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.9 (AS)

Rapporteur Member State : SPAIN
Co-Rapporteur Member State : GREECE

Version History

When	What
July 2022	Initial RAR-RMS Spain
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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.9. ECOTOXICOLOGY DATA

B.9.1. EFFECTS ON BIRDS AND OTHER TERRESTRIAL VERTEBRATES

B.9.1.1. Effects on birds

B.9.1.1.1. Acute oral toxicity to Birds

One acute oral avian toxicity study with Mevalone was previously evaluated as part of the EU review for the EU inclusion of thymol ((DAR, Volume 3, Annex B.9, 2011, B.9.1.1, [redacted] 2007). The data on the representative formulation, Mevalone, are sufficient to address the active substance data requirement. The full summary is provided in in Vol. 3 CP Study B.9.1.1.1/01. A LC_{50} > 10000 mg product/kg bw.(corresponding to >640 mg thymol/kg bw based on the nominal thymol content of 6.4% w/w) was calculated.
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B.9.1.1.2. Short-term dietary toxicity to birds

This study type is no longer required for the risk assessment according to the current EFSA Guidance Document on Risk Assessment for Birds and Mammals (EFSA Journal 2009; 7(12): 1438). No further data are required. Nevertheless, one acute short-term avian dietary toxicity study with Mevalone was previously evaluated as part of the EU review for the EU inclusion of thymol (DAR, Volume 3, Annex B.9, 2011, B.9.1.2), giving an acute dietary LC_{50} value >5866 mg product/kg bw/day (corresponding to >375.4 mg thymol/kg bw, based on the nominal thymol content of 6.4% w/w). However, this study was considered no valid within the peer review, therefore it cannot be used for short-term risk assessment. Furthermore, this study type is no longer required for the risk assessment and the summary has not been provided.
In addition, a literature paper was identified as part of the open literature search, assessing the effects of 15 days of feed supplementation with thymol on Japanese quail. A summary of this paper is provided below as supporting information.

Study B.9.1.1.2/01

Data point:	CA 8.1.1.2/01
Report author	Labaque, M.C., Kembro, J.M., Luna, A., Marin, R.H.
Report year	2013
Report title	Effects of thymol feed supplementation on female Japanese quail (*Coturnix coturnix*) behavioral fear response
Report No	-
Document No	Internal reference: Study 140 Animal Feed Science and Technology Volume 183, Pages 67-72 (2013)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)
Dietary supplementation with thymol has been shown to improve the oxidative stability of eggs and meat during storage. In addition, *in vitro* studies have shown that this compound can act as a positive allosteric modulator of the GABAA receptor, similarly to its analog phenolic compound propofol. Hence, it is conceivable that thymol could also present anxyolitic and/or fear reducing properties, probably also affecting their locomotor activity. Considering that fear-inducing/stressful situations are practically unavoidable during birds' rearing, the thymol feed supplementation could therefore present beneficial consequences in terms of animal welfare. This study evaluates potential fear reducing properties of thymol feed supplementation and its potential effects on locomotor activity by assessing female Japanese quail behavioral responses during brief mechanical restraint

and open field tests. Birds were evaluated after 2 and 15 days of supplementation. During the brief mechanical restraints, the latencies to struggle were significantly (P<0.05) shorter and the number of struggling bouts significantly higher (P<0.05) in the thymol group than in their Control counterparts suggesting a fear reducing effect. No effects on locomotor behavior were detected during open-field testing. The results suggest that dietary supplementation with thymol may help reduce female fear responses when birds are exposed to stressful situation without affecting the bird's locomotor activity.

Materials and methods

Test material 1

Name:	thymol
Formulation type:	Not relevant
Source and lot/batch no.:	E. Merck, Darmstadt, Germany
Active substance content:	Not relevant
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species:	Japanese quail (*Coturnix coturnix*)
Strain/clone:	Not reported
Age at study initiation:	100 days
Weight/length/height at study initiation:	257 ± 9 g (control); 244 ± 4 g (treated)
Source:	Not reported (from a population of a single 240-bird hatch)
Feeding during test:	Yes, fresh commercial feed
Acclimation:	At 28 days of age, birds were randomly housed in 28 groups of 1 male and 3 females into cages. Birds were fed a starter ration (280 g crude protein and 11.72 MJ metabolized energy/kg) and water *ad libitum*.

Test conditions

Test temperature:	23.9-26.7 ºC
Photoperiod:	14 hours light, 10 hours dark
Light intensity:	approx. 180 lux
Test system	
Study type:	Short-term dietary avian feeding test
Duration of study:	15 days
Treatments:	Dietary supplementation at 2 g thymol/kg feed (dose 80 mg of thymol/day/animal)
Analytical determination of test concentrations: No.	
Negative control included:	Yes (5 mL/L ethanol/distilled water)
Positive control included:	No
Parameters measured:	Behaviour after 2 and 15 days of thymol dietary supplementation
Validity criteria:	Not reported

Dietary supplementation with 2 g of thymol per kg of supplemented feed which is equivalent to a dose of 80 mg of thymol/day/animal and control were provided to birds. Mechanical restraint or open-field behavior of female quail from each treatment were evaluated after 2 and 15 days of thymol dietary supplementation. A one-way repeated measure ANOVA was used to determine the effects of dietary supplementation (control and thymol), the days of dietary supplementation (2 and 15 days; the repetition factor) and their interaction on the behavioral variables registered during mechanical restraint and open field tests.

Results

In the mechanical restraint test, the latency to struggle was shorter and the number of struggling bouts was higher in the thymol than in their control counterparts after both 2 and 15 days of supplementation. No further effects (P>0.05) of the thymol treatment were observed after 15 days of dietary supplementation in comparison to only 2 days of supplementation.

In the open-field locomotor activity test, no significant effects of feed supplementation treatment or days of dietary supplementation were observed on any of the variables evaluated (latency to ambulate, % time spent ambulating, distance ambulated; ambulation rate).
No significant body weight changes (P=0.98) were induced by thymol supplementation (244 ± 4 g and 248 ± 4 g, immediately before and 15 days after starting feed supplementation, respectively). Control quail counterparts did not show body weight changes either (257 ± 9 g and 253± 5 g, immediately before and 15 days after starting feed supplementation, respectively).

Assessment and conclusion
Reliability assessment
For full details and justification, please refer to Document KCA 9.4.2/02.
Proposed category: 2 reliable with restrictions
Test item purity not given, and no analytical verification of feed.

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).
Coturnix coturnix (Japanese quail) Dietary supplementation with 2 g thymol/kg feed (equivalent to 80 mg of thymol/day/animal) for 2 to 15 days shows no significant effects on body weight change. In the mechanical restraint test, the latency to struggle was shorter and the number of struggling bouts was higher in the thymol than in their control counterparts after both 2 and 15 days of supplementation. However, in the open-field locomotor activity test, no significant effects of feed supplementation treatment or days of dietary supplementation were observed on any of the variables evaluated (latency to ambulate, % time spent ambulating, distance ambulated; ambulation rate).

Assessment and conclusion by RMS:

Outcome and conclusion of the study:

This is a scientific publication to study the dietary effect of thymol as anxyolitic, fear reducing properties and locomotor activity of Japanese quail (*Coturnix coturnix).*
The test was not conducted under GLP and it does not follow any guidance.
2 g thymol/kg feed was tested, however the test analytical verification to check the actual exposure during the study was not reported.

In agreement with the applicant assessment. The study should be conisdered as **supplementary informaticon.**

B.9.1.1.3. Sub-chronic toxicity and reproduction to birds

It is noted that a data gap for further information to address the long-term risk to birds was identified during the first EU review of thymol (EFSA Journal 2012; 10(11):2916). Further information on natural background levels of thymol was provided as confirmatory data, but this was not considered sufficient by EFSA to enable a comparison between the natural background exposure and the exposure due to the use of the plant protection product (EFSA Supporting publication 2017:EN-1162).

For the renewal of thymol, a waiver is requested for long-term reproductive toxicity data to birds as further vertebrate testing is not justified. Additional weight of evidence to support this waiver is presented below.

Thymol is a naturally occurring phenol compound found in a variety of herbs and foods, particularly citrus fruit. Thymol is present in a variety of herbs including bergamot, thyme and crops such as blackberry, grapefruit, liquorice and celery seed oil. Its concentration ranged from 1 mg/kg in the leaves of bitter orange to 24100 mg/kg in common thyme and 111000 mg/kg in lemon (Table B.9.1.1.3-1).

Table B.9.1.1.3-1. Concentration of thymol in selected fruits, vegetables, herbs and spices[1]

[1] Dr Duke‘s phytochemical and ethnobotanical databases, http://www.ars-grin.gov/duke/highchem.html

Plant species	**Plant part**	**Concentration of thymol (ppm)**
Citrus limon (Lemon)	Pericarp essential oil	111,000
Trachyspermum ammi (Ajwan)	Fruit	33,000
Monarda punctata (Horsemint)	Plant	27,633
Thymus vulgaris (Common thyme)	Plant	24,100
Pycnanthemum nudum (Nude mountain mint)	Shoot	23,348
Monarda fistulosa (Wild bergamot)	Plant	20,850
Cunila origanoides (Stone mint)	Shoot	10,612
Origanum syriacum (Za'Atar)	Shoot	0 - 18, 560
Monarda citriodora (Lemon mint)	Plant	8,337
Satureja montana (Winter savory)	Plant	1,472
Origanum vulgare (Oregano)	Plant	5,000
Satureja obovata (Savory)	Leaf	0 - 7, 765
Monarda citriodora (Lemon mint)	Flower	0 - 6, 175
Origanum vulgare subspecies hirtum (Wild oregano)	Shoot	0 - 6, 165
Thymus zygis (Spanish thyme)	Shoot	0 - 5, 920
Monarda citriodora (Lemon mint)	Leaf	0 - 3, 550
Thymus zygis subspecies sylvestris (Portuguese thyme)	Shoot	350 - 3,550
Monarda russeliana (Russel's monarda)	Plant	1,752
Satureja obovata (Savory)	Leaf	0 - 2, 910
Ocimum gratissimum (Shrubby basil)	Plant	300 - 2,850
Ocimum basilicum (Sweet basil)	Leaf	1,415
Umbellularia californica	Plant	1,320
Monarda media	Plant	1,292
Thymus orospedanus (Oresped Thyme)	Plant	1,278
Thymus capitatus (Sicilian thyme)	Plant	980
Thymus longicaulis	Shoot	0 - 1, 590
Origanum sipyleum	Shoot	0 - 1, 240
Satureja obovata (Savory)	Leaf Shoot	0 - 955 50 - 850
Citrus limon (Lemon)	Leaf essential oil	400
Pycanthemum virginianum (Virginia mountain mint)	Shoot	377
Satureja cilicica (Turkish Savory)	Shoot	0 - 595
Thymus zygis (Spanish thyme)	Shoot	0 - 500
Origanum syriacum (Za' Atar)	Shoot	0 - 385
Thymus mastichina (Spanish marjoram)	Plant	190
Nepeta racemosa (Catmint)	Shoot	0 - 305
Origanum syriacum (Za' Atar)	Shoot	0 - 280
Thymus riatarum (Moroccan thyme)	Shoot	0 - 275
Origanum onites (Oregano)	Shoot	0 - 230
Micromeria juliana	Leaf	0 - 180
Origanum onites (Oregano)	Plant	86
Origanum sipyleum	Shoot	0 - 140
Origanum vulgare var. hirtum (Turkish oregano)	Plant	0 - 120
Thymus serpyllum (Creeping thyme)	Plant	57
Origanum sipyleum	Shoot	0 - 105
Monarda didyma (Oswego tea)	Plant	50
Satureja obovata (Savory)	Leaf	0 - 95
Elsholtzia pilosa (Hairy mint shrub)	Shoot	0 - 85
Origanum onites (Oregano)	Shoot	0 - 85
Satureja douglasii (Douglas' savory)	Plant	39

Plant species	**Plant part**	**Concentration of thymol (ppm)**
Satureja subspicata subsp. Liburnica (Yugoslav savory)	Plant	0 - 75
Origanum minutiflorum (Small flowered oregano)	Shoot	49 - 70
Coridothymus capitatus (Spanish oregano)	Shoot	0 - 65
Thymus x citriodorus (Lemon thyme)	Plant	0 - 50
Satureja cuneifolia (Cuneate Turkish Savory)	Shoot	0 - 45
Citrus reticulata (Tangerine)	Fruit	20
Micromeria congesta	Leaf	35 - 40
Satureja thymbra (Goat oregano)	Shoot	0 - 37
Teucrium micropodioides	Leaf	0 - 32
Mentha longifolia (Biblical mint)	Shoot	15
Thymus broussonettii (Moroccan thyme)	Shoot	0 - 20
Micromeria fructicosa subsp. Barbata (Tea Hyssop)	Shoot	0 - 19
Thymus longicaulis	Shoot	0 - 16
Calamintha nepeta subsp. Glandulosa (Turkish Calamint)	Shoot	0 - 14
Thymus capitatus (Spanish thyme)	Shoot	0 - 9
Hyptis suaveolens (Wild hops)	Shoot	0 - 8
Elsholtzia polystachya (Bush mint)	Leaf	0 - 6.6
Hyssopus officinalis	Shoot	3
Acinos suaveolens	Shoot	0 - 5
Origanum sipyleum	Shoot	0 - 5
Hyptis suaveolens (Wild hops)	Shoot	0 - 4
Citrus aurantium (Bitter orange)	Leaf	1
Teucrium polium var. valentinum	Shoot	0 - 2

In accordance with Regulation (EU) No 283/2013, a test for the effects on reproduction in birds is currently requested if adult birds or nest sites are likely to be exposed during the breeding season. Following field application of the representative formulated product, Mevalone, initial environmental exposure of thymol will decline rapidly in relation to the applied dose due to volatilisation and degradation. It is observed that the DT_{50} in soil for thymol is less than one day and the DT_{50} in air obtained from the Atkinson model is 1.197 hours (Please, see Vol. 3 CA B.8, EFSA Journal 2012;10(11):2916 for details).

Consequently, the duration of exposure under typical conditions will be very limited, particularly in relation to background levels of thymol in the environment. This is also confirmed by the results of the residue trials conducted with Mevalone on grapevines and apples (please see Vol. 3 CA B.7.3 for further details). A total of 11 trials in grapes were conducted in Northern EU countries (Austria, Germany and Northern France) and in Southern EU countries (Spain, Portugal and Italy) in 2006 and 2020. All 2020 trials were conducted according to the critical GAP for the renewal and are therefore relevant to support the use of Mevalone in the EU. In the 2020 season trials in grapes, residues of thymol were not detected or detected up to 0.01 mg/kg in the untreated control samples and not detected or detected up to 0.06 mg/kg in the treated samples. All residues of thymol in grapes had declined to the background levels found in the control samples by 7 days after the last application. Furthermore, a total of 6 trials in apples were conducted in Northern EU countries (Austria, Germany and Northern France) and in Southern EU countries (Spain, Southern France and Italy) in 2020 with an LOQ of 0.01 mg/kg. All trials were conducted according to the critical GAP for the renewal and are therefore relevant to support the use of Mevalone in the EU. Mean residues of thymol in the treated samples were <0.01 to 0.02 mg/kg on the day of application and not detected or below the LOQ of <0.01 mg/kg by 7 days after the last application of Mevalone according to the critical GAP.

This indicates that even the acute exposure will be significantly less than that estimated by the shortcut value (SV). There is thus a clear pattern of exposure with very low acute levels and very short duration so that the long-term residue burden resulting from dietary exposure after application of Mevalone will be very limited.

Following treatment, elevated residues of thymol in bird diet would be present for an extremely short period of time (a matter of hours) over the reproductive period of birds. It is considered that, given the potential for natural exposure to background levels in the diet, especially for herbivores, the exposure following a spray treatment would be negligible and does not merit the sacrifice of vertebrate animals. In contrast, the standard avian reproduction study (OECD test 206) involves at least 20 weeks of continued dietary exposure to the product. The exposure conditions of this test would bear no relation whatsoever to the potential exposure to thymol following application of Mevalone to grapevine and pome fruit.

Thymol is of low acute toxicity to birds (please see Vol. 3 CP B.9.1.1, Table 9.1.1-1). Two studies with the product Mevalone were conducted to address the acute and short-term toxicity. In the avian acute toxicity study with Mevalone and bobwhite quail the acute oral LD_{50} value was > 10000 mg product/kg bw (corresponding to > 640 mg thymol/kg bw based on the nominal thymol content of 6.4% w/w). In an 8-day dietary toxicity study (please see thymol DAR, Volume 3, Annex B.9, 2011, B.9.1.2) with Mevalone there were no deaths or reductions in feed consumption or body weight at the maximum dose tested. The dietary LD_{50} value was equivalent to 5866 mg product/kg bw/day (corresponding to > 375.4 mg thymol/kg bw based on the nominal thymol content of 6.4% w/w). However, this study was considered no valid within the peer review, therefore it cannot be used for short-term risk assessment.
An open literature paper assessing effects of dietary supplementation of thymol for up to 15 days in Japanese quail (*Coturnix coturnix*) is also summarised (Labaque *et al*., 2013; Study B.9.1.1.2/01) above as supporting information, supporting that dietary supplementation with 2 g thymol/kg feed during 2 to 15 days showed no negative effects on feed intake, body weight gain or ambulatory activity. Furthermore, thymol is recognised as a veterinary medicine product for use in all food producing species[2].
Given the characteristics of the active substance, it is highly unlikely that a short-lived substance would result in any effects on reproduction. In the interests of minimising vertebrate testing, it is not justified to conduct a new reproductive avian toxicity study for an active substance that is ubiquitous in the environment and degrades rapidly following application as a plant protection product, and of known low acute oral avian toxicity. As stated in Section 4.3 of the EFSA Guidance Document on Risk Assessment for Birds and Mammals (EFSA Journal 2009; 7 (12): 1438), the lowest of the acute $LD_{50}/10$ and the lowest NOAEL from avian reproduction studies should be used for the long-term screening assessment. Therefore, in the absence of avian reproductive toxicity data the surrogate endpoint of $LD_{50}/10$ is considered an acceptable endpoint for the long-term screening risk assessment. An acceptable reproductive risk to birds for the proposed representative uses was demonstrated at screening assessment even when considering this worst-case surrogate endpoint of $LD_{50}/10$ (Vol. 3 CP Mevalone B.9.2.1.3). Therefore, no further studies on vertebrates are considered to be necessary.

RMS comments:

No long-term toxicity data on birds is available for the active substance thymol or formulation Mevalone. Furthermore, the available short-term study with the formulation was considered as no valid within the peer review. Therefore, a data gap for further information to address the short-term and long-term risk for birds was identified during the first EU review of thymol (EFSA Journal 2012; 10(11):2916).

Since short-term study is no longer required for the risk assessment according to the current EFSA Guidance Document on Risk Assessment for Birds and Mammals (EFSA Journal 2009; 7(12): 1438), no further data are necessary to address short-term risk to birds.

The applicant has requested a waiver for long-term reproductive toxicity data to mammals based on a weight of evidence (see above).

The applicant has also presented data of residues of thymol in a range of plants other than grape or pome (0 – 111000 g/kg, in plant tissue, see Table B.9.1.1.3-1). The data was sourced from "Dr Duke's phytochemical and ethnobotanical databases" (http://www.ars-grin.gov/duke/highchem html). The database is an *ad-hoc* inventory of agriculturally and medicinally relevant plant species and information regarding their phytochemical constituents. The data presented indicates that exposure can be expected when feeding on a range of plants.

However, there are significant uncertainties regarding the reliability of these data: The data do not provided a systematic assessment of the incidence of thymol in plants. In particular, it does not identify how frequently the substances do not occur in plants relevant for the proposed use scenarios, which would result in no background

[2] The European Agency for the Evaluation of Medicinal Products. EMEA/MRL/075/96-FINAL March 1996. Committee for veterinary medicinal products: Thymol: Summary Report. https://www.ema.europa.eu/en/documents/mrl-report/thymol-summary-report-committee-veterinary-medicinal-products_en-0.pdf

exposure. The data are focused on agricultural and medicinal plants and read across to other relevant plant species has not been supported (for example grasses, acting as a food source in crop adjacent areas). Similarly, the reported residues are stated for a range of plant tissues, residues are not necessarily evenly distributed within a plant so the quoted values may not reflect a diet unless composed of the specific plant tissues sampled in the underlying studies.

The range of estimated residues indicates that the dietary exposure, assuming 100% consumption of the food items, would be between 1 – 111000 mg/kg for thymol. In most cases this would result in background exposure greater the predicted exposure following the proposed use.

This data were provided by the applicant during the confirmatory data requirement to address the long-term risk to insectivorous birds (EFSA Supporting publication 2017:EN-1163). The RMS (UK) concluded that :

"The data from "Dr Duke's phytochemical and ethnobotanical databases" indicates that thymol is present in a range of plants. And based on the reported residues under some circumstances background exposure could exceed the predicted exposure based on the proposed use. However, the deficiencies identified above mean that this information is not sufficient to establish a background exposure for birds. The confirmatory data requirements have therefore not been met, and it cannot be concluded that background exposure will exceed the exposure based on the proposed use".

Furthermore, 11 residue trials are available for grapeviens and 6 for apples . Five of the residue trials on grapevines were conducted in 2006 (Bailey, 2007 and 2008). These studies have been evaluated by the Residues section (Vol. 3 CA B.7.3.1). The study by Bailey (2008) included one residue trial conducted in Spain and it was not cover by intended GAP since only three applications were made. Furthermore, the study has been considered not to be acceptable by Residues Section. However, in the study by Bailey (2007) four residue trials were performed (2 in Spain and 2 in Italy) and they were conducted according with the proposed GAP. The study was accepted by Residues Section, but only 2 residue trials (one in Spain and one in Italy) were considered, due to the proximity of the trials, and the other two residue trials must be considered as replicates. In Baley (2007) the residues of thymol on grapes were measured at less than the LOQ (< 0.05 mg/kg), even for the highest tested concentration.

Two studies with 6 residues trials, each on grapes (Chadwick, 2021a) and pomes (Chadwick, 2021b) on SEU and CEU countrie,s were performed. The studies cover the GAP and were accepted by RMS. The mean residues of thymol in grapes were not detected or detected up to 0.01 mg/kg in the untreated control samples and not detected or detected up to 0.06 mg/kg in the treated samples.

However, the data from the residue trials does not address residues on food items other than fruit, e.g. invertebrates and foliage. They do not therefore address the risk to other 'generic focal species' identified at tier 1.

The conclusions for herbivorous birds and insectivorous birds are unaffected as no long-term endpoint are avilable. It should be noted that the data gap identified in the thymol EFSA Conclusion (EFSA Journal 2012) states that : «… the long-term risk to birds and mammals cannot be concluded on the basis of the available data, and a data gap was identified. Further information on the background levels of thymol in the environment might be useful for this purpose". Information on natural background levels of thymol was provided as confirmatory data (Thymol Addendum – Confirmatory Data, August 2016). The RMS (UK) concluded that «... *The confirmatory data requirements are not considered to have been, as it cannot be established that background exposure is greater or similar to predicted exposures.... On the basis of the confirmatory data it cannot be confirmed that additional toxicity data is not required to address the long-term toxicity of the active substance* ». EFSA agreed with RMS conclusion.

The applicant states that due to the high volatilisation (Vp = 3.4 Pa at 20º C) and the rapid degradation (DT_{50} soil 1 < day) initial environmental exposure of thymol will decline rapidly. However, these data are not sufficient to demostrate a negligible exposure. Furthermore, the confirmatory data information on natural background levels of thymol provided as confirmatory datas was not considered sufficient by EFSA to enable a comparison between the natural background exposure and the exposure due to the use of the plant protection product (EFSA Supporting publication 2017:EN-1162). Therefore, under RMS opinion a negligible exposure has not been demonstrated.

Additionally, a literature paper (Labaque *et al*., 2013; Study B.9.1.1.2/01), identified as part of the open literature search, has been included in this document, this study was considered as supporting information by the RMS. In this study, female Japanese quail birds (*Coturnix coturnix japonica*) were fed a diet supplemented with 2 g of thymol per kg of feed, equivalent to a dose of 80 mg of thymol/animal/day. In total, 21 female birds were included in the treated group and 21 female birds in the control group. The Co-RMS has indicated that the

results of this study suggest that there is an effect on the behavior of birds fed with 80 mg of thymol/animal/day even after only 2 days of administration. More specifically, the birds in the treated group exhibited significantly ($p < 0.05$) reduced stress and fear responses compared to the control during short periods of mechanical restriction after 2 and 15 days of consecutive feeding.

The co-RMS agrees with the opinion of RMS that the high volatility of thymol and its rapid degradation in soil and air are not sufficient to demonstrate a negligible exposure. Thus, it cannot be excluded that birds may experience reduced fear and anxiety from exposure to thymol in nature. In that case, reduced fear and anxiety responses could interfere with the normal behavioral activities of birds, such as the fear of predators, leading to less chances of survival. Consequently, the effects on birds' behavior shown in the dietary study by Labaque *et al.* (2013) support the need for further information on the presumable reproductive toxicity of thymol to birds.

Moreover, the applicant proposed the use of $LD_{50}/10$ as surrogate of the long-term endpoint for risk assessment since no avian reproduction study is avaiable and a low acute toxicity was observed (LD50 > mg thymol/kg bw). Birds and Mammals EFSA Guidance (2009) considers the use of the lowest acute $LD_{50}/10$ and the NOAEL from avian reproduction studies for long-term risk assessment. However, no long-term endpoint on birds for thymol is available for comparison. Therefore, the avian reproductive risk could not be considered addressed.

Under RMS opinion the weight of evidence submitted by the applicant is not sufficient to address the avian long term risk. Consequently, **further information should be submitted**.

RMS would appreciate the opinion of the CoRMS regarding the acceptability of the weight of evidence provided to the applicant to address the avian long-term risk.

B.9.1.2. Effects on terrestrial vertebrates other than birds

B.9.1.2.1. Acute oral toxicity to mammals

One acute oral toxicity study was previously evaluated as part of the EU review for the EU inclusion of thymol (DAR, Volume 3, Annex B.9, 2011, B.9.3.1). No further studies are considered necessary for ecotoxicology. The full summary is provided in the mammalian toxicology section (Vol. 3 CA B.9.6.2.1.2). The acute oral LD_{50} value for rat of 980 mg thymol/kg bw is considered valid for use in the ecotoxicological risk assessment.

B.9.1.2.2. Long-term and reproduction toxicity to mammals

It is noted that a data gap for further information to address the long-term risk to mammals was identified during the first EU review of thymol (EFSA Journal 2012; 10(11):2916). Further information on natural background levels of thymol was provided as confirmatory data, but this was not considered sufficient by EFSA to enable a comparison between the natural background exposure and the exposure due to the use of the plant protection product (EFSA Supporting publication 2017:EN-1162).

For the renewal of thymol, a waiver is requested for long-term reproductive toxicity data to mammals as further vertebrate testing is not justified. Additional weight of evidence to support this waiver is presented below.
Thymol is a naturally occurring phenol compound found in a variety of herbs and foods, particularly citrus fruit. Thymol is present in a variety of herbs including bergamot, thyme and crops such as blackberry, grapefruit, liquorice and celery seed oil ranging from 1 mg/kg in the leaves of bitter orange to 24100 mg/kg in common thyme and 111000 mg/kg in lemon (please, see Table B.9.1.1.3-1). Thymol is also used for human consumption as a food additive, as a flavouring, in dental care and in human medicine for topical treatments and for inhalation in respiratory disorders, as well as a veterinary medicine product for use in all food producing species, including horses, pigs, cattle, sheep and dogs[3].
In accordance with Regulation (EU) No 283/2013, a test for the effects on reproduction in mammals is currently requested if adult mammals are likely to be exposed during the breeding season. Following field application of the representative formulated product, Mevalone, initial environmental exposure of thymol will decline rapidly in relation to the applied dose due to volatilisation and degradation. It is observed that the DT_{50} in soil for thymol is less than one day and the DT_{50} in air obtained from the Atkinson model is 1.197 hours (Vol. 3 CA B.8, EFSA Journal 2012;10(11):2916 for details).

[3] The European Agency for the Evaluation of Medicinal Products. EMEA/MRL/075/96-FINAL March 1996. Committee for veterinary medicinal products: Thymol: Summary Report. https://www.ema.europa.eu/en/documents/mrl-report/thymol-summary-report-committee-veterinary-medicinal-products_en-0.pdf

Consequently, the duration of exposure under typical conditions will be very limited, particularly in relation to background levels of thymol in the environment. This is also confirmed by the results of the residue trials conducted with Mevalone on grapevines and apples (please Vol. 3 CA B.7.3). A total of 11 trials in grapes were conducted in Northern EU countries (Austria, Germany and Northern France) and in Southern EU countries (Spain, Portugal and Italy) in 2006 and 2020. All 2020 trials were conducted according to the critical GAP for the renewal and are therefore relevant to support the use of Mevalone in the EU. In the 2020 season trials in grapes, residues of thymol were not detected or detected up to 0.01 mg/kg in the untreated control samples and not detected or detected up to 0.06 mg/kg in the treated samples. All residues of thymol in grapes had declined to the background levels found in the control samples by 7 days after the last application. Furthermore, a total of 6 trials in apples were conducted in Northern EU countries (Austria, Germany and Northern France) and in Southern EU countries (Spain, Southern France and Italy) in 2020 with an LOQ of 0.01 mg/kg. All trials were conducted according to the critical GAP for the renewal and are therefore relevant to support the use of Mevalone in the EU. Mean residues of thymol in the treated samples were <0.01 to 0.02 mg/kg on the day of application and not detected or below the LOQ of <0.01 mg/kg by 7 days after the last application of Mevalone according to the critical GAP.

This indicates that even the acute exposure will be significantly less than that estimated by the shortcut value (SV). There is thus a clear pattern of exposure with very low acute levels and very short duration so that the long-term residue burden resulting from dietary exposure after application of Mevalone will be very limited.

Following treatment, elevated residues of thymol substances in mammal's diet would be present for an extremely short period of time (a matter of hours) over the reproductive period of mammals. It is considered that, given the potential for natural exposure to background levels in the diet, especially for herbivores, the exposure following a spray treatment would be negligible and does not merit the sacrifice of vertebrate animals. In contrast, the standard mammalian two-generation reproduction study (OECD test 416) involves at least 12 weeks of continued dietary exposure to the product. The exposure conditions of this test would bear no relation whatsoever to the potential exposure to thymol following application of Mevalone to grapevine and pome fruit.

The active substance thymol is of low acute toxicity to mammals. One acute oral toxicity study was previously evaluated as part of the EU review for the EU inclusion of thymol (DAR, Volume 3, Annex B.9, 2011, B.9.3.1). The acute oral LD_{50} was 980 mg thymol/kg b.w. (please see Vol. 3 CP Mevalone B.9.1.2, Table 9.1.2-1).

Given the characteristics of the active substance, it is highly unlikely that a short-lived substance would result in any effects on reproduction. In the interests of minimising vertebrate testing, it is not justified to conduct a new reproductive mammalian toxicity study for an active substance that is ubiquitous in the environment and degrades rapidly following application as a plant protection product, and of known low acute oral mammalian toxicity. As stated in Section 4.3 of the EFSA Guidance Document on Risk Assessment for Birds and Mammals (EFSA Journal 2009; 7 (12): 1438), the lowest of the acute $LD_{50}/10$ and the lowest NOAEL from mammalian reproduction studies should be used for the long-term screening assessment. Therefore, in the absence of mammalian reproductive toxicity data the surrogate endpoint of $LD_{50}/10$ is considered an acceptable endpoint for the long-term risk assessment. Please refer to Vol. 3 CP B.2.1.1, for the long-term risk assessment, which demonstrates an acceptable risk to mammals for the proposed representative uses even when considering this worst-case surrogate endpoint of $LD_{50}/10$. Therefore, no further studies on vertebrates are considered to be necessary.

RMS comments:

No multi-generation studies or developmental toxicity studies with the active substance thymol or formulation Mevalone are avilable. However, in accordance with Regulation (EU) No 283/2013, a test for the effects on reproduction in mammals is currently requested if adult mammals are likely to be exposed during the breeding season. Therefore, a data gap for « Further information to address the long-term risk to birds and mammals » was identified.

The applicant has requested a waiver for long-term reproductive toxicity data to mammals based on a weight of evidence (see above).

The data referenced by the notifier regarding concentrations of thymol in terrestrial plants (data presented in Table B.9.1.1.3-1) have been discussed by RMS with regard to the risk assessment for birds under the point B.9.1.1.3. All the same issues regarding suitability to establish a background exposure concentration in bird dietary food items are also relevant for the mammalian assessment. Therefore, it must be concluded that there is insufficient information to establish a background exposure concentration of thymol on the basis of this data.

The data provided does suggest that some exposure will be expected for a range of mammals with an herbivorous component to their diet, feeding on a range of plant species. It is also suggested that for some plant

tissues these residues could be considerable and under certain circunstances background exposure would exceed the predicted exposure from the proposed use.

Furthermore, the available residue trials for grapeviens and apples, discussed by RMS with regard to the risk assessment for birds under the point B.9.1.1.3, are only relevant frugiovorus mammals. The dietary exposure for other feeding guilds (insectivorous, granivorous, omnivorous...) are not cover by residue data. Furthermore, a purely frugivorous generic focal species is not considered at first tier for mammals in vineyard according to EFSA (2009). Also none of the first tier generic focal species relevant to vineyard with mixed diets include a percentage of the diet identified as 'fruit'.
The applicant states that due to the high volatilisation (Vp = 3.4 Pa at 20º C) and the rapid degradation (DT_{50} soil 1 < day) initial environmental exposure of thymol will decline rapidly. However, these data are not sufficient to demostrate a negligible exposure. Furthermore, the confirmatory data information on natural background levels of thymol provided data to address the long-term risk to mammals was not considered sufficient to allow comparison of the background exposure with the predicted exposure based on the proposed use (EFSA Supporting publication 2017:EN-1162). Therefore, under RMS opinion a negligible exposure has not been demonstrated.

A two-generation reproduction study (OECD 416) or developmental toxicity studies (OECD 414) are not available. The applicant proposed the use of $LD_{50}/10$ as surrogate of the long-term endpoint for risk assessment, however, the use of LD50/10 for mammals is not especifically envisaged by EFSA Guidance (2009). Therefore, the long-term risk to mammals could not be considered addressed. Consequently, **further information should be submitted**.
Furthermore, the Co-RMS has proposed that since Developmental toxicity studies on mammals (i.e., rat and rabbit) are available in the DAR of eugenol, May 2011, a read-across approach from the developmental toxicity data of eugenol to geraniol could be performed, since these two substances share common characteristics. The RMS has requested this information to the applicant.

RMS would appreciate the opinion of other MMSS regarding the acceptability of the weight of evidence provided to the applicant to vaiwe the reproductive toxicity data to mammals.

Additioinally, considering that developmental toxicity studies on mammals (i.e., rat and rabbit) are available in the DAR of eugenol, May 2011, the Co-RMS proposed a read-across approach from the developmental toxicity data of eugenol to thymol since these two substances share common characteristics. The RMS has request to the applicant the read-across approach.

B.9.1.3. Active substance bioconcentration in prey of birds and mammals

The log P_{ow} values for thymol are 3.43, 3.44 and 3.41 at pH values 4,7 and 9, respectively (please see Vol. 3 CA B.2 for details) and thus just exceed the trigger value of 3, requiring consideration of the risk to earthworm-eating and fish-eating birds and mammals due to secondary poisoning. However, due to its rapid volatilisation properties and ready biodegradation it is considered unlikely that thymol will be persistent and accumulate in soil or natural water systems. Since thymol is of very low persistence in soil and water, accumulation in worms and fish and exposure of birds and mammals from consumption of these is considered unlikely. According to the EFSA Conclusion Report for thymol (EFSA Journal 2012; 10(11):2916), for bioaccumulation "…it was considered that bioaccumulation in fish is unlikely", and therefore no studies are required. Furthermore, in line with Annex I, Section 4.1.2.8.1 of the CLP Regulation[4], these log P_{ow} values are less than the CLP cut-off criteria of 4, indicating thymol does not show potential to bioaccumulate. Whilst bioconcentration of thymol in prey of birds and mammals is considered unlikely, for completeness conservative secondary poisoning assessments are presented in Vol. 3 CP Mevalone B.9.2.1 and B.9.2.2, using worst-case initial PEC values, worst-case surrogate reproductive endpoints of $LD_{50}/10$ and QSAR-estimated BCF_{fish} values. Acceptable risks to birds and mammals due to secondary poisoning are concluded for the proposed representative uses, even when considering these worst-case parameters.

B.9.1.4. Other data on effects on terrestrial vertebrate wildlife (birds, mammals, reptiles and amphibians)

[4] Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006

The risk assessments for birds and mammals performed according to current EU guidance are presented in Vol. 3 CP Mevalone B.9. At the time of this submission there is no agreed guidance on the requirement for further data or risk assessments on other terrestrial vertebrate wildlife (including reptiles and amphibians). However, as stated in the guidance document for aquatic organisms (EFSA Journal 2013;11(7):3290 Section 7.2.4.) the rainbow trout *Oncorhynchus mykiss* is a good surrogate test species for predicting the acute toxicity of plant protection products for larval stages of amphibian species living in the aquatic compartment of the environment. Using the rainbow trout *Oncorhynchus mykiss* as a surrogate test species for predicting the toxicity of thymol for larval stages of amphibian species a low toxicity can be concluded (B.9.2.1). No relevant data on adverse effects from thymol to other terrestrial wildlife were found during the open literature search. No further data are available nor considered necessary.

B.9.1.5. Potential for endocrine disruption

The available data for thymol do not show any adverse effects that are considered to be EAS-mediated. However, there are no available guideline studies with thymol on fisth to investigate the potential of endocrine disruption of thymol. The available toxicology (mammalian) and ecotoxicology data for thymol showed that the potential for endocrine disruption have not been sufficiently investigated, therefore, further data need to be generated before a conclusion on whether or not the ED criteria are met (Vol. 1, 2.10).

B.9.2. EFFECT ON AQUATIC ORGANISMS

B.9.2.1. Acute toxicity to fish

Two acute fish toxicity studies with thymol were previously evaluated as part of the EU review for the EU inclusion of thymol (DAR, Volume 3, Annex B.9, 2011, B.9.2.1.1). These studies are considered appropriate for the current assessment to support renewal of thymol; full summaries are provided below (Studies B.9.2.1/01 and B.9.2.1/02).
It is also noted that three literature papers were identified during the literature search (B.11.1), which assessed acute effects on various fish species after either 24 hours (CA 9.6.3.4/02) or assessing the very short-term anaesthesia effects of thymol and subsequent recovery (CA 9.6.3.4/22; CA 9.6.3.4/31). Results of these papers are summarised in the Appendix I for completeness, but these are not considered reliable for use in the risk assessment, principally as analytical verification was not reported to confirm the actual exposure during the studies.

Study B.9.2.1/01

Data point:	CA 8.2.1/01
Report author	[redacted]
Report year	2008a
Report title	Acute toxicity of thymol crystals to rainbow trout (*Oncorhynchus mykiss*) in a 96-hour semi-static test
Report No	34281230
Document No	-
Guidelines followed in study	OECD Guideline 203 (1992) EPA Guideline 712-C-96-118: OPPTS 850.1075 (1996)
Deviations from current test guideline	None
Previous evaluation	Yes, evaluated and accepted in the DAR (Thymol, Volume 3, Annex B.9, 2011, B.9.2.1.1) EU-agreed critical endpoint for EU inclusion of thymol (EFSA Journal 2012;10(11):2916)
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary
The 96-hour acute toxicity of thymol to *Oncorhynchus mykiss* was studied under semi-static conditions in accordance with OCED 203. Juveniles of rainbow trout were exposed to thymol at nominal concentrations of 0.625, 1.25, 2.5, 5 and 10 mg thymol/L for 96 hours. Mortality and sublethal effects were observed daily and at

test termination. Analytical verification of test concentrations confirmed samples were 63 – 88% of nominal. Biological results were expressed based on mean measured concentrations.
The 96-hour LC_{50} value for rainbow trout (*Oncorhynchus mykiss*) was calculated to be 3.0 mg thymol/L based on mean measured concentrations. The corresponding NOEC value based on sublethal symptoms was 1.06 mg thymol/L, based on mean measured concentrations. The sublethal effects included tumbling, convulsions, dark coloration, apathy and lateral position.
This study is considered as acceptable and satisfies the guideline requirements in place at the time the study was conducted (OECD test guideline 203, 1992), and also meets the validity criteria in the more recent OECD test guideline 203 update of 2019.

Materials and methods

Test material

Name:	thymol crystals
Source and lot/batch no.:	94747
Active substance content:	99.7% w/w
Expiry date of lot/batch:	March 2008
Storage conditions:	In original container at room temperature (+10 ºC to + 30 ºC, in the dark.

Test organism

Species:	Rainbow trout (*Oncorhynchus mykiss)*
Age at study initiation:	Juveniles
Weight/length at study initiation:	1.23 ± 0.24 g /5.23 ± 0.21 cm (mean ± SD)
Source:	The test fish were obtained from [redacted] Germany.
Feeding during test:	No
Acclimation:	For at least 12 days before the start of the test. The fish were fed with a commercial fish diet food until one day before the start of the test. During the last 7 days prior to test start no fish died in the test fish batch and all fish were healthy.

Test conditions

Hardness:	250 mg/L as calcium carbonate
Test temperature:	13 – 14 ºC
pH:	7.7 – 7.9
Dissolved oxygen:	at least 60% of the air saturation (actual values ≥ 91% (9.1 mg O_2/L))
Photoperiod:	16 h light: 8 h dark
Conductivity:	< 5 µS/cm
Light intensity:	453-552 lux

Groups of seven rainbow trout (*Oncorhynchus mykiss*) were exposed to thymol at nominal concentrations of 0.625, 1.25, 2.5, 5 and 10 mg thymol/L for 96 hours in a semi-static system (22 L glass aquaria) under 16 hours light (453 to 552 lux) and 8 hours dark per day. There was also a control of test medium only, i.e. reconstituted water and a solvent control containing 0.1 mL/L. The test media were renewed after 48 hours. The temperature, which was measured daily in all test units, ranged between 13 and 14ºC and the dissolved oxygen, which was measured in each vessel every 24 hours, was ≥ 91%. Test concentrations were analysed at the start and the end of each renewal period. Mortality and other observations were made after 2, 24, 48, 72 and 96 hours. Statistical analysis was performed. EC_{50} values and 95% confidence limits were calculated, where possible, by Probit analysis. The NOEC and LOEC values were determined directly from the raw data.

