in 90 different languages. Up to hundreds of organisations worldwide contribute to knowledge and data to the AGRIS platform, resulting in a multilingual bibliographic collection of food and agricultural scientific research with special attention to scientific information produced in the global south. Therefore, AGRIS is used by whoever is inclined to find literature on any of FAO's areas of interest.
3. PASCAL	The site is an archive of the PASCAL and FRANCIS bibliographic databases in exact, human and social sciences, produced by the Inist-CNRS from 1972 to 2015. It provides access to more than 20 million references (articles, patents, maps, conferences, books, reports and thesis) in exact sciences and technology, in biological and medical sciences, in art, archaeology, economics, ethnology, geography, history of science and technology, literature, linguistics, administrative and legal sciences, education and religion sciences.
4. PubMed MEDLINE (including TOXNET)	MEDLINE contains information on every area of medicine. More than 30 million records (08/2019). 1946-present.
5. Science Direct	ScienceDirect provides access to more than 16 million articles, 2,500 journals, 370 full open access journals, 39,000 books and 330000 topic pages to help researchers discover more insights, achieve more breakthroughs and move their research forward.
6. Springer	Springer is a leading global scientific, technical and medical portfolio, providing researchers in academia, scientific institutions and corporate R&D departments with quality content through innovative information, products and services. It handles more than 2,900 journals and 300000 books.
7. Wiley	Wiley is one of the largest and most authoritative collections of online journals, books, and research resources, covering life, health, social, and physical sciences.

B.6.10.2.4. Input parameters (search terms) for the literature search

Any documents relating to the essential oils, their synonyms and other common names were collected. The reference collections were queried by name e.g. "thymol" in addition to common variants such as thymic acid, and "thyme campher". Where the number of records for, say, thymol alone exceeded individual reference collection download limits additional specific topics of interest, ecotoxicity, fate, residues, etc., were included. The tables below present the general search terms used:

Table B.6.10.2.4: List search terms used to identify all literature related to the active substance (thymol), its metabolites and plant protection products containing the active substance

Common name	Thymol Thyme camphor Thymic acid Cymophenol, alpha- m-Thymol IPMP Isopropyl cresol Cymophenol, alpha- phenol
(IUPAC and CA)	5-methyl-2-propan-2-ylphenol 2-Isopropyl-5-methylphenol 5-Methyl-2-isopropylphenol 1-Hydroxy-5-methyl-2-isopropylbenzene 1-Methyl-3-hydroxy-4-isopropylbenzene 2-Hydroxy-1-isopropyl-4-methylbenzene 2-Isopropyl-5-methylphenol 3-Hydroxy-1-methyl-4-isopropylbenzene 3-Hydroxy-p-cymene 3-Methyl-6-isopropylphenol 3-p-Cymenol 5-Methyl-2-(1-methylethyl)phenol 5-Methyl-2-isopropyl-1-phenol Methyl-2-isopropylphenol 6-Isopropyl-3-methylphenol 6-Isopropyl-m-cresol

	6-Isopropyl-p-cresol Phenol, 5-methyl-2-(1-methylethyl)- Phenol, 2-isopropyl-5-methyl-
Metabolite(s)	-
Product name	Mevalone, Cedroz, Andromeda, 3Logym ES-00108, Trigemol, Cagenoleta, Eugeti
CAS No.	89-83-8
EC No.	201-944-8

B.6.10.3. Search results

The total numbers of records (before removing duplicates) for the search are summarised in the table below.
The peer-reviewed literature search strategy highlighted 35229 documents of potential interest for thymol to this review before the removal of duplicates occurring because of searching the individual reference collections separately.

Table B.6.10.3/1: Numbers of records (before removing duplicates) for the search

Databases (through online STN)	Number of hits
1. AGRICOLA	88
2. AGRIS (FAO)	204
3. PASCAL	-
4. PubMed MEDLINE (including TOXNET)	18093
5. Science Direct	7643
6. Springer	5276
7. Wiley	3925
Total number of documents retrieved (with duplicates) (global search results related to the active substance, its metabolites and plant protection products containing the active substance)	**35229**

To identify which of these records mentioned the aspects of interest to this particular project a text mining application was built and applied to each document in turn and only those which specifically mentioned, for example fate and behaviour or the specified ecotox terms together with an essential oil or its common variants, were identified as a positive result.
The text mining application comprises a number of different steps each performing a different function in the application and in general terms the approach taken was to Tokenise (identify individual words and features) and Sentence split the documents; use the Gazetteer lists to identify any important key words and phrases such as sediment dwelling organisms; identify the Title and Abstract part of the document; look within the Title and Abstract for patterns matching the natural language expressions and index the results.