Results

Analytical results

The GS-MS analytical method for the determination of thymol in test medium was validated with regards to specificity, linearity, accuracy and precision. Whilst the report does not refer to the current guideline, overall the validation results are in accordance with guideline SANCO/3029/99 rev. 4, 11/07/2000 and are considered fit for purpose. Specificity was demonstrated by the absence of a peak at the characteristic retention time for thymol in the control sample. The analytical calibration was shown to be linear (r = 0.9952) over the range of 0.2 to 6 mg reference item/L. Accuracy was confirmed with recovery determined by fortification of thymol at 1, 2 and 10 mg test reference/L; all recoveries were within the range of 85 – 110% and mean recoveries were within 91 – 107% (i.e. within the guideline range of 70 – 110%). Precision was confirmed with at least four determinations made at each fortification level (1, 2 and 10 mg reference item/L); the relative standard deviation was between 2 – 6% (i.e. within the guideline limit of ≤20%). The limit of quantification (LOQ) was 1 mg thymol/L (i.e. below the biological LC_{50}/NOEC value). The limit of detection (LOD) was 0.05 mg thymol/L in test medium.
A summary of the measured concentrations of thymol in the test media is presented in the tables below.

Table 9.2.1/01-1: Nominal and measured concentrations of thymol in each replicate

Nominal conc. (mg thymol/L)	Replicate	Measured conc. (mg thymol/L)			Percentage of nominal		
		0 h	48 h	Mean*	0 h	48 h	Mean
Water control	1	<LOD	<LOD	n.a.	n.a.	n.a.	n.a.
	2	<LOD	<LOD		n.a.	n.a.	n.a.
Solvent control	1	<LOD	<LOD	n.a.	n.a.	n.a.	n.a.
	2	<LOD	<LOD		n.a.	n.a.	n.a.
0.625	1	<LOQ	<LOQ	n.a.	n.a.	n.a.	n.a.
	2	<LOQ	<LOQ		n.a.	n.a.	
	3	<LOQ	<LOQ		n.a.	n.a.	
	4	<LOQ	<LOQ		n.a.	n.a.	
1.25	1	1.079	1.035	1.065	86	83	85
	2	1.055	1.031		84	82	
	3	1.092	1.082		87	87	
	4	1.102	1.042		88	83	
2.5	1	1.868	1.576	1.805	75	63	72
	2	1.809	1.627		72	65	
	3	1.830	1.971		73	79	
	4	1.835	1.924		73	77	
5	1	3.662	3.540	3.730	73	71	75
	2	3.711	3.459		74	69	
	3	3.713	3.909		74	78	
	4	3.814	4.030		76	81	
10	1	7.592	7.662	7.694	76	77	77
	2	7.846	7.674		78	77	
	3	n.a.	n.a.		n.a.	n.a.	
	4	n.a.	n.a.		n.a.	n.a.	

LOQ = 1 mg thymol/L.
LOD = 0.05 mg thymol/L
* Arithmetic mean of the 4-8 measurements for each treatment group (duplicate samples at 0 and 48 hours)
n.a.: not applicable

Table 9.2.1/01-2: Summary of nominal and mean measured concentrations of thymol over 96-hour test

	Measured concentration (mg thymol/L)						
Nominal conc. (mg thymol/L)	Water control	Solvent control	0.625	1.25	2.5	5	10
Range (min: max)	n.a	n..a	n.a.	1.031-1.102	~~15.2-20.0~~ 1.58-2.0	~~17.0-38.3~~ 3.5-4.0	~~23.9-75.6~~ 7.6-7.8
Median	n.a	n..a	n.a.	1.067	1.832	3.712	7.668
Mean*	n.a	n..a	n.a.	1.065	1.805	3.730	7.694
% of nominal (ref. to mean)	n.a	n..a	n.a.	85.2	72.2	74.6	76.9

* arithmetic mean of the 4-8 measurements for each treatment group (duplicate samples at 0 and 48 hours)
Conc. concentration
n.a.: not applicable

The measured concentrations of the samples of nominal concentrations of 0.625 mg thymol/L were below the NOEC and below the Limit of Quantification. At the start of the test and after renewal of the test media 78% of the nominal test concentrations were found (average for the test concentrations 1.25, 2.5, 5 and 10 mg thymol/L). In the aged test media 77% of the nominal values were found (average for the test concentrations 1.25, 2.5, 5 and 10 mg thymol/l.). Thus, during the test period the fish were exposed to an overall mean measured concentration of 77% of nominal (average for the test concentrations 1.25, 2.5, 5 and 10 mg thymol/L). Thus, results were expressed based on mean measured concentrations.

Biological results
A summary of the effects of thymol on immobilisation of *Oncorhynchus mykiss* after 96 hours exposure is presented in the table below.

Table 9.2.1/01-3: Effect of thymol on mortality of rainbow trout for 96 hours

Nominal conc. (mg thymol/L)	**Mean measured conc. (mg thymol/L)**	**No. organisms at test start**	**Cumulative mortality (no. dead)**				**Cumulative mortality (%)**			
			24 h	**48 h**	**72 h**	**96 h**	**24 h**	**48 h**	**72 h**	**96 h**
Water control	-	7	0	0	0	0	0	0	0	0
Solvent control	-	7	0	0	0	0	0	0	0	0
0.625	<LOQ	7	0	0	0	0	0	0	0	0
1.25	1.065	7	0	0	0	0	0	0	0	0
2.5	1.805	7	0	0	0	0	0	0	0	0
5	3.730	7	5	6	6	6	71	86	86	86
10	7.694	7	7	7	7	7	100	100	100	100

Conc. concentration
n.a.: not applicable

No mortality or sublethal effects were observed in the controls or at nominal test concentrations up to 2.5 mg thymol/L. Sublethal symptoms were observed at nominal test concentrations of 2.5 mg thymol/L and above. The sublethal effects observed at nominally 2.5 mg thymol/L were tumbling during swimming (observed in 29% of fish at 96 hours only), and additional effects observed after 2 hours or more of exposure at nominally 5 mg thymol/L and higher included convulsions, dark coloration, apathy and lateral position. At nominal test concentrations of 5 and 10 mg thymol/L, 86% and 100% mortality, respectively, were recorded after 96 hours. The 96-hour LC_{50} value for thymol to *Oncorhynchus mykiss* was calculated to be 3.0 mg thymol/L and the NOEC was 1.06 mg thymol/L, based on mean measured concentrations.

Validity
All validity criteria were met in accordance with OECD test guideline 203 (1992 and current 2019):
- In the control, the mortality did not exceed one fish at the end of the test. (Actual values: no fish died during the test control or the solvent control).
- The dissolved oxygen concentration in the test media was above 60% of air saturation value during the test (actual values above 91 %).

- Analytical measures were conducted.

Assessment and conclusion

The 96-hour acute toxicity of thymol to *Oncorhynchus mykiss* was studied under semi-static conditions in accordance with OECD 203. The 96-hour LC_{50} value for thymol to *Oncorhynchus mykiss* was calculated to be 3.0 mg thymol/L and the NOEC value was 1.06 mg thymol/L, based on mean measured concentrations. The sublethal effects included were tumbling, dark coloration, apathy, mucous secretion, strong ventilation, distended abdomen and lateral position. This study is considered as acceptable and satisfies the guideline requirements for an acute toxicity study with fish.

Assessment and conclusion by applicant:

The study is acceptable.

Oncorhynchus mykiss 96-hour LC_{50} (mortality) = 3.0 mg thymol/L based on mean measured concentrations.

Assessment and conclusion by RMS:

Outcome and conclusion of the study:

The 96-hour acute toxicity of thymol to *Oncorhynchus mykiss* was studied under semi-static conditions in accordance with OCED 203 (1992).

The range of light intensity applied to the study (453-552 lux) surpassed in 87 lux the lower limit of the range recommended in the OECD test guideline 203 update of 2019 (540-1000 lux). However this little deviation probably does not affect the results.

In table 9.2.1/01-2 there is an error in the range of the measured concentrations 2.5, 5 and 10. The values do not match with those found in the study report and they are expressed in a superior magnitud order. However, the mean, median and the percentages of nominal concentrations match those in the KCA document. The RMS has corrected the values of the range of the measure concentration on Table 9.2.1/01-2 (Please, see table above) according to the study report.

The minimum nominal concentration used (0.625 mg thymol/L) is lower than the LOQ. However, this was not considered an important issue because no mortality or sublethal effects were observed in the controls or at nominal test concentrations up to 2.5 mg thymol/L.

Results were expressed based on mean measured concentrations because most of the measured concentrations were lower than 80 % of the nominal.

All validity criteria were met in accordance with OECD test guideline 203 (update 2019):

- In the control, the mortality did not exceed one fish at the end of the test.
- The dissolved oxygen concentration in the test media was above 60% of air saturation value during the test.
- Analytical measures were conducted.

For all the above mentioned reasons, RMS agrees with the previous evaluation.

The study is considered acceptable.

Agreed endpoints (Based on mean measured concentration):

96h LC_{50} (mortality) = 3.0 mg thymol/L (95% CI 2.4-3.7)
NOEC = 1.06 mg thymol/L

Study B.9.2.1/02

Data point:	CA 8.2.1/02
Report author	[redacted]
Report year	2008b
Report title	Acute toxicity of thymol crystals to Zebra fish (*Danio rerio*) in a 96-hour semi-static test
Report No	34282230
Document No	-
Guidelines followed in study	OECD Guideline 203 (1992) EPA Guideline 712-C-96-118: OPPTS 850.1075 (1996)
Deviations from current test guideline	None
Previous evaluation	Yes, evaluated and accepted in the DAR (Thymol, Volume 3, Annex B.9, 2011, B.9.2.1.1)
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

The 96-hour acute toxicity of thymol to *Danio rerio* was studied under semi-static conditions in accordance with OECD 203. Juveniles of zebra fish were exposed to thymol at nominal concentrations of 2.5, 5.0, 10, 20 and 40 mg thymol/L for 96 hours. Mortality and sub-lethal effects were observed daily and at test termination. Analytical verification of test concentrations confirmed measured concentrations were maintained within 86 – 99% of nominal throughout the exposure period and biological endpoints were based on nominal concentrations. The 96-hour LC_{50} value was calculated to be 7.1 mg thymol/L based on nominal concentrations. The corresponding NOEC value was 2.5 mg thymol/L, based on nominal concentrations. The sub-lethal effects observed included tumbling during swimming, convulsions, apathy and fish lying on side or back.
This study is considered as acceptable and satisfies the guideline requirements in place at the time the study was conducted (OECD test guideline 203, 1992), and also meets the validity criteria in the more recent OECD test guideline 203 update of 2019.

Materials and methods

Test material

Name: thymol crystals
Source and lot/batch no.: 94747
Active substance content: 99.7% w/w
Expiry date of lot/batch: March 2008
Storage conditions: In original container at room temperature (+10 ºC to + 30 ºC, in the dark.

Test organism

Species: Zebra fish (*Danio rerio)*
Age at study initiation: Juveniles
Weight/length at study initiation: 0.21 g ± 0.07/2.35cm ± 0.30 (mean ± SD)
Source: The test fish were obtained from profi aquarium [redacted], Germany.
Feeding during test: No
Acclimation: 12 days before the start of the test. The fish were fed with a commercial fish diet food one day before the start of the test. During the last 7 days prior to test start no fish died in the test fish batch and all fish were healthy.

Test conditions

Hardness: 250 mg/L as calcium carbonate

Test temperature:	22– 24 ºC
pH:	7.7 – 8.0
Dissolved oxygen:	at least 60% of the air saturation (actual values at least 7.6 mg/L; equivalent to 90% at 22 °C, sea level, 1 atm)
Photoperiod:	16 h light: 8 h dark
Conductivity:	<5 µS/cm
Light intensity:	451 – 559 lux

Groups of seven Zebra fish (*Danio rerio*) were exposed to thymol at nominal concentrations of 2.5, 5.0, 10, 20 and 40 mg thymol/L for 96 hours in a semi-static system (22 L glass aquaria) under 16 hours light (200 to 1200 lux) and 8 hours dark per day. There was also a control of test medium only, i.e. reconstituted water and a solvent control containing 0.1 mL/L. The test media were renewed after 48 hours. The temperature, pH and dissolved oxygen were measured daily in all test units. The temperature values ranged between 21 and 25 ºC. The dissolved oxygen concentration in the test media did not fall below 60% of air saturation.

Test concentrations were analysed at the start and the end of each renewal period, except where all test fish were dead at a particular test concentration where aged samples were taken at the time of observation. Mortality and other observations were made after 2, 24, 48, 72 and 96 hours. Statistical analysis was performed using ToxRat Professional v2.09. EC_{50} values and 95% confidence limits were calculated, where possible, by Probit analysis. The NOEC and LOEC values were determined directly from the raw data.

Results

Analytical results

The GS-MS analytical method for the determination of thymol in test medium was validated with regards to specificity, linearity, accuracy and precision. Whilst the report does not refer to the current guideline, overall the validation results are in accordance with guideline SANCO/3029/99 rev. 4, 11/07/2000 and are considered fit for purpose. Specificity was demonstrated by the absence of a peak at the characteristic retention time for thymol in the control sample. The analytical calibration was shown to be linear (r = 0.9965) over the range of 0.5 to 6 mg reference item/L. Accuracy was confirmed with recovery determined by fortification of thymol at 2, 10 and 40 mg test reference/L; all recoveries were within the range of 94 – 107% and mean recoveries were within 97 – 107% (i.e. within the guideline range of 70 – 110%). Precision was confirmed with four determinations made at each fortification level (2, 10 and 40 mg reference item/L); the relative standard deviation was between 1 – 3% (i.e. within the guideline limit of ≤ 20%). The limit of quantification (LOQ) was 2 mg thymol/L (i.e. below the biological LC_{50}/NOEC value). The limit of detection (LOD) was 0.02 mg thymol/L in test medium.

A summary of the measured concentrations of thymol in the test media is presented in the tables below.

Table 9.2.1/02-1: Nominal and measured concentrations of thymol in each replicate

Nominal conc. (mg thymol/L)	Replicate	Measured conc. (mg thymol/L)			Percentage of nominal		
		0 h	**48 h**	**Mean***	**0 h**	**48 h**	**Mean**
Water control	1	<LOD	<LOD	n.a.	n.a.	n.a.	n.a.
	2	<LOD	<LOD		n.a.	n.a.	n.a.
Solvent control	1	<LOD	<LOD	n.a.	n.a.	n.a.	n.a.
	2	<LOD	<LOD		n.a.	n.a.	n.a.
2.5	1	2.450	2.346	2.386	98	94	95
	2	2.386	2.416		95	97	
	3	2.387	2.295		95	92	
	4	2.487	2.320		99	93	
5	1	4.585	4.366	4.573	92	87	91
	2	4.681	4.314		94	86	
	3	4.832	4.472		97	89	
	4	4.838	4.493		97	90	
10		**0 h**	**24 h[a]**	9.017	**0 h**	**24 h[a]**	90
	1	9.109	9.005		91	90	
	2	9.090	8.864		91	89	
20		**0 h**	**2 h[a]**	18.939	**0 h**	**2 h[a]**	95
	1	18.775	18.130		94	91	
	2	18.968	19.882		95	99	
40		**0 h**	**2 h[a]**	38.941 [b]	**0 h**	**2 h[a]**	98[b]
	1	21.176 [b]	38.734		53 [b]	97	
	2	29.901 [b]	39.149		75 [b]	98	

LOQ = 2 mg thymol/L
LOD = 0.02 mg thymol/L
* Arithmetic mean of the 4 -8 measurements for each treatment group (duplicate samples of fresh and aged samples).
n.a.: not applicable
[a] All test fish were dead at this time point s aged samples were taken at the time of observation
[b] mean values calculated for samples from sampling time 2 hours, since test media sampling time 0 hour considered to be inhomogeneous

Table 9.2.1/02-2: Summary of nominal and mean measured concentrations of thymol over 96-hour test

	Measured concentration (mg thymol/L)						
Nominal conc. (mg thymol/L)	Water control	Solvent control	2.5	5	10	20	40
Range (min: max)	n.a	n..a	2.320-2.487	4.314-4.838	8.864-9.109	18.130-19.882	21.176-39.149
Median	n.a	n..a	2.387	4.539	9.048	18.87	38.94[b]
Mean*	n.a	n..a	2.386	4.573	9.017	18.939	38.941[b]
% of nominal (ref. to mean)	n.a	n..a	95	91	90	95	98[b]

* arithmetic mean of the 4-8 measurements for each treatment group (duplicate samples of fresh and aged samples)
[b]average values calculated for samples from sampling time 2 hours, since test media sampling time 0 hour considered to be inhomogeneous
n.a.: not applicable
The measured concentrations in all fresh and aged samples were maintained within 86-99% of nominal values (with the exception of the fresh solutions at the highest test concentration which are not considered reliable due to inhomogeneity, but were confirmed to be within 97-98% of nominal values after 2 hours). Therefore, biological endpoints are reported based on nominal concentrations.

Biological results

A summary of the effects of thymol on mortality of *Danio rerio* after 96 hours exposure is presented in the table below.

Table 9.2.1/02-3: Effect of thymol on mortality of Zebra fish for 96 hours

Nominal conc. (mg thymol/L)	No. organisms at test start	Cumulative mortality (no. dead)				Cumulative mortality (%)			
		24 h	48h	72 h	96 h	24 h	48 h	72 h	96 h
Water control	7	0	0	0	0	0	0	0	0
Solvent control	7	0	0	0	0	0	0	0	0
2.5	7	0	0	0	0	0	0	0	0
5.0	7	0	0	0	0	0	0	0	0
10	7	7	7	7	7	100	100	100	100
20	7	7	7	7	7	100	100	100	100
40	7	7	7	7	7	100	100	100	100

In the controls and in nominal test concentrations of 2.5 and 5.0 mg thymol/L all fish survived until the end of the test. Distinct sub-lethal symptoms (convulsions, apathy and reduced swimming behaviour) were observed at 5.0 mg thymol/L. At 2.5 mg thymol/L symptoms of reduced swimming behaviour were recorded for all fish at the 48 hours assessment, but not at any other assessment. 100% mortality was observed at 10 mg thymol/L and at the higher test concentrations after 24 hours. The 96-hour LC_{50} value for thymol to *Danio rerio* was calculated to be 7.1 mg thymol/L and the NOEC value was 2.5 mg thymol/L, based on nominal concentrations.

Validity

All validity criteria were met in accordance with OECD test guideline 203 (1992 and current 2019):

- In the control, the mortality did not exceed one fish at the end of the test. (Actual values: no fish died during the test in control or solvent control)

- The dissolved oxygen concentration in the test media was above 60% of air saturation value during the test (actual values ≥7.6 mg/L, equivalent to 90%, at 22 °C, sea level, 1 atm).

Assessment and conclusion

The 96-hour acute toxicity of thymol to *Danio rerio* was studied under semi-static conditions in accordance with OECD 203. The 96-hour LC_{50} value was calculated to be 7.1 mg thymol/L based on nominal concentrations. The 96-hour NOEC value based on sub-lethal adverse effects was determined to be 2.5 mg thymol/L based on nominal concentrations. The sub-lethal effects included were tumbling during swimming, convulsions, apathy and fish lying on side or back. This study is considered as acceptable and satisfies the guideline requirements for an acute toxicity study with fish.

Assessment and conclusion by applicant:

The study is acceptable.
Danio rerio 96-hour LC_{50} (mortality) = 7.1 mg thymol/L based on nominal concentrations.

Assessment and conclusion by RMS:

Outcome and conclusion of the study:

The 96-hour acute toxicity of thymol to *Danio rerio* was studied under semi-static conditions in accordance with OECD 203 (1992).

Biological endpoints were based on nominal concentrations because measured concentrations were within 86 – 99% of nominal concentrations throughout the exposure period.

The range of light intensity applied to the study (200-1200 lux) surpassed in 200 lux the lower limit of the range recommended in the OECD test guideline 203 update of 2019 (540-1000 lux). However, this little deviation probably does not affect the results.

All validity criteria were met in accordance with OECD test guideline 203 (update 2019):

- In the control, the mortality did not exceed one fish at the end of the test
- The dissolved oxygen concentration in the test media was above 60% of air saturation value during the test

For all the above mentioned reasons, RMS agrees with the previous evaluation.

The study is considered acceptable.

Agreed endpoints (based on nominal concentration):

96h LC_{50} (mortality) = 7.1 mg thymol/L (95% CI 6.8-7.4)

NOEC = 2.5 mg thymol/L

B.9.2.2. Long-term and chronic toxicity to fish

It is noted that a data gap for further information to address the chronic risk to aquatic organisms was identified during the first EU review of thymol (EFSA Journal 2012; 10(11):2916). Further information on natural background levels of thymol was provided as confirmatory data, but this was not considered sufficient by EFSA to enable a comparison between the natural background exposure and the exposure due to the use of the plant protection product (EFSA Supporting publication 2017:EN-1162).

For the renewal of thymol, a waiver is requested for long-term toxicity data to fish as further vertebrate testing is not justified. Additional weight of evidence to support this waiver is presented below.
Significant long-term exposure of thymol in surface waters is not expected due to its rapid volatilisation properties and ready biodegradation. Following field application of the representative formulated product, Mevalone, initial environmental exposure of thymol will decline rapidly in relation to the applied dose. According to the acute toxicity endpoints obtained from EFSA Journal 2012; 10(11):2916, it is observed that fish are no more sensitive to thymol or the representative product, Mevalone, than *Daphnia magna* (acute LC_{50} and EC_{50} values for fish and *Daphnia* are in same order of magnitude). Furthermore, the acute toxicity endpoints for fish and Daphnia magna are in the same order of magnitude (LC_{50} = 3.0 mg thymol/L and EC_{50} = 4.9 mg thymol/L, respectively for fish and aquatic invertebrates).

Therefore, no new long-term toxicity studies have been carried out for fish to waive further vertebrate testing during active substance renewal. The new long-term study with *Daphnia magna* (Study B.9.2.5.1/01) is expected to be sufficient to address the long-term toxicity for aquatic organisms. The Applicant recognizes the acue endpoint for fish is slightly lower than the toxicity value of Daphnia. However, both endpoints are in the same order of magnitude, so it can be excluded differences in sensitivity between both taxonomic groups. As a realistic worst-case, it can be assumed the chronic toxicity to aquatic invertebrates can also covered the toxicity of fish.

It is also important to note that for thymol, three literature papers were identified as supplementary information during the literature search. These studies assess long-term effects on different fish species for dietary supplementation with thymol (CA 9.6.3.4/05; CA 9.6.3.4/28; and CA 9.6.3.4/29). Based on the results of the

study CA 9.6.3.4/05, thymol enhances hepatic metabolism and enhances productive and reproductive performance and improves bioavailability of nutrients in grass carp. Fishes were fed with thymol up to 300 mg thymol/kg feed for 60 days, and no harmful effects were found. In the study CA 9.6.3.4/28, Nile tilapia was fed with thymol for 63 days with nominal concentration of 500 ppm thymol. It was concluded that the use of thymol did not affect the survival or growth of fish after 63 days. In the study CA 9.6.3.4/29, Nile tilapia fingerlings were fed with thymol for 75 days. The results of the study showed that dietary supplementation of thymol at 1 and 2 mL thymol/kg diet (nominal) for 75 days improved the growth performance, the antioxidant status and the immunity of the Nile tilapia fingerlings.

From these three literature papers, it can be concluded that no adverse effects of thymol on fish are expected after chronic dietary exposure. This line of evidence also supports a low chronic toxicity of Thymol to fish.

It is also important to note that the chronic endpoint for Daphnia magna is likely to be overestimated, since this test was performed under semi-static conditions with three renewals of test medium per week. Please note that the number of intended applications is 4 treatments with an interval of 7 days, a much lower number than the renewals conducted in the chronic study.

According to the Regulation 283/2013 the long-term and chronic toxicity study on fish should be provided since thymol was considered hydrolytically stable at pH 4 and 7. However, it is important to note the current legislation do not consider other relevant routes of degradation/dissipation to trigger the chronic data requirement to aquatic organisms. Please note the main route of dissipation of thymol is via volatilization (DT_{50} in air obtained from the Atkinson model is 1.197 hours).

Considering all the arguments provided above and in the interests of minimizing vertebrates testing, the Applicant thinks a FELS study would not be scientifically justified.

RMS Comments:

No long-term toxicity data on fish is available for the active substance thymol or formulation Mevalone. Therefore, a data gap for further information to address the acute and chronic risk to aquatic organisms was identified during the first EU review of thymol (EFSA Journal 2012; 10(11):2916).

Further information on natural background levels of thymol was provided as confirmatory data, but this was not considered sufficient by EFSA to enable a comparison between the natural background exposure and the exposure due to the use of the plant protection product (EFSA Supporting publication 2017:EN-1162).

The applicant request a waiver for long-term toxicity data to fish since a new long-term study with *Daphnia magna* (Study B.9.2.5.1/01) is available. However, under RMS opinion an uncertainty occurs regarding whether the chronic toxicity data for aquatic invertebrates are sufficient to address the long-term risk to fish since, the acute endpoint of fish (LC_{50} = 3.0 mg thymol/L) is slightly lower than *Daphnia* acute endpoint (EC_{50} = 4.9 mg thymol/L), although both are in the same order of magnitude.

Moreover, RMS respectfully thinks that the chronic endpoint for *Daphnia* is not overestimated, since the study has been conducted according to OECD TG 211 (*Daphnia magna* reproduction test). In this test the endpoint should be set with a stable test substance concentration (i.e. in the range 80 - 120% of nominal or falling below 80% of the measured initial concentration). Furthermore, the guideline states that the frequency of medium renewal will depend on the stability of the test substance, but should be at least three times per week over the maximum renewal period (i.e. 3 days). Therefore, the endpoints is calculated according to the current guidelina and it is not overstimated. Additionally, in PECsw/sed modelling the intended pattern of application is taking into account.

Furthermore, the literature papers included in Vol. 3 CA B.9, Appendix I, were considered of limited reliability by the applicant and so they were not considering further in the corresponding data point. They were included in the monography by RMS, for transparency. Therefore, the RMS respectfully highlights that they were not considered supplementary information.

In particular, the literature studies cited (CA 9.6.3.4/05; CA 9.6.3.4/28; and CA 9.6.3.4/29) were assessed by the applicant and were considered as no reliable and not accepted. Therefore, under RMS opinion the conclusions of these studies cannot be used to support a low chronic tocicity of thymol on fish.

According to the Regulation 283/2013 the long-term and chronic toxicity study on fish is a data requirement. The study should be provided unless it is proved that the substance is stable in water, that is to say there is less than 90% loss of the original substance over 24 hours via hydrolisis. Since, the thymol was considered hydrolytically stable at pH 4 and 7 (Vol. 3 CA Study B.8.2.1.1/01 ; Kelly 2021) the data gap for further

information to address the chronic risk to aquatic organisms has not been addressed. Thus, a new study should be submitted.

Consequently, a **data gap** has been indentified **to submit a chronic toxicity study on fish**.

Considering the suggestion of the Co-RMS, an ELS test (OECD TG 210) could be perform to study the chronic risk to fish and EAS-adversity of the active substace eugenol.

B.9.2.2.1. Fish early life stage toxicity test

No further data are considered necessary

B.9.2.2.2. Fish full-life cycle test

No further data are considered necessary.

B.9.2.2.3. Bioconcentration in fish

The log P_{ow} values for thymol are 3.43, 3.44 and 3.41 at pH values 4,7 and 9, respectively (please see document MCA Section 2, point 2.7 for details) and thus just exceed the trigger value of 3, requiring consideration of bioconcentration in fish. However, due to its rapid volatilisation properties and ready biodegradation it is considered unlikely that thymol will be persistent and accumulate in soil or natural water systems. According to the EFSA Conclusion Report for thymol (EFSA Journal 2012; 10(11):2916), for bioaccumulation "…it was considered that bioaccumulation in fish is unlikely", and therefore no studies are required. Furthermore, in line with Annex I, Section 4.1.2.8.1 of the CLP Regulation[5], these log P_{ow} values are less than the CLP cut-off criteria of 4, indicating thymol does not show potential to bioaccumulate. Whilst bioconcentration of thymol in prey of fish-eating birds and mammals is considered unlikely, for completeness conservative secondary poisoning assessments are presented in Vol. 3 CP Mevalone B.9.2.1 and B.9.2.2, using worst-case initial PEC values, worst-case surrogate reproductive endpoints of $LD_{50}/10$ and QSAR-estimated BCF_{fish} values. Acceptable risks to birds and mammals due to secondary poisoning are concluded for the proposed representative uses, even when considering these worst-case parameters.

B.9.2.3. Potential for endocrine disruption

The available data for thymol do not show any adverse effects that are considered to be EAS-mediated. However, there are no available guideline studies with thymol on fish to investigate the potential of endocrine disruption of thymol. The available toxicology (mammalian) and ecotoxicology data for thymol showed that the potential for endocrine disruption have not been sufficiently investigated, therefore, further data need to be generated before a conclusion on whether or not the ED criteria are met (Vol. 1, 2.10).

B.9.2.4. Acute toxicity to aquatic invertebrates

B.9.2.4.1. Acute toxicity to Daphnia magna

One acute *Daphnia* toxicity study with thymol was previously evaluated as part of the EU review for the EU inclusion of thymol (Thymol DAR, Volume 3, Annex B.9, 2011, B.9.2.1.2). This study is considered appropriate for the current assessment to support renewal of thymol; a full summary is provided below (Study B.9.2.4.1/01).

It is also noted that one literature paper was identified during the literature search (see Document MCA, Section 9 for full details), which assessed acute toxicity of thymol to *Daphnia magna* (CA 9.6.3.4/18). Results of this paper are summarised in Appendix I for completeness, but are not considered reliable for use in the risk assessment, principally as analytical verification was not reported to confirm the actual exposure during the study.

Study B.9.2.4.1/01

[5] Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006

Data point:	CA 8.2.4.1/01
Report author	Grade, R., Wydra, V.
Report year	2007
Report title	Acute toxicity of thymol crystals to *Daphnia magna* in a static 48-hour immobilization test.
Report No	34283220
Document No	-
Guidelines followed in study	OECD Guideline 202 (2004); Commission Directive 92/69/EEC, Annex Part C, C.2 (1992); EPA Guideline 712-C-96-114: OPPTS 850.1010 (1996)
Deviations from current test guideline	None
Previous evaluation	Yes, evaluated and accepted in the DAR (Thymol, Volume 3, Annex B.9, 2011, B.9.2.1.2) EU-agreed critical endpoint for EU inclusion of thymol (EFSA Journal 2012;10(11):2916)
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

The 48-hour acute toxicity of thymol to *Daphnia magna* was studied under static conditions in accordance with OECD Guideline 202 (2004). Daphnids were exposed to nominal concentrations of 0.625, 1.25, 2.5, 5 and 10 mg thymol/L and an untreated control and solvent control for 48 hours. Immobilisation was observed after 24 and 48 hours of exposure. Analytical verification of test concentrations confirmed measured concentrations were maintained within 86 – 111% of nominal throughout the exposure period and biological endpoints were based on nominal concentrations.

The 48-hour EC_{50} value for *Daphnia magna* based on immobilisation was calculated to be 4.9 mg thymol/L based on nominal concentrations. The 48-hour NOEC value based on immobilisation was determined to be 2.5 mg thymol/L based on nominal concentrations.

This study is considered acceptable and satisfies the guideline requirements for an acute toxicity study with freshwater invertebrates.

Materials and methods

Test material

Name: Thymol crystals
Source and lot/batch no.: 94747
Active substance content: 99.7% w/w
Expiry date of lot/batch: March 2008
Storage conditions: In original container, at room temperature (+10 ºC to +30 ºC) in the dark

Test organism

Species: *Daphnia magna* (water flea).
Strain/clone: Clone 5
Age at study initiation: < 24 hours old (6.10 – 21.55 hours old)
Source: Originally sourced from ECT Oekotoxikologie GmbH, Germany. The *daphnids* were bred in the laboratories of IBACON under similar temperature and light conditions as used in the test. The test organisms were not first brood progeny. Once a week the *daphnids* in the stock culture were fed with Tetra Min-extract and on at least all other working days with green algae (*Desmodesmus subspicatus*).
Feeding during test: No
Acclimation: 6 hours under test conditions

Test conditions

Hardness:	250 mg/L as calcium carbonate
Test temperature:	21 ºC.
pH:	7.1 – 7.6; the pH-value did not vary by more than 1.5 units
Dissolved oxygen:	At least 8.2 mg/L
Conductivity:	< 5 µS/cm
Photoperiod:	16 h light: 8 h dark
Light intensity:	110 – 270 lux

Test vessels comprised 150 mL glass beakers, each filled with approximately 125 mL of culture medium and covered with a lid to reduce the loss of water due to evaporation and to avoid the entry of dust into the culture medium. A stock solution was prepared by dissolving 50 mg of the test item in DMF (50.0 mL) to obtain approximately a stock solution of 1 g thymol/L. The stock solutions were diluted in a series of sequential dilutions with the solvent to add the same volumes of solvent (0.1 mL/L) to each test solution. The final nominal concentrations of thymol in the test media were: 0.625, 1.25, 2.5, 5 and 10 mg thymol/L.

Five individual daphnids were added to each test vessel, with four replicate vessels per treatment group. The test solutions were prepared just before introduction of *Daphnia*. Since the active substance is stable in control medium, a static test was performed. Duplicate analytical samples were taken from all test concentrations at test start (0 hours) and after 48 hours. The contents of the test beakers of each treatment were poured together and afterwards duplicate samples from the pooled test media of all test concentrations and solvent control at both sampling times (0 and 48 hours) were analysed. From the control samples only one of the duplicate samples was analysed at both sampling times.
The immobility of daphnids was determined by visual assessments after 24 and 48 hours. Those animals not able to swim within 15 seconds after gentle agitation of the test beaker were considered to be immobile (even if they could still move their antennae). Statistical analysis was performed using ToxRat Professional v2.09. EC_{50} values and 95% confidence limits were calculated, where possible, by Probit analysis. The NOEC and LOEC values were determined with a Williams multiple sequential t-test procedure (comparison to solvent control).

Results

Analytical results
The GC-MS analytical method for the determination of thymol in test medium was validated with regards to specificity, linearity, accuracy and precision. Whilst the report does not refer to the current guideline, overall the validation results presented are in accordance with guideline SANCO/3029/99 rev. 4, 11/07/2000 and are considered fit for purpose. Specificity was demonstrated by the absence of a peak at the characteristic retention time for thymol in the control sample. The analytical calibration was shown to be linear (r = 0.9973) over the range of 0.2 to 6 mg reference item/L. Accuracy was confirmed with recovery determined by fortification of thymol at 0.5, 1, 2, and 10 mg reference item/L; all recoveries at 1, 2 and 10 mg reference item/L were within the range of 93 – 110 % and overall mean recovery was 100% (i.e. within the guideline range of 70-110%). Precision was confirmed with four determinations made at each fortification level; the relative standard deviation at 1, 2 and 10 mg reference item/L was between 1.7 – 2.6% (i.e. within the guideline limit of ≤ 20%). The limit of quantification (LOQ) was 1 mg thymol/L (i.e. below the biological EC_{50}/NOEC value). The limit of detection (LOD) was 0.02 mg thymol/L in the test medium.
The measured concentrations of thymol during the 48-hour exposure period of the *Daphnia magna* toxicity study are summarised in the table below.

Table 9.2.4.1/01-1: Nominal and measured concentrations of thymol in each replicate

Nominal conc. (mg thymol/L)	Duplicate samples	Measured conc. (mg thymol/L)			Percentage of nominal		
		0 h	48 h	Mean*	0 h	48 h	Mean*
Water control	1	<LOD	<LOD	n.a.	n.a	n.a.	n.a
Solvent control	1	<LOD	n.a.	n.a.	n.a.	n.a.	n.a.
0.625	1	<LOQ	<LOQ	n.a.	n.a.	n.a.	n.a.
	2	<LOQ	<LOQ		n.a.	n.a.	
1.25	1	1.318	1.077	1.223	105	86	98
	2	1.382	1.114		111	89	
2.5	1	2.493	2.201	2.325	100	88	93
	2	2.413	2.193		97	88	
5	1	5.361	4.757	5.072	107	95	101
	2	5.267	4.904		105	98	
10	1	10.061	9.606	9.916	101	96	99
	2	10.209	9.789		102	98	

LOQ = 1 mg thymol/L
LOD = 0.02 mg thymol /L
* arithmetic mean of the 4 measurements for each treatment group (duplicate samples at 0 and 48 hours)
n.a.: not applicable
conc. concentration

Table 9.2.4.1/01-2: Summary of nominal and mean measured concentrations of thymol for 48 hours

	Measured concentration (mg thymol/L)						
Nominal conc. (mg thymol/L)	Water control	Solvent control	0.625	1.25	2.5	5	10
Range (min: max)	n.a.	n.a.	n.a.	1.077-1.382	2.193-2.493	4.757-5.361	9.606-10.209
Median	n.a.	n.a.	n.a.	1.216	2.307	5.085	9.925
Mean*	n.a.	n.a.	n.a.	1.223	2.325	5.072	9.916
% of nominal (ref. to mean)	n.a.	n.a.	n.a.	98	93	101	99

* arithmetic mean of the 4 measurements for each treatment group (duplicate samples at 0 and 48 hours)
n.a.: not applicable
conc. concentration

At the start of the test 104% of the nominal test concentrations were found (average for nominal concentrations of 1.25, 2.5, 5 and 10 mg thymol/L). At the end of the test, 92% of the nominal values were found (average for nominal concentrations of 1.25, 2.5, 5 and 10 mg thymol/L). Thus, during the test period the *Daphnia* were exposed to an overall mean measured concentration of 98% of nominal (average for nominal concentrations of 1.25. 2.5, 5 and 10 mg thymol/L). The active substance was found in concentrations close to the nominal concentrations at the start and the end of the test. Analytical verification of test concentrations confirmed that measured concentrations of all fresh and aged samples were between 86 – 111% of nominal. Therefore, biological endpoints are reported below based on nominal concentrations.

Biological results

A summary of the test effects of thymol on immobilisation of *Daphnia magna* after 48 hours exposure is presented in the table below.

Table 9.2.4.1/01-3: Summary of effects of thymol on immobilisation of *Daphnia magna* over 48 hours

Nominal conc. (mg thymol/L)	No. organisms at test start	Cumulative immobilised		Cumulative (%) immobilised	
		24 h	48 h	24 h	48 h
Water control	20	0	0	0	0
Solvent control	20	1	1	5	5
0.625	20	0	0	0	0
1.25	20	0	0	0	0
2.5	20	0	1	0	5
5.0	20	5**	9	25	45*
10	20	20*	20	100	100*

*significant in comparison to solvent control according to Williams multiple sequential t-test procedure

** all animals trapped at the surface of the medium

No immobilisation was observed in the control and in the three lowest test concentrations (0.625, 1.25 and 2.5 mg thymol/L). A single specimen was immobile in the solvent control and at a test concentration of 2.5 mg thymol/L after 48 hours; however, these observations were not considered to be treatment-related. Significant immobilisation was observed in the two highest test concentrations after 48 hours (45% and 100% at test concentrations of 5.0 and 10 mg thymol/L, respectively). As a result, the following endpoints were obtained, the 48-hour EC_{50} (immobilisation) = 4.9 mg thymol/L and the NOEC (48-hour) = 2.5 mg thymol/L, based on nominal concentrations.

Validity
All validity criteria were met in accordance with OECD test guideline 202 (2004):
- Immobilisation in the control group was ≤ 10% (actual value: 0% in control; 5% in solvent control).

- The dissolved oxygen concentration at the end of the test was ≥3 mg/L in all test vessels (actual values: ≥ 8.2 mg O_2/L).

- Analytical measurement of test concentrations was included.

Assessment and conclusion

The 48-hour acute toxicity of thymol to *Daphnia magna* was studied under static conditions in accordance with OECD 202. The 48-hour EC_{50} value was calculated to be 4.9 mg thymol/L based on nominal concentrations. This study is considered as acceptable and satisfies the guideline requirements for an acute toxicity study with freshwater invertebrates.

Assessment and conclusion by applicant:

The study is acceptable.

Daphnia magna 48-hour EC_{50} (immobilisation) = 4.9 mg thymol/L based on nominal concentrations.

Assessment and conclusion by RMS:

Outcome and conclusion of the study:

This *Daphnia* toxicity study with thymol was previously evaluated as part of the EU review for the EU inclusion of thymol. The EU-agreed EC_{50} value of 4.9 mg thymol/L for *Daphnia magna*.

Four nominal concentrations of thymol were tested (0.625, 1.25, 2.5, 5.0 and 10 mg thymol/L) plus two controls (water and solvent control) in a static test.

The minimum nominal concentration used (0.625 mg thymol/L) is lower than the LOQ. However, this was not considered an important issue because no immobility daphnids were in test solutions up to 2.5 mg thymol/L.

The active substance was found in concentrations close to the nominal concentrations at the start and the end of the test (between 86 – 111% of nominal). Therefore, biological endpoints were reported based on nominal concentrations.

All validity criteria were met in accordance with OECD test guideline 202 (2004):

- Immobilisation in the control group was ≤ 10%
- The dissolved oxygen concentration at the end of the test was ≥ 3 mg/L in all test vessels
- Analytical measurement of test concentrations was included.

RMS agrees with the previous evaluation. The study is considered acceptable.

Agreed endpoints (based on nominal concentrations):

48h EC_{50} (immobilisation) = 4.9 mg thymol/L
48 h-NOEC (48h) = 2.5 mg thymol/L.

B.9.2.4.1. Acute toxicity to an additional aquatic invertebrate species

Since the active substance does not have an insecticidal mode of action, no further data are required.

B.9.2.5. Long-term and chronic toxicity to aquatic invertebrates

B.9.2.5.1. Reproductive and development toxicity to Daphnia magna

A new chronic toxicity study with *Daphnia magna* has been provided to support the renewal of thymol; a full summary is provided below (Study 9.2.5.1/01).

Study B.9.2.5.1/01

Data point:	CA 8.2.5.1/01
Report author	Egeler, P.
Report year	2021
Report title	Thymol: A Study on the Chronic Toxicity to *Daphnia magna*
Report No	20GC2DB
Document No	-
Guidelines followed in study	OECD Test Guideline 211 (2012)
Deviations from current test guideline	None
Previous evaluation	No
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

The chronic toxicity of thymol to *Daphnia magna* was studied under static-renewal conditions in accordance with OECD Test Guideline 211 (2012). Daphnids were exposed to nominal concentrations of 0.0563, 0.141, 0.352, 0.880 and 2.20 mg thymol/L (corresponding to mean measured concentrations of 0.0344, 0.137, 0.300, 0.767 and 2.00 mg thymol/L) and an untreated control (Elendt medium M4) for 21 days.

Adult daphnids were observed daily for immobility, presence of eggs and mortality. They were transferred to fresh media 3 times per week. Any juveniles produced were counted and removed daily.

Analytical verification of the test item concentrations indicated they were stable in the test solutions >80% of the nominal concentrations, for all but the lowest test concentration. Therefore, biological endpoints are based on mean measured concentrations.

The 21-day EC_{10}, EC_{20} and EC_{50} values for *Daphnia magna*, based on the total number of living offspring per surviving parental daphnids were determined to be 0.292 mg thymol/L, 0.554 mg thymol/L and 1.88 mg thymol/L (mean measured), respectively.

The 21-day NOEC value for *Daphnia magna*, based on the total number of living offspring per surviving *Daphnia magna* parental daphnids, was determined to be 0.137 mg thymol/L and the corresponding 21-day LOEC value was determined to be 0.300 mg thymol/L (mean measured).

This study is considered acceptable and satisfies the requirements for a chronic toxicity study with freshwater invertebrates (OECD test guideline 211, 2012).

Materials and methods

Test material

Name:	Thymol
Chemical Name:	5-Methyl-2-isopropylphenol; 5-Methyl-2-(1-methylethyl)phenol; 2-Isopropyl-5-methylphenol
Formulation type:	not applicable
Source and lot/batch no.:	THY/02/2019-20
Active substance content:	99.58%
Appearance:	Colourless to white crystals
Expiry date of lot/batch:	31 July 2021
Storage conditions:	Store in cool, dry, well-ventilated and dark place away from direct sunlight, in tightly closed container; stored at test facility in closed container at ambient temperature in the dark

Test organism

Species:	*Daphnia magna* (Straus) (water flea).
Strain/clone:	M10
Age at study initiation:	<24 hours old
Source:	Originally supplied by KU Leuven, Belgium, cultured at ECT Oekotoxikologie GmbH since December 22, 2011
Feeding during test:	Three times per week with fresh algae suspension

Acclimation:	Not applicable

Test conditions

Test medium:	Elendt medium M4
Hardness:	250 - 263 mg/L as $CaCO_3$
Test temperature:	20.6 – 21.5 °C*
pH:	7.7 – 9.0*
Dissolved oxygen:	8.9 – 11.7 mg/L*
Photoperiod:	16 hours of light / 8 hours of dark
Light intensity:	15.13 – 16.74 µE m^{-2} s^{-1}

*Measured (x 2) on days 0, 2, 5, 7, 9, 14 and 16 of the test

Ten replicate vessels, each a glass beaker containing a single daphnid in 50-60 mL medium, were allocated to each test concentration and control. Test solutions were renewed three times per week (semi-static test system). The daphnids were fed three times per week after transfer to fresh test solutions. Daily observations were made of the parental daphnids in all test vessels; immobile parental daphnids were removed upon recording. From day 8, onwards, the live offspring (F1 generation) was counted daily and removed from the vessels. Observations of abnormal behaviour of the test animals were recorded.

Samples of test media were taken at the start and end of media renewal cycles, during the 21-day exposure period, and analysed for thymol by GC-MS. Water quality parameters (dissolved oxygen concentration, pH, water hardness and temperature (manual measurement)) were determined once per test week in fresh and aged solutions of the control and the highest test item concentration.

The Cochran-Armitage test procedure was applied with immobility at 21 days to detect an increasing trend in responses (Alpha: 0.050; one-sided greater). Determination of EC_x values for parental immobility by Probit analysis was not possible due to the poor concentration-response relationship. Fisher's Exact Binomial Test with Bonferroni correction was used to determine the threshold concentration for parental immobility. Non-linear regression analysis (3-parameter normal Cumulative Distribution Function (CDF)) was used to determine EC_x values for reproduction. Prior to threshold concentration testing, a qualitative trend analysis by contrasts was applied to check for monotonicity of the concentration-response relationship. The Williams Multiple Sequential t-test Procedure ($p \leq 0.05$) was used to determine the threshold concentrations for reproduction.
The statistical software package ToxRat Professional 3.3.0 (ToxRat Solutions GmbH, Naheweg 15, D-52477 Alsdorf) was used for these calculations using the nominal concentrations.

Results

Analytical results
The GC-MS analytical method for the determination of thymol in test medium was validated with regards to specificity, linearity, accuracy and precision in accordance with guideline SANCO/3029/99 rev. 4, 11/07/2000. Specificity was demonstrated by the absence of a peak at the characteristic retention time for thymol in the control sample. The analytical calibration was shown to be linear ($r^2 \geq 0.9991$) over the range of 0.0084 mg thymol/L to 0.200 mg thymol/L. Accuracy was confirmed with recovery determined by fortification of thymol at 0.028 and 2.20 mg thymol/L; all recoveries were within the range of 91-105% and mean recoveries were within 96-98% (i.e. within the guideline range of 70-110%). Precision was confirmed with five determinations made at each fortification level; the relative standard deviation was between 1.9-5.5% (i.e. within the guideline limit of ≥20%). The limit of quantification (LOQ) was 0.0280 mg thymol/L (i.e. below the biological NOEC value). The limit of detection (LOD) was 0.084 mg thymol/L in test medium. All samples were analysed within 24 h after extraction, therefore the stability of thymol in the final extracts was not assessed.
The measured concentrations of thymol during the 21-day exposure period of the *Daphnia magna* toxicity study are summarised in the tables below.

Table 9.2.5.1/01-1: Nominal and measured concentrations of thymol in each replicate during the 21-day study

Nominal concentration (mg thymol/L)	Measured concentration (mg thymol/L)							Percentage of nominal (%)						
	Day						Mean [a]	Day						Mean [a]
	2F	5A	7F	9A	14F	16A		2F	5A	7F	9A	14F	16A	
Control	<LOD	-	-	-	-	<LOD	-	-	-	-	-	-	-	-
0.0563	0.0507	0.043	0.0523	0.0404	<LOD[b]	<LOD[b]	0.0344	90.1	76.4	92.9	71.8	n.a.	n.a.	61.1
0.141	0.131	0.119	0.135	0.122	0.177	0.153	0.137	92.9	84.4	95.7	86.5	126	109	97.2
0.352	0.330	0.290	0.340	0.270	0.270	0.290	0.300	93.8	82.4	96.6	76.7	76.7	82.4	85.2
0.880	0.840	0.680	0.840	0.730	0.800	0.733	0.767	95.5	77.3	95.5	83.0	90.9	83.3	87.2
2.20	2.110	1.990	2.030	1.880	2.110	1.81	2.00	95.9	90.5	92.3	85.5	95.9	82.3	90.9

[a] time weighted mean

[b] Measured concentrations <LOD indicate a possible error in preparation of the C1 solution on days 14 and 16. To calculate time-weighted mean measured concentrations (TWM) for the C1 treatment group, the measured concentrations days 14 and 16 were replaced by 1/2 of the LOD (0.0084 mg/L/2 = 0.0042 mg/L), equivalent to 7% of the nominal concentration of 0.0563 mg/L. This is considered a robust approach as it is expected that this error occurred only for the single renewal period of days 14-16 of the study.

F: fresh media; A: aged media

Table 9.2.5.1/01-2: Summary of nominal and mean measured concentrations of thymol during the 21-day study

	Measured concentration (mg thymol/L)					
Nominal concentration (mg thymol l/L)	Control	0.563	0.141	0.352	0.880	2.20
Range (min - max)	-	<LOD – 0.0507	0.119 – 0.135	0.027– 0.330	0.680– 0.800	1.810 – 2.110
Mean*	-	0.0344	0.137	0.300	0.767	2.00
% of nominal	-	61.1	97.2	85.2	87.2	90.9

* time weighted mean

The analytical verification of the test item concentrations confirmed that measured concentrations were stable for all but the lowest concentration, which was calculated to be below 80% of nominal concentrations. The biological endpoints are therefore based on mean measured (time-weighted) concentrations.

Biological results

Summaries of the effects of thymol on parental daphnid survival/immobility and the fecundity of the introduced and surviving parent daphnids are presented in the tables below.

Table 9.2.5.1/01-3: Total mobility/immobility of parental daphnids at the end of the 21-day study

Nominal concentration (mg thymol/L)	Mean measured concentration (mg thymol/L)	Number of daphnids			Immobility [%]
		Introduced	Mobile	Immobile	
Control	-	10	10	0	0.0
0.0563	0.0344	10	10	0	0.0
0.141	0.137	10	10	0	0.0
0.352	0.300	10	10	0	0.0
0.880	0.767	10	10	0	0.0
2.20	2.00	10	9	1	10.0

For immobile parental daphnids, a concentration-response relationship could not be determined. A few sublethal effects were observed in the living parental daphnids at all concentration levels, but no concentration-response relationship based on sublethal effects in living parental daphnids was determined.

Table 9.2.5.1/01-4: Summary of effects of thymol on the total number of living offspring per surviving *Daphnia magna* parent after 21-days' exposure

Nominal concentration (mg thymol/L)	Mean measured concentration (mg thymol/L)	Replicate	Cumulative number of live juveniles per surviving parent at 21 days		
			Per test vessel	Mean	% reduction relative to control*
Control	-	1	112	135.4	-
		2	127		
		3	109		
		4	122		
		5	149		
		6	164		
		7	124		
		8	130		
		9	145		
		10	172		
0.0563	0.0344	1	136	135.7	-0.2
		2	146		
		3	129		
		4	177		
		5	135		

Nominal concentration (mg thymol/L)	Mean measured concentration (mg thymol/L)	Replicate	Cumulative number of live juveniles per surviving parent at 21 days		
			Per test vessel	Mean	% reduction relative to control*
		6	117		
		7	144		
		8	127		
		9	101		
		10	145		
0.141	0.137	1	161	139.1	-2.7
		2	137		
		3	103		
		4	139		
		5	158		
		6	151		
		7	99		
		8	138		
		9	134		
		10	171		
0.352	0.300	1	128	114.8**	15.2
		2	85		
		3	152		
		4	112		
		5	75		
		6	102		
		7	109		
		8	121		
		9	126		
		10	138		
0.880	0.767	1	103	103.9**	23.3
		2	96		
		3	135		
		4	89		
		5	106		
		6	119		
		7	116		
		8	68		
		9	121		
		10	86		
2.20	2.00	1	55	65.0**	52.0
		2	73		
		3	-		
		4	45		
		5	57		
		6	72		
		7	67		
		8	71		
		9	109		
		10	36		

- = inadvertent mortality (unknown cause): offspring excluded from statistical analysis

* % offspring reduction compared to control (negative values = higher number than control)

** Statistically significant difference compared to the control (Williams t test, alpha 0.050, one-sided)

The total number of living offspring was evaluated per surviving parental daphnid and per introduced parent daphnid, which did not die accidentally or inadvertently during the test. Endpoints are based on the former parameter.

A new chronic toxicity study with *Daphnia magna* has been provided to support the renewal of thymol

Assessment and conclusion

The 21day chronic toxicity of thymol to *Daphnia magna* was studied under static-renewal conditions in accordance with OECD test guideline 211 (2012).
The 21-day EC_{10}, EC_{20} and EC_{50} values for *Daphnia magna*, based on the total number of living offspring per surviving parental daphnids were determined to be 0.292 mg thymol/L, 0.554 mg thymol/L and 1.88 mg thymol/L (mean measured), respectively.
The 21-day NOEC value for *Daphnia magna*, based on the total number of living offspring per surviving *Daphnia magna* parental daphnids, was determined to be 0.137 mg thymol/L and the corresponding 21-day LOEC value was determined to be 0.300 mg thymol/L (mean measured).

Assessment and conclusion by applicant:

The study is acceptable.

Daphnia magna:

21-day EC_{10} (reproduction) = 0.292 mg thymol/L (mean measured), based on the total number of living offspring per surviving parental daphnids

21-day EC_{20} (reproduction) = 0.554 mg thymol/L (mean measured), based on the total number of living offspring per surviving parental daphnids

21-day NOEC (reproduction) = 0.137 mg thymol/L (mean measured), based on the total number of living offspring per surviving parental daphnids

Assessment and conclusion by RMS:

Outcome and conclusion of the study:

A new 21 days chronic toxicity study with *Daphnia magna* has been provided to support the renewal of thymol according to OECD Guideline 211 (2012).
Five nominal test item concentration (0.0563, 0.141, 0.352, 0.880 and 2.20 mg thymol/L) plus a control was tested under semi-static conditions.
The test item concentrations were stable in the test solutions at >80% of nominal, except for the lowest test concentration, where the time-weighted mean (TWM) of the measured concentrations was < 80% of nominal concentrations (61.1%). Therefore the biological endpoints were based on TWM of the measured concentrations : 0.0344, 0.137, 0.300, 0.767 and 2.00 mg test item/L.

The valididty criteria were met :

- Mortality of the parent animals (female Daphnia) in the controls at the end of the test should be ≤20% (0.0%)
- Mean number of live offspring produced per surviving parent animal in the controls at the end of the test should be ≥60 (135.4).

RMS agrees to the applicant conclusions. The study is considered acceptable.

Agreed endpoints :
21-day EC_{10} (reproduction) = 0.292 mg thymol/L (mean measured), based on the total number of living offspring per surviving parental daphnids
21-day EC_{20} (reproduction) = 0.554 mg thymol/L (mean measured), based on the total number of living offspring per surviving parental daphnids
21-day NOEC (reproduction) = 0.137 mg thymol/L (mean measured), based on the total number of living offspring per surviving parental daphnids

B.9.2.5.1. Reproductive and developmental toxicity to an additional aquatic invertebrate species

Since the active substance does not have an insecticidal mode of action, no further data are required.

B.9.2.5.2. Development and emergence in Chironomus riparius

Since the active substance does not have an insecticidal mode of action, no further data are required.

B.9.2.5.3. Sediment dwelling organisms

Accumulation of thymol in aquatic sediment is not expected due to its rapid volatilisation properties and ready biodegradation. No further toxicity data on sediment dwelling organisms are required, as previously agreed during the EU inclusion of thymol (EFSA Journal 2012; 10(11):2916).

B.9.2.6. Effects on algal growth

B.9.2.6.1. Effects on growth of green algae

One green algal study with thymol was previously evaluated as part of the EU review for the EU inclusion of thymol (DAR, Volume 3, Annex B.9, 2011, B.9.2.1.3). This study is considered appropriate for the current assessment to support renewal of thymol; a full summary is provided below (Study B.9.2.6.1/01).

Study B.9.2.6.1/01

Data point:	CA 8.2.6.1/01
Report author	Hoffman, K., Wydra, V.
Report year	2011 (revised final report) (KCA 8.2.6.1/01a) Original report: Grade, R., Wydra, V., 2008c (KCA 8.2.6.1/01b)
Report title	Toxicity of thymol crystals to *Pseudokirchneriella subcapitata* in an Algal Growth Inhibition Test.
Report No	34284210
Document No	-
Guidelines followed in study	OECD Guideline for Testing of Chemicals, Section 2, No. 201: "Freshwater Alga and Cyanobacteria, Growth inhibition Test", (2006) EPA Guideline 712-C-96-164: OPPTS 850.5400, “Algal Toxicity, Tiers I and II”,(l996)
Deviations from current test guideline	No
Previous evaluation	Yes, evaluated and accepted in the DAR (Thymol, Volume 3, Annex B.9, 2011, B.9.2.1.3) EU-agreed critical endpoint for EU inclusion of thymol (EFSA Journal 2012;10(11):2916)
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

The purpose of this test was to determine the inhibitory effect of the test item thymol on the growth of the freshwater green algal species *Pseudokirchneriella subcapitata.* The 96-hour acute toxicity of thymol to *Pseudokirchneriella subcapitata* was studied in an algal growth inhibition test in accordance with OECD 201. Test species were exposed to control, solvent control, and test chemical at nominal concentrations of 1.0, 3.2, 10, 32 and 100 mg thymol/L for 96 hours. Exponentially growing cultures of this unicellular green algal species were exposed to various concentrations of thymol under defined conditions. The inhibition of growth in relation to control cultures was determined over a test period of 96 hours, and thus over several algal generations. Analytical verification of test concentrations confirmed measured concentrations were maintained

within 69 – 100% of nominals throughout the 96-hour test period. Biological endpoints are reported based on mean measured concentrations.
The 72-hour E_rC_{50} (growth rate) value was calculated to be 11.1 mg thymol/L based on mean measured concentrations. The 72- hour NOEC value based on growth rate was determined to be 8.10 mg thymol/L based on mean measured concentrations. This study is considered as acceptable and satisfies the guideline requirements for an inhibition study with algae species (OECD test guideline 201 (2011) and OCSPP 850.5400, 2012).

Materials and methods

Test material

Name: thymol crystals
Source and lot/batch no.: 94747
Active substance content: 99.7 % w/w
Expiry date of lot/batch: March 2008 (sponsor information)
Storage conditions: In original container in the refrigerator +10 °C to +30 °C), in the dark.
Test organism
Species: Algae (*Pseudokirchneriella subcapitata)*
Strain/clone: Strain No. 61, 8l SAG
Source: Supplied by the "Sammlung von Algenkulturen, Pflanzenphysiologisches Institut der Universität Göttingen", 37073 Göttingen, Germany. The algae were cultivated in the laboratories of Ibacon under standardised conditions according to the test guidelines.
Feeding during test: No
Acclimation: Algal cells were taken from an exponentially growing pre-culture, which was set up 3 days prior to the test start under the same conditions as in the test.

Test conditions

Hardness: 24 mg/L as calcium carbonate
Test temperature: 23 – 24 ºC
pH: 7.9 – 8.4
Conductivity: < 5 µS/cm
Photoperiod: Continuous illumination
Light intensity: 7120 – 7700; 7392 Lux (mean value)

The toxicity of thymol to *Pseudokirchneriella subcapitata* was tested in an algal growth inhibition test. A stock solution of thymol was prepared by dissolving it in the solvent dimethylformamide (DMF); the stock solution was then serially diluted with DMF to obtain a set of solvent solutions. The final test concentrations were prepared by adding these solvent solutions to the culture media.

There were five nominal test concentrations: 1.0, 3.2, 10, 32 and 100 mg thymol/L, a control containing culture medium only, and a solvent control containing 100 mg DMF/L.

Exponentially growing cultures of *P. subcapitata* were inoculated at 5000 cells/mL and cultured for 96 hours at temperatures of 23 – 24ºC. Light intensity ranged between 7120 and 7700 lux. The test vessels were 50 mL Erlenmeyer flasks, each containing 30 mL culture medium. Six replicate cultures of the control containing culture medium only and the solvent control containing 100 mg DMF/L and three replicates of each test concentration were used.

Measured concentrations of thymol were determined at 0 and 96 hours. Algal cell numbers were determined spectrophotometrically after approximately 24, 48, 72 and 96 hours. Cell densities were determined by means of a regression between absorption and cell densities, which had been calculated previously using a light microscope on a dilution series of control samples. A sample was taken from the test concentration of 100 mg thymol/L and examined microscopically in order to assess any effect on the algal cells. Statistical analysis was performed using ToxRat Professional, version 2.10.05. EC_{50} values and 95% confidence limits were calculated, where possible, by Probit analysis. The NOEC and LOEC values were determined using Williams test (area

under the growth curve and yield) or Bonferroni t-test (growth rate) to test for significant differences compared to the control values.

Results

Analytical results

The GC-MS analytical method for the determination of thymol in test medium was validated with regards to specificity, linearity, accuracy and precision. Whilst the report does not refer to the current guideline, overall the validation results are in accordance with guideline SANCO/3029/99 rev. 4, 11/07/2000 and are considered fit for purpose. Specificity was demonstrated by the absence of a peak at the characteristic retention time for thymol in the control sample. The analytical calibration was shown to be linear (r = 0.9977) over the range of 0.3 – 10 mg thymol/L. Accuracy was confirmed with recovery determined by fortification of thymol at 0.8 (<LOQ), 3, 10 and 100 mg reference item/L; all recoveries were within the range of 82 – 106% % for 3 – 10 mg reference item/L (i.e. within the guideline range of 70-110%). Precision was confirmed with four determinations made at each fortification level; the relative standard deviation was between 3 – 5 % (i.e. within the guideline limit of ≤ 20%). The limit of quantification (LOQ) was 3 mg thymol/L (i.e. below the biological E_rC_{50}/NOEC value). The limit of detection (LOD) was 0.779 mg thymol/L in test medium.

A summary of the measured concentrations of thymol in the test media is presented in the tables below.

Table 9.2.6.1/01-1: Nominal and measured concentrations of thymol in each replicate

Nominal conc. (mg thymol/L)	Mean measured (mg thymol/L)	Duplicate samples	Measured conc. (mg thymol/L)			Percentage of nominal		
			0 h	96h	Mean*	0 h	96 h	Mean*
Water control	Water control	1	<LOD	<LOD	n.a.	n.a	n.a.	n.a
Solvent control	Solvent control	1	<LOD	<LOD	n.a.	n.a.	n.a.	n.a.
		2	<LOD	<LOD		n.a.	n.a.	
1	<LOQ	1	<LOD	<LOQ	n.a.	n.a.	n.a.	n.a.
		2	<LOD	<LOQ		n.a.	n.a.	
3.2	2.722	1	3.204	2.359	2.722	100	74	84
		2	2.998	2.325		94	73	
10	8.155	1	9.124	7.219	8.155	91	72	81
		2	9.399	6.877		94	69	
32	27.225	1	30.554	23.557	27.225	95	74	84
		2	31.878	22.912		100	72	
100	79.242	1	82.452	75.291	79.242	82	75	79
		2	86.870	72.355		87	72	

LOQ = 3 mg thymol/L
LOD = 0.779 mg thymol/L
* arithmetic mean of the 4 measurements for each treatment group (duplicate samples at 0 and 96 hours)
n.a.: not applicable

Table 9.2.6.1/01-2: Summary of nominal and mean measured concentrations of thymol over 96 hours

	Measured concentration (mg thymol/L)						
Nominal concentration (mg thymol./L)	Water control	Solvent control	1	3.2	10	32	100
Mean measured (mg thymol/L)	n.a.	n.a.	<LOQ	2.722	8.155	27.225	79.242
Range (min: max)	n.a.	n.a.	n.a.	2.325-3.204	7.219-9.399	22.912-31.878	72.355-86.870
Median	n.a.	n.a.	n.a.	2.678	8.171	27.055	78.871
Mean*	n.a.	n.a.	n.a.	2.722	8.155	27.225	79.242
% of nominal (ref. to mean)	n.a.	n.a.	n.a.	84	81	84	79

* Arithmetic mean of the 4 measurements for each treatment group (duplicate samples at 0 and 96 hours)
n.a.: not applicable

Analytical verification of the highest four test concentrations confirmed measured concentrations were maintained within 69 – 100% of nominals throughout the 96-hour test period. As detailed in the revised report (2011), the test concentration of 1.0 mg thymol/L could not be quantified with the available analytical method. Therefore, the overall geometric mean value was assumed as concentration for this treatment level. Since the geometric mean values in the treatment levels of 32 – 100 mg thymol/L were all close to 80% of nominals, this approach was considered to be justified. This step was necessary, since for the re-evaluation of the statistical data a concentration has to be introduced also for the lowest treatment level.
Biological endpoints are reported based on mean measured concentrations.

Biological results
A summary of the effects of thymol on biomass, growth rate and yield of *Pseudokirchneriella subcapitata* after 96 hours exposure is presented in the tables below.

Table 9.2.6.1/01-3: Algae cell densities during the test period of 96 hours

Nominal conc. (mg thymol/L)	Mean measured (mg thymol/L)	Rep	Cell density (10000 cells/mL)					Mean biomass[a]	Mean biomass[b]
			0 h	24 h	48 h	72 h	96 h		
Control	-	1	0.5	2.7056	11.782	50.525	165.16	159.3	43.8
		2	0.5	2.7056	11.950	52.710	161.21		
		3	0.5	2.6216	11.866	42.205	152.55		
		4	0.5	2.5375	10.437	36.742	156.84		
		5	0.5	2.6216	10.101	37.919	161.21		
		6	0.5	2.5375	10.521	42.793	159.3		
Solvent control	-	1	0.5	2.7056	10.525	42.373	160.53	158.6	43.5
		2	0.5	2.9577	10942	43.045	159.02		
		3	0.5	2.8737	11.362	44.222	151.29		
		4	0.5	2.7056	11.110	42.793	165.58		
		5	0.5	2.8737	10.521	44.306	158.52		
		6	0.5	2.6216	11.950	44.306	158.6		
1.0	<LOQ	1	0.5	2.2854	11.278	41.869	160.96	161.8	43.5
		2	0.5	2.4535	7.8321	44.306	163.73		
		3	0.5	2.5375	10.942	44.474	160.7		
3.2	2.722	1	0.5	2.4535	6.0673	42.205	158.35	155.2	36.4
		2	0.5	2.3695	8.3364	30.859	157.17		
		3	0.5	2.2854	6.5715	36.238	150.11		
10	8.155	1	0.5	2.1173	4.7226	8.5885	68.090	70.6	9.3
		2	0.5	2.2854	4.9747	9.4289	77.754		
		3	0.5	2.4535	4.3864	9.9332	65.821		
32	27.225	1	0.5	1.8652	1.1088	1.9493	10.605	6.2	0.7
		2	0.5	2.1173	0.4365	-0.4039	1.6971		
		3	0.5	1.6971	0.6886	0.6046	6.2354		
100	79.242	1	0.5	1.3610	0.7727	0.9408	5.7311	6.1	1.1
		2	0.5	1.8652	0.9408	2.0333	5.8992		
		3	0.5	0.5206	0.6886	0.1844	6.5715		

Conc.: concentration; Rep.: replicate
a mean values after 96 hours
b mean values after 72 hours
at the start of 5000 cells/mL

Table 9.2.6.1/01-4: Effect of thymol on the growth rate of *Pseudokirchneriella subcapitata* during the test period

Nominal conc. (mg thymol/L)	Mean measured (mg thymol/L)	Growth rates µ [1/day]				% of inhibition of µ[b]			
		0 – 24 hours	0 – 48 hours	0 - 72 hours	0 - 96 hours	0 – 24 hours	0 – 48 hours	0 - 72 hours	0 - 96 hours
Control	-	1.657	1.549	1.488	1.441	-	-	-	-
Solvent control	-	1.718	1.548	1.489	1.440	0.0	0.0	0.0	0.0
1.0	<LOQ	1.578	1.492	1.489	1.445	8.1	3.6	0.0	-0.4
3.2	2.722	1.555	1.314	1.427	1.434	9.5	15.1	4.2	0.4
10	8.155	1.518	1.119	0.974	1.237	11.7	27.7	34.5	14.1
32	27.225	1.327	0.163	-0.774	0.567	22.7	89.4	152.0	60.6
100	79.242	0.786	0.231	0.115	0.624	54.3	85.1	92.3	56.7

[b] % inhibition relative to the control. Negative values indicate an increase relative to the control.

Table 9.2.6.1/01-5: Effect of thymol on the yield of *Pseudokirchneriella subcapitata* during the test period

Nominal conc. (mg thymol/L)	Mean measured (mg thymol/L)	Yield y [mg/L]				% of inhibition of y[b]			
		0 – 24 hours	0 – 48 hours	0 - 72 hours	0 - 96 hours	0 – 24 hours	0 – 48 hours	0 - 72 hours	0 - 96 hours
Control	-	2.122	10.61	43.316	158.788	-	-	-	-
Solvent control	-	2.290	10.568	43.008	158.116	0.0	0.0	0.0	0.0
1.0	<LOQ	1.925	9.517	43.050	161.296	15.9	9.9	-0.1	-2.0
3.2	2.722	1.869	6.492	35.934	154.712	18.4	38.6	16.4	2.2
10	8.155	1.785	4.195	8.817	70.055	22.0	60.3	79.5	55.7
32	27.225	1.393	0.245	0.351	5.679	39.2	97.7	99.2	96.4
100	79.242	0.749	0.301	0.553	5.567	67.3	97.2	98.7	96.5

[b] % inhibition relative to the control. Negative values indicate an increase relative to the control.

Table 9.2.6.1/01-6: Effect of thymol on area under the growth curves during the test period

Nominal conc. (mg thymol/L)	Mean measured (mg thymol/L)	Areas under the Growth curves				% of inhibition of A[b]			
		0 – 24 hours	0 – 48 hours	0 - 72 hours	0 - 96 hours	0 – 24 hours	0 – 48 hours	0 - 72 hours	0 - 96 hours
Control	-	1.061	7.424	34.389	135.441	-	-	-	-
Solvent control	-	1.145	7.574	34.361	134.932	0.0	0.0	0.0	0.0
1.0	<LOQ	0.963	6.684	32.967	121.651	15.9	11.7	4.1	-0.2
3.2	2.722	0.935	5.115	26.328	49.824	18.4	32.5	23.4	9.8
10	8.155	0.893	3.883	10.388	4.829	22.0	48.7	69.8	63.1
32	27.225	0.697	1.516	1.814	4.386	39.2	80.0	94.7	96.4
100	79.242	0.374	0.899	1.326		67.3	88.1	96.1	96.7

[b] % inhibition relative to the control. Negative values indicate an increase relative to the control.

The control and the solvent control did not show any significant difference in cell densities over the 96-hour test period (Student t-test).

The 72-hour E_XC_{50} values for *Pseudokirchneriella subcapitata* were calculated to be (based on mean measured concentrations) (in line with the EFSA list of endpoints; EFSA Journal 2012; 10(11):2916):
The 72-hour E_rC_{50} (growth rate) was calculated to be 11.1 mg thymol/L (CI: 8.33 – 15.1 mg thymol/L (mean measured).
The 72-hour E_bC_{50} (biomass) was calculated to be 5.14 mg thymol/L (CI: 4.67 – 5.66 mg thymol/L (mean measured).
The 72-hour E_yC_{50} (yield) was calculated to be 4.89 mg thymol/L (CI: 4.36 – 5.47 mg thymol/L (mean measured).

The 72-hour E_rC_{10} (growth rate) = 3.81 mg thymol/L (CI: 1.59 – 5.61 mg thymol/L) (mean measured).
The 72-hour E_bC_{10} (biomass) = 1.54 mg thymol/L (CI: 1.22 – 1.84 mg thymol/L) (nominal values).
The 72-hour E_yC_{10} (yield) = 2.23 mg thymol/L (CI: 1.75 – 2.64 mg thymol/L) (nominal values).

The 72-hour E_rC_{20} (growth rate) = 5.50 mg thymol/L (CI: 2.96 – 7.49 mg thymol/L) (mean measured).
The 72-hour E_bC_{20} (biomass) = 2.33 mg thymol/L (CI: 1.97 – 2.67 mg thymol/L) (nominal values).
The 72-hour E_yC_{20} (yield) = 2.92 mg thymol/L (CI: 2.42 – 3.35 mg thymol/L) (nominal values).

The 72-hour NOEC values for *Pseudokirchneriella subcapitata* were determined to be:

The 72-hour NOE_rC (growth rate) was determined to be 8.10 mg thymol/L (mean measured).
The 72-hour NOE_bC (biomass) was determined to be 0.82 mg thymol/L (mean measured).
The 72-hour NOE_yC (yield) was determined to be 0.82 mg thymol/L (mean measured).

The 72-hour LOEC values for *Pseudokirchneriella subcapitata* were determined to be:

The 72-hour LOE_rC (growth rate) was determined to be 26.9 mg thymol/L (mean measured).
The 72-hour LOE_bC (biomass) was determined to be 2.69 mg thymol/L (mean measured).
The 72-hour LOE_yC (yield) was determined to be 2.69 mg thymol/L (mean measured).

For completeness, the 96-hour $E_XC_{50/10}$ values for *Pseudokirchneriella subcapitata* were calculated to be:

The 96-hour E_rC_{50} (growth rate) was calculated to be 35.2 mg thymol/L (CI: 22.6 – 68.2 mg thymol/L) (mean measured).
The 96-hour E_bC_{50} (biomass) was calculated to be 6.47 mg thymol/L (CI: 6.10 – 6.86 mg thymol/L) (mean measured).
The 96-hour E_yC_{50} (yield) was calculated to be 7.52 mg thymol/L (CI: 7.09 – 7.93 mg thymol/L) (mean measured).

The 96-hour E_rC_{10} (growth rate) = 3.75 mg thymol/L (CI: 0.72 – 7.59 mg thymol/L) (mean measured).
The 96-hour E_bC_{10} (biomass) = 2.66 mg thymol/L (CI: 2.29 – 2.99 mg thymol/L) (mean measured).
The 96-hour E_yC_{10} (yield) = 3.68 mg thymol/L (CI: 2.85 – 4.31 mg thymol/L) (mean measured).

The 96-hour NOEC values for *Pseudokirchneriella subcapitata* were determined to be:

The 96-hour NOE_rC (growth rate) was determined to be 2.69 mg thymol/L (mean measured).
The 96-hour NOE_bC (biomass) was determined to be 0.82 mg thymol/L (mean measured).
The 96-hour NOE_yC (yield) was determined to be 2.69 mg thymol/L L for yield (mean measured).

The 96-hour LOEC values for *Pseudokirchneriella subcapitata* were determined to be:

The 96-hour LOE_rC (growth rate) was determined to be 8.10 mg thymol/L(nominal values).
The 96-hour LOE_bC (biomass) was determined to be 2.69 mg thymol/L (nominal values).
The 96-hour LOE_yC (yield) was determined to be 8.10 mg thymol/L (nominal values)

Validity
All validity criteria were met in accordance with OECD test guideline 201 (2006):

- The biomass in the control cultures increased exponentially by a factor of at least 16 within 72 hours (actual value: 87.6-fold increase within 72 hours)
- The mean coefficient of variation for section-by-section specific growth rates in the control cultures did not exceed 35% (actual values: 10.5% 0 – 72h)).
- The coefficient of variation of average specific growth rates during the whole test period in control replicates did not exceed 7% (actual values: 3.3 (0 – 72h)).

Assessment and conclusion

In a 96-hour toxicity study, cultures of *Pseudokirchneriella subcapitata* were exposed to thymol at nominal concentrations of 1, 3.2, 10, 32 and 100 mg thymol/L under static-dose response test conditions in accordance with OECD 201.

Analytical verification of test concentrations confirmed measured concentrations were maintained within 69 – 100% of nominals throughout the 96-hour test period. Biological endpoints are reported based on mean measured concentrations.

The 72-hour E_rC_{50} (growth rate) was estimated to be 11.1 mg thymol/L (mean measured), the 72-hour E_rC_{50} (biomass) was estimated to be 5.14 mg mg thymol/L (mean measured) and the 72-hour E_yC_{50} (yield) was estimated to be 4.89 mg thymol/L (mean measured). This study is considered acceptable and valid.

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

The study is acceptable.

Pseudokirchneriella subcapitata

72-hour E_rC_{50} (growth rate) = 11.1 mg thymol/L (mean measured).
72-hour E_bC_{50} (biomass) = 5.14 mg thymol/L (mean measured).
72-hour E_yC_{50} (yield) = 4.89 mg thymol/L (mean measured).

72-hour E_rC_{10} (growth rate) = 3.81 mg thymol/L (mean measured).
72-hour E_bC_{10} (biomass) = 1.54 mg thymol/L (mean measured).
72-hour E_yC_{10} (yield) = 2.23 mg thymol/L (mean measured).

72-hour E_rC_{20} (growth rate) = 5.50 mg thymol/L (mean measured).
72-hour E_bC_{20} (biomass) = 2.33 mg thymol/L (mean measured).
72-hour E_yC_{20} (yield) = 2.92 mg thymol/L (mean measured).

Assessment and conclusion by RMS:

Outcome and conclusion of the study:

This study was previously evaluated as part of the EU review for the inclusion of thymol.
Analytical verification of test concentrations confirmed measured concentrations were maintained within 69 – 100% of nominals throughout the 96-hour test period. Therefore, the biological endpoints are based on mean measured concentrations.

All validity criteria were met in accordance with OECD test guideline 201 (2006):

- The biomass in the control cultures increased exponentially by a factor of at least 16 within 72 hours (87.6).
- The mean coefficient of variation for section-by-section specific growth rates in the control cultures did not exceed 35% (10.5%, 0 – 72h).
- The coefficient of variation of average specific growth rates during the whole test period in control replicates did not exceed 7% (3.3%; (0 – 72h).

RMS agrees with the assessment of the applicant. The study is considered acceptable

Agreed endpoints:

72-hour ErC_{50} = 11.1 mg thymol/L (mean measured).
72-hour EbC_{50} = 5.14 mg thymol/L (mean measured).
72-hour EyC_{50} = 4.89 mg thymol/L (mean measured).

72-hour ErC_{10} = 3.81 mg thymol/L (mean measured).
72-hour EbC_{10} = 1.54 mg thymol/L (mean measured).
72-hour EyC_{10} = 2.23 mg thymol/L (mean measured).

72-hour ErC_{20} = 5.50 mg thymol/L (mean measured).
72-hour EbC_{20} = 2.33 mg thymol/L (mean measured).
72-hour EyC_{20} = 2.92 mg thymol/L (mean measured).

B.9.2.7. Effects on aquatic macrophytes

Thymol does not exhibit herbicidal activity or a plant growth regulator mode of action. Therefore, data on the effects on aquatic macrophytes are not required in accordance with Regulation (EU) No 283/2013.

B.9.2.8. Further testing on aquatic organisms

No further studies are considered necessary.

B.9.3. EFFECTS ON ARTHROPODS

B.9.3.1. Effects on bees

Several literature papers were identified during the literature search (B.9.11.1), assessing the short-term and long-term effects of thymol (or veterinary/bee-health formulations containing thymol) on bees. The most relevant of these literature papers are summarised below, Studies B.9.3.1/01 to B.9.3.1/04. The remaining papers are summarised in the Appendix I for completeness, but as discussed further in B.11.1 some of these papers are considered of limited relevance or reliability to the risk assessment of bees. Nevertheless, overall these papers all support a low risk of thymol to bees.

Study B.9.3.1/01

Data point:	CA 8.3.1/01
Report author	Giacomelli, A., Pietropaoli, M., Carvelli,A., Iacoponi, F., Formato, G.
Report year	2016
Report title	Combination of thymol treatment (Apiguard®) and caging the queen technique to fight *Varroa destructor*
Report No	-
Document No	Internal reference: Study 85 Apidologie Volume 47, Pages 606-616
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)
Guaranteeing high acaricide efficacy to control *Varroa destructor* is fundamental for colony survival. In this study, we verified the efficacy and impact of a commercial thymol-based veterinary product (Apiguard®) on colony honey bee populations when used alone or combined with the biotechnical method of caging honey bee queens to create an artificial brood interruption period in the colony. Apiguard® killed 76.1% of the mites while queen caging killed 40.6% of the mites. The combination of Apiguard® administration with queen caging killed 96.8% of the mites. Comparing bee numbers before and after treatment, Apiguard® treated colonies with caged queens had 48.7% fewer bees compared to before treatment, while Apiguard® alone reduced the number of adult bees by 13.6%. None of the treatments in the different groups resulted in elevated queen mortality.

Materials and methods

Test material 1

Name:	Apiguard (thymol)
Formulation type:	Slow release gel
Source and lot/batch no.:	Vita Europe Ltd (UK)
Active substance content:	Each aluminium tray contains 12.5 g of thymol in 50 g of gel
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species:	Honey bees, *Apis mellifera.*
Strain/clone:	Not reported
Age at study initiation:	Not reported
Weight/length/height at study initiation:	Not reported
Source:	Not reported
Feeding during test:	Not reported
Acclimation:	Not reported

Test conditions

Test temperature:	16.8-29.7 ºC Apiary 1 and 17.9-29.2 ºC Apiary 2
Photoperiod:	Not reported
Light intensity:	Not reported
Test system	
Study type:	Field trial in Central Italy with treatment of honey bee colonies with Apiguard trays

Duration of study:	Approx. 1 month
Treatments:	46 colonies were randomly divided into four different groups: (1) 10 colonies were treated with one tray of Apiguard® twice consecutively for a period of 10 days per tray ("Apiguard" group); (2) queens were caged in 12 colonies for 20 days using VARCONTROL® cages ("Queen caging" group); (3) 15 colonies were treated with Apiguard® as before and queens were caged for 20 days ("Apiguard plus queen caging"); and (4) 9 hives were left untreated to understand natural mite mortality ("Control").
Analytical determination of test concentrations: No	
Negative control included:	Yes
Positive control included:	No
Parameters measured:	Adult honey bee mortality (main purpose of study was to assess mite infestation, but this not discussed further here as not relevant to ecotoxicological assessment of bees).
Validity criteria:	Not reported

46 honey bee colonies were monitored: 24 in Apiary 1 and 22 in Apiary 2. The 46 colonies were randomly divided into four different groups: (1) 10 colonies were treated with one tray of Apiguard® twice consecutively for a period of 10 days per tray ("Apiguard group"); (2) queens were caged in 12 colonies for 20 days using VARCONTROL ® cages ("Queen caging" group); (3) 15 colonies were treated with Apiguard® as before and queens were caged for 20 days ("Apiguard® plus queen caging"); and (4) 9 hives were left untreated to understand natural mite mortality ("Control"). Colonies were housed in 10 frame Dadant-Blatt bee hives, had a similar strength and were free of any other symptomatic disease, except for varroatosis. The infestation levels between two apiaries and different treatment groups were similar. The infestation recorded in the groups ranged from 0.04 to 0.06 adult Varroa per bee. To verify the homogeneity of initial Varroa infestation of the selected colonies in the two apiaries, the natural mite fall was recorded for two weeks.
To determine the impact of Apiguard and queen caging on the number of adult honey bees in the treated hives, the study estimated the colony populations (adult bees) at the beginning of the treatments (day 15) and on day 34.