After removing duplicates and text mining process, the total number of hits potentially relevant is presented below:

Table B.6.10.3/2: Number of documents identified as potentially relevant after text mining and removal of duplicates

All databases	Number of hits*
Mammalian Toxicity	**494**
Residues	**303**
Environmental fate and behaviour	**510**
Ecotox	**1120**

** Total number of documents retrieved after removal of duplicates/triplicates (using general and section specific terms)*

B.6.10.4. Study selection

The remaining summary records were assessed for relevance in a two-step process:

- Firstly, a rapid assessment for relevance was conducted based on the titles and abstracts, to exclude summary records, which were considered irrelevant, and the justification was recorded.

- Secondly, a detailed assessment relevance and reliability of full-text documents was conducted for any remaining summary records that were not excluded in the first step.

Table B.6.10.4. Criteria for relevance

Data requirement	Justification for Search Strategy
Toxicological and metabolism studies (OECD IIA 5.1 to 5.10 and OECD IIIA 7.1 and 7.6)	
ADME (OECD IIA 5.1)	1. Defined test material 2. In vivo tests in relevant test species for toxicological testing (i.e rat, mouse, rabbit, dog) 3. In vitro tests 4. PBPK modelling 5. Specific endpoint can be clearly related to this data requirement
Acute toxicity, irritation, sensitisation (a.s.) (OECD IIA 5.2)	1. Defined test material 2. Relevant test species for toxicological testing (i.e rat, mouse, rabbit, dog, guinea pig) 3. Relevant (physiologic) route of exposure 4. Specific endpoint can be clearly related to this data requirement
Short-term toxicity (OECD IIA 5.3)	1. Defined test material 2. Relevant test species for toxicological testing 3.Relevant (physiologic) route of exposure 4. Specific endpoint can be clearly related to this data requirement
Genotoxicity (OECD IIA 5.4)	1. Defined test material 2. In vitro tests 3. In vivo tests in relevant test species for toxicological testing 4. Specific endpoint can be clearly related to this data requirement
Long-term toxicity and carcinogenicity (OECD IIA 5.5)	1. Defined test material 2. Relevant test species for toxicological testing (i.e. rat, mouse) 3. Relevant (physiologic) route of exposure 4. Specific endpoint can be clearly related to this data requirement
Reproductive toxicity (OECD IIA 5.6)	1. Defined test material 2. Relevant test species for toxicological testing 3. Relevant (physiologic) route of exposure 4. Specific endpoint can be clearly related to this data requirement
Neurotoxicity (OECD IIA 5.7)	1. Defined test material 2. In vivo tests in relevant test species for toxicological testing 3. Relevant (physiologic) route of exposure 4. Specific endpoint can be clearly related to this data requirement
Studies on metabolites and impurities (OECD IIA 5.8)	1. Defined test material 2. In vitro tests 3. In vivo tests in relevant test species for toxicological testing 4. Relevant (physiologic) route of exposure 5.Specific endpoint can be clearly related to this data requirement
Supplementary studies on active substance (e.g. immunotoxicity studies on mixture effects) (OECD IIA 5.10)	1. Defined test material 2. Relevant test species for toxicological testing 3.Specific endpoint can be clearly related to this data requirement
Endocrine disrupting properties (OECD IIA 5.10)	1. Defined test material 2. In vitro tests 3. In vivo tests in relevant test species for toxicological testing (i.e rat, mouse, rabbit, dog) 4. Relevant (physiologic) route of exposure 5. Specific endpoint can be clearly related to this data

Data requirement	Justification for Search Strategy
Toxicological and metabolism studies (OECD IIA 5.1 to 5.10 and OECD IIIA 7.1 and 7.6)	
	requirement
Medical data (OECD IIA 5.9)	1. Defined test material 2. Epidemiological studies 3. Poisonings, clinical cases 4. Relevant (physiologic) route of exposure
Acute toxicity, irritation, sensitisation (PPP) (OECD IIIA 7.1)	1. Defined test material 2. Relevant test species for toxicological testing (i.e rat, mouse, rabbit, dog, guinea pig) 3. Relevant (physiologic) route of exposure 4. Specific endpoint can be clearly related to this data requirement
Exposure assessment (PPP) (OECD IIIA 7.3-7.5)	1. Defined test material 2. Field studies 3. Calculations 4. Specific endpoint can be clearly related to this data requirement
Dermal absorption (PPP) (OECD IIIA 7.6)	1. Defined test material 2. In vitro tests 3. In vivo tests in relevant test species for toxicological testing (i.e. rat) 4. Specific endpoint can be clearly related to this data requirement

B.6.10.4.1. Rapid assessment

The total number of publications excluded after rapid assessment for relevance and the number of publications for which a detailed assessment of the full-text was required are summarised in the table below.

Table B.6.10.4.1: Numbers of publications excluded/identified after rapid assessment for relevance for thymol

Study selection process	Toxicology (including human exposure)	Residues	Environmental fate and behaviour	Ecotoxicology
Number of publications excluded after rapid assessment for relevance (non-relevant, excluded literature)	481	284	507	983
Number of publications for which detailed assessment of full-text required after rapid assessment for relevance	13	19	3	137

B.6.10.4.2. Detailed assessment

The total number of publications excluded and the final number of relevant papers identified after detailed assessment of full-texts for relevance are summarised in the tables below.

Table B.6.10.4.2/1: Numbers of publications excluded/identified after detailed assessment for relevance for thymol

Study selection process	Toxicology (including human exposure)	Residues	Environmental fate and behaviour	Ecotoxicology
Number of publications excluded from further consideration after detailed assessment for relevance (non-relevant, excluded literature)	3	9	0	93
Number of publications considered relevant following the two-step process (i.e. after detailed assessment of full-text)	**10**	**10**	**3**	**44**

All publications excluded and included following the detailed assessment are presented in the tables below, and a reason was added if they were not included in the dossier.

Table B.6.10.4.2/2: Publications excluded from the risk assessment after detailed assessment of full-text documents – results for thymol in toxicology and human exposure

Author(s)	Year	Title	Source	Reason for not including in dossier (based on relevance criteria)
Baldissera M., Souza C.F., De Matos A.F.I.M., Doleski P.H., Baldisserotto B., Da Silva A.S., Monteiro S.G. (CA 9.6.5.1/01)	2018	Blood-brain barrier breakdown, memory impairment and neurotoxicity caused in mice submitted to orally treatment with thymol	Environmental Toxicology and Pharmacology, 62:114–119	Study not relevant or reliable due to several methodological deficiencies, e.g. behavioural testing not conducted pre-exposure and limited vs OECD guideline requirements, small group sizes (n=6, single sex), relevance of *in vitro* investigations to risk assessment unclear, no in-life observations and no histopathology.
Chikhoune A., Stouvenel L., Iguer-Ouada M., Hazzit M., Schmitt A., Lorès P., Wolf J.P., Aissat K., Auger J., Vaiman D., Touré A. (CA 9.6.5.1/02)	2015	*In vitro* effects of *Thymus munbyanus* essential oil and thymol on human sperm motility and function	Reproductive BioMedicine Online, 31:411-420	Non-relevant method of administration therefore not relevant to hazard assessment.
Chrastinova Lubica, Laukova Andrea, Madarova Michaela, Gancarcikova Sona; Sopkova Drahomira, Andrejcakova Zuzana, Placha Iveta, Bacova Kristina, Zitterl-Eglseer Karin.	2020	Effect of Thymol Addition and Withdrawal on Some Blood Parameters Antioxidative Defence System and Fatty Acid Profile in Rabbit Muscle.	Animals (Basel). 2020 Jul 22; 10(8): 1248. doi: 10.3390/ani1008 1248	Study not relevant or reliable due to several methodological deficiencies.