Results
The percent survival of adult bees was: 86.4% (ranging from 65.3% to 112.9%) in the "Apiguard®" group, 61.1% (ranging from 32.4% to 91.3%) in the "Queen caging"group, 51.3% (ranging from 34.8% to 67.3%) in the "Apiguard® plus queen caging" group, and 79.9% (ranging from 41.9% to 123.1%) in the "Control "group. The difference in adult bee population reduction between the "Apiguard® plus queen caging" and "Apiguard®" groups was statistically significant (P <0.001). Likewise, the difference in adult bee population reduction between the "Apiguard®" and "Queen caging" groups was statistically significant (P <0.001). There were no significant differences in the number of adult bees between the "Apiguard®" and "Control" groups (P =0.999) or between the "Apiguard® plus queen caging" and the "Queen caging" groups (P =0.999). None of the treatments resulted in queen mortality in the four experimental groups.

Assessment and conclusion
Reliability assessment
For full details and justification, please refer to Document KCA 9.4.2/02.
Proposed category: 2 reliable with restrictions
Study tested a slow-release gel formulation containing thymol, but exact exposure of thymol to bees is unknown (and no analytical verification included).

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).
In a field trial, honey bee colonies in Central Italy were treated with the anti-*Varroa* treatment "Apiguard®". Apiguard is a slow-release gel, added in aluminium trays to the honey bee hive; each tray contains 12.5 g of thymol in 50 g of gel. This study included (but was not limited to) 10 colonies treated with one tray of Apiguard® twice consecutively for a period of 10 days per tray ("Apiguard" group); and 9 hives left untreated to understand natural mite mortality ("Control"). There were no significant differences in the number of adult bees between the "Apiguard®" and "Control" groups on day 34 (survival of 86.4 and 79.9%, respectively).

Assessment and conclusion by RMS:

Outcome and conclusion of the study:

This is a scientific publication to study the effect of thymol (applied as a formulated product Apiguard®) into honey bee colonies as a *Varroa* treatment.
The test was not conducted under GLP and it does not follow any guidance.
No statisitcally significant differences in the number of adult bees were found between control bees and the bees treated with thymol. However, RMS is concerned about the reliability of these results to support bees risk assessment, since the bees exposure is not oral or contact, but the exposure is by evaporation of the thymol from the trays added to honey bees hive, each tray contains 12.5 g of thymol in 50 g of gel.

Under RMS opinion the study could be considered as supplementary information.

Study B.9.3.1/02

Data point:	CA 8.3.1/02
Report author	Glavan, G., Novak, S., Božič, J., Kokalj, A.J.
Report year	2020
Report title	Comparison of sublethal effects of natural acaricides carvacrol and thymol on honeybees
Report No	-
Document No	Internal reference: Study 97 Pesticide Biochemistry and Physiology 166, 104567, Pages 1-9 (2020)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)

The parasitic mite *Varroa destructor* is a threat to beekeeping colonies. Among naturally derived acaricides, the monoterpenoid essential oil compound thymol is used in beekeeping for varroa mite control, but adverse impacts on honeybees has been already documented. Carvacrol, another monoterpenoid, also has a high acaricidal potential and could thus be promising for regular use in beekeeping, but information is scarce regarding the effects of prolonged systemic administration of carvacrol on honeybees. In this study, we evaluate and compared the sublethal effects of long term consumption of carvacrol and thymol on Carnolian honeybee workers (*Apis mellifera carnica*). Survival and feeding rate were determined preliminary to assess sublethal concentrations.

The sublethal effects were analysed by the activity of the acetylcholinesterase (AChE), enzyme involved in the control of neurotransmission, and the activity of detoxifying enzyme glutathione S-transferase (GST) in heads and thoraces. We found that, thymol and carvacrol, caused mortality only at the highest concentrations tested, 1% and 5% respectively. As demonstrated by others, both substances could be effective against *varroa* at concentrations ten times lower than those causing significant honeybee mortality. However, we demonstrated sublethal effects at the 0.05% carvacrol and thymol exposure concentrations evidenced as increased activity of AChE and GST in the honeybee heads. In conclusion, prolonged treatment with thymol and carvacrol affects bee nervous system and induce detoxification processes possibly resulting in a limited use for acaricidal purposes. We postulate that under the same chronic exposure conditions carvacrol and thymol will have similar sublethal effects on honeybees.

Materials and methods

Test material 1

Name:	Thymol
Formulation type:	Not relevant
Source and lot/batch no.:	Merck (Darmstadt, Germany)
Active substance content:	≥ 98%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species:	Carnolian honeybees *Apis mellifera carnica*
Strain/clone:	Pollman 1879
Age at study initiation:	Summer adult worker honeybees
Source:	Collected from the same healthy colony, made by mixing two colonies, at the Department of Biology, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia. Colonies were maintained according to good honeybee practice and were not treated with any chemical substances for 1 month prior to the experiment.
Feeding during test:	Yes, protein cake constituted of 4% chicken egg yolk, 8% yeast, 13% tofu, 73% powdered sugar without additives and 2% olive oil (all cake ingredients were the products of organic farming), plus dechlorinated water, both provided *ad libitum.*
Acclimation:	Yes, 34 °C and 60% relative humidity

Test conditions

Test temperature:	34 ± 0.5 °C
Relative humidity:	60%
Photoperiod:	Continuous darkness
Test system	
Study type:	7-day oral toxicity test, with test item administered in a contaminated protein cake.
Duration of study:	7 days, feeders and their contents were renewed with freshly prepared food every 48 hours during the exposure period
Treatments:	control; 0.05%, 0.5% and 1% (w/w; weight of substance/ weight of food) offered as a contaminated protein cake
Analytical determination of test concentrations: No	
Negative control included:	Yes
Positive control included:	No
Parameters measured:	Mortality and feeding rate. Molecular biomarkers (enzymatic activity) were also measured, but are not discussed further in this summary as not considered relevant.
Validity criteria:	Mortality in controls did not exceed 15% at the end of the test

Since thymol is volatile and insoluble in sucrose solution, the test substance was fed as a contaminated protein cake that consisted of 4% chicken egg yolk, 8% yeast, 13% tofu, 73% powdered sugar without additives and 2% olive oil. Thymol was dissolved in warm olive oil before being added to the cake. For the oral exposure, the feeders, which were graduated 5 mL sterile syringes for single use with sealed ends, were inserted vertically and extended to the bottom of the cage and filled with the test cake mixture. A hole the size of a bee was made in the middle of the syringe with a scalpel that enabled the bees to freely access the protein cake. Fifteen honeybees per cage were exposed in 3 cages per concentration (total of 12 cages) in two sets of experiments (to give 6 cages per concentration).

Results

After 7 days oral exposure (*via* a dosed protein cake), negligible mortality of honeybees (below 10%) was noticed up to 0.05% of thymol. Very low mortality (around 20%) was evidenced in the case of 0.5% of thymol. The concentration of 1% of thymol caused around 45% of honeybees to die, compared to approximately 10% in the control group (based on Figure 1 of the paper). Feeding of honeybees on food dosed with essential oils was evaluated as an average feeding rate/ honeybee/ day. A significant increase in feeding was evidenced at 0.5% of thymol in comparison to control, while the highest concentration of thymol (1%) had no effect on the feeding in comparison to control.

No effects on mortality or feeding rate were observed at concentrations up to 0.05% thymol (based on nominal concentrations).

Assessment and conclusion

Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 2 reliable with restrictions

Positive control (toxic reference) not included. No analytical verification of dosed feed (as usually required in a 10-day chronic oral honeybee test according to OECD 245).

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).

Apis mellifera (honey bee) 7-day oral exposure *via* dosed protein cake:

No effects on mortality or feeding rate at 0.05% (w/w; weight of substance/ weight of food) thymol based on nominal concentrations.

At 0.5% w/w thymol (nominal), approx. 10% corrected mortality, and slight but significant increase in feeding rate.

At 1% w/w thymol (nominal), approx. 35% corrected mortality, and no effects on feeding rate.

Assessment and conclusion by RMS:

Outcome and conclusion of the study:

This is a scientific publication to study the effect of natural acaricides (thymol and carvacol) on honeybees. Mortality, feeding rate and sublethal effects (activity of the acetylcholinesterase (AChE) and of detoxifying enzyme glutathione S-transferase (GST)) were studied. The study was performed on Carnolian honeybee workers (*Apis mellifera carnica*).
The test was not conducted under GLP and it does not follow any guidance. The validity criteria of the current Guideline to study the chronic oral toxicity test on bees (OECD 245, 2017) cannot be checked. Additionally, a positive control was not tested. The study lasted for 7 days.
Three test item concentration were tested : 0.05, 0.5 and 1 g thymol/100g of food offered as a contaminated protein cake plus a control.
The test analytical verification to check the actual exposure during the study was not reported.
Effects on mortality were observed : 10% at 0.5% w/w thymol and 35% at 1% w/w. However, a significan effect on feeding rate was only observed at 0.5% (w/w) of thymol.
With regards to the sublethal effects, AChE and GST activities were increased in the heads in all exposure groups above 0.05% of thymol. AChE activity in thoraces was not altered, while GST activity was increased only at the highest tested concentration (1%).

Therefore, acute and chronic effects on bees were observed due to oral exposure of thymol, mainly in the highest tested concentrations. However, because of the deficiencies of the study it should be considered as supplementary information.

Study B.9.3.1/03

Data point:	CA 8.3.1/03
Report author	Gashout, H.A., Goodwin, P.H., Guzman-Novoa, E.
Report year	2018
Report title	Lethality of synthetic and natural acaricides to worker honey bees (*Apis mellifera*) and their impact on the expression of health and detoxification-related genes
Report No	-
Document No	Internal reference: Study 232 Environmental Science and Pollution Research, Volume 25, Pages 34730–34739 (2018)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)
In this study, honey bees (*Apis mellifera* L.) were exposed to LD_{05} and LD_{50} doses of five commonly used acaricides for controlling the parasitic mite, *Varroa destructor*. LD_{50} values at 48 h post-treatment showed that tau-fluvalinate was the most toxic, followed by amitraz, coumaphos, thymol, and formic acid. However, the hazard ratios, which estimate the hive risk level based on a ratio of a standard dose of acaricide per hive to the LD_{50} of the acaricide, revealed that tau-fluvalinate was the most hazardous followed by formic acid, coumaphos, amitraz, and thymol. The expression of the honey bee acetylcholinesterase gene increased after treatment with the LD_{05} and LD_{50} acaricide doses and could distinguish three patterns in the timing and level of increased expression between acaricides: one for amitraz, one for tau-fluvalinate and formic acid, and one for coumaphos and thymol. Conversely, changes in cytochrome P450 gene expression could also be detected in response to all five acaricides, but there were no significant differences between them. Changes in vitellogenin gene expression could only detect the effects of taufluvalinate, amitraz, or coumaphos treatment, which were not significantly

different from each other. Among the acaricides tested, coumaphos, amitraz, and thymol appear to be the safest acaricides based on their hazard ratios, and a good marker to detect differences between the effects of sub-lethal doses of acaricides is monitoring changes in acetylcholinesterase gene expression.

Materials and methods

Test material

Name:	Thymol
Formulation type:	Not relevant
Source and lot/batch no.:	Fisher Scientific (Ottawa, Ontario, Canada)
Active substance content:	Not reported
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species:	Honey bee (*Apis mellifera* L.*)*
Strain/clone:	Buckfast strain
Age at study initiation:	newly emerged bees, same age
Weight/length/height at study initiation:	Not reported
Source:	Honey Bee Research Center of the University of Guelph in Ontario, Canada
Feeding during test:	Yes. 50% sugar/distilled water solution
Acclimation:	placed in wooden emergence cages and incubated overnight at 32 ± 2 ºC and 60 ± 5% relative humidity. The next morning, newly emerged bees were used for experiments.

Test conditions

Test temperature:	32 ± 2 ºC
Relative humidity:	60 ± 5%
Photoperiod:	Not reported
Light intensity:	Not reported

Test system

Study type:	Acute contact toxicity test
Duration of study:	48 hours
Treatments:	0.5, 5, 50, 250 and 750 µg thymol/bee
Analytical determination of test concentrations:	No
Negative control included:	Yes, a control and a solvent control
Positive control included:	No
Parameters measured:	Mortality
Validity criteria:	Not reported

To estimate LD_{50} and LD_{05} values, thymol was serially diluted in 95% ethanol for final concentrations of 150.0, 50.0, 10.0, 1.0, and 0.1 mg/mL. The doses of thymol applied to the bees were 750, 250, 50, 5, and 0.5 µg/bee. Thymol and the solvent used were administered individually as a single 10-µg topical dose in 5 µL to the dorsal surface of each worker's thorax using a micropipette. Bees were held between two fingers until the 5 µL of the acaricide was absorbed onto the thorax. For controls, a set of workers were treated with 5 µL of 95% ethanol, while the non-treated control was not exposed to ethanol or thymol. Each treated group consisted of 25 bees. After receiving the topical treatment, each group of bees was placed and maintained in a wooden cage (12.7 × 8.5 × 14.5 cm) with a wire-screened wall. The cages were provided with two gravity-top feeders. The number of dead bees was recorded at 2, 24 and 48 hours post-treatment. The experiment was repeated three times.
Mortality rates were calculated for each treatment and the data were arcsine-square root transformed to normalize means and then subjected to analysis of variance. The LD_{50}, LD_{05} values, inverse 95% confidence limits, slopes, intercepts, and r^2 values were calculated for the different acaricides by Probit analysis using the US Environmental Protection Agency Statistical Program (version 1.5) (Lindberg et al. 2000), which adjusts for the non-treated control mortality (USEPA 1992).

Results
At 2 hours post treatment with thymol, mortality was not significantly different from that of control bees. At 24 and 48 hours post treatment, mortality caused by thymol was now significantly different than those of both controls.
48h-contact-LD_{50} value was reported to be 51.250 µg thymol/bee (95% confidence limits: 37.469 – 68.775 µg thymol/bee) (based on nominal concentrations).
Assessment and conclusion
Reliability assessment
For full details and justification, please refer to Document KCA 9.4.2/02.
Proposed category: 2 reliable with restrictions
Study did not follow a specific guideline but is well reported. Test item purity not reported and no positive control.

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).
Apis mellifera: 48h-contact LD_{50} = 51.250 µg thymol/bee (95% confidence limits: 37.469-68.775 µg thymol/bee) based on nominal concentrations.

Assessment and conclusion by RMS:

Outcome and conclusion of the study:

This is a scientific publication to compare the most commonly used synthetic acaricides (tau-fluvalinate, amitraz, and coumaphos) to natural acaricides (thymol and formic acid) for survival (LD_5 and LD_{50} were determined). Furthermore, in a second trial, bees were treated with LD_5 and LD_{50} doses to determine the expression of health and detoxification-related genes.
The test was not conducted under GLP and it does not follow any guidance. Therefore, the validity criteria cannot be checked. The exposure of the bees was through contact.
Five test item concentration were tested to calculate LD_5 and LD_{50} (0.5, 5, 50, 250 and 750 µg thymol/bee) plus a control and a solvent control. A treatment with a toxic standard was not tested. Moreover, the test analytical verification to check the actual exposure during the study was not reported.
A LD50 for thymol of 51.250 µg thymol/bee was conculated. However, due to the deficiencies of the study, this endpoint cannot be used as

Therefore, acute and chronic effects on bees were observed due to oral exposure of thymol, mainly in the highest tested concentrations. However, because of the deficiencies of the study this endpoint cannot be used to EU Regulatory purpose.

Therefore, in agreement with the applican conclusion, this study should be considered as **supplementary information.**

Study B.9.3.1/04

Data point:	CA 8.3.1/04
Report author	Maistrello, L., Lodesani, M., Costa, C., Leonardi, F., Marani, G., Caldon, M., Mutinelli, F., Granato, A.
Report year	2008
Report title	Screening of natural compounds for the control of nosema disease in honeybees (*Apis mellifera*)
Report No	-
Document No	Internal reference: Study 293 Apidologie volume 39, Pages 436-445 (2008)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)
The potential of some natural compounds (thymol, vetiver essential oil, lysozyme, resveratrol) for the control of nosema infection in honeybees was evaluated. A first trial aimed at screening substances, in candy preparations, on the basis of their toxicity to honeybees and bees' dietary preferences. None of the tested substances showed an increased bee mortality or decreased bee preference, and were therefore considered suitable for further testing. In the second trial the effects of the natural compounds on nosema diseased honeybees were evaluated: bees were individually dosed with nosema spores and fed candies prepared with the screened substances. The results showed that bees fed with thymol and resveratrol candies had significantly lower infection rates, and bees supplied with resveratrol prepared candy also lived significantly longer. We suggest that thymol and resveratrol could be useful in alternative strategies for the control of nosema disease.

Materials and methods

Test material

Name:	thymol
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma
Active substance content:	minimum of 99.5%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species:	Honey bees, *Apis mellifera ligustica*
Strain/clone:	Not reported
Age at study initiation:	Newly emerged, nosema-free worker honey bees
Weight/length/height at study initiation:	Not reported
Source:	CRA-API apiary, Reggio Emilia, Italy.
Feeding during test:	Yes, Bee candy (powdered sugar (85%), sterilized honey (10%) and water (5%)
Acclimation:	Combs with capped cells close to emergence were brought into the laboratory and kept in a thermostat (34°C) for 6 hours. Later were kept in wooden cages (10 x 10 x 20 cm).

Test conditions

Test temperature:	33 ºC
Relative humidity:	70%
Photoperiod:	constant darkness
Light intensity:	Not reported
Test system	
Study type:	Chronic toxicity test
Duration of study:	11 days
Treatments:	0.06 and 0.12 mg thymol/ g f.s.
Analytical determination of test concentrations: No	
Negative control included:	Yes, control candy (CE) containing ethanol (3.2 µL/g) and control candy without ethanol
Positive control included:	No
Parameters measured:	Mortality
Validity criteria:	Not reported

The natural compounds were added to the honey during preparation of the candy and ethanol was used to aid their solubility; a control candy containing ethanol (3.2 µL/g) was set up as well as control candy without ethanol. The treated candy, contained in a 3 cm diameter Petri dish (9 g), was positioned upside-down on a 1 cm diameter hole on top of the cage. A gravity feeder containing water was also fitted on the cages. A minimum number of two cages per product and concentration were set up yielding a total of 30 test cages. On the 4th, 7th and 11th day from the beginning of the experiment, the candy dishes were weighed to estimate the feed intake and dead bees in each cage were counted and removed.
All analyses were carried out using Statistica- StatSoft v. 7.1.

Results
Average mortality of the caged bees fed with the experimental candies after 11 days was 54% and no significant differences were detected among the groups. The daily bee intake of candy was 49 mg on average and no significant differences were detected in the statistical comparison. The results showed that thymol caused no toxic effects on the adult bees, or anti-feedant properties, as candy intake was similar throughout the experimental groups.

Assessment and conclusion
Reliability assessment
For full details and justification, please refer to Document KCA 9.4.2/02.
Proposed category: 2 reliable with restrictions
No analytical verification is included; and no positive control.

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

The study is considered as supporting information only (reliable with restrictions).
Apis mellifera: thymol caused no toxic effects on the adult bees at nominally 0.12 mg thymol/g feeding solution after 11 days' exposure.

Assessment and conclusion by RMS:

Outcome and conclusion of the study:

This is a scientific publication to evaluate the potential of some natural compounds (thymol, vetiver essential oil, lysozyme, resveratrol) for the control of nosema infection in honeybees.
A first trial evaluates the toxicity the tested substance to honeybees and bees' dietary preferences. A second trial study the effects of of the natural compounds on nosema diseased honeybees.
Two thymol concentrations were tested to study its toxicity (0.12 and 2.5 mg a.s./g food) plus a control and a solvent control, during 11 days. A toxicity control was not tested.
The test was not conducted under GLP and it does not follow any guidance. Therefore, the validity criteria cannot be checked. Moreover, the test analytical verification to check the actual exposure during the study was not reported.
Average mortality of bees fed with the experimental candies after 11 days was 54% and no significant differences were detected among treatments. However, a high mortality was observed in the controls: > 50% and > 30% for control and solvent control, respectively. Please, see the following figure for further information.

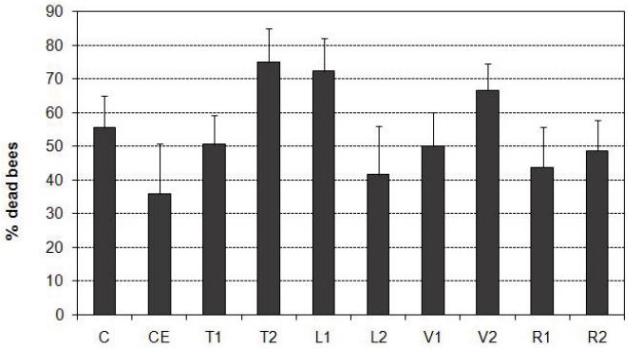

Figure 1. Average (+SE) percentage of dead bees per differently fed group (C = Control, CE = Control Ethanol, T1 = Thymol 0.12 mg/g, T2 = Thymol 0.06 mg/g, L1 = Lysozyme 2.5 mg/g, L2 = Lysozyme 0.5 mg/g, V1 = Vetiver oil 1.2 mg/g, V2 = Vetiver oil 0.6 mg/g, R1 = Resveratrol 0.01 mg/g, R2 = Resveratrol 0.001 mg/g).

According to OECD Guideline 245 (2017, Honey bee (*Apis mellifera L.*), Chronic Oral Toxicity Test (10-day feeding), the study is valid if "*The average mortality across replicates for the untreated control and solvent control groups is ≤ 15 % at the end of the test (10 days following start of exposure); when a solvent control is included, the average mortality across replicates for the solvent control should also be ≤ 15 %*".
The study was not conducted following a guideline. and thus, it would not be necessary that meet specific validity criteria. However, considering high mortality in the controls the RMS is concerning with regards to the reliability of the study.

Therefore, considering this concern the study is **not accepted**.

B.9.3.1.1. Acute toxicity to bees

B.9.3.1.1.1. Acute oral toxicity

The data on the representative formulation, Mevalone, are sufficient to address the active substance data requirement. An acute oral honey bee toxicity study with Mevalone was previously evaluated as part of the EU review for the EU inclusion of thymol (DAR, Volume 3, Annex B.9, 2011, B.9.4.1). A full summary is provided in Vol. 3 CP Mevalone B.9.5.1.1.1/01.

B.9.3.1.1.2. Acute contact toxicity

The data on the representative formulation, Mevalone, are sufficient to address the active substance data requirement. An acute contact honey bee toxicity study with Mevalone was previously evaluated as part of the EU review for the EU inclusion of thymol (DAR, Volume 3, Annex B.9, 2011, B.9.4.1). A full summary is provided in Vol. 3 CP Mevalone B.9.5.1.1.1/01.

B.9.3.1.2. Chronic toxicity to bees

New data on the representative formulation, Mevalone are sufficient to address the active substance data requirement. A new chronic oral honey bee toxicity study with Mevalone has been provided to meet new data requirements under Regulation (EU) No 284/2013; a full summary is provided in Vol. 3 CP B.9.5.1.2/01.
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B.9.3.1.3. Effects on honeybee development and other honeybee life stages

New data on the representative formulation, Mevalone are sufficient to address the active substance data requirement. A new repeated exposure honey bee larval toxicity study with Mevalone has been provided to meet new data requirements under Regulation (EU) No 284/2013; a full summary is provided in Vol. 3 CP B.9.5.1.3/01.

B.9.3.1.4. Sub-lethal effects

No further studies are considered necessary.

B.9.3.2. Effects on non-target arthropods other than bees

Two literature papers were identified during the literature search (see B.9.11.1 for full details), assessing the effects of topical/contact exposure of thymol on *Chrysoperla* (lacewing) and *Podisus* (predatory bug) species. Results of these papers are summarised in Appendix I for completeness (CA 9.6.3.4/01 and CA 9.6.3.4/17), but these are considered of limited relevance and reliability for use in the risk assessment, principally as a non-standard study design was used and test item purity was not reported. The critical endpoints summarised in Vol. 3 CP Mevalone B.9.3.2.1 from the standard glass plate (IOBC) studies with *Typhlodromus* and *Aphidius* species, testing the representative product, Mevalone, are considered more relevant and reliable for the risk assessment. No further studies are considered necessary.

B.9.3.2.1. Effects on Aphidius rhopalosiphi

The data on the representative formulation, Mevalone are expected to be sufficient to address the active substance data requirement. An *Aphidius rhopalosiphi* glass plate laboratory study with Mevalone was previously evaluated as part of the EU review for the EU inclusion of thymol (DAR, Volume 3, Annex B.9, 2011, B.9.5.1). A full summary is provided in Vol. 3 CP B.9.3.2.1.

B.9.3.2.2. Effects on Typhlodromus pyri

The data on the representative formulation, Mevalone are expected to be sufficient to address the active substance data requirement. A *Typhlodromus pyri* glass plate laboratory study with Mevalone was previously evaluated as part of the EU review for the EU inclusion of thymol (DAR, Volume 3, Annex B.9, 2011, B.9.5.2). A full summary is provided in Vol. 3 CP B.9.3.2.1.

B.9.4. EFFECTS ON NON-TARGET SOIL MESO- AND MACROFAUNA

B.9.4.1. Earthworm – sub-lethal effects

The data on the representative formulation, Mevalone are expected to be sufficient to address the active substance data requirement. One acute earthworm toxicity study with Mevalone was previously evaluated as part of the EU review for the EU inclusion of thymol (DAR, Volume 3, Annex B.9, 2011, B.9.6.1). The requirement for acute toxicity data for earthworms is now obsolete under Regulation (EU) No 283/2013. This study is therefore not directly relevant for the current assessment, but is provided in Vol. 3 CP B.9.7.1 as supporting information to support renewal of thymol. In addition, a new chronic toxicity study with formulation Mevalone has been provided to meet new data requirements under Regulation (EU) No 284/2013; a full summary is provided in Vol 3 CP B.9.7.1.1/02.

B.9.4.2. Effects on non-target soil meso- and macrofauna (other than earthworms)

B.9.4.2.1. Species level testing

The data on the representative formulation, Mevalone, are expected to be sufficient to address the active substance data requirement. It is noted that *Hypoaspis acuelifer* and *Folsomia candida* studies are not formally required as there is no direct application to soil and a low risk is concluded at Tier 1 with *T. pyri* and *A. rhopalosiphi*, but a new *Folsomia candida* study is provided for completeness. A new chronic toxicity study in *Folsomia candida* with formulation Mevalone has therefore been provided, a full summary is provided in CP B.9.7.2.1.1/01.

B.9.5. EFFECTS ON SOIL NITROGEN TRANSFORMATION

The data on the representative formulation, Mevalone, are expected to be sufficient to address the active substance data requirement. One mineralisation study with Mevalone was previously evaluated as part of the EU review for the EU inclusion of thymol (DAR, Volume 3, Annex B.9, 2011, B.9.8.1). A full summary is provided Vol. 3 CP B.9.9.

B.9.6. EFFECTS ON TERRESTRIAL NON-TARGET HIGHER PLANTS

B.9.6.1. Summary of screening data

The available screening data on the representative formulation, Mevalone, are expected to be sufficient to address the active substance data requirement. A summary is provided in Vol. 3 CP B.9.11.
In addition, it is noted that several literature papers were identified during the literature search (see B.9.11.1 for full details), assessing the effects of thymol on various plant terrestrial species. Results of these papers are summarised in Appendix I for completeness, but as discussed further in B.9.11.1 these are considered of limited relevance and reliability for use in the risk assessment, principally as non-standard study designs were used. The available screening data summarised in Vol. 3 CP B.11, testing the representative product, Mevalone, are considered more relevant and reliable for the risk assessment of terrestrial non-target higher plants. No further studies are considered necessary.

B.9.6.2. Testing on non-target plants

Since the screening data confirmed that the representative formulation, Mevalone, does not have an herbicidal mode of action, no further data are required.

B.9.7. EFFECTS ON OTHER TERRESTRIAL ORGANISMS (FLORA AND FAUNA)

No further studies are considered necessary.

B.9.8. EFFECTS ON BIOLOGICAL METHODS FOR SEWAGE TREATMENT

A new study with the representative formulation Mevalone has been provided to meet new data requirements under Regulation (EU) No 283/2013 for the active substance; a full summary is provided below.

Study B.9.8/01

Data point:	CA 8.8/01
Report author	Hammesfahr, U.
Report year	2020
Report title	Mevalone: Toxicity to Activated Sludge in a Respiration Inhibition Test
Report No	155781171
Document No	-
Guidelines followed in study	OECD Guideline 209 (2010) and ISO 8192 (2007)
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted at EU level
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

The influence of the product Mevalone on the activity of activated sludge was evaluated by measuring the respiration rate under defined conditions. The respiration rate (oxygen consumption) of an aerobic activated sludge fed with a standard amount of synthetic sewage feed was measured in the presence of various concentrations of the product after an incubation period of 3 hours.

The test was performed in accordance with OECD guideline 209 (2010). Five test concentrations were tested: 10, 32, 100, 320 and 1000 mg product/L. The product Mevalone showed significant effects on total and heterotrophic respiration at the highest tested concentration of 1000 mg product/L, corresponding to 31.5 mg eugenol/L, 69.3 mg geraniol/L and 62.3 mg thymol/L.

The critical (lowest) 3-hour NOEC value, based on nitrification respiration, was determined to be 32 mg product/L, corresponding to 1.0 mg eugenol/L, 2.2 mg geraniol/L, and 2.0 mg thymol/L. The critical (lowest) 3-hour EC_{50} value, based on nitrification respiration, was calculated to be 204.9 mg product/L (CI: 115.5 – 361.7 mg product/L), corresponding to 6.5 mg eugenol/L, 14.2 mg geraniol/L and 12.8 mg thymol/L.

This study is considered as acceptable and satisfies the requirements established in OECD Guideline 209 (2010).

Materials and methods

Test material

Name: Mevalone
Formulation type: CS
Source and lot/batch no.: 11001
Active substance content: 3.15%, 6.93 and 6.23% w/w (analysed) for eugenol, geraniol and thymol respectively
Expiry date of lot/batch: August 31, 2021
Storage conditions: At 20 ± 5 °C in the dark

Toxic reference

Name: 3,5-Dichlorophenol
Formulation type: Not relevant
Source and lot/batch no.: MKBZ0947V
Active substance content: 100%
Expiry date of lot/batch: June 28, 2022
Storage conditions: At 20 ± 5 °C in the dark

Nitrification inhibitor

Name: N-allylthiourea (ATU)
Formulation type: Not relevant
Source and lot/batch no.: S7709958
Active substance content: 100%
Expiry date of lot/batch: Not reported

Storage conditions: At 20 ± 5 °C in the dark

Test organism

Species: Activated sludge, microorganisms
Source: From a domestic waste water treatment plant (municipal sewage treatment plant Bensheim, Germany).

Test conditions

Test temperature: 20 ± 2°C during pre-incubation (3 hours) and evaluation period. Recorded continuously
pH: At start 6.6 - 6.8 at the end 6.8 - 6.9
Oxygen concentration: At start 4.4 – 5.0 at the end 6.0- 8.2 mg O_2/L

Five nominal test concentrations were tested: 10, 32, 100, 320 and 1000 mg product/L, with five replicates for each test concentration. The reference item 3,5-Dichlorophenol was tested at the nominal test concentrations of 1, 4, and 16 mg/L (five replicates for each test concentration) under otherwise identical test conditions. Six controls (pure water, synthetic sewage feed and inoculum, but without addition of the product) were tested in parallel. A nitrification inhibitor N-allylthiourea (ATU) was tested in the same way with six separate controls and at the identical nominal concentrations of the product and also the reference item 3,5-dichlorophenol under otherwise identical test conditions.

For each replicate a test solution with a final volume of 500 mL was tested per treatment in a glass flask. 16 mL synthetic sewage feed and an adequate amount of the product or an adequate volume of the stock solution of the reference item were filled up with pure water to 250 mL before the start of the test. At the start of the test 250 mL activated sludge inoculum with a sludge concentration of 3.0 g/L suspended solids was added, first to two controls, then to the test solutions of the reference item in increasing concentrations, to a further two controls, then to the product in increasing concentrations and finally to an additional two controls. During the 3 hour aeration period the flasks were stirred on a magnetic stirrer to maintain sludge flocs in suspension. For the measurement of the respiration rate a well-mixed sample of each test medium was poured into a Karlsruher flask after exactly 3 hours incubation time. The oxygen concentration was then measured with an oxygen electrode and recorded for about ten minutes. During measurement, the samples were continuously stirred on a magnetic stirrer.

For the statistical determination of NOEC values, data were analysed using a Williams Multiple Sequential t-test (α= 0.05, one-sided smaller). Regression analysis was used to calculate ECx values. The software used to perform the statistical analysis was ToxRat Professional, Version 3.3.0, ToxRat Solutions GmbH.

Results

Biological results
The influence of Mevalone on the total respiration rate of activated sludge is presented below:

Table 9.8/01-1. Influence of Mevalone and the Reference Item 3,5-Dichlorophenol on the Inhibition of the Total Respiration Rate of Aerobic Waste Water Microorganisms after 3 Hours of Exposure

Treatment	Nominal Conc.	Respiration Rate [mean][a]	Standard deviation[a]	Inhibition [mean][a]	Significant effect compared to control[b]
	[mg/L]	[mg O_2/g*h]		[%]	
Control	---	22.9	3.3		
3,5-DCP	1	13.6	3.1	40.7	*
	4	12.5	2.6	45.4	*
	16	4.9	0.4	78.7	*
Mevalone	10	26.8	3.0	-17.2	n.s.
	32	28.7	2.2	-25.6	n.s.
	100	26.2	1.9	-14.5	n.s.
	320	22.4	1.7	2.0	n.s.
	1000	6.6	0.7	71.0	*

Coefficient of Variation of control: 14.6%
a: mean value of 5 replicates, 6 replicates in case of control
b: significance according to Williams Multiple Sequential t-test, one-sided smaller, α = 0.05 (* = significant; n. s.: not significant)
Inhibition [mean] % = 100-(Respiration Rate [mean] [mg O2/g*h]*100 / mean value respiration rate in controls)

In comparison to the inoculum controls total respiration was not inhibited up to and including a product concentration of 320 mg product/L. The highest product concentration of 1000 mg product/L strongly inhibited the total respiration.
For total respiration, the 3-hour NOEC value was determined to be at a Mevalone concentration of 320 mg product/L, corresponding to 10.1 mg eugenol/L, 22.2 mg geraniol/L and 19.9 mg thymol/L.
The 3-hour EC_{10} value for total respiration was established to be 282.6 mg product/L (CI: 193.4 – 413.1 mg product/L), corresponding to 8.9 mg eugenol/L, 19.6 mg geraniol/L and 17.6 mg thymol/L.
The 3-hour EC_{20} value for total respiration was established to be 376.5 mg product/L (CI: 263.7 – 543.4 mg product/L), corresponding to 8.9 mg eugenol/L, 19.6 mg geraniol/L and 17.6 mg thymol/L.
The 3-hour EC_{50} value for total respiration was established to be 651.4 mg product/L (CI: 422.0 – >1000 mg product/L), corresponding to 20.5 mg eugenol/L, 45.1 mg geraniol/L and 40.6 mg thymol/L.

The influence of Mevalone on the heterotrophic respiration rate of activated sludge is presented below:

Table 9.8/01-2. Influence of Mevalone and the Reference Item 3,5-Dichlorophenol on the Inhibition of the Heterotrophic Respiration Rate of Aerobic Waste Water Microorganisms after 3 Hours of Exposure

Treatment	Nominal Conc.	Respiration Rate [mean][a]	Standard deviation[a]	Inhibition [mean][a]	Significant effect compared to control[b]
	[mg/L]	[mg O_2/g*h]		[%]	
Control	---	14.7	3.8	---	
3,5-DCP	1	12.0	2.2	18.2	n.s.
	4	16.3	1.2	-11.3	n.s.
	16	5.0	0.6	65.6	*
Mevalone	10	18.8	2.3	-28.2	n.s.
	32	19.8	1.9	-34.7	n.s.
	100	21.0	1.4	-43.0	n.s.
	320	18.3	1.6	-24.9	n.s.
	1000	9.4	2.2	36.2	*

Coefficient of Variation of control: 26.1%
a: mean value of 5 replicates, 6 replicates in case of control
b: significance according to Williams Multiple Sequential t-test, one sided smaller, α = 0.05 (test item) or Welsh t-test after Bonferroni Holm, one sided smaller, α = 0.05 (reference item), * = significant; n. s.: not significant
Inhibition [mean] % = 100-(Respiration Rate [mean] [mg O2/g*h]*100 / mean value respiration rate in controls)

In comparison to the inoculum controls the heterotrophic respiration was not inhibited up to and including a product concentration of 320 mg product/L. The highest product concentration of 1000 mg product/L inhibited the total respiration.
For heterotrophic respiration, the 3-hour NOEC value was determined to be at a product concentration of 320 mg product/L, corresponding to 10.1 mg eugenol/L, 22.2 mg geraniol/L and 19.9 mg thymol/L.
The 3-hour EC_{10} value based on heterotrophic respiration was determined to be 599.5 mg product/L (CI: 8.2 – >1000 mg product/L), corresponding to 18.9 mg eugenol/L, 41.5 mg geraniol/L and 37.3 mg thymol/L.
The 3-hour EC_{20} value based on heterotrophic respiration was determined to be 717.0 mg product/L (CI: 9.8 – >1000 mg product/L), corresponding to 22.6 mg eugenol/L, 49.7 mg geraniol/L and 44.7 mg thymol/L.
The 3-hour EC_{50} value based on heterotrophic respiration was determined to be 1010.0 mg product/L (CI: 3.0 – >1000 mg product/L), corresponding to 31.8 mg eugenol/L, 70.0 mg geraniol/L and 62.9 mg thymol/L.

The influence of Mevalone on the respiration rate based on nitrification of activated sludge is presented below:

Table 9.8/01-3. Influence of Mevalone and the Reference Item 3,5-Dichlorophenol on the Inhibition of the Nitrification Respiration Rate of Aerobic Waste Water Microorganisms after 3 Hours of Exposure

Treatment	Nominal Concentration	Respiration Rate [mean][a]	Standard Deviation[a]	Inhibition [mean][a]	Significant effect compared to control[d]
	[mg/L]	[mg O_2/g*h]		[%]	
Control	---	8.2	3.3	---	---
3,5-DCP	1	1.6	3.1	81.0	---
	4	-3.8	2.6	146.8	---
	16	-0.2	0.4	102.0	---
Mevalone	10	8.0	3.0	2.3	n.s.
	32	8.9	2.2	-9.1	n.s.
	100	5.2	1.9	36.5	*
	320	4.1	1.7	50.2	*
	1000	-2.7	0.7	133.2	*
Coefficient of Variation of control: 40.8%					
[a]: mean value of 5 replicates, 6 replicates in case of control					
[d] significance according Williams Multiple t-test, one-sided smaller, α = 0.05 (* = significant; n. s.: not significant)					
Inhibition [mean] % = 100-(Respiration Rate [mean] [mg O_2/g*h]*100 / mean value respiration rate in controls)					

In comparison to the inoculum controls, the respiration rates of the activated sludge based on nitrification were inhibited starting from a product concentration of 100 mg product/L.
For nitrification respiration the 3-hour NOEC value was determined to be at a product concentration of 32 mg product/L, corresponding to 1.0 mg eugenol/L, 2.2 mg geraniol/L and 2.0 mg thymol/L.
The 3-hour EC_{10} value based on nitrification respiration was determined to be 64.7 mg product/L (CI: 40.3 – 103.9 mg product/L), corresponding to 2.0 mg eugenol/L, 4.5 mg geraniol/L and4.0 mg thymol/L.
The 3-hour EC_{20} value based on nitrification respiration was determined to be 96.1 mg product/L (CI: 60.9 – 153.3 mg product/L), corresponding to 3.0 mg eugenol/L, 6.7 mg geraniol/L and 6.0 mg thymol/L.
The 3-hour EC_{50} value based on nitrification respiration was determined to be 204.9 mg product/L (CI: 115.5 – 361.7 mg product/L), corresponding to 6.5 mg eugenol/L, 14.2 mg geraniol/L and 12.8 mg thymol/L.
Validity
All validity criteria were met in accordance with OECD guideline 209 (2010):
- The blank controls oxygen uptake was ≥20 mg oxygen per one gram of activated sludge (dry weight of suspended solids) in an hour (actual value 22.9 mg O_2/g/h).
- The coefficient of variation of oxygen uptake rate in control replicates was ≤30% at the end of the definitive test (actual values 14.6% for the total respiration and 26.1% for the heterotrophic respiration).
- The reference item 3,5-dichlorophenol EC_{50} values were within the range of 2 mg/L to 25 mg/L for total respiration (actual value 2.8 mg/L), 5 mg/L to 40 mg/L for heterotrophic respiration (actual value 15.0 mg/L) and 0.1 mg/L to 10 mg/L for nitrification respiration (actual value 0.8 mg/L).

Assessment and conclusion
In a 3-hour activated sludge respiration inhibition test, Mevalone showed significant effects on total and heterotrophic respiration at concentrations of 1000 mg product/L, corresponding to 31.5 mg eugenol/L, 69.3 mg geraniol/L and 62.3 mg thymol/L. For nitrification respiration, significant effects were determined starting from a test item concentration of 100 mg product/L, corresponding to 3.2 mg eugenol/L, 6.9 mg geraniol/L, and 6.2 mg thymol/L.
For total and heterotrophic respiration, the 3-hour NOEC value was determined to be at a test item concentration of 320 mg product/L, corresponding to 10.1 mg eugenol/L, 22.2 mg geraniol/L and 19.9 mg thymol/L. For nitrification respiration the 3-hour NOEC value was determined to be at a test item concentration of 32 mg product/L, corresponding to 1.0 mg eugenol/L, 2.2 mg geraniol/L and 2.0 mg thymol/L.
The critical (lowest) 3-hour NOEC value, based on nitrification respiration, was determined to be 32 mg product/L, corresponding to 1.0 mg eugenol/L, 2.2 mg geraniol/L, and 2.0 mg thymol/L. The critical (lowest) 3-hour EC_{50} value, based on nitrification respiration, was calculated to be 204.9 mg product/L (CI: 115.5 – 361.7 mg product/L), corresponding to 6.5 mg eugenol/L, 14.2 mg geraniol/L and12.8 mg thymol/L.This study is considered as acceptable and satisfies the requirements established in OECD Guideline 209 (2010).
This study is considered as acceptable and satisfies the requirements established in OECD Guideline 209 (2010).