Table B.6.10.4.2/3: Relevant studies included in the supplementary dossier after detailed assessment of full-text documents for relevance: sorted by data requirements in toxicology and human exposure

Data requirement	Author(s)	Year	Title	Source
CA 5.1.1/06	Kohlert C., Schindler G., Marz R., Abel G., Brinkhaus B., Derendorf H., Grafe E-U., Veit M.	2002	Systemic availability and pharmacokinetics of thymol in humans	Journal of Clinical Pharmacology, 42: 731-737.
CA 5.1.2/01	Xie K., Tashkin D.P., Luo M.Z., Zhang J.Y.	2019	Pharmacokinetic study of thymol after intravenous injection and high-dose inhalation in mouse model	Pharmacology Research & Perspectives, 00:e00515
CA 5.4.1/07 Previously evaluated in DAR 2011	Buyukleyla M., Rencuzogullari E.	2009	The effects of thymol on sister chromatid exchange, chromosome aberration and micronucleus in human lymphocytes	Ecotoxicology and Environmental Safety, 72(3): 943-947

Data requirement	Author(s)	Year	Title	Source
CA 5.4.1/09	Someya H., Higo Y., Ohno M., Tsutsui T.W., Tsutsui T.	2008	Clastogenic activity of seven endodontic medications used in dental practice in human dental pulp cells	Mutation Research, 650: 39-47
CA 5.4.1/10	Llana-Ruiz-Cabello M., Maisanaba S., Puerto M., Prieto A.I., Pichardo S., Jos A., Camean A.M.	2014	Evaluation of the mutagenicity and genotoxic potential of carvacrol and thymol using the Ames Salmonella test and alkaline, Endo III and FPG-modified comet assays with the human cell line Caco-2	Food and Chemical Toxicology, 72: 122–128
CA 5.4.1/11	Maisanaba S., Prieto A.I., Puerto M., Gutierrez-Praena D., Demir E., Marcos R., Camean A.M.	2015	*In vitro* genotoxicity testing of carvacrol and thymol using the micronucleus and mouse lymphoma assays	Mutation Research, 784-785: 37–44
CA 5.4.1/12	Aydin S., Basaran A.A., Basaran N.	2005	The effects of thyme volatiles on the induction of DNA damage by the heterocyclic amine IQ and mitomycin C	Mutation Research, 58: 43–53
CA 5.4.2/02 Previously evaluated in DAR 2011	Azirak S. Rencuzogullari E.	2008	The *in vivo* genotoxic effects of carvacrol and thymol in rat bone marrow cells	Environmental Toxicology, 23(6): 728-735.
CA 5.8.3/02	Chen J., Ahn K.C., Gee N.A., Gee S.J., Hammock B.D., Lasley B.L.	2007	Antiandrogenic properties of parabens and other phenolic containing small molecules in personal care products	Toxicology and Applied Pharmacology, 221(3): 278–284.
CA 5.8.3/03	Michalíkova K., Linhartova L., Ezechias M., Cajthaml T.	2019	Assessment of agonistic and antagonistic properties of widely used oral care antimicrobial substances toward steroid estrogenic and androgenic receptors	Chemosphere, 217: 534-541.

Assessment and conclusion by RMS:

The RMS has checked the review of literature data submitted by the applicant and considers that in general term it is acceptable.

However, according to the EFSA Guidance Document published in EFSA Journal 2011; 9(2): 2092, the RMS has noticed the following data have not been presented separately for each database, as it is indicated in the EFSA Guidance, 5.2.3 section (see also Table 2 and Appendix A.4):

- The complete search strategy or strategies used, including all the search terms, text-words, subject index headings and the relationship between the search terms. The RMS regards that the applicant has used the same search strategy for all the databases.
- The total number of summary records retrieved from each database after removing duplicates.

Once checked the steps performed by the applicant after overall assessment of the dossier of thymol for its renewal, RMS is agreed with this search but disagreed with the study selection in the following studies:

- Baldissera, M. *et al.* (2018). "Blood-brain barrier breakdown, memory impairment and neurotoxicity caused in mice submitted to orally treatment with thymol": It was rejected after detailed assessment because according to the applicant, the study was not relevant or reliable due to several methodological deficiencies, e.g. behavioural testing not conducted pre-exposure and limited vs OECD guideline

requirements, small group sizes (n=6, single sex), relevance of *in vitro* investigations to risk assessment unclear, no in-life observations and no histopathology. In the RMS opinion, this study provides supportive information about the neurotoxicity potential of thymol for which no specific studies were submitted, and should be included in this DRAR.