Assessment and conclusion by applicant:

The study is acceptable.
The critical (lowest) 3-hour NOEC value, based on nitrification respiration, was determined to be 32 mg product/L, corresponding to 1.0 mg eugenol/L, 2.2 mg geraniol/L and 2.0 mg thymol/L.
The critical (lowest) 3-hour EC_{50} value, based on nitrification respiration, was calculated to be 204.9 mg product/L (CI: 115.5 – 361.7 mg product/L), corresponding to 6.5 mg eugenol/L, 14.2 mg geraniol/L and 12.8 mg thymol/L.

Assessment and conclusion by RMS:

Outcome and conclusion of the study:

The test was performed according to OECD 209 (2010). The validity criteria are met:
- Control O_2 uptake was 22.9 mg O_2/g/h (it should be at least 20 mg O_2/g/h);
- The coefficient of variation in the controls was 14.6% for the total respiration and 26.%1 for heterotrophic respiration (it should be less than 30%);
- The EC_{50} for 3,5-DCP was:
  - For the total respiration: 2.8 mg/L (the expected range is 2 – 25 mg/L).
  - For heterotrophic respiration: 15.0 mg/L (the expected range is 5 - 40 mg/L).
  - For the oxygen uptake due to nitrification: 0.8 mg/L (the expected range is 0.1 - 10 mg/L).

The study was conducted with the formulated product (Mevalone) instead of the active substance.

The study is considered acceptable.

Agreed endpoints

Based on nitrification respiration:

3-h EC_{50} = 204.9 (CI: 115.5-361.7) mg Mevolane/L; 12.8 mg thymol/L
3-h EC_{10} = 64.7 (CI: 40.3-103.9) mg Mevolane/L; 4.0 mg thymol/L
3-h EC_{20} = 96.1 (CI: 60.9-153.3) mg Mevolane/L; 6.0 mg thymol/L
3-h NOEC = 32 mg Mevolane/L; 2.0 mg thymol/L;

B.9.9. MONITORING DATA

No further studies are considered necessary.

B.9.10. BIOLOGICAL ACTIVITY OF METABOLITES POTENTIALLY OCCURRING IN GROUNDWATER

No further studies are considered necessary since the residue definition does not include any relevant metabolite in groundwater.

B.9.11. REFERENCES RELIED ON

B.9.11.1. Literature search

B.9.11.1.1. Summary

This data point reviewed the 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.

It has been written 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, in line with Section 5.2.2 of EFSA Journal 2011;9(2):2092:

- 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, in line with Section 5.3 of EFSA Journal 2011;9(2):2092:

- 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.

All literature papers that were considered relevant to the risk assessment following this process are summarised in the corresponding Vol. 3 CA/CP B.9, where an assessment of their reliability is also presented in line with Section 5.4 of EFSA Journal 2011;9(2):2092.

The results of this search were as follows:

Study selection process	**Thymol**			
	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)	35 229			
Total number of documents retrieved (without duplicates) after focused search strategy (combining terms for active substance and specific data requirements)	494	303	510	1120
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***

* Includes 41 papers, plus 3 papers for which full-text was not available. In absence of the full-text, these 3 papers are considered relevant and the available abstract has been provided as a summary (but cannot be assessed for reliability).

For ecotoxicology, 5 papers have been considered as relevant and reliable following the two-step process. For 39 remaining papers, since the reliability of relevant papers is limited to the ecotoxicological risk assessment of thymol, they have not been considered further in this document and brief summaries of these papers are provided below in the Appendix I.

Aim is to find scientific peer-reviewed open literature, as required by Article 8(5) of Regulation (EC) No 1107/2009 on the placing of plant protection products on the market, on thymol dealing with toxicological and toxicokinetic studies, operator exposure, residues, fate and behaviour in the environment and ecotoxicological articles which are published within the last fifteen years from a wide range of reference collections consulted through online wide and various databases.

Details on these various reference collections are provided hereafter (B.11.1.2) fulfilling requirements from EFSA and AGES on the bibliographic database (in terms of quality and quantity). The search strategy leading to the inclusion or exclusion of publications is described below (see CA 9.2, CA 9.3 and CA 9.4) and summarized in two workflow charts (B.9.11.1.3 and B.9.11.1.4).

B.9.11.1.2. Search strategy

The following initial search strategy was used, in line with Section 5.2.2 of EFSA Journal 2011;9(2):2092:

- 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 9.11.1.2.2 below.

The literature search was conducted between 2nd July 2020 and 16/17 February 2021. 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.9.11.1.2.1. Bibliographic database used in the literature review

In line with Section 5.2.1 of EFSA Journal 2011;9(2):2092, 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 9.11.1.2.1-1: Bibliographic databases used in the literature search

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 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 millions 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 330,000 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 300,000 book.
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.9.11.1.2.2. 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 9.11.1.2.2-1: List of 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
Chemical name (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

Often an event or outcome is not explicitly described by the subject at the title or abstract level and it would be difficult to adequately describe the individual ecotox effects one can envisage using key words and/or subject headings in a complex search query. This granular information was captured during the text processing phase using customised gazetteer lists such as the extracts given in Table 9.11.1.2.2-2 below for aquatic invertebrates and birds. These case insensitive lists contain both generic terms such as “mollusc(s)” and both common and scientific names at the species level, e.g., pond snail (Lymnaea). In total the gazetteer list of terms describing aquatic invertebrates contained 47 entities and the birds gazetteer contained 63 entities (All subject gazetteer list files used in this project have been provided to Staphyt as a separate zip file KCA 9.2.4/01). On another hand, the subject lists were crossed with Effects lists for each section.

The tables below present examples of the subject and effect section specific search terms used:

Table 9.11.1.2.2-2: List of subject search terms used specific to a section (example)

Aquatic Invertebrate list (selected entries)	**Birds list (selected entries)**
chironomid	Anas
chironomids	avian
Chironomus	bird
Crassostrea	birds
crustacea	blackcap
crustacean	blackcaps
crustaceans	bluetit
Daphnia	bluetits
daphnid	bobwhite
…	…

Table 9.11.1.2.2-3: List of effect search terms used specific to a section (example)

Effect (ecotox)
absorption
acute
adrenal
adsorption
adverse
allergen
allergenic
allergens
allergic
…

B.9.11.1.3. 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 for first search (done in 2020), 33 715 documents, for second search (done in 2021), 1 514 documents, total 35 229 documents of potential interest for thymol to this review before the removal of duplicates occurring as a result of searching the individual reference collections separately.

Table 9.11.1.3-1: Numbers of records (before removing duplicates) for the search (based on search terms according to Table 9.11.1.2.2-1)

Databases (through online STN)	Number of hits Thymol First search	Number of hits Thymol Second search	Number of hits Thymol Total
1. AGRICOLA	-	88	88
2. AGRIS (FAO)	190	14	204
3. PASCAL	-	-	-
4. PubMed MEDLINE (including TOXNET)	17 870	223	18 093
5. Science Direct	7 424	219	7 643
6. Springer	4 701	575	5 276
7. Wiley	3 530	395	3 925
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)	**33 715**	**1 514**	**35 229**

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 behavior 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 for, say, the ecotoxicity of thymol, an example being "Acaricidal activities of *Santolina africana* and *Hertia cheirifolia* essential oils against the two-spotted spider mite (*Tetranychus urticae*)."; and index the results.
After removing duplicates and text mining process, the total number of hits potentially relevant is presented below:

Table 9.11.1.3-2: Number of documents identified as potentially relevant after text mining and removal of duplicates

All databases	Number of hits* Thymol
Mammalian Toxicity	494
Residues	303
Environmental fate and behaviour	510
Ecotox	**1120**

*Total number of documents retrieved after removal of duplicates/triplicates (using general plus section specific terms)

B.9.11.1.4. Study Selection

The remaining summary records were assessed for relevance in a two-step process, in line with Section 5.3 of EFSA Journal 2011;9(2):2092:

- 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 of the relevance and reliability of full-text documents was conducted for any remaining summary records that were not excluded in the first step.

All literature papers that were considered ecotoxicological relevant to the risk assessment following this two-step process are summarised in Vol. 3 CA/CP B.9, where an assessment of their reliability is also presented in line with Section 5.4 of EFSA Journal 2011;9(2):2092.

Crietria for relevance is included in the following table

Data requirement	Justification for Search Strategy
Ecotoxicological studies (OECD IIA 8.1 to 8.8 and IIIA 10.1 to 10.7)	
Effects on birds (OECD IIA 8.1.1 and IIA 8.1-3 – 8.1.5; OECD IIIA 10.1.1) Effects on aquatic organisms (OECD II 8.2 and IIIA 10.2) Effects on other terrestrial vertebrates (OECD IIA 8.1.2 and IIA 8.1-3 – 8.1.5; IIIA 10.1.2, IIIA 10.1.3) Effects on bees (OECD IIA 8.3.1 and OECD IIIA 10.3.1) Effects on other arthropod species (OECD IIA 8.3.2 and OECD IIIA 10.3.2) Effects on earthworms (OECD IIA 8.4.1 and OECD IIIA 10.4.1) Effects on soil non-target macro-organisms (OECD IIA 8.4.2 and OECD IIIA 10.4.2) Effects on soil non-target micro-organisms (OECD IIA 8.5 and OECD IIIA 10.5)	**Relevance check based on information given in the title and the abstract:** 1. Ecotoxicological studies conducted with the active substance, metabolites or product containing the active substance (defined test material) addressing any of the data requirements. 2. Field studies relevant for European conditions (climate, species, …) 3. Papers which are dealing with the effects on mammals were not included in the relevance assessment because this was considered to be covered in the section toxicology (see 2.4.1 Toxicology). However, studies on other non-target vertebrate species (e.g. fish, birds, other mammals than relevant for toxicological testing) or studies which are not covered by the data requirements of the section toxicology, e.g. field studies, avoidance studies, were considered in the relevance and reliability check. 4. Literature reviews were excluded where clear from the title and abstract. 5. Literature coping with the general production/presence of essential oils in plants were excluded. 6. Literature coping with effects only at a molecular level (e.g. gene expression) were excluded, unless where potentially related to endocrine activity. **Relevance check based on full-text**: 1. Literature reviews were excluded. 2. Literature coping with combined and/or mixture toxicity were excluded (no tests were conducted with the active substance and/or a solo formulation). 3. Papers were excluded if they only assessed effects of plant essential oils containing several constituents. If an active substance test item had a purity of <70% of the active substance of interest (i.e. <70% eugenol, geraniol or thymol), literature were excluded.

Data requirement	Justification for Search Strategy
Effects on other non-target organisms (flora and fauna) believed to be at risk (OECD IIA 8.6 and OECD IIIA 10.7) Effects on biological methods of sewage treatment (OECD IIA 8.8)	4. Literature coping with effects on target pests (i.e. efficacy data), including effects on fungi, insect pests (mites, mosquitoes, ticks) and nematodes were excluded.

B.9.11.1.4.1. 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 9.11.1.4.1-1: Numbers of publications excluded/identified after rapid assessment for relevance for thymol

Study selection process	Thymol			
	Toxicology (including human exposure)	Residues	Environmental fate and behaviour	Ecotoxicology
Number of publications excluded after rapid assessment for relevance (non-relevant, excluded literature)	445	271	494	899
Number of publications for which detailed assessment of full-text required after rapid assessment for relevance	12	17	3	129

B.9.11.1.4.2. 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 911.1.4.2-1: Numbers of publications excluded/identified after detailed assessment for relevance for thymol

Study selection process	Thymol			
	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)	2	8	0	92
Number of publications considered relevant following the two-step process (i.e. after detailed assessment of full-text)	**10**	**9**	**3**	**37***

* Includes 34 papers, plus 3 papers for which full-text was not available. In absence of the full-text, these 4 papers are considered relevant and the available abstract has been provided as a summary (but cannot be assessed for reliability).

All publications excluded following the detailed assessment are presented in the tables below, with a reason for not including in the dossier.

Table 9.11.1.4.2-2: Publications excluded from the risk assessment after detailed assessment of full-text documents – results for thymol (Ecotoxicology)

Author(s)	Year	Title	Source	Reason for not including in dossier (based on relevance criteria)
Zhou Xiaolong ; Zhao Duoyong ; He Weizhong ; Liu Shenghong ; Liu Hejiang ; Feng Ting ; Wang Cheng ; Wu Lipeng ; Huo Xin (No 7 in CA 9.4/02)	2017	Acaricidal Activity and Synergistic Effect of Thyme Oil Constituents against Carmine Spider Mite (Tetranychus Cinnabarinus (Boisduval)).	Molecules. 2017 Nov 1;22(11):1873. doi: 10.3390/molecules22111873	Assessed effects only on target pests
Chi-Hoon Lee; Bo-Kyung Sung; Hoi-Seon Lee (No 12 in CA 9.4/02)	2006	Acaricidal activity of fennel seed oils and their main components against Tyrophagus putrescentiae a stored-food mite	Journal of Stored Products Research Volume 42 Issue 1 2006 Pages 8-14	Assessed effects only on target pests
Laryssa Xavier Araújo; Tatiane Pinheiro Lopes Novato; Viviane Zeringota; Renata Silva Matos; Tatiane Oliveira Souza Senra; Ralph Maturano; Márcia Cristina Azevedo Prata; Erik Daemon; Caio Márcio Oliveira Monteiro (No 14 in CA 9.4/02)	2015	Acaricidal activity of thymol against larvae of Rhipicephalus microplus (Acari: Ixodidae) under semi-natural conditions	Parasitology Research volume 114 pages 3271 – 3276 (2015)	Assessed effects only on target pests
Natalia Damiani; Liesel B. Gende; Pedro Bailac; Jorge A. Marcangeli; MartÃn J. Eguaras (No 15 in CA 9.4/02)	2009	Acaricidal and insecticidal activity of essential oils on Varroa destructor (Acari: Varroidae) and Apis mellifera (Hymenoptera: Apidae)	Parasitology Research volume Â 106 Â pages 145 â€“ 152 (2009)	No assessment of test item with ≥70% purity

Author(s)	Year	Title	Source	Reason for not including in dossier (based on relevance criteria)
Mohamed E. I. Badawy; Sailan A. A. El-Arami; Samir A. M. Abdelgaleil (No 16 in CA 9.4/02)	2010	Acaricidal and quantitative structure activity relationship of monoterpenes against the two-spotted spider mite Tetranychus urticae	Experimental and Applied Acarology volume 52 pages 261 – 274 (2010)	Assessed effects only on target pests
Born Flávia de Souza ; da Camara Claudio Augusto Gomes ; de Melo João Paulo Ramos ; de Moraes Marcilio Martins (No 17 in CA 9.4/02)	2018	Acaricidal property of the essential oil from Lippia gracilis against Tetranychus urticae and a natural enemy Neoseiulus californicus under greenhouse conditions.	Exp Appl Acarol. 2018 Aug;75(4):491-502. doi: 10.1007/s10493-018-0286-3.	Assessed effects only on target pests
Su Li-Chong ; Huang Chin-Gi ; Chang Shang-Tzen ; Yang Shu-Hui ; Hsu Shan-Hui ; Wu Wen-Jer ; Huang Rong-Nan (No 26 in CA 9.4/02)	2014	An improved bioassay facilitates the screening of repellents against cat flea Ctenocephalides felis (Siphonaptera: Pulicidae).	Pest Manag Sci. 2014 Feb;70(2):264-70. doi: 10.1002/ps.3554.	Assessed effects only on target pests
Y.?P. LUO; T.?H. HUANG; N.?Y. TIEN; Y.?P. LUO; T.?H. HUANG; N.?Y. TIEN (No 27 in CA 9.4/02)	2015	An in vitro bioassay for the quantitative evaluation of mosquito repellents against Stegomyia aegypti (=Aedes aegypti) mosquitoes using a novel cocktail meal	Medical and Veterinary Entomology Volume 29 Issue 3	No assessment of test item with ≥70% purity
Silva R A ; Vieira L S ; Camurça-Vasconcelos A L F ; Bevilaqua C M L ; Morais S M ; Maciel M V ; Costa C T C ; Macedo I T F ; Oliveira L M B ; Braga R R (No 41 in CA 9.4/02)	2007	Anthelmintic activity of Croton zehntneri and Lippia sidoides essential oils.	Vet Parasitol. 2007 Sep 30;148(3-4):288-94. doi: 10.1016/j.vetpar.2007.06.012.	No assessment of test item with ≥70% purity

Author(s)	Year	Title	Source	Reason for not including in dossier (based on relevance criteria)
Marjanovi? S. ?or?e Bogunovi? Danica Milovanovi? Mirjana Marinkovi? Darko et al. (No 44 in CA 9.4/02)	2019	Antihelminic activity of carvacrol thymol cinnamaldehyde and p-cymen against the free-living nematode Caenorhabditis elegans and rat pinworm Syphacia muris	[Acta Veterinaria]; 2019/RS/RS2019_0.rdf	Assessed effects only on target pests
Lee Sung-Chan ; Seo Seon-Mi ; Huh Min-Jung ; Kwon Jun-Hyeong ; Nam Il ; Park Ji-Hong ; Park Il-Kwon (No 54 in CA 9.4/02)	2020	Behavioral and Electrophysiological Effects of Ajowan (Trachyspermum ammi Sprague) (Apiales: Apiaceae) Essential Oil and Its Constituents on Nymphal and Adult Bean Bugs Riptortus clavatus (Thunberg) (Hemiptera: Alydidae).	Insects. 2020 Feb 4;11(2):104. doi: 10.3390/insects11020104	Assessed effects only on target pests
Delia M. Pinto-Zevallos b; Maria de Fátima Arrigoni-Blank a; Elizangela Mércia Cruz Oliveira a; Taís Santos Sampaio a; Adriano Pimentel Farias a; Caroline Rabelo Coelho c; Arie Fitzgerald Blank a; Maria Clezia dos Santos a; Adenir Vieira Teodoro b; Mariana Santos Menezes a (No 57 in CA 9.4/02)	2019	Bioactivity of essential oil from Lippia gracilis Schauer against two major coconut pest mites and toxicity to a non-target predator	Crop Protection Volume 125 November 2019 104913	No assessment of test item with ≥70% purity
Tabanca Nurhayat ; Bernier Ulrich R ; Ali Abbas ; Wang Mei ; Demirci Betul ; Blythe	2013	Bioassay-guided investigation of two Monarda essential oils as repellents of yellow fever	J Agric Food Chem. 2013 Sep 11;61(36):8573-80. doi: 10.1021/jf402182h.	Assessed effects only on target pests

Author(s)	**Year**	**Title**	**Source**	**Reason for not including in dossier (based on relevance criteria)**
Eugene K ; Khan Shabana I ; Baser K Husnu Can ; Khan Ikhlas A (No 58 in CA 9.4/02)		mosquito Aedes aegypti.		
Mohaddeseh Abouhosseini Tabari; Mohammad Reza Youssefi; Alireza Barimani; Atefeh Araghi (No69 in CA 9.4/02)	2015	Carvacrol as a potent natural acaricide against Dermanyssus gallinae	Parasitology Research volume 114 pages 3801 – 3806 (2015)	Assessed effects only on target pests
Giatropoulos Athanassios ; Kimbaris Athanasios ; Michaelakis ?ntonios ; Papachristos Dimitrios P ; Polissiou Moschos G ; Emmanouel Nickolaos (No 72 in CA 9.4/02)	2018	Chemical composition and assessment of larvicidal and repellent capacity of 14 Lamiaceae essential oils against Aedes albopictus.	Parasitol Res. 2018 Jun;117(6):1953-1964. doi: 10.1007/s00436-018-5892-9.	Assessed effects only on target pests
Mancini Emilia ; Camele Ippolito ; Elshafie Hazem S ; De Martino Laura ; Pellegrino Carlo ; Grulova Daniela ; De Feo Vincenzo (No 74 in CA 9.4/02)	2014	Chemical composition and biological activity of the essential oil of Origanum vulgare ssp. hirtum from different areas in the Southern Apennines (Italy).	Chem Biodivers. 2014 Apr;11(4):639-51. doi: 10.1002/cbdv.201300326	No assessment of test item with ≥70% purity
Ouassila Khalfi; Naima Sahraoui; Fatiha Bentahar; Chahrazed Boutekedjiret; Ouassila Khalfi; Naima Sahraoui; Fatiha Bentahar; Chahrazed Boutekedjiret (No 75 in CA 9.4/02)	2008	Chemical composition and insecticidal properties of Origanum glandulosum (Desf.) essential oil from Algeria - Khalfi - 2008 - Journal of the Science of Food and Agriculture - Wiley Online Library	Journal of the Science of Food and Agriculture Volume 88 Issue 9	No assessment of test item with ≥70% purity
Farzam Masoumi; Mohammad Reza Youssefi; Mohaddeseh Abouhosseini Tabari	2016	Combination of carvacrol and thymol against the poultry red mite (Dermanyssus gallinae)	Parasitology Research volume 115 pages 4239 – 4243 (2016)	Assessed effects only on target pests

Author(s)	Year	Title	Source	Reason for not including in dossier (based on relevance criteria)
(No 84 in CA 9.4/02)				
Tabanca Nurhayat ; Avonto Cristina ; Wang Mei ; Parcher Jon F ; Ali Abbas ; Demirci Betul ; Raman Vijayasankar ; Khan Ikhlas A (No 89 in CA 9.4/02)	2013	Comparative investigation of Umbellularia californica and Laurus nobilis leaf essential oils and identification of constituents active against Aedes aegypti.	J Agric Food Chem. 2013 Dec 18;61(50):12283-91. doi: 10.1021/jf4052682.	Assessed effects only on target pests
Opender Koul; Rajwinder Singh 1; Birpal Kaur; Dharamvir Kanda (No 92 in CA 9.4/02)	2013	Comparative study on the behavioral response and acute toxicity of some essential oil compounds and their binary mixtures to larvae of Helicoverpa armigera Spodoptera litura and Chilo partellus	Industrial Crops and Products Volume 49 August 2013 Pages 428-436	Assessed effects only on target pests
Araújo I N ; Alves P B ; Cavalcanti S C H ; Niculau E dos S ; Blank A F ; Câmara C A G (No 98 in CA 9.4/02)	2010	Composition and acaricidal activity of Lippia sidoides essential oil against two-spotted spider mite (Tetranychus urticae Koch).	Bioresour Technol. 2010 Jan;101(2):829-32. doi: 10.1016/j.biortech.2009.08.053.	Assessed effects only on target pests
Juliana Navarro-Rocha a; María Fe Andrés b; Carmen E. Díaz c; Jesus Burillo a; Azucena González-Coloma b (No 99 in CA 9.4/02)	2020	Composition and biocidal properties of essential oil from pre-domesticated Spanish Satureja Montana	Industrial Crops and Products Volume 145 March 2020 111958	Assessed effects only on target pests
Werdin González Jorge O ; Gutiérrez María M ; Murray Ana P ; Ferrero Adriana A (No 100 in CA 9.4/02)	2011	Composition and biological activity of essential oils from Labiatae against Nezara viridula (Hemiptera: Pentatomidae) soybean pest.	Pest Manag Sci. 2011 Aug;67(8):948-55. doi: 10.1002/ps.2138.	Assessed effects only on target pests

Author(s)	**Year**	**Title**	**Source**	**Reason for not including in dossier (based on relevance criteria)**
Mahmood R. (National Agricultural Research Centre Islamabad (Pakistan). Honeybee Research Inst.) Wagchoure E.S. (National Agricultural Research Centre Islamabad (Pakistan). Honeybee Research Inst.) Atta-ul-Mohsin (PMAS University of Arid Agriculture Rawalpindi (Pakistan). Dept. of Entomology) Raja S. et al. (No 105 in CA 9.4/02)	2013	Control of ectoparasitic mites in honeybee (Apis mellifera L.) colonies by using thymol and oxalic acid	[Pakistan Journal of Zoology (Pakistan)]; 2013/PK/PK2013_0 rdf	Assessed only combined/mixture toxicity
Humberto Boncristiani a b; Robyn Underwood c 1; Ryan Schwarz a; Jay D. Evans a; Jeffery Pettis a; Dennis vanEngelsdorp c (No 118 in CA 9.4/02)	2012	Direct effect of acaricides on pathogen loads and gene expression levels in honey bees Apis mellifera	Journal of Insect Physiology Volume 58 Issue 5 May 2012 Pages 613-620	Assessed effects only at a molecular level
Nevin Keskin 1; Erkay Özgör 2 (No 132 in CA 9.4/02)	2018	Effect of thymol and oxalic acid according to application method against Varroa destructor in Honeybees	Journal of Biotechnology Volume 280 Supplement 30 August 2018 Page S48	Assessed only combined/mixture toxicity
Kristina W. Rothchild; Lynn S. Adler; Rebecca E. Irwin; Ben M. Sadd; Philip C. Stevenson; Evan C. Palmer?Young; Kristina W. Rothchild; Lynn S. Adler; Rebecca E. Irwin; Ben M. Sadd; Philip C. Stevenson; Evan C.	2018	Effects of short?term exposure to naturally occurring thymol concentrations on transmission of a bumble bee parasite - Rothchild - 2018 - Ecological Entomology - Wiley Online Library	Ecological Entomology Volume 43 Issue 5	No assessment of effects on non-target organisms

Author(s)	Year	Title	Source	Reason for not including in dossier (based on relevance criteria)
Palmer?Young (No 137 in CA 9.4/02)				
Monique B. Morselli a; Matheus D. Baldissera b; Carine F. Souza b; João H. Reis a; Bernardo Baldisserotto b; Alison A. Sousa a; Fernando Zimmer a; Diogo L.A. Lopes a; Tiago G. Petrolli c; Aleksandro S. Da Silva a (No 142 in CA 9.4/02)	2020	Effects of thymol supplementation on performance mortality and branchial energetic metabolism in grass carp experimentally infected by Aeromonas hydrophila	Microbial Pathogenesis Volume 139 February 2020 103915	Assessed effects only at a molecular level
Xiaopeng Lu a; Hua Weng c; Chao Li a; Jun He a b; Xing Zhang a b; Zhiqing Ma a b (No 148 in CA 9.4/02)	2020	Efficacy of essential oil from Mosla chinensis Maxim. cv. Jiangxiangru and its three main components against insect pests	Industrial Crops and Products Volume 147 May 2020 112237	Assessed effects only on target pests
Roman Pavela a; Filippo Maggi b; Giulio Lupidi b; Kevin Cianfaglione c d; Xavier Dauvergne c; Maurizio Bruno e; Giovanni Benelli f (No 152 in CA 9.4/02)	2017	Efficacy of sea fennel (Crithmum maritimum L. Apiaceae) essential oils against Culex quinquefasciatus Say and Spodoptera littoralis (Boisd.)	Industrial Crops and Products Volume 109 15 December 2017 Pages 603-610	Assessed effects only on target pests
Moghadamnia Ali Akbar ; Sut Stefania ; Dall'Acqua Stefano ; Benelli Giovanni ; Maggi Filippo ; Youssefi Mohammad Reza ; Tabari Mohaddeseh Abouhosseini ; Esfandiari Aryan ; Kazemi Sohrab (No 155 in CA 9.4/02)	2019	Efficacy of Two Monoterpenoids Carvacrol and Thymol and Their Combinations against Eggs and Larvae of the West Nile Vector Culex pipiens.	Molecules. 2019 May 15;24(10):1867. doi: 10.3390/molecules24101867	Assessed effects only on target pests
Z.L. Zheng a b; Justin	2009	Evaluation of oregano	Aquaculture Volume 292	Assessed effects only at a

Author(s)	Year	Title	Source	Reason for not including in dossier (based on relevance criteria)
Y.W. Tan c; H.Y. Liu a; X.H. Zhou a; X. Xiang a; K.Y. Wang b (No 171 in CA 9.4/02)		essential oil (Origanum heracleoticum L.) on growth antioxidant effect and resistance against Aeromonas hydrophila in channel catfish (Ictalurus punctatus)	Issues 3–4 15 July 2009 Pages 214-218	molecular level
Adriana Maria da Silveira Novelino; Erik Daemon; Geraldo Luiz Gonçalves Soares (No 173 in CA 9.4/02)	2007	Evaluation of the acaricide effect of thymol menthol salicylic acid and methyl salicylate on Boophilus microplus (Canestrini 1887) (Acari: Ixodidae) larvae	Parasitology Research volume 101 Article number: 809 (2007)	Assessed effects only on target pests
Novato Tatiane Pinheiro Lopes ; Araújo Laryssa Xavier ; de Monteiro Caio Márcio Oliveira ; Maturano Ralph ; Senra Tatiane de Oliveira Souza ; da Silva Matos Renata ; Gomes Geovany Amorim ; de Carvalho Mario Geraldo ; Daemon Erik (No 174 in CA 9.4/02)	2015	Evaluation of the combined effect of thymol carvacrol and (E)-cinnamaldehyde on Amblyomma sculptum (Acari: Ixodidae) and Dermacentor nitens (Acari: Ixodidae) larvae.	Vet Parasitol. 2015 Sep 15;212(3-4):331-5. doi: 10.1016/j.vetpar.2015.08.021.	Assessed effects only on target pests
Ma Wei-Bin ; Feng Jun-Tao ; Jiang Zhi-Li ; Wu Hua ; Ma Zhi-Qing ; Zhang Xing (No 190 in CA 9.4/02)	2014	Fumigant activity of eleven essential oil compounds and their selected binary mixtures against Culex pipiens pallens (Diptera: Culicidae).	Parasitol Res. 2014 Oct;113(10):3631-7. doi: 10.1007/s00436-014-4028-0.	Assessed effects only on target pests
F. Erler (No 191 in CA 9.4/02)	2005	Fumigant activity of six monoterpenoids from aromatic plants in Turkey	Journal of Plant Diseases and Protection volume 112 pages 602 –	Assessed effects only on target pests

Author(s)	Year	Title	Source	Reason for not including in dossier (based on relevance criteria)
		against the two stored-product pests confused flour beetle Tribolium confusum and Mediterranean flour moth Ephestia kuehniella	611 (2005)	
Chae Song-Hwa ; Kim Soon-Il ; Yeon Seong Hum ; Perumalsamy Haribalan ; Ahn Young-Joon (No 194 in CA 9.4/02)	2014	Fumigant toxicity of summer savory and lemon balm oil constituents and efficacy of spray formulations containing the oils to B- and neonicotinoid-resistant Q-biotypes of Bemisia tabaci (Homoptera: Aleyrodidae).	J Econ Entomol. 2014 Feb;107(1):286-92. doi: 10.1603/ec13287	Assessed effects only on target pests
Hanan A. Gashout a b; Ernesto Guzman-Novoa b; Paul H. Goodwin b; Adriana Correa-Benítez c (No 209 in CA 9.4/02)	2020	Impact of sublethal exposure to synthetic and natural acaricides on honey bee (Apis mellifera) memory and expression of genes related to memory	Journal of Insect Physiology Volume 121 February–March 2020 104014	Assessed effects only at a molecular level
Gomes Geovany Amorim ; Zeringóta Viviane ; Franco Cristiane Teixeira ; de Oliveira Débora Ramos ; Melo Diego ; de Carvalho Mário Geraldo ; Daemon Erik ; de Oliveira Monteiro Caio Márcio ; Novato Tatiane (No 210 in CA 9.4/02)	2018	In vitro assessment of the acaricidal activity of carvacrol thymol eugenol and their acetylated derivatives on Rhipicephalus microplus (Acari: Ixodidae).	Vet Parasitol. 2018 Aug 30;260:1-4. doi: 10.1016/j.vetpar.2018.07.009.	Assessed effects only at a molecular level
T. D'Addabbo; I. Birò; X. Malov; N. Sasanelli; A.	2009	Influence of arbuscular mycorrhizal fungi on the	Helminthologia volume 46	Assessed effects only on target pests

Author(s)	Year	Title	Source	Reason for not including in dossier (based on relevance criteria)
Anton; T. Takács (No 214 in CA 9.4/02)		nematicidal properties of leaf extracts of Thymus vulgaris L.	Article number: 230 (2009)	
Bisrat Daniel ; Jung Chuleui (No 221 in CA 9.4/02)	2020	Insecticidal Toxicities of Three Main Constituents Derived from Trachyspermum ammi (L.) Sprague ex Turrill Fruits against the Small Hive Beetles Aethina tumida Murray.	Molecules. 2020 Mar 1;25(5):1100. doi: 10.3390/molecules25051100	Assessed effects only on target pests
Pandiyan G Navaneetha ; Mathew Nisha ; Munusamy Sundharesan (No 227 in CA 9.4/02)	2019	Larvicidal activity of selected essential oil in synergized combinations against Aedes aegypti.	Ecotoxicol Environ Saf. 2019 Jun 15;174:549-556. doi: 10.1016/j.ecoenv.2019.03.019.	Assessed effects only on target pests
Carvalho Mário G de ; Figueiredo Marcela B ; Gomes Geovany A ; Santangelo Jayme M ; Pontes Emerson G ; Azambuja Patricia ; Garcia Elói S (No 231 in CA 9.4/02)	2017	Lethal and sublethal effects of essential oil of Lippia sidoides (Verbenaceae) and monoterpenes on Chagas' disease vector Rhodnius prolixus.	Mem Inst Oswaldo Cruz. 2017 Jan 1;112(1):63-69. doi: 10.1590/0074-02760160388.	Assessed effects only on target pests
Rocha G F ; Del Vesco A P ; Santana T P ; Santos T S ; Cerqueira A S ; Zancanela V T ; Fernandes R P M ; Oliveira Júnior G M (No 233 in CA 9.4/02)	2020	Lippia gracilis Schauer essential oil as a growth promoter for Japanese quail.	Animal. 2020 Apr 29:1-9. doi: 10.1017/S1751731120000816	No assessment of test item with ≥70% purity
Alexandros Papachristoforou a b c 1; Chrisovalantis Papaefthimiou a; Georgia	2011	Monitoring the gravitational reflex of the ectoparasitic mite Varroa destructor: A novel	Pesticide Biochemistry and Physiology Volume 101 Issue 2 October 2011 Pages 109-117	Assessed effects only on target pests

Author(s)	Year	Title	Source	Reason for not including in dossier (based on relevance criteria)
Zafeiridou a; Vasiliki Goundy a; Max Watkins d; George Theophilidis a (No 244 in CA 9.4/02)		bioassay for assessing toxic effects of acaricides		
Govindarajan Marimuthu ; Sivakumar Rajamohan ; Rajeswary Mohan ; Veerakumar Kaliyan (No 246 in CA 9.4/02)	2013	Mosquito larvicidal activity of thymol from essential oil of Coleus aromaticus Benth. against Culex tritaeniorhynchus Aedes albopictus and Anopheles subpictus (Diptera: Culicidae).	Parasitol Res. 2013 Nov;112(11):3713-21. doi: 10.1007/s00436-013-3557-2.	Assessed effects only on target pests
Jason Lei; Michael Leser; Essam Enan (No 249 in CA 9.4/02)	2010	Nematicidal activity of two monoterpenoids and SER-2 tyramine receptor of Caenorhabditis elegans	Biochemical Pharmacology Volume 79 Issue 7 1 April 2010 Pages 1062-1071	Assessed effects only on target pests
Andrés Maria Fe ; González-Coloma Azucena ; Muñoz Ruben ; De la Peña Felipe ; Julio Luis Fernando ; Burillo Jesus (No 250 in CA 9.4/02)	2018	Nematicidal potential of hydrolates from the semi industrial vapor-pressure extraction of Spanish aromatic plants.	Environ Sci Pollut Res Int. 2018 Oct;25(30):29834-29840. doi: 10.1007/s11356-017-9429-z.	Assessed effects only on target pests
Rajwinder Singh; Opender Koul; Pushpinder J. Rup; Jawala Jindal; Rajwinder Singh; Opender Koul; Pushpinder J. Rup; Jawala Jindal (No 260 in CA 9.4/02)	2011	Oviposition and feeding behavior of the maize borer Chilo partellus in response to eight essential oil allelochemicals	Entomologia Experimentalis et Applicata Volume 138 Issue 1	Assessed effects only on target pests
Norihide Nakada; Toshikatsu Tanishima; Hiroyuki Shinohara; Kentaro Kiri; Hideshige Takada (No 262 in CA 9.4/02)	2006	Pharmaceutical chemicals and endocrine disrupters in municipal wastewater in Tokyo and their removal during activated sludge treatment	Water Research Volume 40 Issue 17 October 2006 Pages 3297-3303	No assessment of effects on non-target organisms

Author(s)	**Year**	**Title**	**Source**	**Reason for not including in dossier (based on relevance criteria)**
Roya Razavizadeh; Farnaz Farahzadianpoor; Fatemeh Adabavazeh; Setsuko Komatsu (No 266 in CA 9.4/02)	2019	Physiological and morphological analyses of Thymus vulgaris L. in vitro cultures under polyethylene glycol (PEG)-induced osmotic stress	In Vitro Cellular & Developmental Biology - Plant volume 55 pages 342 – 357 (2019)	Assessed effects only at a molecular level
Arlene Sobrinho Ventura; Tarcila Sousa de Castro Silva; Ricardo Basso Zanon; Luis Antonio Kioshi Aoki Inoue; Claudia Andrea Lima Cardoso (No 267 in CA 9.4/02)	2019	Physiological and pharmacokinetic responses in neotropical Piaractus mesopotamicus to the essential oil from Lippia sidoides (Verbenaceae) as an anesthetic	International Aquatic Research volume 11 pages 1 – 12 (2019)	Assessed only combined/mixture toxicity
Ibáñez María Dolores ; Blázquez María Amparo (No 270 in CA 9.4/02)	2018	Phytotoxicity of Essential Oils on Selected Weeds: Potential Hazard on Food Crops.	Plants (Basel). 2018 Sep 22;7(4):79. doi: 10.3390/plants7040079	No assessment of test item with ≥70% purity
M.E. Bolio-González; A. Ortega-Pacheco; A. Alzina-López; E.J. Gutiérrez-Ruiz; E. Gutiérrez-Blanco; A.J. Aguilar-Caballero; J.A. Rosado-Aguilar; K. Arjona-Cambranes; J.F.J. Torres-Acosta; R.I. Rodríguez-Vivas (No 271 in CA 9.4/02)	2017	Plant products and secondary metabolites with acaricide activity against ticks	Veterinary Parasitology Volume 238 30 April 2017 Pages 66-76	Assessed effects only on target pests
Alayrangues Julie ; Hotier Lucie ; Massou Isabelle ; Bertrand Yolaine ; Armengaud Catherine (No 276 in CA 9.4/02)	2016	Prolonged effects of in-hive monoterpenoids on the honey bee Apis mellifera.	Ecotoxicology. 2016 Jul;25(5):856-62. doi: 10.1007/s10646-016-1642-x.	Assessed only combined/mixture toxicity

Author(s)	**Year**	**Title**	**Source**	**Reason for not including in dossier (based on relevance criteria)**
Renkema Justin M ; Buitenhuis Rosemarije ; Hallett Rebecca H (No 279 in CA 9.4/02)	2017	Reduced Drosophila suzukii Infestation in Berries Using Deterrent Compounds and Laminate Polymer Flakes.	Insects. 2017 Oct 31;8(4):117. doi: 10.3390/insects8040117	Assessed effects only on target pests
Stashenko Elena ; Nerio Luz Stella ; Olivero-Verbel Jesus (No 281 in CA 9.4/02)	2010	Repellent activity of essential oils: a review.	Bioresour Technol. 2010 Jan;101(1):372-8. doi: 10.1016/j.biortech.2009.07.048.	Assessed effects only on target pests
Zohreh Emami Bistgani a b; Fatemeh Sefidkon a (No 284 in CA 9.4/02)	2019	Review on ethnobotany phytochemical molecular and pharmacological activity of Thymus daenensis Celak.	Biocatalysis and Agricultural Biotechnology Volume 22 November 2019 101400	Literature reviews excluded
Montserrat Anguita; Jaume Galobart; Orsolya Holczknecht; Jordi Tarrés?Call; Elisa Pettenati; Fabiola Pizzo; Paola Manini; EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Vasileios Bampidis; Giovanna Azimonti; Maria de Lourdes Bastos; Henrik Christensen; Birgit Dusemund; Maryline Kouba; Mojca Kos Durjava; Marta López?Alonso; Secundino López Puente; Francesca Marcon; Baltasar Mayo; Alena Pechová; Mariana	2019	Safety and efficacy of AviPlus® as a feed additive for turkeys for fattening turkeys reared for breeding and suckling piglets - - 2019 - EFSA Journal - Wiley Online Library	EFSA Journal Volume 17 Issue 7	Assessed only combined/mixture toxicity

Author(s)	Year	Title	Source	Reason for not including in dossier (based on relevance criteria)
Petkova; Fernando Ramos; Yolanda Sanz; Roberto Edoardo Villa; Ruud Woutersen; Montserrat Anguita; Jaume Galobart; Orsolya Holczknecht; Jordi Tarrés?Call; Elisa Pettenati; Fabiola Pizzo; Paola Manini; EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Vasileios Bampidis; Giovanna Azimonti; Maria de Lourdes Bastos; Henrik Christensen; Birgit Dusemund; Maryline Kouba; Mojca Kos Durjava; Marta López?Alonso; Secundino López Puente; Francesca Marcon; Baltasar Mayo; Alena Pechová; Mariana Petkova; Fernando Ramos; Yolanda Sanz; Roberto Edoardo Villa; Ruud Woutersen (No 286 in CA 9.4/02)				
Zhang Leshuai W ; Koci Juraj ; Jeffery Brett ; Riviere Jim E ; Monteiro-Riviere Nancy A (No 288 in CA 9.4/02)	2015	Safety assessment of potential food ingredients in canine hepatocytes.	Food Chem Toxicol. 2015 Apr;78:105-15. doi: 10.1016/j.fct.2015.02.003.	Mammalian toxicology study not assessed in ecotox section
EFSA Panel on Additives	2012	Scientific Opinion on the	EFSA Journal Volume 10	Assessed only

Author(s)	Year	Title	Source	Reason for not including in dossier (based on relevance criteria)
and Products or Substances used in Animal Feed (FEEDAP); EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) (No 291 in CA 9.4/02)		safety and efficacy of AviPlus® as feed additive for chickens and minor avian species for fattening and reared for laying and minor porcine species (weaned)	Issue 5	combined/mixture toxicity
Silva Lima Aldilene ; Milhomem Marlise Neves ; Santos Monteiro Odair ; Arruda Alana Carynne Pereira ; de Castro Joaquim Antônio Martins ; Fernandes Yan Michel Lopes ; Maia José Guilherme Soares ; Costa-Junior Lívio Martins (No 294 in CA 9.4/02)	2018	Seasonal analysis and acaricidal activity of the thymol-type essential oil of Ocimum gratissimum and its major constituents against Rhipicephalus microplus (Acari: Ixodidae).	Parasitol Res. 2018 Jan;117(1):59-65. doi: 10.1007/s00436-017-5662-0.	Assessed effects only on target pests
Baki Ayd?n a; Luis André L. Barbas b (No 295 in CA 9.4/02)	2020	Sedative and anesthetic properties of essential oils and their active compounds in fish: A review	Aquaculture Volume 520 15 April 2020 734999	Literature reviews excluded
Paola Melendez-Renteria Virginia Nevarez-Moorillon R. Rodriguez-Herrera Cristobal N. Aguilar (No 301 in CA 9.4/02)	2012	Solid State Fermentation of Mexican Oregano (Lippia Berlandieri Schauer) Waste	[American Journal of Agricultural and Biological Science]; 2012/DJ/DJ2012_1.rdf	No assessment of effects on non-target organisms
Blank Arie Fitzgerald ; Picanço Marcelo Coutinho ; Araújo Ana Paula Albano ; Cristaldo Paulo Fellipe ; Bacci Leandro ; Santos Abraão Almeida ;	2017	Sub-lethal effects of essential oil of Lippia sidoides on drywood termite Cryptotermes brevis (Blattodea: Termitoidea).	Ecotoxicol Environ Saf. 2017 Nov;145:436-441. doi: 10.1016/j.ecoenv.2017.07.057.	Assessed effects only on target pests

Author(s)	Year	Title	Source	Reason for not including in dossier (based on relevance criteria)
de Oliveira Bruna Maria Santos ; Melo Carlisson Ramos ; Lima Ana Paula Santana ; Santana Emile Dayara Rabelo (No 303 in CA 9.4/02)				
L. X. ARAÚJO; T. P. L. NOVATO; V. ZERINGOTA; R. MATURANO; D. MELO; B. C. DA SILVA; E. DAEMON; M. G. DE CARVALHO; C. M. O. MONTEIRO; L. X. ARAÚJO; T. P. L. NOVATO; V. ZERINGOTA; R. MATURANO; D. MELO; B. C. DA SILVA; E. DAEMON; M. G. DE CARVALHO; C. M. O. MONTEIRO (No 304 in CA 9.4/02)	2016	Synergism of thymol carvacrol and eugenol in larvae of the cattle tick Rhipicephalus microplus and brown dog tick Rhipicephalus sanguineus	Medical and Veterinary Entomology Volume 30 Issue 4	Assessed effects only on target pests
Evan C. Palmer?Young; Ben M. Sadd; Rebecca E. Irwin; Lynn S. Adler; Evan C. Palmer?Young; Ben M. Sadd; Rebecca E. Irwin; Lynn S. Adler (No 305 in CA 9.4/02)	2017	Synergistic effects of floral phytochemicals against a bumble bee parasite - Palmer?Young - 2017 - Ecology and Evolution - Wiley Online Library	Ecology and Evolution Volume 7 Issue 6	No assessment of effects on non-target organisms
Gaire Sudip ; Scharf Michael E ; Gondhalekar Ameya D (No 306 in CA 9.4/02)	2020	Synergistic Toxicity Interactions between Plant Essential Oil Components Against the Common Bed Bug (Cimex lectularius	Insects. 2020 Feb 19;11(2):133. doi: 10.3390/insects11020133	Assessed effects only on target pests

Author(s)	**Year**	**Title**	**Source**	**Reason for not including in dossier (based on relevance criteria)**
		L.).		
Atcharee Ratwatthananon; Nutchaya Kumrungsee; Vasakorn Bullangpoti; Wanchai Pluempanupat; Sorachat Tharamak; Thitaree Yooboon; Anchulee Pengsook; Atcharee Ratwatthananon; Nutchaya Kumrungsee; Vasakorn Bullangpoti; Wanchai Pluempanupat; Sorachat Tharamak; Thitaree Yooboon; Anchulee Pengsook (No 307 in CA 9.4/02)	2020	Synthesis of thymyl esters and their insecticidal activity against Spodoptera litura (Lepidoptera: Noctuidae)	Pest Management Science Volume 76 Issue 3	No assessment of test item with ≥70% purity
Lim Eu Gene ; Roh Hyun Sik ; Coudron Thomas A ; Park Chung Gyoo (No 309 in CA 9.4/02)	2011	Temperature-dependent fumigant activity of essential oils against twospotted spider mite (Acari: Tetranychidae).	J Econ Entomol. 2011 Apr;104(2):414-9. doi: 10.1603/ec10249	Assessed effects only on target pests
Ricci Donata ; Epifano Francesco ; Fraternale Daniele (No 323 in CA 9.4/02)	2017	The Essential Oil of Monarda didyma L. (Lamiaceae) Exerts Phytotoxic Activity in Vitro against Various Weed Seed.	Molecules. 2017 Feb 2;22(2):222. doi: 10.3390/molecules22020222	No assessment of test item with ≥70% purity
Mahmoud Alagawany; Mayada R. Farag; Ayman S. Salah; Mohamed A. Mahmoud; Mahmoud Alagawany; Mayada R. Farag; Ayman S. Salah; Mohamed A. Mahmoud (No 331 in CA 9.4/02)	2020	The role of oregano herb and its derivatives as immunomodulators in fish - Alagawany - - Reviews in Aquaculture - Wiley Online Library	Reviews in Aquaculture Early View	Literature reviews excluded

Author(s)	**Year**	**Title**	**Source**	**Reason for not including in dossier (based on relevance criteria)**
Waliwitiya Ranil ; Nicholson Russell A ; Kennedy Christopher J ; Lowenberger Carl A (No 332 in CA 9.4/02)	2012	The synergistic effects of insecticidal essential oils and piperonyl butoxide on biotransformational enzyme activities in Aedes aegypti (Diptera: Culicidae).	J Med Entomol. 2012 May;49(3):614-23. doi: 10.1603/me10272	Assessed effects only on target pests
Ooi Ei Lin ; Zhou Zhigang ; Ran Chao ; Hu Jun ; Liu Wenshu ; Liu Zhi ; He Suxu ; Dan Bui Chau Truc ; Diem Nguyen Ngoc (No 334 in CA 9.4/02)	2016	Thymol and Carvacrol Affect Hybrid Tilapia through the Combination of Direct Stimulation and an Intestinal Microbiota-Mediated Effect: Insights from a Germ-Free Zebrafish Model.	J Nutr. 2016 May;146(5):1132-40. doi: 10.3945/jn.115.229377.	Assessed effects only at a molecular level
Jean-Luc Carayon; Nathan Téné; Elsa Bonnafé; Julie Alayrangues; Lucie Hotier; Catherine Armengaud; Michel Treilhou (No 336 in CA 9.4/02)	2014	Thymol as an alternative to pesticides: persistence and effects of Apilife Var on the phototactic behavior of the honeybee Apis mellifera	Environmental Science and Pollution Research volume 21 pages 4934 – 4939 (2014)	Assessed only combined/mixture toxicity
Ahmad K.J. (Beekeeping and Hill Fruit Pests Research Station Rawalpindi (Pakistan)) Razzaq A. (Beekeeping and Hill Fruit Pests Research Station Rawalpindi (Pakistan)) Abbasi K.H. (Beekeeping and Hill Fruit Pests Research Station Rawalpindi (Pakistan)) Shafiq M. (Beekeeping and Hill Fruit Pests Research	2014	Thymol as control agent of mites (Varroa destructor) on honeybees (Apis mellifera)	[Pakistan Journal of Agricultural Research (Pakistan)]; 2014/PK/PK2014_0 rdf	Assessed effects only on target pests

Author(s)	Year	Title	Source	Reason for not including in dossier (based on relevance criteria)
Station Rawalpindi (Pakistan)) et al. (No 337 in CA 9.4/02)				
Porá??ová Janka ; Camele Ippolito ; De Feo Vincenzo ; Gru?ová Daniela ; Caputo Lucia ; Elshafie Hazem S ; Baranová Beáta ; De Martino Laura ; Sedlák Vincent ; Goga?ová Zuzana (No 338 in CA 9.4/02)	2020	Thymol Chemotype Origanum vulgare L. Essential Oil as a Potential Selective Bio-Based Herbicide on Monocot Plant Species.	Molecules. 2020 Jan 29;25(3):595. doi: 10.3390/molecules25030595	Assessed only combined/mixture toxicity
Yan-Wei Cheng a 1; Xian-Wang Kong b 1; Ning Wang c; Ting-Ting Wang d; Jian Chen d; Zhi Qi Shi d (No 339 in CA 9.4/02)	2020	Thymol confers tolerance to salt stress by activating anti-oxidative defense and modulating Na+ homeostasis in rice root	Ecotoxicology and Environmental Safety Volume 188 30 January 2020 109894	No assessment of effects on non-target organisms
Renata Silva Matos a b; Patrícia Rosa de Oliveira a; Letícia Coelho d; Luiza Gabriella Ferreira de Paula d; Viviane Zeringota d; Bianca Carvalho Silva b; Caio Monteiro c; Erik Daemon b; Maria Izabel Camargo-Mathias a (No 341 in CA 9.4/02)	2020	Thymol: Effects on reproductive biology and Gene's organ morphology in Rhipicephalus sanguineus sensu lato engorged females (Acari: Ixodidae)	Ticks and Tick-borne Diseases Volume 11 Issue 1 January 2020 101308	Assessed effects only at a molecular level
Sanjay Singh Bhandari a; Mahaveer Prasad Kabra b (No 342 in CA 9.4/02)	2014	To evaluate anti-anxiety activity of thymol	Journal of Acute Disease Volume 3 Issue 2 2014 Pages 136-140	Assessed effects only on target pests
Zhang Zhilin ; Xie Yongjian ; Wang Yong ; Lin Zhufeng ; Wang Lihua	2017	Toxicities of monoterpenes against housefly Musca domestica	Environ Sci Pollut Res Int. 2017 Nov;24(31):24708-24713.	Assessed effects only on target pests

Author(s)	Year	Title	Source	Reason for not including in dossier (based on relevance criteria)
; Li Guoyuan (No 345 in CA 9.4/02)		L. (Diptera: Muscidae).	doi: 10.1007/s11356-017-0219-4.	
Gaire Sudip ; Scharf Michael E ; Gondhalekar Ameya D (No 347 in CA 9.4/02)	2019	Toxicity and neurophysiological impacts of plant essential oil components on bed bugs (Cimicidae: Hemiptera).	Sci Rep. 2019 Mar 8;9(1):3961. doi: 10.1038/s41598-019-40275-5	Assessed effects only on target pests
Piri Amir ; Sahebzadeh Najmeh ; Zibaee Arash ; Sendi Jalal Jalali ; Shamakhi Leila ; Shahriari Morteza (No 348 in CA 9.4/02)	2020	Toxicity and physiological effects of ajwain (Carum copticum Apiaceae) essential oil and its major constituents against Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae).	Chemosphere. 2020 Oct;256:127103. doi: 10.1016/j.chemosphere.2020.127103.	Assessed effects only on target pests
Soon-Il Kim; June-Sun Yoon; Je Won Jung; Ki-Bae Hong; Young-Joon Ahn; Hyung Wook Kwon (No 349 in CA 9.4/02)	2010	Toxicity and repellency of origanum essential oil and its components against Tribolium castaneum (Coleoptera: Tenebrionidae) adults	Journal of Asia-Pacific Entomology Volume 13 Issue 4 December 2010 Pages 369-373	Assessed effects only on target pests
Marcelo C. Picanço b; Abraão A. Santos a; Izailda B. Santos b; Mirian F. Pimentel b; Ane C.C. Santos a; Arie F. Blank a; Ana Paula A. Araújo c; Paulo F. Cristaldo a; Leandro Bacci a; Carlisson R. Melo a (No 353 in CA 9.4/02)	2018	Toxicity of essential oils of Lippia gracilis chemotypes and their major compounds on Diaphania hyalinata and non-target species	Crop Protection Volume 104 February 2018 Pages 47-51	No assessment of test item with ≥70% purity
Asmaa Z. El-Sharkawy b; Neama A. Abdel-Hamid b; Sameeh A. Mansour a	2015	Toxicity of essential plant oils in comparison with conventional insecticides	Industrial Crops and Products Volume 63 January 2015 Pages 92-99	No assessment of test item with ≥70% purity

Author(s)	Year	Title	Source	Reason for not including in dossier (based on relevance criteria)
(No 354 in CA 9.4/02)		against the desert locust Schistocerca gregaria (Forskål)		
V. Rozman a; I. Kalinovic a; Z. Korunic b (No 357 in CA 9.4/02)	2007	Toxicity of naturally occurring compounds of Lamiaceae and Lauraceae to three stored-product insects	Journal of Stored Products Research Volume 43 Issue 4 2007 Pages 349-355	Assessed effects only on target pests
Park Il-Kwon ; Kim Junheo N ; Lee Yeon-Suk ; Lee Sang-Gil ; Ahn Young-Joon ; Shin Sang-Chul (No 358 in CA 9.4/02)	2008	Toxicity of plant essential oils and their components against Lycoriella ingenua (Diptera: Sciaridae).	J Econ Entomol. 2008 Feb;101(1):139-44. doi: 10.1603/0022-0493(2008)101[139:topeoa]2.0.co;2	Assessed effects only on target pests
Samira Goudarzvande Chegini; Habib Abbasipour; Jaber Karimi; Alireza Askarianzadeh (No 360 in CA 9.4/02)	2018	Toxicity of Shirazi thyme Zataria multiflora essential oil to the tomato leaf miner Tuta absoluta (Lepidoptera: Gelechiidae)	International Journal of Tropical Insect Science volume 38 pages 340 – 347 (2018)	Assessed effects only on target pests
Rajwinder Singh; Opender Koul; Pushpinder J. Rup; Jawala Jindal (No 361 in CA 9.4/02)	2009	Toxicity of some essential oil constituents and their binary mixtures against Chilo pattellas (Lepidoptera: Pyralidae)	International Journal of Tropical Insect Science volume 29 pages 93 – 101 (2009)	Assessed effects only on target pests
Llana-Ruiz-Cabello María ; Puerto María ; Maisanaba Sara ; Guzmán-Guillén Remedios ; Pichardo Silvia ; Cameán Ana M (No 382 in CA 9.4/02)	2018	Use of micronucleus and comet assay to evaluate evaluate the genotoxicity of oregano essential oil (Origanum vulgare l. Virens) in rats orally exposed for 90 days.	J Toxicol Environ Health A. 2018;81(12):525-533. doi: 10.1080/15287394.2018.1447522.	Mammalian toxicology study not assessed in ecotox section
Juliana Navarro-Rocha a; Alejandro F. Barrero b; Jesús Burillo a; A. Sonia	2018	Valorization of essential oils from two populations (wild and commercial) of	Industrial Crops and Products Volume 116 June 2018 Pages 41-45	Assessed effects only on target pests

Author(s)	Year	Title	Source	Reason for not including in dossier (based on relevance criteria)
Olmeda c; Azucena González-Coloma d (No 387 in CA 9.4/02)		Geranium macrorrhizum L.		

All relevant publications identified following the detailed assessment are presented in the tables below, in order of data requirement and author, respectively. These relevant papers and reliable are summarised in the corresponding Vol. 3 CA/CP B.9, where an assessment of their reliability is also presented in line with Section 5.4 of EFSA Journal 2011;9(2):2092.

For ecotoxicology, 5 papers have been considered as relevant and reliable following the two-step process and have been summarized in this document (Labaque *et al*., 2013, Study B.9.1.1.2/01; Giacomelli *et al*., 2016, Study B.9.3.1/01; Glavan et al., 2020, Study B.9.3.1/02; Gashout *et al*., 2018, Study B.9.3.1/03; and Maistrello *et al*., 2008 Study B.9.3.1/04). For 39 remaining papers, since the reliability of relevant papers is limited to the ecotoxicological risk assessment of thymol, they have not been considered further in Vol. 3 CA/CP B.9 and brief summaries of these papers are provided below in the Appendix I.

Table 9.11.1.4.2-3: Relevant studies included in the supplementary dossier after detailed assessment of full-text documents for relevance: sorted by data requirement(s) for thymol

Data requirement (indicated by the corresponding CA and CP data point)	Author(s)	Year	Title	Source
ECOTOXICOLOGY				
CA 8.1.1.2/01	M.C. Lábaque a; J.M. Kembro a b; A. Luna a; R.H. Marin a b	2013	Effects of thymol feed supplementation on female Japanese quail (Coturnix coturnix) behavioral fear response	Animal Feed Science and Technology Volume 183 Issues 1–2 28 June 2013 Pages 67-72
CA 8.3.1/01	Francesca Iacoponi; Giovanni Formato; Alessandra Giacomelli; Marco Pietropaoli; Andrea Carvelli	2016	Combination of thymol treatment (Apiguard®) and caging the queen technique to fight Varroa destructor	Apidologie volume 47 pages 606 – 616 (2016)
CA 8.3.1/02	Glavan Gordana ; Novak Sara ; Boži?? Janko ; Jemec Kokalj Anita	2020	Comparison of sublethal effects of natural acaricides carvacrol and thymol on honeybees.	Pestic Biochem Physiol. 2020 Jun;166:104567. doi: 10.1016/j.pestbp.2020.104567.
CA 8.3.1/03	Hanan A. Gashout; Paul H. Goodwin; Ernesto Guzman-Novoa	2018	Lethality of synthetic and natural acaricides to worker honey bees (Apis mellifera) and their impact on the expression of health and detoxification-related genes	Environmental Science and Pollution Research volume 25 pages 34730 – 34739 (2018)
CA 8.3.1/04	Lara Maistrello; Marco Lodesani; Cecilia Costa; Francesco Leonardi; Giovanna Marani; Mauro Caldon; Franco Mutinelli; Anna Granato	2008	Screening of natural compounds for the control of nosema disease in honeybees (Apis mellifera)	Apidologie volume 39 pages 436 – 445 (2008)
CA 9.6.3.4/01 [CA 8.3.2]	Castilhos R V ; Grützmacher A D ; Coats J R	2018	Acute Toxicity and Sublethal Effects of Terpenoids and Essential Oils on the Predator Chrysoperla externa (Neuroptera: Chrysopidae).	Neotrop Entomol. 2018 Apr;47(2):311-317. doi: 10.1007/s13744-017-0547-6.
CA 9.6.3.4/02 [CA 8.2.1]	Nutthalak Laksanavilat; Puntipa Junhirun; Vasakorn	2018	Acute toxicity of essential oil compounds (thymol and 18-	Agriculture and Natural Resources Volume 52 Issue 2 April 2018 Pages

Data requirement (indicated by the corresponding CA and CP data point)	Author(s)	Year	Title	Source
	Bullangpoti; Warasinee Mujchariyakul		cineole) to insectivorous guppy Poecilia reticulata Peters 1859	190-194
CA 9.6.3.4/03 [CA 8.3.1]	Fanny Mondet; Mark Goodwin; Alison Mercer	2011	Age-related changes in the behavioural response of honeybees to Apiguard ® a thymol-based treatment used to control the mite Varroa destructor	Journal of Comparative Physiology A volume 197 Article number: 1055 (2011)
CA 9.6.3.4/04 [CA 8.6]	Kordali Saban ; Cakir Ahmet ; Ozer Hakan ; Cakmakci Ramazan ; Kesdek Memis ; Mete Ebru	2008	Antifungal phytotoxic and insecticidal properties of essential oil isolated from Turkish Origanum acutidens and its three components carvacrol thymol and p-cymene.	Bioresour Technol. 2008 Dec;99(18):8788-95. doi: 10.1016/j.biortech.2008.04.048.
CA 9.6.3.4/05 [CA 8.2.2]	Monique B. Morselli; João H. Reis; Matheus D. Baldissera; Carine F. Souza; Bernardo Baldisserotto; Tiago G. Petrolli; Diovani Paiano; Diogo L. A. Lopes; Aleksandro S. Da Silva	2020	Benefits of thymol supplementation on performance the hepatic antioxidant system and energetic metabolism in grass carp	Fish Physiology and Biochemistry volume 46 pages 305 – 314 (2020)
CA 9.6.3.4/06 [CA 8.6]	Nobuhiro Sekine; Sakae Shibutani	2013	Chemical structures of p - menthane monoterpenes with special reference to their effect on seed germination and termite mortality	Journal of Wood Science volume 59 pages 229 – 237 (2013)
CA 9.6.3.4/07 [CA 8.3.1]	Konstantinos M. Kasiotis; Zampia D. Tzouganaki; Kyriaki Machera	2018	Chromatographic determination of monoterpenes and other acaricides in honeybees: Prevalence and possible synergies	Science of The Total Environment Volume 625 1 June 2018 Pages 96-105
CA 9.6.3.4/08 [CA 8.3.1]	Nanetti A. (Consiglio per la Ricerca e la Sperimentazione in Agricoltura (CRA) - Istituto	2007	Comparison between six thymol based formulations in the control of Varroa destructor	[APOidea - Rivista Italiana di Apicoltura (Italy)]; 2007/IT/IT2007_0 rdf

Data requirement (indicated by the corresponding CA and CP data point)	Author(s)	Year	Title	Source
	Nazionale di Apicoltura Bologna (Italy)) Bacandritsos N. (National Agricultural Research Foundation (NAGREF) Athens (Greece). Inst. of Veterinary Research) Papanastasiou I. (Consiglio per la Ricerca e la Sperimentazione in Agricoltura (CRA) - Istituto Nazionale di Apicoltura Bologna (Italy)) Saitanis C. (Athens Univ. (Greece). Lab. of Ecology and Environmental Sciences) et al.		(Anderson et Trueman) in Greece [Apis mellifera L.]	
CA 9.6.3.4/09 [CA 8.3.1]	Raffique M.K. (Government of the Punjab Mian Channu (Pakistan). Dept. of Plant Protection) Mahmood R. (National Agricultural Research Centre Islamabad (Pakistan). Honeybee Research Inst.) Aslam M. (PMAS Arid Agriculture Univ. Rawalpindi (Pakistan)) Sarwar G.	2013	Control of Tropilaelaps clareae mite by using formic acid and thymol in honey bee Apis mellifera L. colonies	[Pakistan Journal of Zoology (Pakistan)]; 2013/PK/PK2013_0 rdf
CA 9.6.3.4/10 [CA 8.3.1]	Cecilia Costa; Marco Lodesani; Lara Maistrello	2010	Effect of thymol and resveratrol administered with candy or syrup on the development of Nosema ceranae and on the longevity of honeybees (Apis mellifera L.) in laboratory conditions	Apidologie volume 41 pages 141 – 150 (2010)
CA 9.6.3.4/11 [CA 8.3.1]	Théotime Colin; Meng Yong Lim; Stephen R. Quarrell; Geoff R. Allen; Andrew B. Barron	2019	Effects of thymol on European honey bee hygienic behaviour	Apidologie volume 50 pages 141 – 152 (2019)

Data requirement (indicated by the corresponding CA and CP data point)	Author(s)	Year	Title	Source
CA 9.6.3.4/12 [CA 8.3.1]	Sönmez D.	2019	Efficacy of thymol and thymol active ingredient products against of parasitic mite (Varroa destructor Anderson&Trueman)	2019/TR/TR2019_0 rdf
CA 9.6.3.4/13 [CA 8.3.1]	Marrufo Olivares Jorge Carlos Medina Medina Luis Abdelmir de Jesús May-Itzá William	2012	Eficacia de un gel a base de timol en el control del ácaro Varroa destructor que infesta colonias de abejas Apis mellifera bajo condiciones tropicales en Yucatán México	[Veterinaria Mexico]; 2012/DJ/DJ2012_0.rdf
CA 9.6.3.4/14 [CA 8.3.1]	Maria Dimou; Andreas Thrasyvoulou; Chrysoula Tananaki; Georgios Goras; Nicola Huggett; Emmanouel Karazafiris	2014	Evaluation of the impact of Exomite Pro™ on Varroa mite (Varroa destructor) populations and honeybee (Apis mellifera) colonies: efficacy side effects and residues	Parasitology Research volume 113 pages 1251 – 1259 (2014)
CA 9.6.3.4/15 [CA 8.3.1]	Michel Treilhou; Catherine Armengaud; Maelys Bergougnoux	2013	Exposure to thymol decreased phototactic behaviour in the honeybee (Apis mellifera) in laboratory conditions	Apidologie volume 44 pages 82 – 89 (2013)
CA 9.6.3.4/16 [CA 8.6]	Waliwitiya Ranil ; Isman Murray B ; Vernon Robert S ; Riseman Andrew	2005	Insecticidal activity of selected monoterpenoids and rosemary oil to Agriotes obscurus (Coleoptera: Elateridae).	J Econ Entomol. 2005 Oct;98(5):1560-5. doi: 10.1093/jee/98.5.1560
CA 9.6.3.4/17 [CA 8.3.2]	Emile D.R. Santana a; Ane C.C. Santos a; Jefferson E. Silva a; Genésio T. Ribeiro c; Aline M. Pinheiro a; Ítala T.B.F. Santos c; Arie F. Blank a; Ana Paula A. Araújo b; Leandro Bacci a; Ana P.S. Lima a	2020	Insecticide activity of botanical compounds against Spodoptera frugiperda and selectivity to the predatory bug Podisus nigrispinus	Crop Protection Volume 136 October 2020 105230
CA 9.6.3.4/18 [CA 8.2.4.1]	Park Hye-Mi ; Park Il-Kwon ;	2012	Larvicidal activity of ajowan (	J Agric Food Chem. 2012 Jun

Data requirement (indicated by the corresponding CA and CP data point)	Author(s)	Year	Title	Source
	Seo Seon-Mi		Trachyspermum ammi) and Peru balsam (Myroxylon pereira) oils and blends of their constituents against mosquito Aedes aegypti acute toxicity on water flea Daphnia magna and aqueous residue.	13;60(23):5909-14. doi: 10.1021/jf301296d.
CA 9.6.3.4/19 [CA 8.3.1]	Jean?Baptiste Ferdy; Jeremy Tabart; Angelique Vetillard; Gael Charpentier; Cyril Vidau; Jean?Baptiste Ferdy; Jeremy Tabart; Angelique Vetillard; Gael Charpentier; Cyril Vidau	2014	Lethal and sub?lethal effects of thymol on honeybee (Apis mellifera) larvae reared in vitro	Pest Management Science Volume 70 Issue 1
CA 9.6.3.4/20 [CA 8.3.1]	Théotime Colin a; William G. Meikle b; Amy M. Paten c; Andrew B. Barron a	2019	Long-term dynamics of honey bee colonies following exposure to chemical stress	Science of The Total Environment Volume 677 10 August 2019 Pages 660-670
CA 9.6.3.4/21 [CA 8.6]	Fabrizio Araniti a; Begoña Miras-Moreno b; Luigi Lucini b; Marco Landi c; Maria Rosa Abenavoli a	2020	Metabolomic proteomic and physiological insights into the potential mode of action of thymol a phytotoxic natural monoterpenoid phenol	Plant Physiology and Biochemistry Volume 153 August 2020 Pages 141-153
CA 9.6.3.4/22 [CA 8.2.1]	Bianchini A E ; Garlet Q I ; da Cunha J A ; Bandeira G Junior ; Brusque I C M ; Salbego J ; Heinzmann B M ; Baldisserotto B	2017	Monoterpenoids (thymol carvacrol and S-(+)-linalool) with anesthetic activity in silver catfish (Rhamdia quelen): evaluation of acetylcholinesterase and GABAergic activity.	Braz J Med Biol Res. 2017 Oct 19;50(12):e6346. doi: 10.1590/1414-431X20176346
CA 9.6.3.4/23 [CA 8.2.6]	Sandra G. Gómez de Saravia; Silvia E. Rastelli; Guillermo Blustein; Marisa R. Viera	2018	Natural compounds as potential algaecides for waterborne paints	Journal of Coatings Technology and Research volume 15 pages 1191 – 1200 (2018)
CA 9.6.3.4/24 [CA 8.6]	P. Nasrollahi; S. M. Razavi; A. Ghasemian; S. Zahri	2018	Physiological and Biochemical Responses of Lettuce to Thymol as Allelochemical	Russian Journal of Plant Physiology volume 65 pages 598 – 603 (2018)
CA 9.6.3.4/25 [CA 8.6]	Pinheiro Patrícia Fontes ;	2015	Phytotoxicity and Cytotoxicity	J Agric Food Chem. 2015 Oct

Data requirement (indicated by the corresponding CA and CP data point)	Author(s)	Year	Title	Source
	Costa Adilson Vidal ; Alves Thammyres de Assis ; Galter Iasmini Nicoli ; Pinheiro Carlos Alexandre ; Pereira Alexandre Fontes ; Oliveira Carlos Magno Ramos ; Fontes Milene Miranda Praça		of Essential Oil from Leaves of Plectranthus amboinicus Carvacrol and Thymol in Plant Bioassays.	21;63(41):8981-90. doi: 10.1021/acs.jafc.5b03049.
CA 9.6.3.4/26 [CA 8.3.1]	Abd-El-Megeed S.M. Ain-Shams Univ. Cairo (EGY). Fac. of Agric [Corporate Author]	2013	Queen honey bee characteristics and their relation to colony performance	2013/EG/EG2013_0 rdf
CA 9.6.3.4/27 [CA 8.6]	Bruno Dufau Mattos a 1; Lucas Rafael da Silva b 1; Irisdoris Rodrigues de Souza b; Washington Luiz Esteves Magalhães c; Daniela Morais Leme b	2019	Slow delivery of biocide from nanostructured microscaled particles reduces its phytoxicity: A model investigation	Journal of Hazardous Materials Volume 367 5 April 2019 Pages 513-519
CA 9.6.3.4/28 [CA 8.2.2]	Margaret Aanyu a b; Mónica B. Betancor a; Óscar Monroig c	2020	The effects of combined phytogenics on growth and nutritional physiology of Nile tilapia Oreochromis niloticus	Aquaculture Volume 519 30 March 2020 734867
CA 9.6.3.4/29 [CA 8.2.2]	Shimaa A. Amer a; Abdallah E. Metwally a; Shimaa A.A. Ahmed b	2018	The influence of dietary supplementation of cinnamaldehyde and thymol on the growth performance immunity and antioxidant status of monosex Nile tilapia fingerlings (Oreochromis niloticus)	The Egyptian Journal of Aquatic Research Volume 44 Issue 3 September 2018 Pages 251-256
CA 9.6.3.4/30 [CA 8.3.1]	Théotime Colin; Jenny A. Plath; Simon Klein; Peta Vine; Jean-Marc Devaud; Mathieu Lihoreau; William G. Meikle; Andrew B. Barron	2020	The miticide thymol in combination with trace levels of the neonicotinoid imidacloprid reduces visual learning performance in honey bees (Apis mellifera)	Apidologie (2020)

Data requirement (indicated by the corresponding CA and CP data point)	Author(s)	Year	Title	Source
CA 9.6.3.4/31 [CA 8.2.1]	Morteza Yousefi a; Seyyed Morteza Hoseini b; Yury Anatolyevich Vatnikov a; Alexandr Alexeevich Nikishov a; Evgeny Vladimirovich Kulikov a	2018	Thymol as a new anesthetic in common carp (Cyprinus carpio): Efficacy and physiological effects in comparison with eugenol	Aquaculture Volume 495 1 October 2018 Pages 376-383
CA 9.6.3.4/32 [CA 8.6]	Milene Miranda Praça-Fontes a c; Larissa Fonseca Andrade-Vieira d; Kellen Barelo Corrêa b; Thayllon de Assis Alves f; Franceli Aparecida da Cruz d; Valdemar Lacerda Júnior e; Adésio Ferreira f; Taís Cristina Bastos Soares a g; Thammyres de Assis Alves a 1; Patrícia Fontes Pinheiro b 1	2018	Toxicity of thymol carvacrol and their respective phenoxyacetic acids in Lactuca sativa and Sorghum bicolor	Industrial Crops and Products Volume 114 April 2018 Pages 59-67
CA 9.6.3.4/33[CA 8.3.1]	Théotime Colin; Casey C. Forster; Jack Westacott; Xiaobo Wu; William G. Meikle; Andrew B. Barron	2021	Effects of late miticide treatments on foraging and colony productivity of European honey bees (Apis mellifera).	Apidologie (2021)
CA 9.6.3.4/34[CA 8.2.1]	Baki Aydin; Nihat Orhan	2021	Effects of thymol and carvacrol anesthesia on the electrocardiographic and behavioral responses of the doctor fish Garra rufa.	Aquaculture Volume 533 25 February 2021 736134
CA 9.6.3.4/35[CA 8.3.1]	Sabahi Qodratollah ; Morfin Nuria ; Emsen Berna ; Gashout Hanan A ; Kelly Paul G ; Otto Stephanie ; Merrill A Rod ; Guzman-Novoa Ernesto	2020	Evaluation of Dry and Wet Formulations of Oxalic Acid Thymol and Oregano Oil for Varroa Mite (Acari: Varroidae) Control in Honey Bee (Hymenoptera: Apidae) Colonies.	J Econ Entomol. 2020 Dec 9;113(6):2588-2594. doi: 10.1093/jee/toaa218
CA 9.6.3.4/36[CA 8.3.1]	Wallace Borges Matos a; Ane Caroline Celestino Santos a;	2021	Potential source of ecofriendly insecticides: Essential oil	Ecotoxicology and Environmental Safety Volume 209 February 2021 111764

Data requirement (indicated by the corresponding CA and CP data point)	Author(s)	Year	Title	Source
	Ana Paula Santana Lima a; Emile Dayara Rabelo Santana a; Jefferson Elias Silva a; Arie Fitzgerald Blank b; Ana Paula Albano Araújo c; Leandro Bacci a b		induces avoidance and cause lower impairment on the activity of a stingless bee than organosynthetic insecticides in laboratory.	
CA 9.6.3.4/37[CA 8.3.1]	Hanan A. Gashout; Ernesto Guzman-Novoa; Paul H. Goodwin	2020	Synthetic and natural acaricides impair hygienic and foraging behaviors of honey bees.	Apidologie volume 51 pages 1155 – 1165 (2020)
CA 9.6.3.4/38[CA 8.2.1]	Vieira Raquel ; Venâncio Carlos ; Félix Luís	2021	Teratogenic Oxidative Stress and Behavioural Outcomes of Three Fungicides of Natural Origin (Equisetum arvense Mimosa tenuiflora Thymol) on Zebrafish (Danio rerio).	Toxics. 2021 Jan 9;9(1):8. doi: 10.3390/toxics9010008
CA 9.6.3.4/39[CA 8.2.1]	Krawczyk-Santos Anna Paula ; Taveira Stephânia Fleury ; de Oliveira Gisele Augusto Rodrigues ; Pereira Iúri Barbosa ; Carvalho Endrew Henrique de Sousa ; Rodrigues Laís de Brito ; Mattos Bruno Dufau ; Magalhães Washington Luiz Esteves ; Leme Daniela Morais	2021	Thymol-Loaded Biogenic Silica Nanoparticles in an Aquatic Environment: The Impact of Particle Aggregation on Ecotoxicity.	Environ Toxicol Chem. 2021 Feb;40(2):333-341. doi: 10.1002/etc.4938.

B.9.11.1.1. Conclusion

In this literature search for the active substance thymol, 35 229 references were identified and evaluated for their potential relevance for data requirements "toxicological and toxicokinetic studies, human exposure, residues, fate and behaviour in the environment and ecotoxicological studies".
After rapid and detailed assessments for relevance, the following publications were considered to be relevant:

	Number of publications considered to be relevant Thymol
Toxicology, including human exposure	10
Residues	10
Environmental fate	3
Ecotoxicology	**44***

* Includes 41 papers, plus 3 papers for which full-text was not available. In absence of the full-text, these 3 papers are considered relevant and the available abstract has been provided as a summary (but cannot be assessed for reliability).

All relevant and reliable publications identified following the detailed assessment are summarised in the corresponding Vol. 3 CA/CP, where an assessment of their reliability is also presented in line with Section 5.4 of EFSA Journal 2011;9(2):2092.
For ecotoxicology, 5 papers have been considered as relevant and reliable following the two-step process and have been summarized in this document (Labaque et al., 2013, Study B.9.1.1.2/01; Giacomelli *et al.*, 2016, , Study B.9.3.1/01; Glavan *et al*., 2020, Study B.9.3.1/02; Gashout *et al*., 2018, Study B.9.3.1/03; and Maistrello *et al.*, 2005, Study B.9.3.1/04). For 39 remaining papers, since the reliability of relevant papers is limited to the ecotoxicological risk assessment of thymol, they have not been considered further in this document and brief summaries of these papers are provided below in Appendix I.

Assessment and conclusion by RMS:

The RMS has checked the review of literature data submitted by the applicant and considers the databases used for the search are acceptable, and the search strategy as well. Furthermore, the justifications given by the applicant for non-relevance seemed acceptable.

Therefore, RMS considered acceptable the review literature submitted by the applicant.

B.9.11.2. Reference relied on

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?
Study B.9.1.1.2/01 CA 8.1.1.2/01	Labaque, M.C., Kembro, J.M., Luna, A., Marin, R.H.	2013	Effects of thymol feed supplementation on female Japanese quail (*Coturnix coturnix*) behavioral fear response [Internal reference: Study 140] Animal Feed Science and Technology Volume 183, Pages 67-72 (2013) Not GLP Published	Y	N	-	Public	N
Study B.9.2.1/01 CA 8.2.1/01	[redacted]	2008a	Acute toxicity of thymol crystals to rainbow trout (*Oncorhynchus mykiss*) in a 96-hour semi-static test Report No. 34281230 [redacted] Germany GLP Unpublished	Y	N	-	Eden Research plc	Y In DAR IIA 8.2.1.1/01
Study B.9.2.1/02 CA 8.2.1/02	[redacted]	2008b	Acute toxicity of thymol crystals to zebra fish (*Danio rerio*) in a 96-hour semi-static test Report No. 34282230 [redacted] Germany GLP Unpublished	Y	N	-	Eden Research plc	Y In DAR IIA 8.2.1.1/02
Study B.9.2.4.1/01 CA	Grade, R., Wydra, V.	2007	Acute toxicity of thymol crystals to *Daphnia magna* in a static 48-hour	N	N	-	Eden Research plc	Y In DAR 8.3.1.1/01

8.2.4.1/01			immobilization test Report No. 34283220 IBACON GmbH, Germany GLP Unpublished					
Study B.9.2.5.1/01 CA 8.2.5.1/01	Egeler, P.	2021	Thymol: A study on the chronic toxicity to *Daphnia magna* Report No. 20GC2DB ECT Oekotoxikologie GmbH, Germany GLP Unpublished	N	Y	Study not previously submitted	Eden Research plc	N
Study B.9.2.6.1/01 CA 8.2.6.1/01a	Hoffmann, K., Wydra, V.	2011	Revised final report: Toxicity of thymol crystals to *Pseudokirchneriella subcapitata* in an algal growth inhibition test Report No. 34284210 IBACON GmbH, Germany GLP Unpublished	N	N	-	Eden Research plc	Y In DAR IIA 8.4/01
Study B.9.2.6.1/01 CA 8.2.6.1/01c	Grade, R., Wydra, V.	2008c	Original report: Toxicity of thymol crystals to *Pseudokirchneriella subcapitata* in an algal growth inhibition test Report No. 34284210 IBACON GmbH, Germany GLP Unpublished	N	N	-	Eden Research plc	Y In DAR IIA 8.4/01
Study 9.3.1/01 CA 8.3.1/01	Giacomelli, A., Pietropaoli, M., Carvelli,A., Iacoponi, F., Formato, G.	2016	Combination of thymol treatment (Apiguard®) and caging the queen technique to fight *Varroa destructor* [Internal reference: Study 85] Apidologie Volume 47, Pages 606-616 Not GLP Published	N	N	-	Public	N
Study 9.3.1/02	Glavan, G., Novak, S.,	2020	Comparison of	N	N	-	Public	N

CA 8.3.1/02	Božič, J., Kokalj, A.J.		sublethal effects of natural acaricides carvacrol and thymol on honeybees [Internal reference: Study 97] Pesticide Biochemistry and Physiology 166, 104567, Pages 1-9 (2020) Not GLP Published					
Study 9.3.1/03 CA 8.3.1/03	Gashout, H.A., Goodwin, P.H., Guzman-Novoa, E.	2018	Lethality of synthetic and natural acaricides to worker honey bees (*Apis mellifera*) and their impact on the expression of health and detoxification-related genes [Internal reference: Study 232] Environmental Science and Pollution Research, Volume 25, Pages 34730–34739 (2018) Not GLP Published	N	N	-	Public	N
Study 9.3.1/04 CA 8.3.1/04	Maistrello, L., Lodesani, M., Costa, C., Leonardi, F., Marani, G., Caldon, M., Mutinelli, F., Granato, A.	2008	Screening of natural compounds for the control of nosema disease in honeybees (*Apis mellifera*) Internal reference: Study 293 Apidologie volume 39, Pages 436-445 (2008) Not GLP Published	N	N	-	Public	N
Study 9.8/01 CA 8.8/01	Hammesfahr, U.	2020	Mevalone: Toxicity to activated sludge in a respiration inhibition test Report No. 155781171 IBACON GmbH, Germany GLP Unpublished	N	Y	Study not previously submitted	Eden Research plc	N

[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).

APPENDIX I.

STUDY SUMMARIES OF RELEVANT PUBLICATIONS ACCORDING FULL-TEXT

As some potentially relevant parameters were included in the following study, a reliability assessment was also conducted. Following detailed assessment, overall it was concluded that this studies are of limited reliability for use in the ecotoxicological risk assessment of thymol. Whilst some may be considered as supporting information, they are of limited added value to the risk assessment and therefore have not been considered further in the corresponding data point. Since these studies are considered as not relaible it has not been evaluated by the RMS, a brief summary of this papers with the applicant's assessment has been included below for transparency.

Data point:	CA 9.6.3.4/01 [CA 8.3.2]
Report author	Castilhos, R. V., Grützmacher, A. D., Coats, J. R.
Report year	2017
Report title	Acute Toxicity and Sublethal Effects of Terpenoids and Essential Oils on the Predator *Chrysoperla externa* (Neuroptera: Chrysopidae)
Report No	-
Document No	Internal reference: Study 21 Neotropical entomology volume 47, Pages 311-317
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)

The search for new safer insecticides has increased in recent agriculture. Botanical compounds such as terpenoids and plant essential oils with insecticidal activity could represent important tools in pest management, and their risk assessment against non-target organisms is necessary since they may serve as a precursor for the synthesis of new insecticide active ingredients. For this study, the acute toxicity and sublethal effects of seven terpenoids and three essential oils with recognized insecticidal activity were evaluated on the predator *Chrysoperla externa* (Hagen) (Neuroptera: Chrysopidae) in laboratory bioassays. Results indicate that these compounds feature relative selectivity to the predator *C. externa*; however, sublethal effects on reproduction were recorded for some compounds. The phenolic monoterpenoids carvacrol and thymol were more acutely toxic than other terpenoids screened, with LD_{50} <20,000 µg/g; however, they were less toxic than natural pyrethrins (toxicity standard) in these bioassays. Sublethal effects on fecundity and fertility were observed for R-(+)-limonene, while oregano oil only affected fecundity. The compounds evaluated here have potential to be used as insecticides and can serve as backbone for future synthesis of selective active ingredients; however, a complete risk assessment to *C. externa* and other non-target organisms is necessary for their incorporation in future crop protection paradigms.