- Chikhoune A., *et al*. (2015). "*In vitro* effects of *Thymus munbyanus* essential oil and thymol on human sperm motility and function": It was rejected after detailed assessment because according to the applicant, the study was performed with non-relevant method of administration. Nevertheless, the RMS considers that in the absence of *in vivo* data about sperm motility, in vitro information should be added to the DRAR.
- Bacova, K. *et al. (*wrongly referenced in table B.6.10.4.2/2 above, extracted from the applicant´s literature review, as Crastinova *et al.)* (2020). "Effect of thymol addition and withdrawal on some blood parameters, antioxidative defence system and fatty acid profile in rabbit muscle": It was rejected after detailed assessment because according to the applicant, the study was not relevant or reliable due to several methodological deficiencies. However, the RMS considers that it should be included as supplementary information as provides information about the presence of thymol in plasma and tissues after oral administration in diet to rabbits, and about its effect on the biochemical parameters.

LIST OF REFERENCES

Data Point	**Author(s)**	**Year**	**Title** **Report No.** **Document No.** **Source (where different from company)** **GLP/ Officially recognised testing facilities[2,3]** **Published or not**	**Vertebrate study Y/N**	**Data protection claimed Y/N**	**Justification if data protection is claimed**	**Owner**	**Previously used[1]** **Y/N** **If yes, for which data point?**
CA 5.1.1/01	Austgulen L.T., Solheim E., Scheline R.R.	1987	Metabolism in Rats of *p*-Cymene Derivatives: Carvacrol and Thymol Pharmacology and Toxicology, 61:98-102. Not GLP Published	Y	N	-	Public	Y In DAR IIA 5.1
CA 5.1.1/02	Takada M., Agata I., Sakamoto M., Yagi N., Hayashi N.	1979	On the Metabolic Detoxication of Thymol in Rabbit and Man. The Journal of Toxicological Sciences, 4:341-350. Not GLP Published	Y	N	-	Public	Y In DAR IIA 5.1
CA 5.1.1/03	EMEA - Committee for veterinary medicinal products	1996	Thymol - Summary report EMEA/MRL/075 /96-FINAL, March 1996 Not GLP	N	N	-	Public	Y In DAR IIA 5.1

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If yes, for which data point?
			Published					
CA 5.1.1/04	Mattia A., Sipes G.I.	2005	WHO food additives series 46: phenol and phenol derivatives Prepared by the Fifty-fifth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) Not GLP Published	N	N	-	Public	Y In DAR IIA 5.1
CA 5.1.1/05	[redacted]	2009	Thymol: Oral (Gavage) Administration Pharmacokinetic Study in the Mouse [redacted], [redacted]UK Report No. 8202028 GLP Not published	Y	N	-	Eden Research plc	Y In DAR IIA 5.1
CA 5.1.1/06	Kohlert C., Schindler G., Marz R., Abel G., Brinkhaus B., Derendorf H., Grafe E-U., Veit M.	2002	Systemic availability and pharmacokinetics of thymol in humans Journal of Clinical Pharmacology, 42:731-737 Not GLP Published	N	N	-	Public	N
CA 5.1.1/07	Xie K., Tashkin D.P., Luo M.Z., Zhang J.Y.	2019	Pharmacokinetic study of thymol after intravenous injection and high-dose inhalation in mouse model Pharmacology	Y	N	-	Public	N

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If yes, for which data point?
			Research & Perspectives, 00:e00515 Not GLP Published					
CA 5.2.1/01	Jenner, P.M., Hagan, E.C., Taylor, J.M., Cook, E.L. and Fitzhugh, O.G.	1964	Food Flavourings and Compounds of Related Structure I. Acute Oral Toxicity. *Fd Cosmet. Toxicol*., 2, 327-343. GLP: No Published	Y	N	n/a	Public	Y KIIA 5.2.1 (DAR 2011)
CA 5.2.1/02	Hasegawa R. Nakaji Y., Kurokawa Y., Tobe M.	1989	Acute toxicity tests on 113 environmental chemicals. *Science Reports of the Research Institutes, Tohoku University*, 36(1-4): 10-16 GLP: No Published	Y	N	n/a	Public	Y KIIA 5.2.1 (DAR 2011)
CA 5.2.1/03	Escobar A.	2006	Final Report on the Safety Assessment of Sodium p-Chloro-m-Cresol, p-Chloro-m-Cresol, Chlorothymol, Mixed Cresols, m-Cresol, o-Cresol, p-Cresol, Isopropyl Cresols, Thymol, o-Cymen-5-ol, and Carvacrol *International Journal of Toxicology*, 25(Suppl. 1):29–	Y	N	n/a	Public	Y KIIA 5.2.1 KIIA 5.2.2 KIIA 5.2.4 KIIA 5.2.6 KIIA 5.8.2 KIIA 5.8.3 (DAR 2011)