Materials and methods

Test material

Name:	Thymol
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma-Aldrich
Active substance content:	Not reported
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species:	*Chrysoperla externa* (Neuroptera: Chrysopidae).
Strain/clone:	Not reported
Age at study initiation:	Third instar larvae approximately 6 days old
Weight/length/height at study initiation:	Average weight 2.86 ± 0.34 mg
Source:	Pesticide Toxicology Laboratory in the Department of Entomology at Iowa State University (Ames, IA, USA)
Feeding during test:	Not reported for the sublethal test.
Acclimation:	Not reported

Test conditions (acute test)

Test temperature:	24 ºC.
Photoperiod:	14 light: 10 dark.
Humidity:	60-70%.
Test system 1	
Study type:	Acute toxicity test
Duration of study:	72 hours
Treatments:	At least five concentrations (actual concentrations not reported) applied topically at a volume of 0.5 µL solution.
Analytical determination of test concentrations: No	
Negative control included:	Yes (acetone)
Positive control included:	Yes, an extract containing 20% natural pyrethrins (pyrethrins I and II, cinerins I and II, and jasmolins I and II) (Pet Chemicals Inc., Miami Springs, FL) was used as toxicity standard for comparison
Parameters measured:	Mortality
Validity criteria:	Not reported
Test system 2	
Study type:	Sublethal effects on fecundity and fertility
Duration of study:	24 hours
Treatments:	One concentration (actual concentration not reported) at LD_{10} from acute test applied topically at a volume of 0.5 µL solution.
Analytical determination of test concentrations: No	
Negative control included:	Yes (acetone)
Positive control included:	Not reported
Parameters measured:	Fecundity (no. of eggs/female/day) and fertility (% of hatched larvae)
Validity criteria:	Not reported

Third instar larvae of *C. externa* with approximately 6 days old were treated on the dorsal abdomen with 0.5 µL of a solution (containing thymol) delivered by a Hamilton handle micro applicator. The compounds were diluted in certified acetone, in at least five concentrations in a range previously established for each compound in range-finding tests. In the control treatment, larvae were topically treated with 0.5 µL of certified acetone only. An extract containing 20% natural pyrethrins (pyrethrins I and II, cinerins I and II, and jasmolins I and II) (Pet Chemicals Inc., Miami Springs, FL) was used as toxicity standard for comparison. Four replications (five to six larvae per replication) were used for each concentration. Before being treated, the larvae were weighed on a digital scale in order to determine live weight. Mortality was recorded 72 h after treatment and larvae were considered dead when no response was observed after stimulation by a slight touch of a camel hair brush.

For assessing the sublethal effects of terpenoids and essential oils on fecundity and fertility of C. externa, third instar larvae were treated topically with 0.5 µL of the LD10 of the compounds that showed acute toxicity. A total of 24 larvae were used for each treatment. The surviving adults that originated from treated larvae were collected, transferred to cages, and used for reproductive performance assessment. Four couples were selected for each treatment. One week after the first oviposition, four samples were collected from eggs deposited in a period of 24 h. The number of eggs in each sample was counted to determine the average fecundity (no. of eggs/female/day), and the eggs were then incubated until hatching to determine the fertility rate (percentage of hatched larvae).

The LD50 of each compound was calculated by Probit analysis using SAS 9.3 (SAS Institute 2011). Normality of fecundity and fertility data was assessed by Shapiro-Wilk test and the homogeneity of variance was assessed

by Bartlett test. Data were subjected to analysis of variance (ANOVA), and treatments were compared by Dunnett test with α = 0.05

Results

The acute (72-hour) topical/contact LD_{50} value for third instar larvae of *Chrysoperla externa* was reported to be 19 115 µg thymol/g (Confidence Intervals: (15 297–23 479 µg thymol/g).

When exposed topically to thymol at a dose equivalent to the acute LD_{10}, no significant effect on reproduction was found for thymol since the number of eggs/female/day was similar to the control. Fertility rates were higher than 70% and thus similar to the control.

Assessment and conclusion

Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 2 reliable with restrictions.

Purity of test item not reported. Actual concentrations tested not reported.

The concentration tested is not clear.

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).

Acute (72-hour) topical/contact LD_{50} for third instar larvae of *Chrysoperla externa* = 19 115 µg thymol/g (95% confidence limits: 15 297–23 479 µg thymol/g).

When exposed topically to thymol at a dose equivalent to the acute LD_{10}, no significant effect on reproduction.

Data point:	CA 9.6.3.4/02 [CA 8.2.1]
Report author	Bullangpoti, V., Mujchariyakul, W., Laksanavilat, N., Junhirun, P.,
Report year	2018
Report title	Acute toxicity of essential oil compounds (thymol and 1,8-cineole) to insectivorous guppy, *Poecilia reticulata* Peters, 1859
Report No	-
Document No	Internal reference: Study 22 Agriculture and Natural Resources volume 52, pages 190-194 (2018)
Guidelines followed in study	American Water Works Association (AWWA) and the Water Environment Federation (WEF). (1998) Organisation for Economic Co-operation and Development (1993) (assumed to be OECD 203 but guideline number not reported)
Deviations from current test guideline	Yes – 24-hour exposure, rather than 96 hours; no analytical verification (see further details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	No

Abstract (copied from original literature)

Thymol and 1,8-cineole are now known bioinsecticidal monoterpenes occurring in many essential oils. The present study determined their toxicity on insectivorous guppy fish, *Poecilia reticulata* Peters, 1859 known to feed on mosquito larvae. The toxicity was recorded post 24 h treatment in experimental aquaria. The estimated median lethal concentration (LC_{50}) values of thymol and 1,8-cineole for female fish were 12.51 and 3997.07 mg/L, respectively, and for males the concentration required was 10.99 and 1701.93 mg/L, respectively. There was significant inhibition of acetylcholinesterase (AChE) and carboxylesterase (CarE) in treated male fish after 1,8-cineole treatment, whereas thymol induced CarE in both sexes but induced only AChE in females. Overall, thymol and 1,8-cineole were moderately toxic to guppies compared to synthetic pesticides.

Materials and methods

Test material 1

Name:	thymol
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma- Aldrich (St. Louis, USA)
Active substance content:	97-99%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species:	*Poecilia reticulata* (guppy)
Strain/clone:	Not reported
Age at study initiation:	2 months old
Weight/length/height at study initiation:	mean 0.20 g (range 15-25 g) /2.50 cm (range 3.8-5.1 cm)
Source:	local breeder in Bangkok, Thailand
Feeding during test:	No
Acclimation:	24 hours

Test conditions

Hardness:	Not reported
Test temperature:	26.0 ± 1.0 ºC
pH:	7.0
Dissolved oxygen:	7.2-7.9 mg/L
Conductivity:	Not reported
Photoperiod:	Not reported
Light intensity:	Not reported

Test system

Study type:	Acute toxicity test
Duration of study:	24 hours
Treatments:	Nominal concentrations 0-20 mg thymol/L, but unclear how many concentrations tested.
Analytical determination of test concentrations:	No
Negative control included:	Yes (0.5% triton X100 in acetone)
Positive control included:	No
Parameters measured:	Mortality/ behaviour. Molecular biomarkers (enzymatic activity) were also measured, but are not discussed further in this summary as not considered relevant.
Validity criteria:	Not reported

Groups of experimental animals, each consisting of 10 individuals, were selected at random and placed into aerated aquaria. After 24 hours of adaptation, 1 mL of different concentrations of essential oil compounds was added to the experimental aquaria making total concentrations of 0-20 mg/L for thymol. All determinations were repeated five times for each concentration. A control group was provided with 1 mL of 0.5% triton X100 in acetone (AR grade) in a test aquarium. The fish were not fed for the duration of the experiment. Mortality was recorded 24 hours after the start of the tests. Dead individuals were removed immediately. Behavioral changes were followed closely. The median lethal concentration (LC_{50}) and 95% confidence limits were calculated using the Statplus for Mac software program (AnalystSoft Inc., Walnut, Canada).

Results

The calculated 24-hour acute LC_{50} values of thymol for the *Poecilia reticulata* (guppy) female fish was 12.51 mg thymol/L (95% confidence interval (CI): 6.86-19.02), and for males was 10.99 mg thymol/L (CI: 5.70-13.94), based on nominal concentrations. No mortality occurred in the control group.

The control group showed normal behavior during the test period. The changes in behavioral response started after dosing with thymol (minimum effect concentration not reported), where paralytic, erratic swimming, rapid gill movement and motionless stature of fish were observed. Fish were seen adhering to the bottom of aquaria at all treatment concentrations. At a dose of more than 10 mg/L of thymol, the paralysis occurred within the first hour of treatment in aquaria.

Assessment and conclusion

Reliability assessment
For full details and justification, please refer to Document KCA 9.4.2/02.
Proposed category: 3 not reliable
Analytical verification of test concentrations was not reported, nor was all nominal concentrations tested.

Assessment and conclusion by applicant:

The study is not acceptable (not reliable).
Poecilia reticulata (guppy) 24-hour LC_{50} (females) = 12.51 mg thymol/L (95% confidence limits: 6.86-19.02 mg thymol/L) based on nominal concentrations
Poecilia reticulata (guppy) 24-hour LC_{50} (males) =10.99 mg thymol/L (95% confidence limits: 5.70-13.94 mg thymol/L) based on nominal concentrations.

Data point:	CA 9.6.3.4/03 [CA 8.3.1]
Report author	Mondet, F., Goodwin, M., Mercer, A.
Report year	2011
Report title	Age-related changes in the behavioural response of honeybees to Apiguard®, a thymol-based treatment used to control the mite *Varroa destructor*
Report No	-
Document No	Internal reference: Study 24 Journal of comparative physiology volume 197, Pages 1055-1062 (2011)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	No

Abstract (copied from original literature)
The parasitic mite *Varroa destructor* is responsible for heavy losses in honey bee colonies and represents a major threat to the beekeeping industry. Essential oils offer an attractive alternative to the use of synthetic chemicals for the control of varroa. Amongst them, thymol appears to be particularly promising. However, treatments using thymol as their active substance, such as the gel formulation Apiguard®, are suspected to have adverse effects on honey bee colonies. In this study, laboratory assays are used to investigate the effects of Apiguard® exposure on honey bee behaviour. Our results reveal that honey bee responses to this anti-varroa treatment change with honey bee age. While 2-day-old bees respond neutrally to Apiguard®, older bees generally avoid the Apiguard® gel. Responses of forager bees were particularly striking. Foragers appear to be repelled by Apiguard®. Touching their antennae with Apiguard® induces robust fanning behaviour. Our data suggest, however, that forager bees exposed to Apiguard® in the hive can become habituated to this treatment. These results offer interesting new perspectives on the effects of Apiguard® on honey bee behaviour and serve to highlight age-related changes in honey bee responses to gustatory, as well as olfactory cues.
Materials and methods

Test material 1

Name:	Apiguard®
Formulation type:	Slow release gel
Source and lot/batch no.:	Not reported
Active substance content:	25% thymol w/w
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species: Honey bees
Strain/clone: Not reported
Age at study initiation: 2-4 day old and pollen foragers
Weight/length/height at study initiation: as applicable (mean and range, SD)
Source: Not reported
Feeding during test: Yes, bees were fed 30% sucrose solution and left for 1 h before testing them
Acclimation: Not reported

Test conditions

Test temperature: 35 ºC

Photoperiod: Not reported
Light intensity: Not reported
Test system
Study type: Behavioural responses to Apiguard® in laboratory tests in bees reared with or without Apiguard® in a hive
Duration of study: <1 hour
Treatments: Not reported in terms of thymol concentration
Analytical determination of test concentrations: No.
Negative control included: Yes (agar)
Positive control included: No
Parameters measured: behaviour
Validity criteria: Not reported
Fanning in response to Apiguard was examined more closely using forager bees. The bees were cold-anesthetized after collection from the hive, and mounted on a toothpick by gluing the most distal segment of each leg to the toothpick using Super Glue. Six bees were examined in each session, and 24 bees from Apiguard treated hives were analysed in total. A toothpick impregnated with fresh Apiguard gel was used to touch different parts of the bee's body to see whether it would trigger a fanning response. Bees were tested one at a time and contact was made with each leg, the wings, the mandibles, the proboscis and the antennae. Apiguard gel that had been allowed to air dry and become hard was also touched to the antennae. Stimulations of different body parts were performed in random order with an interval of 10 min between each stimulus. For each stimulation, the presence or absence of fanning was recorded. Apiguard was delivered also in the form of an odour puff. A piece of filter paper containing Apiguard (3 µg) was placed in a 20 mL syringe, which was then used to deliver a 5 sec puff of thymol-filled air to the bee's antennae.

Results

Forager bees that were exposed to Apiguard often displayed fanning behaviour. To examine this further, we investigated which body part might be responsible for mediating this response. We found that touching the mandibles, proboscis, legs, or wings of a forager bee with a toothpick carrying Apiguard elicited no response, but that fanning could be reliably induced by touching the antennae of the bee with Apiguard. To determine whether this fanning behaviour was triggered by the texture of the gel, we repeated the experiment with Apiguard gel that had been allowed to dry out. Antennal contact with dry Apiguard gel also elicited a strong fanning response. Interestingly, Apiguard delivered in the form of an odour puff did not trigger fanning behaviour, nor did contacting the antennae with control gel. Fanning behaviour in response to Apiguard contact was also age dependent. Fanning was observed only rarely in 2-day-old bees, whereas in 4-day-old bees and foragers the behaviour was robust, highly predictable and could be abolished almost entirely if antennal contact with Apiguard gel was prevented by covering the gel with wire mesh. Behavioural responses to Apiguard may be affected by prolonged exposure to this anti-varroa agent in the hive. The absence of Apiguard avoidance displayed by young adult bees and the habituation of more mature bees to volatiles released by this anti-varroa treatment indicated that bees become habituated to vapours released from the gel, including thymol.

Assessment and conclusion

Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.
Proposed category: 3 not reliable
Study only tested an anti-*Varroa* gel formulation containing 25% w/w thymol, but other components of formulation are unknown. Concentrations of formulation or thymol in the trays received in the hive are not clearly reported.

Assessment and conclusion by applicant:

The study is considered as not reliable.

Young adult bees and more mature bees become habituated to vapours released from the gel Apiguard, including thymol.

Data point:	CA 9.6.3.4/04 [CA 8.6]
Report author	Kordali, S., Cakir, A, Ozer, H., Cakmakci, R., Kesdek, M., Mete, E.
Report year	2008
Report title	Antifungal, phytotoxic and insecticidal properties of essential oil isolated from Turkish *Origanum acutidens* and its three components, carvacrol, thymol and p-cymene
Report No	-
Document No	Internal reference: Study 43 Bioresource Technology Volume 99, Pages 8788–8795 (2008)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)
The chemical composition of essential oil isolated by hydrodistillation from the aerial parts of *Origanum acutidens* was analyzed by GC–MS. Carvacrol (87.0%), p-cymene (2.0%), linalool acetate (1.7%), borneol (1.6%) and b-caryophyllene (1.3%) were found to be as main constituents. Antifungal, phytotoxic and insecticidal activities of the oil and its aromatic monoterpene constituents, carvacrol, p-cymene and thymol were also determined. The antifungal assays showed that *O. acutidens* oil, carvacrol and thymol completely inhibited mycelial growth of 17 phytopathogenic fungi and their antifungal effects were higher than commercial fungicide, benomyl. However, p-cymene possessed lower antifungal activity. The oil, carvacrol and thymol completely inhibited the seed germination and seedling growth of *Amaranthus retroflexus*, *Chenopodium album* and *Rumex crispus* and also showed a potent phytotoxic effect against these plants. However, p-cymene did not show any phytotoxic effect. Furthermore, *O. acutidens* oil showed 68.3% and 36.7% mortality against *Sitophilus granarius* and *Tribolium confusum* adults, respectively. The findings of the present study suggest that antifungal and herbicidal properties of the oil can be attributed to its major component, carvacrol, and these agents have a potential to be used as fungicide, herbicide as well as insecticide.

Materials and methods

Test material

Name:	thymol
Formulation type:	Not relevant
Source and lot/batch no.:	Fluka
Active substance content:	> 99% purity
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Note, tests on agricultural pathogenic fungi and pest insects are excluded from this summary as these are not relevant to the ecotoxicological risk assessment of non-target organisms.

Test organisms 1

Species: *Amaranthus retroflexus*, *Chenopodium album* and *Rumex crispus*

Strain/clone: Not reported
Age at study initiation: seeds
Weight/length/height at study initiation: not applicable as seeds
Source: Erzurum region (Turkey). To avoid possible inhibition caused by toxins from fungi or bacteria, the seeds were surface- sterilized with 15% sodium hypochlorite for 20 min and then rinsed with abundant distilled water.
Feeding during test: No
Acclimation: Two layers of filter paper were placed on the bottom of each Petri dish (9 cm diameter) and then 50 seeds of *A. retroflexus*, *C. album* and *R. crispus* were placed on the filter paper. Then, 10 mL of distilled water was added to each Petri dish. Lids of Petri dishes were closed with Whatman No. 1 filter paper wrapping up tightly with a transparent ribbon.

Test conditions 1

Test temperature: 23 ± 2 °C on a growth chamber
Humidity: 80%
Photoperiod: 12 hours light / 12 hours darkness
Light intensity: fluorescent light
Test system 1
Study type: Seedling emergence test and seedling growth test
Duration of study: 10 days
Treatments: - To determine the herbicidal effects of vapors: 10 mg/Petri dish content of thymol, dissolved in DMSO (1:1; w/v) was applied to filter paper placed on the lid of the Petri dish.
- To determine the contact herbicidal effect, thymol was dissolved in DMSO–water solution (1%; v/v). The final concentration of the treatments was 1.0 mg/mL, with 10 mL applied on filter paper in the bottom of the Petri dishes (onto which the seeds were placed on top).
Analytical determination of test concentrations: No
Negative control included: Yes
Positive control included: Yes, 2,4-D, isooctyl ester (10 µL/Petri dishes)
Parameters measured: the number of germinated seeds and seedling lengths were measured
Validity criteria: Not reported

Test organisms 2

Species: *Amaranthus retroflexus*, *Chenopodium album* and *Rumex crispus*
Strain/clone: Not reported
Age at study initiation: seeds were sown into the pots
Weight/length/height at study initiation: 3-4 real leaves stage for spraying
Source: Not mentioned (assumed the same as above). To avoid possible contaminants caused by fungi or bacteria, weeds of the plant samples were surface-sterilized with 15% sodium hypochlorite for 20 min and then rinsed with abundant distilled water. Seeds were sown in pots (10 x 10 cm) filled with 550 g soil (organic material ratio: 2.02%; pH 7.5) and exposure of test item was to germinated plants (3-4 leaf stage).
Feeding during test: No
Acclimation: Emergence occurs in the same conditions of exposure.

Test conditions 2

Test temperature: 23 ± 2 °C on a growth chamber
Humidity: 80%
Photoperiod: 12 hours light / 12 hours darkness
Light intensity: not reported
Test system 2

Study type:	Phytotoxicity assays
Duration of study:	48 hours
Treatments:	Thymol was dissolved in 10 mL of DMSO–water solution (1% v/v). The final concentration of the treatment was 1.0 mg/mL. These emulsions (10 mL for each pot) were sprayed uniformly with a glass atomizer on the surface of whole plants in each pot in the stage of 3–4 real leaves.
Analytical determination of test concentrations: No	
Negative control included:	Yes, 10 mL of DMSO–water solution (1%),
Positive control included:	Yes, 2,4-D, isooctyl ester (10 mg for each pot))
Parameters measured:	Killed plants were counted and recorded at 24 and 48 hours after sample applications
Validity criteria:	Not reported

Results

Herbicidal and phytotoxic effects

The results obtained from volatile and contact phase effect assays showed that the thymol (10.0 mg/Petri dish) completely inhibited the germination and seedling growth (i.e. 0% germination) of all three tested weed species, *Amaranthus retroflexus, Chenopodium album* and *Rumex crispus* (applied to filter paper in Petri dishes, in the absence of a soil substrate) compared to 38.0, 50.7 and 72.0% germination in the negative controls.

Thymol (10 mg/pot) showed potent phytotoxic effects against all plant species tested *Amaranthus retroflexus, Chenopodium album* and *Rumex crispus*. 48 hours after the application, 10 mg/pot concentration of the thymol induced 89.9%, 77.8 and 48.0% phytotoxic effects (as % mean mortality) on *Amaranthus retroflexus, Chenopodium album* and *Rumex crispus* respectively compared to 0.7, 1.9 and 0.7% in the negative controls. For comparison, the positive control (2,4-D, isooctyl ester at 10 mg/pot) completely killed exposed *R. crispus* and *C. album* after 48 hours and showed 75.8% mortality for *A. retroflexus*.

Assessment and conclusion

Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 2 reliable with restrictions

No analytical verification of test solutions and no clear endpoints to use for the risk assessment (routes of exposure in the Petri dish tests are quite different to standard non-target terrestrial plant tests, and concentrations/rates tested are only reported in terms of mg per Petri fish or pot).

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).

It was shown that thymol (applied to filter paper in Petri dishes) at 10 mg/Petri dish completely inhibited seed emergence of three weed species: *Amaranthus retroflexus, Chenopodium album* and *Rumex crispus.*

It was shown that thymol (applied to seedlings grown in soil) at 10 mg/pot led to phytotoxic effects (89.9%, 77.8 and 48.0% mortality after 48 hours) on three weed species: *A. retroflexus, C. album* and *R. crispus*.

Data point:	CA 9.6.3.4/05 [CA 8.2.2]
Report author	Morselli, M.B., Reis, J.H., Baldissera, M.D., Souza, C. F., Baldisserotto, B., Petrolli, T. G., Paiano, D., Lopes, D. L. A., and Da Silva, A. S.
Report year	2020
Report title	Benefits of thymol supplementation on performance, the hepatic antioxidant system, and energetic metabolism in grass carp
Report No	-
Document No	Internal reference: Study 56 Fish Physiol Biochem Volume 46, Pages 305–314 (2020)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	No

Abstract (copied from original literature)

Thymol is an herbal food additive used to improve animal performance. Thymol acts via its potential to enhance productive and reproductive performance, and by improving bioavailability of nutrients in fish. Nevertheless, the exact mechanisms associated with these phenomena remain poorly understood, although recent evidence has suggested the involvement of the phosphotransfer network and antioxidant status. Therefore, the aim of this study was to determine whether the improvement of the antioxidant/oxidant status and the phosphoryl transfer network may be involved in enhanced growth performance in grass carp (*Ctenopharyngodon idella*) fed with various levels of thymol (100, 200, and 300 mg/kg feed). Thymol supplementation (100 mg/kg feed) produced higher body weight and weight gain for 60-day post-feeding compared to the control group. Specific growth rate was higher; while feed conversion ratio was lower in fish that consumed 100 mg of thymol/kg compared to other groups. Hepatic lactate dehydrogenase activity and lipid peroxidation levels were lower in the thymol supplemented group (100 mg/kg feed) than in the control group, while reactive oxygen species were lower in all supplemented groups than in the control group. Hepatic superoxide dismutase (300 mg/kg feed) and glutathione peroxidase (100, 200, and 300 mg/kg feed) activities, as well as antioxidant capacity against peroxyl radicals (100 mg/kg feed) were higher in these groups than in the control group. Based on these data, we conclude that 100 mg thymol/kg dietary supplementation increased growth performance of fingerling grass carp. Finally, hepatic adenylate kinase activity was lower in the thymol supplemented group (100 mg/kg feed) than in the control group. Thymol supplementation (100 mg/kg feed) improved hepatic energy metabolism, while practically all tested concentrations of thymol enhanced hepatic antioxidant status, all of which may be pathways involved in increased growth performance in fingerling grass carp.

Materials and methods

Test material

Name: Thymol (2-isopropyl-5-methylphenol)
Formulation type: Not relevant for active substance, granulated thymol
Source and lot/batch no.: Sigma-Aldrich® (São Paulo, Brazil)
Active substance content: Not reported
Expiry date of lot/batch: Not reported
Storage conditions: Not reported

Test organism

Species: grass carp (*Ctenopharyngodon idella*)
Strain/clone: Not reported
Age at study initiation: fingerlings
Weight/length/height at study initiation: 7.33 ± 0.12 g; 8.13 ± 0.12 cm
Source: Not reported.

Feeding during test:	Yes. The basal diet was based on corn, poultry viscera meal, feather meal, and soybean meal, which were crushed and mixed with other ingredients, providing all nutritional requirements for fish. Fish were fed on one of the tested diets at a feeding rate of 6% of total biomass for 60 consecutive days. The feed protocol was divided into two equal portions and offered to fish twice daily at 09:00 h and 17:00 h.
Acclimation:	10 days in 250-L fiberglass tanks with continuous aeration under controlled water variables in fresh water. The water parameters remained stable throughout all acclimation period: temperature (23.1 ± 0.2 °C), pH (6.9 ± 0.4), dissolved oxygen (6.8 ± 0.2 mg/L), total ammonia (0.81 ± 0.03 mg/L), and non-ionized ammonia (0.0041 ± 0.00028 mg/L).

Test conditions

Only acclimation conditions were reported in the paper and were as described below:

Hardness:	Not reported
Test temperature:	23.1 ± 0.2 °C
pH:	6.9 ± 0.4
Dissolved oxygen:	6.8 ± 0.2 mg/L
Conductivity:	total ammonia (0.81 ± 0.03 mg/L), and non-ionized ammonia (0.0041 ± 0.00028 mg/L)
Photoperiod:	Not reported
Light intensity:	Not reported
Test system	
Study type:	Feeding toxicity test
Duration of study:	60 days
Treatments:	0.0, 100, 200, and 300 mg thymol/kg of feed, four groups in quadruplicate with ten fish per aquarium (60 L)
Analytical determination of test concentrations: No	
Negative control included:	Yes (basal diet)
Positive control included:	No
Parameters measured:	On day 60 post-feeding, all animals were weighed: body weight, total body length, weight gain, and added body length were calculated for the total period (60 days). Specific growth rate and feed conversion ratio were calculated. On day 61 the liver was removed and dissected in a glass dish over ice to evaluate the various parameters. Molecular biomarkers (including hepatic reactive oxygen species, and enzymatic activity (e.g. glutathione S-transferase, hexokinase (HK) and glucose 6-phosphate dehydrogenase)) were measured, but are not discussed further in this summary as not considered relevant.
Validity criteria:	Not reported

Results

Growth performance

No significant difference was observed between groups regarding length and feed consumption. However, after 60 days body weight and body weight gain were higher in the group that was fed on 100 mg thymol/kg feed compared to all other groups. The body weight in the fish increased over 60 days by 8.7% (control), 47.1% (100 mg thymol/kg feed), 10.9% (200 mg thymol/kg feed), and 15.4% (300 mg thymol/kg feed). The specific growth rate was higher while the feed conversion ratio was lower in fish that consumed 100 mg thymol/kg feed compared to other groups.

Assessment and conclusion

Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported.

Assessment and conclusion by applicant:

The study is not acceptable (not reliable).

Ctenopharyngodon idella (grass carp): In fish fed on diets supplemented with thymol (purity not reported) for 60 days, body weight increased over 60 days by 47.1% (100 mg thymol/kg feed), 10.9% (200 mg thymol/kg feed), and 15.4% (300 mg thymol/kg feed) compared to 8.7% in the control, based on nominal concentrations of thymol in the diet.

Data point:	CA 9.6.3.4/06 [CA 8.6]
Report author	Sekine, N.; Shibutani, S.
Report year	2013
Report title	Chemical structures of p -menthane monoterpenes with special reference to their effect on seed germination and termite mortality
Report No	-
Document No	Internal reference: Study 78 Journal of Wood Science volume 59 pages 229 – 237 (2013)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)
A series of p-menthane monoterpenes was investigated to confirm any correlation between their bioactivity (effect on seed germination and termite mortality) and chemical structure. The germination percentages of *Brassica rapa* seeds at a concentration of 0.1 mg/Petri dish of (+)-pulegone, isopulegol, piperitone, (-)-dihydrocarveol, terpinen-4-ol and (-)-menthol were found to be 21.6, 27.3, 27.3, 29.1, 42.9 and 43.4, respectively. The lethal concentration 50 values of carvacrol, (+)-pulegone, thymol, (-)-menthol and (-)-terpinen-4-ol for termites (*Reticulitermes speratus*) were 0.34, 0.50, 0.65, 0.92 and 1.26 (mg/Petri dish), respectively. Of all the compounds tested, phenols produced the highest levels of termite mortality, with ketones and alcohols also showing bioactivity. An assessment of the bioactivity revealed that the presence of a phenol group was effective for termite mortality, with a carbonyl group also showing strong bioactivity. The presence of an alcohol or isopropyl group in a ring also contributed to the bioactivity, whereas the presence of an isopropenyl group at the same position, however, exhibited an inhibitory effect on seed germination. In conclusion, the bioactivity of the p-menthane monoterpenoids was dependent upon the presence and position of certain functional groups and the degree of saturation in the functional group of the side chain.

Materials and methods

Test material 1

Name:	Thymol
Formulation type:	Not relevant
Source and lot/batch no.:	Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan)
Active substance content:	Not reported
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism 1

Species:	*Brassica rapa*

Strain/clone: var. *perviridis*
Age at study initiation: Seed stage
Weight/length/height at study initiation: Not relevant
Source: Provided by Takii & Co. Ltd. (Kyoto, Japan)
Feeding during test: Not relevant
Acclimation: Not relevant

Test organism 2

Species: *Raphanus sativus*
Strain/clone: var. *sativus*
Age at study initiation: Seed stage

Weight/length/height at study initiation: Not relevant
Source: Provided by Takii & Co. Ltd. (Kyoto, Japan)
Feeding during test: Not relevant
Acclimation: Not relevant

Test organism 3

Species: *Reticulitermes speratus*, termites
Strain/clone: Not reported
Age at study initiation: Not reported
Weight/length/height at study initiation: Not reported
Source: Collected from the Institute of Wood Technology at the Akita Prefectural University of Japan, in July 2009. The colonies were maintained in a room at 20 ± 2°C for one and a half years prior to the initiation of the test.
Feeding during test: Not reported
Acclimation: Not reported

Test conditions 1 (seed germination test)

Test temperature: 24 ± 2°C
Photoperiod: Sequential 10 h photoperiods and 14 h dark periods
Light intensity: Not reported
Test system 1
Study type: Seed germination test (application to filter paper in a Petri dish)
Duration of study: 3 days
Treatments: 10.0, 1.0 and 0.1 mg thymol/Petri dish (nominal)
Analytical determination of test concentrations: No
Negative control included: Yes (acetone)
Positive control included: No
Parameters measured: Germination percent (%) which is the number of germinated seeds after 2 days (or 1 day) of the tests of each sample/number of germinated seeds after 3 days of the tests of control x 100
Validity criteria: Not reported
The effects of thymol on germination were tested on the *R. sativus* and *B. rapa* seeds. A sample of each of the compounds (50, 5 and 0.5 mg) was dissolved separately in acetone (10 mL), and a portion (2.0 mL) of the resulting solutions was poured onto a filter paper (90 mm diameter) in a Petri dish. In a control Petri dish, acetone (2.0 mL) was only used. The acetone solvent was then removed by evaporation over a period of 20 min in a fume hood. Deionized water (10 mL) was then added to each of the Petri dishes together with 20 seeds of *R. sativus* or *B. rapa*. Each of the compound tests and control treatments were performed in triplicate. The Petri dishes were then covered and placed in a growth chamber for 3 days (sequential 10 h photoperiods and 14 h dark periods).

Test conditions 2 (termites)

Test temperature: 25 ± 1°C
Photoperiod / Light intensity: dark during the test (1 hour)
Test system 2
Study type: Termite acute toxicity test (application to filter paper in a Petri dish)
Duration of study: 1 hour
Treatments: 2.0, 1.0 and 0.5 mg thymol/Petri dish (nominal)
Analytical determination of test concentrations: No
Negative control included: Yes (acetone)
Positive control included: No
Parameters measured: Mortality
Validity criteria: Not reported
Application to filter papers was comparable to that described above for the seed germination test (with exception of different rates applied). Application was to Petri dishes together with 20 active termites (workers). The Petri dishes were then covered and placed in an incubator at for the entire test period (1 hour).

Results

Seed germination test

When applied to filter paper at nominally 10 mg thymol/Petri dish, there was no (0%) germination of *R. sativus* and *B. rapa* seeds compared to 91.3 and 86.4 % germination in the controls for the two plant species, respectively.

For *R. sativus***,** applications of thymol at 0.1 and 1.0 mg thymol/Petri dish caused significant inhibition of seed germination compared to controls, although at the lowest rate of 0.1 mg thymol/Petri dish the standard deviation between replicates was large (*R. sativus* % germination was 91.3, 68.9 and approximately 42% in the control, 0.1 and 1.0 mg thymol/Petri dish groups, respectively (based on Figure 2 of paper)).

For *B. rapa***,** applications of thymol at 1.0 mg thymol/Petri dish caused significant inhibition of seed germination compared to controls, but there was no significant effect for 0.1 mg thymol/Petri dish (*B. rapa* % germination was 86.4, approximately 80 and approximately 38% in the control, 0.1 and 1.0 mg thymol/Petri dish groups, respectively (based on Figure 3 of paper)).

Termite toxicity test

The 1-hour LC_{50} value for *Reticulitermes speratus* termites was reported to be 0.65 mg thymol/Petri dish (95% confidence limits 0.59 – 0.70) (based on nominal rates).

Assessment and conclusion

Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.
Proposed category: 2 reliable with restrictions
Test item purity not reported and no analytical verification of test solutions applied. No positive controls included, and LC_{50} value for termites calculated based on data for only three test rates.

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).

Seed germination test (application to filter paper in Petri dish)

Raphanus sativus var. *sativus:* after 2-3 days, significant effect on germination following exposure to 0.1, 1.0 and 10.0 mg thymol/Petri dish (nominal) compared to controls.

Brassica rapa var. *perviridis:* after 2-3 days, significant effect on germination following exposure to 1.0 and 10.0 mg thymol/Petri dish compared to controls, but no effect at 0.1 mg thymol/Petri dish (nominal).

Termite toxicity test (application to filter paper in Petri dish)

Reticulitermes speratus (termite) 1-hour LC_{50} = 0.65 mg thymol/Petri dish (95% confidence limits: 0.59-0.70 mg thymol/Petri dish) based on nominal application rates

Data point:	CA 9.6.3.4/07 [CA 8.3.1]
Report author	Kasiotis K.M., Tzouganaki, Z.D., Machera K.
Report year	2018
Report title	Chromatographic determination of monoterpenes and other acaricides in honeybees: Prevalence and possible synergies
Report No	-
Document No	Internal reference: Study 79 Science of The Total Environment Volume 625, Pages 96 - 105 (June 2018)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)
In this study, the first targeted GC–MS/MS method for the detection and quantification of monoterpenic phenols, thymol and carvacrol in honeybees, employing a simplified sample preparation protocol, using ethyl acetate as the extraction solvent, is reported. The method was then applied to honeybees' samples after reported death incidents to evaluate the levels of the afore mentioned compounds in the course of 2015 early 2017. In parallel, other regularly used acaricides, namely amitaz, tau-fluvalinate, and coumaphos were also monitored using an LC-ESI-MS/MS multiresidue method based on modified QuEChERS technique. Breakdown products of amitraz; DMF and DMPF and coumaphos oxon were also investigated. The predominant acaricides detected were coumaphos, thymol, metabolites DMF and DMPF, and in less extent tau-fluvalinate, with concentrations for compounds varying from the low ng/g scale up to approximately 60,000 ng/g $_{\text{bee body weight}}$. The highest concentrations were observed for coumaphos and thymol. Preliminary risk assessment using hazard quotient (HQ) as the criterion, showed negligible risk from acaricides as individual components of bees. However, potential synergistic effects between acaricides or acaricides and other pollutants should not be disregarded.

Materials and methods
This paper is not strictly relevant to the ecotoxicological risk assessment in terms of assessing effects on non-target organisms, but for completeness, has been briefly summarised and included in this section of the dossier as supporting information. In this study, thymol concentration levels in bees after reported death incidents in Greece between 2015-2017 were examined in order to contribute to the domain of thymol's toxicity to honeybees after its application as a *Varroa* mite control agent.

Reference material as analytical standard

Name:	Thymol
Formulation type:	Not relevant
Source and lot/batch no.:	Acros Organics
Active substance content:	99%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species:	Honeybee (assumed *Apis mellifera)*
Strain/clone:	Not reported
Source:	Bees used as control sample were obtained from Agricultural University of Athens experimental apiaries that were not subjected to thymol or other acaricide treatments. The exposed honeybee samples were sent for analysis from beekeepers all over Greece.

Test system

Study type:	Monitoring study - thymol concentration levels in bees after reported death incidents from beekeepers all over Greece.
Duration of study:	Not relevant. Monitoring study of dead honeybees after treatment against *Varroa*.
Treatments:	Not reported – monitoring study in which the exposed honeybee samples were sent for analysis from beekeepers all over Greece.
Analytical determination:	Yes (method validation : based on the SANTE guideline SANTE/11945/2015,2015).
Parameters measured:	Thymol concentration levels in bees after reported death incidents

Results

The analysis of dead honeybee samples sent from beekeepers across Greece in 2015 – 2017 demonstrated that thymol was evidenced in 14 out of 47 samples (i.e. 30% of samples analysed). Concentrations of thymol in these samples varied from 14.4 to 40900 ng thymol/$g_{bee\ bw}$.

Assessment and conclusion

Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: Not reliable for assessing ecotoxicological effects on bees, but considered 2 reliable with restrictions as a monitoring study on residue levels

No guideline is used. In this monitoring study there was no information about the actual exposure of the honeybees prior to death, but it is implied from the paper that all exposure occurred as a result of the use of thymol as a *Varroa* treatment, not as a plant protection product. The analytical method is considered reliable.

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).

The analysis of dead honeybee samples sent from beekeepers across Greece in 2015 – 2017 demonstrated that thymol was evidenced in 14 out of 47 samples (i.e. 30% of samples analysed). Concentrations of thymol in these samples varied from 14.4 to 40900 ng thymol/$g_{bee\ bw}$.

Data point:	CA 9.6.3.4/08 [CA 8.3.1]
Report author	Nanetti A., Bacandritsos N., Papanastasiou I., Saitanis C. *et al.*
Report year	2007
Report title	Comparison between six thymol-based formulations in the control of *Varroa destructor* (Anderson et Trueman) in Greece [*Apis mellifera L.*]
Report No	-
Document No	Internal reference: Study 95 Agris FAO Volume 3, Pages 126-133 (2007)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Cannot be assessed as full text not available

Abstract (copied from original literature; Full text was not available)

The present study was undertaken to evaluate the efficacy of six formulations containing thymol against the Varroa mite under real beekeeping conditions of the Attica region Greece. Three of them were commercially available (Api Life Var Apiguard Thymovar) while the other ones were prepared for the purposes of the experiment (crystals gel with clove and vaseline oil). The treatments were made in April-June 2002. To fit the usual beekeeping practice of the area the colonies were forced to a broodless condition that was attained by queen caging and was exploited to promote the treatment efficacy. The commercial formulations yielded high average efficacy ranging between 90.4 and 90.6%. Crystal gel and vaseline formulations killed 91.8, 88.4, and 84.9% of the mites respectively. The mixture of thymol and vaseline was significantly less effective than the

other formulations. All the queens survived the caging period although they were placed rather close to the point of treatment. External observations of treated and control colonies did not put into evidence serious problems with tolerability. Thymol crystals induced some clear behavioural disturbance which was temporary and did not seem strong enough to exclude the formulation from the field praxis. Apiguard induced lower disturbance to the colonies if compared to the other commercial acaricides. The acaricides taken into consideration fit the need of practicable highly effective and fairly tolerable treatments against Varroa mites during the warm period of the beekeeping season. The easy and cheap preparation makes the non-commercial formulations worth of further consideration and opens the perspective to include them into the beekeeping practice.

Assessment and conclusion by applicant:

The study cannot be fully assessed as full text not available.

Data point:	CA 9.6.3.4/09 [CA 8.3.1]
Report author	Raffique, M.K.; Mahmoud, R.; Aslam, M.; Sarwar, G.
Report year	2012
Report title	Control of *Tropilaelaps clareae* Mite by Using Formic Acid and Thymol in Honey Bee *Apis mellifera L.* Colonies
Report No	-
Document No	Internal reference: Study 106 Pakistan J. Zoo., Volume 44, Issue 4, Pages 1129-1135, (2012)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	No

Abstract (copied from original literature)

The proposed research work was conducted in March, 2008 at Honeybee Research Institute of National Agricultural Research Centre, Islamabad on *Apis mellifera lingustica* honeybee colonies infested with the *Tropilaelaps clareae*. Treatments were given by dividing experimental 36 colonies into two groups (Formic acid and Thymol). Formic acid (70%) and Thymol were applied in six treatments including control (T0=0.0 ml, Tl=5ml, T2=l0ml, T3=l5ml, T4=20ml and T5= 25ml) and (T0=0.0g, Tl=5g, T2=l0g, T3=l5g, T4=20g, and T5=25g) respectively with three replications for five weeks. The treatments were given randomly by using complete randomized design (CRD). The maximum number 782 and 5l8 of mites collected in mite collection trays treated with 25 ml formic acid and 25 g of thymol. The highest efficacy of formic acid and thymol against *T. clareae* on honeybee was 97 and 95.3%. ln formic acid group T5 (25 ml) yielded the maximum honey 22.54 kg/colony. In thymol group, T5 (25g) yielded the maximum honey 20.7 kg/colony. From all the results it was clearly observed that all the treatments of formic acid and thymol are effective against the *T. clareae*. It was observed that during whole experiment no queens were lost and there was no adult honeybee mortality in any of the colonies.