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If yes, for which data point?
			127 GLP: No Published					
CA 5.2.2/01	US-EPA	2006	Thymol, Exemption for a requirement of a tolerance Federal register, 71(11) 18th January, 2006 GLP: No Published	Y	N	n/a	Public	Y KIIA 5.2.2 KIIA 5.2.3 KIIA 5.6.1 (DAR 2011)
CA 5.2.5/01	McOmie W.A., Anderson H.H., Estess F.M.	1949	Comparative toxicity of certain t-Butyl substituted cresols and xylenols. Journal of the American Pharmaceutical Association, 38:366-369 GLP: No Published	Y	N	n/a	Public	Y KIIA 5.2.5 (DAR 2011)
CA 5.2.6/01	Klecak G., Geleick H., Frey J.R.	1977	Screening of fragrance materials for allergenicity in the guinea pig. *Journal of the Society of Cosmetic Chemists*, 28:53-64. GLP: No Published	Y	N	n/a	Public	KIIA 5.2.6 (DAR 2011)
CA 5.3.2/01 CA 5.3.1 CA 5.6.1	[redacted]	1996	Combined repeat dose and reproductive/developmental toxicity screening test of thymol by oral	Y	N	n/a	-	Y KIIA 5.3.1, 5.3.2 KIIA 5.6 (DAR 2011)

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If yes, for which data point?
			administration in rats. Report No. 4L421 GLP: Yes Not published					
CA 5.3.2/02	Hagan E.C., Hansen W.H., Fitzhugh O.G., Jenner P.M., Jones W.I.	1967	Food flavouring and compounds of related structure II. Subacute and chronic toxicity. *Food and Cosmetics Toxicology*, 5:141-157. GLP: No Published	Y	N	n/a	Public	Y KIIA 5.3.1, 5.3.2 (DAR 2011)
CA 5.4.1/01	Stammati, A., Bonsi, P., Zucco, F., Moezelaar, R., Alakomi, H.L. and von Wright, A.	1999	Toxicity of selected plant volatiles in microbial and mammalian short-term assays. Food and Chemical Toxicology, 37:813-823. Not GLP Published	N	N	-	Public	Y IIA 5.4.1 (DAR 2011)
CA 5.4.1/02	Azizan, A. and Blevins, R.D.	1995	Mutagenicity and antimutagenicity testing of six chemicals associated with the pungent properties of specific spices as revealed by the Ames *Salmonella*/Microsomal assay. Archives of Environmental Contamination and Toxicology,	N	N	-	Public	Y IIA 5.4.1 (DAR 2011)

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If yes, for which data point?
			28: 248-258 Not GLP Published					
CA 5.4.1/03	Florin, I., Rutberg, L., Curvall, M. and Enzell, C.R.	1980	Screening of tobacco smoke constituents for mutagenicity using the Ames test. Toxicology, 15 (3): 219-232. Not GLP Published	N	N	-	Public	Y IIA 5.4.1 (DAR 2011)
CA 5.4.1/04	Shibuya, T., Sakamoto, K., Kawakami, K., Hara, T., Shimizu, Y., Matsuki, Y. Nakagomi, M. and Iida, S.	1996	Reverse mutation test of thymol on bacteria. Hatano Research Institute, Food and Drug Safety Center, Kanagawa, Japan. Not GLP Published	N	N	-	Public	Y IIA 5.4.1 (DAR 2011)
CA 5.4.1/05	Hikiba, H., Watanabe, E., Barrett, J.C. and Tsutsui, T.	2005	Ability of fourteen chemical agents used in dental practice to induce chromosome aberrations in Syrian hamster embryo cells. Journal of Pharmaceutical Sciences, 97: 146-152. Not GLP Published	N	N	-	Public	Y IIA 5.4.1 (DAR 2011)
CA 5.4.1/06	Tanaka, N., Yamakage, K., Kusakabe, H.,	1996	*In vitro* chromosomal aberration test of thymol on cultured Chinese hamster cells.	N	N	-	Public	Y IIA 5.4.1 (DAR 2011)

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If yes, for which data point?
	Hashimoto, K., Nagao, T. and Ohta, R.		Hatano Research Institute, Food and Drug Safety Center, Kanagawa, Japan. GLP Published					
CA 5.4.1/07	Buyuklcyla, M. and Rencuzogullari, E.	2009	The effects of thymol on sister chromatid exchange, chromosome aberration and micronucleus in human lymphocytes. Ecotoxicology and Environmental Safety, 72(3): 943-947. Not GLP Published	N	N	-	Public	Y IIA 5.4.1 (DAR 2011)
CA 5.4.1/08	Lloyd M.	2011	Mutation at the *hprt* locus of mouse lymphoma L5178Y cells (MLA) using the Microtitre[R] fluctuation technique. Covance Laboratories Ltd., Harrogate, UK Report No. 8226097. GLP Not published	N	N	-	Eden Research plc	Y IIA 5.4.1 (DAR 2011)
CA 5.4.1/09	Someya, H., Higo, Y., Ohno, M., Tsutsui, T.W. and Tsutsui, T.	2008	Clastogenic activity of seven endodontic medications used in dental practice in human dental pulp cells. Mutation Research, 650:39–47.	N	N	-	Public	N

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If yes, for which data point?
			Not GLP Published					
CA 5.4.1/10	Llana-Ruiz-Cabello, M., Maisanaba, S., Puerto, M., Prieto, A.I., Pichardo, S., Jos A., Cameán, A.M.	2014	Evaluation of the mutagenicity and genotoxic potential of carvacrol and thymol using the Ames *Salmonella* test and alkaline, Endo III and FPG-modified comet assays with the human cell line Caco-2. Food and Chemical Toxicology, 72: 122–128. Not GLP Published	N	N	-	Public	N
CA 5.4.1/11	Maisanaba, S., Prieto, A.I., Puerto, M., Gutiérrez-Praena, D., Demir, E., Marcos, R. and Cameán A.M.	2015	*In vitro* genotoxicity testing of carvacrol and thymol using the micronucleus and mouse lymphoma assays. Mutation Research, 784-785: 37–44. Not GLP Published	N	N	-	Public	N
CA 5.4.1/12	Aydin, S., Başaran, A.A. and Başaran, N.	2005	The effects of thyme volatiles on the induction of DNA damage by the heterocyclic amine IQ and mitomycin C. Mutation Research, 58: 43–53. Not GLP Published	N	N	-	Public	N
CA	Shibuya,	1996	Micronucleus test	Y	N	-	Public	Y

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If yes, for which data point?
5.4.2/01	T., Horiya, N., Katoh, M., Hara, T., Sekino, S., Matsuki, Y., Iida, S. and Nakagomi, M.		of thymol using mice. Hatano Research Institute, Food and Drug Safety Center, Kanagawa, Japan. Not GLP Published					IIA 5.4.2 (DAR 2011)
CA 5.4.2/02	Azirak, S. and Rencuzogullari, E.	2008	The *in vivo* genotoxic effects of carvacrol and thymol in rat bone marrow cells. Environmental Toxicology, 23(6): 728-735. Not GLP Published	Y	N	-	Public	Y IIA 5.4.2 (DAR 2011)
CA 5.4.2/03	[redacted].	2009	Induction of chromosome aberrations in the bone marrow of treated rats. [redacted] UK. Report No. 8201846. GLP Not published	Y	N	-	Eden Research plc	Y IIA 5.4.2 (DAR 2011)
CA 5.6.1/03	Haeri S., Minaie B., Amin G., Nikfar S., Khorasani R., Esmaily H., Salehnia A., Abdollahi M.	2006	Effect of *Satureja khuzestanica* essential oil on male rat fertility. Fitoterapia, 77:495-499 GLP: No Published	Y	N	n/a	Public	Y KIIA 5.6 (DAR 2011)
CA 5.7.1/01	Baldissera M., Souza C.F.,	2018	Blood-brain barrier breakdown,	Y	N	n/a	Public	N

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If yes, for which data point?
	De Matos A.F.I.M., Doleski P.H., Baldisserotto B. Da Silva A.S., Monteiro S.G.		memory impairment and neurotoxicity caused in mice submitted to orally treatment with thymol. Environmental Toxicology and Pharmacology, 62 (2018): 114–119. GLP: No Published					
CA 5.8.2/01	Viana G.S.B., Matos F.F., Araujo W.L., Matos F.J.A., Craveiro A.A.	1981	Essential oil of *Lippia grata*: pharmacological effects and main constituents. Quarterly Journal of Crude Drug Research, 19(1):1-10 GLP: No Published	Y	N	n/a	Public	Y KIIA 5.8.1 (DAR 2011)
CA 5.8.2/02	Chikhoune, A., Stouvenel, L., Iguer-Ouada, M., Hazzit, M., Schmitt, A., Lorès, P., Wolf, J.P., Aissat, K., Auger, J., Vaiman, D. and Touré, A.	2015	*In-vitro* effects of *Thymus munbyanus* essential oil and thymol on human sperm motility and function. Reproductive BioMedicine Online, 31 (3): 411-420. GLP: No Published	N	N	n/a	Public	N
CA 5.8.2/03	Bacova, K., Zitterl-Eglseer, K., Chrastinova, L.,	2020	Effect of thymol addition and withdrawal on some blood parameters, antioxidative	Y	N	n/a	Public	N

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If yes, for which data point?
	Laukova, A., Madarova, M., Gancarcikova, S., Sopkova, D., Andrejcakova, Z. and Placha, I.		defence system and fatty acid profile in rabbit muscle. Animals, Vol. 10, Issue 8, 1248. GLP: No Published					
CA 5.8.3/01a, b	Staphyt Ltd, Eden Research plc.	2021	Thymol: Assessment of endocrine disrupting properties Report no. 21/12 Not GLP Not published	N	N	-	Eden research plc	N
CA 5.8.3/02	Chen J., Ahn K.C., Gee N.A., Gee S.J., Hammock B.D., Lasley B.L.	2007	Antiandrogenic properties of parabens and other phenolic containing small molecules in personal care products Toxicology and Applied. Pharmacology 221(3):278–284 Not GLP Published	N	N	-	Public	N
CA 5.8.3/03	Michalíkova K., Linhartova L., Ezechias M., Cajthaml T.	2019	Assessment of agonistic and antagonistic properties of widely used oral care antimicrobial substances toward steroid estrogenic and androgenic receptors Chemosphere 217:534-541 Not GLP Published	N	N	-	Public	N

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities[2,3] Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used[1] Y/N If yes, for which data point?
CA 5.9.1/01	Volpe, M.J.	2021	Manufacturer Medical Report GLP: No Not published	N	N	n/a	Eden Research plc	N
CA 5.9.2/01 CA 5.9.3/01	BG Chemie	2000	Thymol Toxicological evaluation, No.259 GLP: No Published	N	N	n/a	Public	Y KIIA 5.9.2 KIIA 5.9.3 (DAR 2011)
CA 5.9.3/02	Soo Hoo GW, Hinds RL, Dinovo E, Renner SW.	2003	Fatal large-volume mouthwash ingestion in an adult: a review and the possible role of phenolic compound toxicity. J. Intensive Care Med. 2003 May-Jun; 18(3): 150-5. GLP: No Published	N	N	n/a	Public	Y Summarised as BG Chemie (2000), but not referenced

[1] In order to facilitate the compilation of the final list of the tests and studies relied upon and the corresponding data protection, indicate whether the study was used in the previous DAR/RAR or, when the information is available, whether the study was already submitted in the framework of national authorisations.
[2] See Art.3 of Annex of Regulation No 283/2013 and 284/2013
[3] The RMS shall check that the GLP statement has been properly signed in the study report, that the study results are properly reported in accordance with GLP standards and following the relevant guidance by OECD on the review of the GLP status of non-clinical safety data (currently under development).