Materials and methods

Test material 1

Name:	Thymol
Formulation type:	Not relevant
Source and lot/batch no.:	Not reported
Active substance content:	Not reported
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species:	Honeybee (*Apis mellifera lingustica*)
Strain/clone:	*lingustica*
Age at study initiation:	Not relevant (full colony)
Source:	Honeybee Research Institute of National Agricultural Research Centre, Islamabad
Feeding during test:	Not reported
Acclimation:	Not reported

Note, the principle aim of the study was to assess effects on the mite species, *Tropilaelaps clareae*, but this aspect of the study is not discussed further in this summary as effects on *T. clareae* (a target pest) are not relevant for the ecotoxicological assessment of non-target organisms.

Test conditions

Test temperature:	Not reported
Photoperiod:	Not reported
Light intensity:	Not reported
Test system	
Study type:	Field study testing the efficacy of thymol for mite control in honey bee colonies (efficacy on mites not relevant for ecotoxicological assessment, but the observed effects on the honey bee colonies are reported in this summary as honeybees relevant as non-target organisms)
Duration of study:	five weeks
Treatments:	control, 5 g, l0 g, l5 g, 20 g, 25 g applied as thymol crystals in Petri dishes placed on top of the brood frames under the top cover of hives at weekly intervals for five weeks
Analytical determination of test concentrations: No	
Negative control included:	Yes
Positive control included:	No
Parameters measured:	Honeybee mortality and behaviour, including assessments of general colony health (queen loss and damage to bee brood) and honey yield
Validity criteria:	Not reported

Results

No adverse effects on honeybee colonies were observed for all thymol treatments when applied at up to and including 25 g thymol (based on nominal rates) at weekly intervals over the exposure period of 5 weeks. This included no loss of queens, no severe damage to bee brood and no increase in bee mortality rate during the test. Honey yield increased compared to the untreated control with increasing rates of thymol treatment, which was associated with the corresponding reduction in mite infestation on bees.

Assessment and conclusion

Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable, study aim was to check effects of thymol on mites and not honeybees. Nevertheless, no adverse effects on honeybee colonies were reported at the highest concentration tested. Since there is not enough information on the test conditions, test item purity/source, or methods/frequency of colony assessments, this study is considered as non-reliable.

Assessment and conclusion by applicant:

The study is not acceptable (not reliable).

No adverse effects reported on honeybee colonies at a rate of 25 g thymol (nominal) applied as thymol crystals in Petri dishes placed on top of the brood frames under the top cover of hives at weekly intervals for five weeks.

Data point:	CA 9.6.3.4/10 [CA 8.3.1]
Report author	Costa, C., Lodesani, M., Maistrello, L.
Report year	2010
Report title	Effect of thymol and resveratrol administered with candy or syrup on the development of *Nosema ceranae* and on the longevity of honeybees (*Apis mellifera L.*) in laboratory conditions
Report No	-
Document No	Internal reference: Study 133 Apidologie Volume 41, Pages 141–150 (2010)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)
The aim of this study was to evaluate the effect of thymol and resveratrol administered in two different formulation modes (candy and syrup) on the development of *Nosema ceranae* and on the longevity of honey bees. Emerging bees from a nosema-free apiary were individually infected with 1 µL of sucrose syrup containing 18000 spores of *N. ceranae*, placed in cages, and kept in an incubator at 33 ◦C and 65% RH. The experimental groups were fed candy or syrup prepared with thymol (100 ppm) or resveratrol (10 ppm). Infection levels were monitored over a 25 day period by removal and dissection of two live bees per cage. On day 25, post-infection bees fed with thymol syrup had significantly lower levels of infection (60 ± 9 million spores/bee) compared to control bees (138 ± 7 million spores/bee). Bees fed with thymol or resveratrol syrup lived significantly longer (23 and 25 days, respectively) than bees fed with control syrup (20 days). Thymol treated syrup appears to be promising in the control of nosema infection.

Materials and methods

Test material 1

Name:	Thymol
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma
Active substance content:	99.5%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species:	*Apis mellifera* L, Honey bees.
Strain/clone:	Reported as 'presumably *A. m. ligustica'*
Age at study initiation:	Newly emerged honey bees
Source:	CRA-API apiary in Reggio Emilia, Italy
Feeding during test:	Yes, *ad libitum*. The syrup was administered to the bees via gravity feeders fitted into each cage, initially containing 4 mL syrup, which was replaced every 3 days.
Acclimation:	Newly emerged bees were manually collected and individually fed with 1 µL of the inoculated sucrose syrup suspension before being placed in the

cages.

Test conditions

Test temperature: incubated at 33 ºC and 65 % RH
Photoperiod: Dark
Light intensity: Not reported (dark)
Test system
Study type: Chronic toxicity laboratory test
Duration of study: 25 days
Treatments: Two different modes of administration of thymol was tested: syrup and candy. A 50% w/v sucrose solution was used to prepare the treated syrups and bee candy was prepared by mixing icing sugar (85%), honey produced in the test facility's apiary (10%), and water (5%). Ethanol (3,2 µL/g) was used to aid solubility of thymol and added to the control syrup and candy. 0.1 mg thymol/g feeding solution (nominal)
Analytical determination of test concentrations: No
Negative control included: Yes
Positive control included: No
Parameters measured: Mortality
Validity criteria: Not reported
Treated candy or syrup were administered to honey bee workers artificially infected *with N. ceranae*, and thereafter kept in small wooden, glass sided cages measuring 10 × 10 × 20 cm, provided with a small frame and foundation, and gravity feeders and/or feeding dishes. Each cage contained approximately 30 bees and was incubated at 33°C and 65% RH in the dark. Each treatment was replicated four times, thus yielding a total of 24 cages: 2 administrations (syrup and candy) × 3 groups (2 active ingredients (thymol and resveratrol) + 1 control) × 4 replicates. The control cages consisted in infected bees fed with untreated syrup and candy.

Results

Results from survival analysis showed that 13 days after the beginning of the experiment, about 90% of the bees were still alive in all groups. During the following days (upto 25 days), differences among treatments became apparent, as the bees fed with thymol treated syrup lived significantly longer than bees fed with control syrup and with treated and control candy.
No significant differences were observed in intake of candies or syrups containing thymol compared to controls

Assessment and conclusion

Reliability assessment
For full details and justification, please refer to Document KCA 9.4.2/02.
Proposed category: 2 reliable with restrictions
No analytical verification or positive control as routinely required for chronic oral honey bee laboratory tests.

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).

Apis mellifera L. In a laboratory test, honey bees were exposed to thymol in 50% sucrose solution or bee candy at nominally 0.1 mg thymol/g feeding solution for upto 25 days. Bees fed with thymol treated syrup lived significantly longer than bees fed with control syrup and with treated and control candy. No significant differences were observed in intake of candies or syrups containing thymol compared to controls.

Data point:	CA 9.6.3.4/11 [CA 8.3.1]
Report author	Colin, T., Lim, M.Y., Quarrell, S. R., Allen, G. R., Barron, A. B.
Report year	2019
Report title	Effects of thymol on European honey bee hygienic behaviour
Report No	-
Document No	Internal reference: Study 141 Apidologie volume 50, Pages 141–152 (2019)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)
The parasitic mite *Varroa destructor* is a major threat to the European honey bee *Apis mellifera*. Beekeepers apply the miticide thymol directly within the hives to kill this parasitic mite. Thymol is repellent to bees and causes them to ventilate the hive, yet its impact on bee hygienic behaviours that prevent the spread of diseases has never been studied. We measured the efficiency of colonies at removing dead adult bees, uncapping dead pupal cells and removing dead brood in two miticide-free Australian environments where the mite is absent. Thymol increased the uncapping and removal of dead brood by 24 to 36% after 48 h at both locations but had no effect on the removal of dead adult bees. The increased removal of brood could enhance the effect of thymol on *V. destructor*, especially if bees preferentially remove cells infected with the mite.

Materials and methods

Test material

Name: Apiguard®
Formulation type: Slow-release gel
Source and lot/batch no.: Vita Europe Ltd
Active substance content: 25% thymol (12.5 g thymol in a each Apiguard® aluminium tray)
Expiry date of lot/batch: Not reported
Storage conditions: Not reported

Test organism

Species: *Apis mellifera*, honey bees.
Strain/clone: Golden Italian strain.
Source: Colonies located at housed at the University of Tasmania's apiary at the Horticultural Science Research Centre, Sandy Bay, Australia; and the Wheen Bee Foundation, in Richmond, New South Wales, Australia.
Feeding during test: Not relevant
Acclimation: Not relevant
Test system
Study type: Field trial in which honey bee colonies were treated with the *Varroa*-treatment formulation "Apiguard®"
Duration of study: From March 27 to May and from April 13 to May 25.
Treatments: Apiguard® trays (containing 12.5 g thymol each) were administered in autumn as per the manufacturer's instructions by introducing one tray on top of the brood frames. Trays were replaced after 2 weeks. The second tray was also removed after 2 weeks
Analytical determination of test concentrations: No
Negative control included: Yes
Positive control included: No
Parameters measured: Behaviour
Validity criteria: Not reported

Two experiments were performed to test the impact of Apiguard® on the hygienic performances of the hives. The frozen brood assay to assess uncapping and brood removal and the dead bee removal assay to assess the undertaking of dead bees. All analyses were conducted using R v.3.3.1. The efficiency of hives at uncapping cells and removing dead brood and bees was analysed using mixed effects logistic regressions (GLMM) with the package lme4 v.1.1–14.

Results

There was no difference in brood removal between the control and Apiguard® groups within each location. The second assay, after Apiguard® was introduced into the hives, Apiguard® -treated hives removed 84.1 ± 6.3% of the freeze-killed brood and were more efficient in removing dead brood than the control hives (66.2 ± 12.5%) in Sydney. In Hobart, hives removed 48.8 ± 5.3% but there was no significant difference between treatments (42.99 ± 9.28% in control hives and 53.66 ± 9.56% in Apiguard® hives). In the third assay, Apiguard® -treated hives removed 49.2 ± 6% of the dead brood and were more efficient than the control hives (36.7 ± 5.6%) in Hobart, but there was no significant difference between treatments in Sydney where bees removed 34 ± 1% of the dead brood (53.25 ± 7.92% in control hives and 55.10 ± 8.80% in Apiguard® hives) None of the hives removed more than 95% of the freeze-killed brood, the hive that performed the best removed 88% of the brood in Hobart.

Assessment and conclusion

Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 2 reliable with restrictions

Study tested a slow-release gel formulation containing thymol, but exact exposure of thymol to bees is unknown (and no analytical verification included).

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).

In a field trial, honey bee colonies in Australia were treated with the anti-*Varroa* treatment "Apiguard®". Apiguard is a slow-release gel, added in aluminium trays to the honey bee hive; each tray contains 12.5 g of thymol. In this study, Apiguard trays (containing 12.5 g thymol each) were administered in autumn as per the manufacturer's instructions by introducing one tray on top of the brood frames. Trays were replaced after 2 weeks. The second tray was also removed after 2 weeks. Apiguard increased the uncapping and removal of dead brood by 24 to 36% after 48 hours at both locations but had no effect on the removal of dead adult bees. The increased removal of brood could enhance the effect of Apiguard on *V. destructor*, especially if bees preferentially remove cells infected with the mite.

Data point:	CA 9.6.3.4/12 [CA 8.3.1]
Report author	Sönmez D.
Report year	2019
Report title	Efficacy of thymol and thymol active ingredient products against of parasitic mite (*Varroa destructor* Anderson & Trueman)
Report No	-
Document No	Internal reference: Study 154 Agris FAO (2019)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Cannot be assessed as full text not available

Abstract (copied from original literature; Full text was not available)

Effects of powdered thymol, Thymovar and Apiguard for the control of the honey bee parasitic mite *Varroa destructor* and colony growth parameters were evaluated in this research. The best results were obtained with powdered thymol (92.03%) and in Thymovar (83.60%). The efficacy of Apiguard was 76.08%. Applications of powdered thymol and thymol active ingredient products used in this research did not show any negative effects on the brood area and bee populations.

Assessment and conclusion by applicant:

The study cannot be fully assessed as full text not available.

Data point:	CA 9.6.3.4/13 [CA 8.3.1]
Report author	May-Itzá, W.J.; Medina, L.A.M.; Olivares, J.C.M.
Report year	2007
Report title	Effectiveness of a thymol based gel for the control of *Varroa destructor* mite that infests *Apis mellifera* honey bee colonies, under tropical conditions in Yucatan, Mexico
Report No	-
Document No	Internal reference: Study 159 Vet. Méx., Volume 38, Pages 1-8 (2007)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)

The effectiveness of a thymol based gel (12.5 g of thymol in 50 g of gel) for controlling *Varroa destructor* in commercial honey bee (*Apis mellifera*) colonies under tropical conditions was evaluated. Three groups of honey bee colonies were used, the first group received one tray containing thymol gel (G1), the second group received two trays with thymol gel (G2) and the third group (control) received no treatment (G3).The application of the gels was repeated twice with two weeks between treatments. In total G1 received 2 trays and G2 received 4 trays of thymol gels, thus the honey bee colonies and the mites were exposed to the thymol gels during 30 days. The mite infestation levels in adult bees and capped brood were registered before and after the application of the treatments in order to evaluate the effectiveness of the thymol-based gels. The effectiveness of the treatments estimated for adult bees was 97% and 93% for G1 and G2, respectively, and in the capped brood the effectiveness was 94% and 95% for both groups respectively. The results show that the application of one

thymol gel (G1) tray with a second application in a 15-day interval (total of two trays) can eliminate ≈95% of the mites in honey bee colonies under tropical conditions and thus can be used as an alternative method for the apiculturist of this region to control this parasite.

Materials and methods

Test material

Name:	Product name not given (assumed to be Apiguard based on details on application doses given in the paper, but cannot be confirmed)
Formulation type:	Gel
Source and lot/batch no.:	Not reported
Active substance content:	Not reported (thymol based gel administered in honey bee colonies in an aluminium tray at 12.5 g of thymol in 50 g of gel)
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species:	Honeybee (*Apis mellifera*)
Strain/clone:	Not reported
Age at study initiation:	Adult
Source:	Faculty of Veterinary Medicine and Animal Husbandry of the Autonomous University of Yucatan, in Xmatkuil, at 15.5 km from Merida, Yucatan, Mexico
Feeding during test:	Not reported
Acclimation:	Not reported

Test conditions

Test temperature:	Not reported
Photoperiod:	Not reported
Light intensity:	Not reported
Test system	
Study type:	Field trial in Mexico with treatment of honey bee colonies with (assumed) Apiguard trays
Duration of study:	30 days
Treatments:	Fifteen colonies were divided in three experimental groups: 1) group of colonies (n=5) who received two trays of thymol gel; the first tray was set at the beginning of the research (simple dose) and the second 15 days later (G1); 2) groups of colonies who received four trays of thymol gel; two trays were set at the beginning of the research (double dose) and another two, 15 days after (G2); 3) group of colonies to whom no acaricide was applied during the same period of evaluation of the gels, considered as control group (G3).
Analytical determination of test concentrations: No	
Negative control included:	Yes
Positive control included:	No
Parameters measured:	Behaviour
Validity criteria:	Not reported

The aim of the study was to verify the efficacy of thymol against *Varroa* mites. Nevertheless, the effect on bees has been reported. Fifteen colonies were divided in the three experimental groups G1, G2 and G3.

Results

In this research it was observed that the application of only one dose of thymol gel and its repetition at 15 days presented no negative effect over the adult bee population, the brood or queen. Nevertheless, in G2, where two

trays of thymol gel were applied the same day, it was observed that two colonies swarmed and, in the rest, bees jammed in the entrance of the beehive due to greater concentration of thymol in the colony, which exerts a repellent action in the adult bee population.

Assessment and conclusion

Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 2 reliable with restrictions

Study tested a slow-release gel formulation containing thymol, but exact exposure of thymol to bees is unknown (and no analytical verification included).

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).

In a field trial, honey bee colonies in Mexico were treated with an anti-*Varroa* treatment gel formulation. The application of only one dose of thymol gel (12.5 g thymol in 50 g gel) and its repetition at 15 days presented no negative effect over the adult bee population, the brood or queen. Concentrations of thymol (in the gel formulation) above 50 g thymol/bee colony showed a repellent effect in the adult bee population.

Data point:	CA 9.6.3.4/14 [CA 8.3.1]
Report author	Tananaki,C., Goras, G., Huggett, N., Karazafiris, E., Dimou, M., Thrasyvoulou, A.
Report year	2014
Report title	Evaluation of the impact of Exomite Pro™ on Varroa mite (*Varroa destructor*) populations and honeybee (*Apis mellifera*) colonies: efficacy, side effects and residues
Report No	-
Document No	Internal reference: Study 176 Parasitology Research Volume 113, Pages 1251-1259 (2014)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)

In this research, we examined the application of thymol-based powder, directly over the top of the brood frames in colonies with different population in a 2-year study. The efficacy against mites, the side effects on bees and the contamination of honey were studied comparably to thymol-based gel treatment. In one-store colonies, thymol-based powder treatment gave average efficacy 64.5% and did not differ significantly from thymol-based gel treatment (65.4%). The natural mortality in control colonies was 41.4% and the corrected efficacy (E_T) during 2 years was 39.4 and 40.9 %, respectively. In two-store bee colonies, the application of thymol-based powder on top of each hive body gave higher E_T (45.4%) than on top of the double body hive (40.4%), without statistically significant differences. The average concentration of thymol residues in honey 24 days after the application was 368 and 1119 µg/kg for the honey of colonies treated with thymol-based powder and thymol-base gel, respectively.

Materials and methods

Test material 1

Name:	Exomite Pro™
Formulation type:	thymol-based powder
Source and lot/batch no.:	Not reported
Active substance content:	thymol 6%
Expiry date of lot/batch:	Not reported

Storage conditions: Not reported

Test material 2

Name: Apiguard
Formulation type: thymol-based gel
Source and lot/batch no.: Not reported
Active substance content: Not reported
Expiry date of lot/batch: Not reported
Storage conditions: Not reported

Test organism

Species: *Apis mellifera*, honey bee
Strain/clone: *Apis mellifera macedonica*
Age at study initiation: Not reported
Weight/length/height at study initiation: Not applicable
Source: Apiary of laboratory of Apiculture, School of Agriculture, of Aristotle University
Feeding during test: Not reported
Acclimation: Not reported

Test conditions

Temperature: first year: mean 17.5ºC (10.4°C – 25.6°C),
second year, mean: 16.8°C (11.7°C – 23.2°C
Photoperiod: Not reported
Light intensity: Not reported
Test system
Study type: Field trial with honey bee colonies treated with *Varroa* treatments containing thymol
Duration of study: 2 years
Treatments: First year: 20 g Entostat (6% thymol) (nominal) or 50 g Apiguard per colony (equivalent to 12.5 g thymol) (nominal)
Second year: 40 g of thymol-based powder on top of double body hive or 20 g of powder on top of each hive's body
Analytical determination of test concentrations: No
Negative control included: Yes
Positive control included: No
Parameters measured: Mortality, number of brood cells, honey combs, residues of thymol in honey
Validity criteria: Not reported
In the first year (2008), 45 colonies situated in a single brood chamber were divided into three test groups and randomly underwent one of the following three treatments: thymol-based powder (n=15), thymol-based gel (n=15) and control (n=15). The thymol-based powder treatment was applied as a dust found in sachet. Each sachet contained 20 g of Entostat™ powder with 6 % thymol and 0.5 % silica flow agent. The foil sachet was opened, and the powder contents sprinkled over the top of the brood frames, ensuring the whole area was covered. A second treatment was re-applied 14 days after the initial one. One aluminum tray of 50 g thymol-based gel (Apiguard) per colony was placed over the brood area for two weeks, followed by the addition of a second tray for a further two weeks. Each tray contained 12.5 g thymol in a slow release gel.
In the second year (2009), 30 bee colonies having two hive- body population were divided into three groups of ten hives. The first group (A) was treated by sprinkling 40 g of thymol-based powder on top of double body hive. The second group (B) was treated by sprinkling 20 g of powder on top of each hive's body. The two application methods were compared to non-treated control colonies (group C).
Results
First year:

The decline of brood in the colonies treated with thymol-based powder and thymol-based gel resulted to greater reduction of brood population compared to the control colonies. However, the reduction of brood had no further negative impact on the experimental colonies, which overwintered successfully. Two of the colonies treated with thymol-based powder absconded. These colonies had initially very high mite infestation (47.7 and 34.6%) and nine-comb bee population. Thymol-based powder caused higher bee mortality than thymol-based gel and control regime. Nevertheless, the daily average of dead bees (16.0 ± 15.6) cannot be considered as a serious disadvantage of thymol powder treatment since up to 20 dead bees per day is generally considered as an acceptable number. The higher mortality of adult bees was observed a day after the first treatment when on average, 165 and 29 dead bees were counted in front of the colonies treated by thymol-based powder and thymol-based gel, respectively. During the second treatment, the number of dead bees was 42 for thymol-based powder and 1.5 for thymol-based gel. Soon after the administration of thymol-based powder, a perceivable bustle was noticed in the colonies. In a few cases, the bees were escaping and two colonies were absconded. The magnitude of bustle did not seem to be related to the colony strength.

Second year:

Due to the late season and the natural constraint of egg laying, brood and bee population in all cases declined. The decrease was similar in all groups. During the pre-treatment period, the total number of dead bees found in the three groups did not differ significantly. During the treatment period, a higher mortality of bees was noticed, especially in colonies that were treated on top of each hive's body. The average mortality in the colonies that received thymol-based powder on top of each hive's body (group B) was 16.8 ±17.0 bees per day and differed statistically significant from control group that had 4.3 ± 1.9 dead bees per day ($F = 5.37$, $p<0.000$). It was also higher compared to the colonies that received a single dose on top of the double body hive (group A) (8.3 ± 3 .8), although the differences between them were not significant. The daily number of dead bees increased noticeably 1 day after the first treatment. When thymol-based powder was applied on top of each super, the number of dead bees reached 211.9 ± 146.8 in one day with a maximum of 476 and a minimum of 90. In the case of the single dose, the daily number of dead bees was 72.1 ± 41.6 with a maximum of 139 and a minimum of 19 dead bees per day. The mortality in the control group remained in the usual levels (7.0 ± 2.9). During the second treatment, the number of dead bees dropped in both groups A and B, but it still remained significantly higher than the control group.

Thymol residues were not detected in honey samples collected from hives before treatment, but they were found in three out of five honey samples which were collected 12 days later from the hives of the first two groups. During the last sampling, residues were found in the 80 and 60 % of the samples collected from hives which had been treated with thymol-based gel and thymol-based powder, respectively. The average concentration for the first formulation at the first sampling was 716.1 µg/kg and for the second one was 1119.9 µg/ kg. Increase of the thymol's residues was also observed in the honey samples that collected 12 (188.7 µg/kg) and 24 days (368.0 µg/kg) after treatment with thymol-based powder. The average concentration of thymol residues was about three times higher in thymol-based gel group than in thymol-based powder group. It is characteristic that in the case of thymol-based gel, three samples exceeded the Swiss tolerance value of 0.8 mg/kg, while in the group treated with powder, only one sample did. Large variability in honey residues among hives of the same group was observed, probably caused by the different size of brood and bees' population that colonies had, which affected the thymol's vaporization.

The reduction of brood had no further negative impact on the colonies. Hence, significant losses of adult bees were observed only one day after treatment. After that, bee losses fell to expected numbers. No differences were found in the efficacy between thymol-based powder and thymol-based gel, and the adverse effects that were observed (decrease in brood and population) are not considered as serious drawback and can be overcome by simulative feeding during or after treatment. Finally, regarding the residues, the thymol-based gel contaminates the honey at higher concentrations than the thymol-based powder.

Assessment and conclusion

Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 2 reliable with restrictions

Study tested a slow-release gel formulation and a powder containing thymol, but exact exposure of thymol to bees is unknown (and no analytical verification included).

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).

Apis mellifera: In a field trial, honey bee colonies in Greece were treated with an anti-*Varroa* treatment gel or powder; First year: 20 g Entostat (6% thymol) (nominal) or 50 g Apiguard per colony (equivalent to 12.5 g thymol) (nominal); Second year: 40 g of thymol-based powder on top of double body hive or 20 g of powder on top of each hive's body

The effects on mortality, brood cells and population (honey combs) were observed during the first year of study. Mortality was higher than the controls after one day after the first treatment. The effects on mortality dropped one day after the second treatment, but was significantly higher than the control. The reduction of brood cells and population had no further negative impact on the survival of colonies during the second year of study.

Data point:	CA 9.6.3.4/15 [CA 8.3.1]
Report author	Bergougnoux, M., Treilhou, M., Armengaud, C.
Report year	2013
Report title	Exposure to thymol decreased phototactic behaviour in the honeybee (*Apis mellifera*) in laboratory conditions
Report No	-
Document No	Internal reference: Study 180 Apidologie volume 44, Pages 82-89 (2013)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	No

Abstract (copied from original literature)

The effects of the terpenoid thymol were evaluated on the phototactic behaviour of the adult honeybee (*Apis mellifera*) 1 and 24 hours after a topical application. The behaviour was quantified under different light intensities by measuring the time spent in the light source area and in areas opposite the source. Stimuli of 200 lux induced positive phototaxis of the bees. Thymol administered at 1 ng/bee had no effect on the phototaxic behaviour while bees that had received 10 or 100 ng thymol 1 hour before the test were less attracted by the 200-lux stimulus. The effect of thymol increased when the phototaxic behaviour was tested 24 hours after the topical application. However, with a light intensity of 400 lux the dose 10 ng/bee was ineffective and for 600 lux the phototaxic behaviour of the bees was not modified by the exposure to thymol.

Materials and methods

Test material

Name:	thymol
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma
Active substance content:	99.5 %
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species:	Honey bee, *Apis mellifera*
Strain/clone:	Not relevant

Age at study initiation:	Adults
Source:	Not reported. The colony and the hive had not received any treatment specially miticide treatment for 1 year before the beginning of the experiment.
Feeding during test:	Not reported
Acclimation:	Bees were fed with 10 µL of a 30% sucrose solution, then starved for 3 hours before the test.

Test conditions

Test temperature:	33 ºC
Photoperiod:	Not reported
Light intensity:	200, 400 and 600 lux
Test system	
Study type:	Acute contact toxicity test
Duration of study:	48 hours
Treatments:	control group (0.1 % acetone) and thymol groups composed of bees treated at 1, 10 and 100 ng thymol/bee (nominal)
Analytical determination of test concentrations: No	
Positive control included:	No
Parameters measured:	Mortality, phototaxis behaviour
Validity criteria:	Not reported

Thymol was first dissolved in acetone then diluted in water. Acetone at 0.1 % was present uniformly in all the solutions used for the topical application. Four experimental groups were conducted in parallel: control group (0.1 % acetone) and thymol groups composed of bees treated at 1, 10 and 100 ng/bee. Each group was composed of six bees. Experiments conducted with 200 lux were repeated three times (18 bees in total for each group) and two separate experiments were performed with 400 and 600 lux (12 bees in total for each group). Differences among treated groups were assessed by analysis of variance (ANOVA) and Scheffe's post hoc tests. Differences were deemed statistically significant at $P<0.05$. Normality and equal variances of data used for ANOVA were confirmed.

Results

Mortality was not significantly different between groups. In the present experimental conditions, the mean percentage of dead bees in each group was 44 ± 27 (control), 48 ± 19 (1 ng/bee), 62 ± 27 (10 ng/bee) and 44 ± 26 (100 ng/bee) after the topical application.

The results on the locomotor activity indicated that in low (200 lux), medium (400 lux) and high (600 lux) intensity light conditions, thymol had no effect on locomotor activity 1 hour after a topical application. However, 24 hours after treatment 10 and 100 ng thymol-treated bees were more active than control bees under the low-intensity stimulus (200 lux). At 1 ng/bee, thymol had no significant effect on locomotion or phototaxis. However, bees exposed to 10 or 100 ng were less attracted than control bees to light stimuli of low intensity.

Assessment and conclusion

Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

High mortality in control (44%) , so largely above the trigger of 10% in OECD 214 guideline (even if this guideline was not followed).

Assessment and conclusion by applicant:

The study is not reliable.

Apis mellifera: After acute contact exposure, mortality was not significantly different between groups compared to the control and up to 100 ng thymol/bee.

Data point:	CA 9.6.3.4/16 [CA 8.6]
Report author	Waliwitiya, R., Isman, M. B., Vernon, R.S., Riseman, A.
Report year	2020
Report title	Insecticidal Activity of Selected Monoterpenoids and Rosemary Oil to *Agriotes obscurus* (Coleoptera: Elateridae)
Report No	-
Document No	Internal reference: Study 218 Journal of Economic Entomology volume 98, Pages 1560-1665 (2020)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)
Acute toxicities of three naturally occurring monoterpenoid essential oil constituents and the essential oil of rosemary were tested against late instars of *Agriotes obscurus* (L.) (Coleoptera: Elateridae). Both contact and volatile toxicities of thymol, citronellal, eugenol, and rosemary oil were determined. Also, phytotoxicity of these compounds was evaluated on corn germination and seedling development. Thymol had the greatest contact toxicity (LD_{50} = 196.0 µg/larva), whereas citronellal and eugenol were less toxic (LD_{50} = 404.9 and 516.5 µg/larva, respectively). Rosemary oil did not show any significant contact toxicity, even at 1600 µg/larva. In terms of volatile toxicity, citronellal was the most toxic to wireworm larvae (LC_{50} =6.3 µg/cm^3) followed by rosemary oil (LC_{50}=15.9 µg/cm^3), thymol (LC_{50} =17.1 µg/cm^3), and eugenol (LC_{50} = 20.9 µg/cm^3). Thymol, eugenol, and citronellal significantly inhibited corn seed germination and development, whereas rosemary oil had only minimal phytotoxic effects.

Materials and methods

Test material 1

Name: thymol
Formulation type: Not relevant
Source and lot/batch no.: Sigma, St. Louis, MO
Active substance content: > 98%
Expiry date of lot/batch: Not reported
Storage conditions: Not reported

Test material 2

Name: eugenol
Formulation type: Not relevant
Source and lot/batch no.: Arylessence. Inc., Marietta, GA
Active substance content: > 95%
Expiry date of lot/batch: Not reported
Storage conditions: Not reported

Test organism

Species: Corn seed
Strain/clone: Pioneer Brand Hybrid #39T68

Age at study initiation: Not relevant
Weight/length/height at study initiation: Not relevant
Source: Pioneer Hi-Bred Ltd
Feeding during test: Not relevant
Acclimation: Corn seed were presoaked in water for 24 hours.

Note, study also tested effects on the beetle larvae, *Agriotes obscurus*, but this not discussed further in this summary as it's a target pest species so not relevant to the ecotoxicological assessment of non-target organisms.

Test conditions

Test temperature: 22 ºC
Photoperiod: Not reported
Light intensity: Not reported
Test system
Study type: Phytotoxicity test
Duration of study: 6 days
Treatments: 200, 400, 800, 1,600, and 3,200 µg eugenol and thymol/seed (nominal)
Analytical determination of test concentrations: No
Negative control included: Yes (methanol)
Positive control included: No
Parameters measured: Germination percentage, cotyledon length, and radicle length
Validity criteria: Not reported

Corn seeds were coated with 20 µL of the test solutions using a micropipettor. 10 corn seeds were exposed to concentrations of 200, 400, 800, 1600, and 3200 µg eugenol and thymol/seed with three complete replications. Plastic pots (14 cm in diameter) with cleaned sand were used to test the phytotoxicity of the compounds, eugenol and thymol to corn. After treating seeds, they were planted 2.5 cm in depth in the sand. Pure methanol was used as the control treatment. ANOVA procedures were performed by SPSS software.

Results

Differences in corn seed germination percentage, cotyledon length, and radicle length were observed among the treatments ($P > 0.005$) and concentrations ($P > 0.0001$). Thymol and eugenol inhibited germination and reduced cotyledon and radical growth as concentration increased. Thymol and eugenol, applied to corn seed at 400 µg/seed, caused no phytotoxicity.

The seed germination was reduced by 3%, 3%, 30%, 23% and 80% when exposed at 200, 400, 800, 1600 and 3200 µg thymol/seed, respectively. The cotyledon length was reduced by about 11% and 56% when exposed respectively to 1600 and 3200 µg thymol/seed. The radicle length was reduced by about 18% and 63% when exposed respectively to 1600 and 3200 µg thymol/seed.

The seed germination was reduced by 0%, 3%, 10%, 10% and 43% when exposed at 200, 400, 800, 1600 and 3200 µg eugenol/seed, respectively. The cotyledon length was reduced by about 13% and 24% when exposed respectively to 1600 and 3200 µg eugenol/seed. The radicle length was reduced by about 11% and 25% when exposed respectively to 1600 and 3200 µg eugenol/seed.

Thymol and eugenol, applied to corn seed at 400 µg/seed, caused no phytotoxicity.

Thymol, applied to corn seed at 3200 µg/seed, causes a reduction in germination of 80% and in growth (more than 50% inhibition on cotyledon and radicle lengths).

Eugenol applied to corn seed at 3200 µg/seed, causes a reduction of 43% in germination and in growth (about 25% inhibition on cotyledon and radicle lengths).

Assessment and conclusion

Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.
Proposed category: 2 reliable with restrictions
No analytical verification of test concentrations.

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).
Corn seed. No effects on seed germination at concentrations of 400 µg thymol/seed.
Corn seed. No effects on seed germination at concentrations of 400 µg eugenol/seed.

Data point:	CA 9.6.3.4/17 [CA 8.3.2]
Report author	Lima, A.P.S., Santana, E.D.R., Santos, A.C.C, Silva, J.E., Ribeiro, G.T., Pinheiro, A.M., Santos, I.T.B.F., Blank, A.F., Araujo, A.P.A., Bacci, L.
Report year	2020
Report title	Insecticide activity of botanical compounds against *Spodoptera frugiperda* and selectivity to the predatory bug *Podisus nigrispinus*
Report No	-
Document No	Internal reference: Study 222 Crop Protection Volume 136, 105230 (2020)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)
Pest control based exclusively on the use of organosynthetic insecticides can cause several problems including the reduction of populations of natural enemies. Thus, the use of selective insecticides is essential for efficient pest control. In this study, we evaluated the lethal effects of the essential oil of *Lippia sidoides* and its major compound thymol on the fall armyworm, *Spodoptera frugiperda*, as well as the selectivity and behavioral effects of these compounds on *Podisus nigrispinus*, an important predator of defoliating caterpillars. The botanical compounds were more selective to the predator than the insecticide deltamethrin, which was about 2.7 times more toxic to *P. nigrispinus* than to *S. frugiperda*. The insecticide deltamethrin caused rapid mortality to nymphs of *P. nigrispinus* with LT_{50} = 0.36 hour. Additionally, the mortality of individuals exposed to this treatment reached 100% in less than 3 hours. The essential oil (LT_{50} = 119 hours) and the thymol (LT_{50} = 93 hours) acted more slowly on the predator. On the contrary, for the pest, the botanical compounds acted faster than the synthetic insecticide. Nymphs exposed to deltamethrin and thymol showed behavioral changes, as there was an increase and decrease in speed and distance covered, respectively. All insects stayed longer on the untreated side of the arenas. The present study reveals the potential of the essential oil and thymol for the control of the *S. frugiperda* pest and, at the same time, the selectivity of these compounds over *P. nigrispinus.*

Materials and methods

Test material

Name:	thymol
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma- Aldrich
Active substance content:	Not reported
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species:	Predatory bug, *Podisus nigrispinus*
Strain/clone:	Not reported
Age at study initiation:	third instar nymphs
Weight/length/height at study initiation:	Not reported
Source:	Laboratories of the Federal University of Sergipe (UFS), São Cristóvão-SE, Brazil.
Feeding during test:	Yes *ad libitum* with pupae of *T. molitor*
Acclimation:	*P. nigrispinus* adults were kept in cages and fed with pupae of the alternative prey *Tenebrio molitor* L. (Coleoptera: enebrionidae). The postures deposited by the females were collected daily and transferred to Petri dishes (9 x 1.5 cm) containing cotton moistened with distilled water. After hatching, nymphs were fed with pupae of *T. molitor* until adulthood, when they were transferred to the cages.
Test conditions	
Temperature:	25°C ± 0.5°C
Humidity:	70 ± 10%
Photoperiod:	12 hours
Light intensity:	Not reported
Test system	
Study type:	Acute toxicity test
Duration of study:	48 hours
Treatments:	topical application on prothoraic region (0.5 µL thymol), no information on the tested concentrations.

Analytical determination of test concentrations: No

Negative control included:	Yes (acetone used as solvent)
Positive control included:	Yes (deltamethrin)
Parameters measured:	Mortality
Validity criteria:	Not reported

The bioassays for determination of lethal doses were conducted with groups of ten third instar nymphs of *P. nisgrispinus.* The treatments were topically applied (0.5 µL) on the prothoracic region of each insect using a microsyringe. Four replicates per insect and treatment were used. The acetone solvent was used as control.

The mortality data of *P. nigrispinus* was subjected to Probit analysis to determine the mortality dose curves for each treatment, using the procedure PROC PROBIT (SAS Institute, 2008).

Results

P. nigrispinus nymphs showed rapid reduction in survival when exposed to deltamethrin. LD_{50} value was reported to be 10.27 mg thymol/g (CI: 8.37 - 11.54 mg thymol/g) (based on nominal concentrations).

Assessment and conclusion

Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 2 reliable with restrictions

Purity of test item not given.

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

The study is considered as supporting information only (reliable with restrictions).

Podisus nigrispinus: LD_{50} = 10.27 mg thymol/g (95% confidence limits: 8.37 - 11.54 mg thymol/g) based on nominal concentrations

Data point:	CA 9.6.3.4/18 [CA 8.2.4.1]
Report author	Seo, S.-M., Park, H.-M., Park, I.-K.
Report year	2012
Report title	Larvicidal activity of ajowan (*Trachyspermum ammi*) and Peru balsam (*Myroxylon Pereira*) Oils and Blends of their constituents against Mosquito, *Aedes aegypti*, Acute toxicity on water flea, *Daphnia magna*, and aqueous residue
Report No	-
Document No	Internal reference: Study 225 Agricultural and food chemistry, Volume 60, Pages 5909-5914 (2012)
Guidelines followed in study	EPA-821-R-02-012
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	No

Abstract (copied from original literature)
This study evaluated the larvicidal activity of 20 plants essential oils and components from ajowan (*Trachyspermum ammi*) and Peru balsam (*Myroxylon Pereira*) oils against the mosquito, *Aedes aegypti.* Of the 20 plant essential oils, ajowan and Peru balsam oils at 0.1 mg/mL exhibited 100 and 97.5% larval mortality, respectively. At this same concentration, the individual constituents, (+)-camphene, benzoic acid, thymol, carvacrol, benzyl benzonate, and benzyl *trans*-cinnamate, caused 100% mortality. The toxicity of blends of constituents identified in two active oils indicated that thymol and benzyl benzoate were major contributors of the larvicidal activity of the artificial blend. This study also tested the acute toxicity of these two active oils and their major constituents against the water flea *Daphnia magna*. Peru balsam oil and benzyl *trans*-cinnamate were the most toxic to *D. mag*na. Two days after the treatment, residues of ajowan and Peru balsam oils in water were 36.2 and 85.1%, respectively. Less than 50% of benzyl *trans*-cinnamate and thymol were detected in the water at 2 days after treatment. The results show that the essential oils of ajowan and Peru balsam and some of their constituents have potential as botanical insecticides against *A. aegypti* mosquito larvae.

Materials and methods

Test material

Name: Thymol
Formulation type: Not relevant
Source and lot/batch no.: Fluka (Buchs, Switzerland)
Active substance content: 99%
Expiry date of lot/batch: Not reported
Storage conditions: Not reported

Test organism

Species: Water flea (*Daphnia magna)*
Strain/clone: Not reported
Age at study initiation: neonates, 24 hours-old
Weight/length/height at study initiation: not reported
Source: National Academy of agricultural science, Suwon, Republic of Korea
Feeding during test: Not reported
Acclimation: Not reported

Test conditions

Hardness:	Not reported
Test temperature:	20 ± 1ºC
pH:	Not reported
Dissolved oxygen:	Not reported
Conductivity:	Not reported
Photoperiod:	16:8 hours light/dark cycle
Light intensity:	Not reported

Test system

Study type:	Acute toxicity test
Duration of study:	48 h
Treatments:	1.0, 1.8, 3.24, 5.8, 10.5 mg thymol/L

Analytical determination of test concentrations: No

Negative control included:	Yes, control tanks received only acetone.
Positive control included:	No
Parameters measured:	Mortality
Validity criteria:	Not reported

The acute toxicity of thymol to *Daphnia magna* has been tested. Five concentrations were tested 20 neonates were used per test, and each test was repeated four times.
The LC_{50} values were calculated using probit analysis.

Results
Results for the ajowan oil are considered not relevant as this is a mixture of essential oils containing 44.17% of thymol. Thymol was tested on its own, thus only the results for thymol are reported.
The 48h-LC_{50} *Daphnia magna* value was reported to be 5.94 mg thymol/L (95% confidence limits: 5.45-6.53 mg thymol/L) (based on nominal concentrations).

Assessment and conclusion
Reliability assessment
For full details and justification, please refer to Document KCA 9.4.2/02.
Proposed category: 3 not reliable because the analytical verification of test concentrations was not reported.

Assessment and conclusion by applicant:

The study is not acceptable (not reliable).

Daphnia magna: 48h-LC_{50} = 5.94 mg thymol/L (95% confidence limits: 5.45-6.53 mg thymol/L) based on nominal concentrations

Data point:	CA 9.6.3.4/19 [8.3.1]
Report author	Charpentier, G., Vidau, C., Ferdy, J.B., Tabart, J., Vetillard, A.
Report year	2014
Report title	Lethal and sub-lethal effects of thymol on honeybee (*Apis mellifera*) larvae reared *in vitro*
Report No	-
Document No	Internal reference: Study 230 Pest Management Science, Volume 70, Pages 140-147
Guidelines followed in study	Modification of Aupinel *et al*. (2005), Improvement of artificial feeding in a standard *in vitro* method for rearing *Apis mellifera* larvae. *Bull Insect* **58**:107–111 (2005).
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)
BACKGROUND: Thymol offers an attractive alternative to synthetic chemicals to keep *Varroa* under control. However, thymol accumulates in bee products and is suspected of having adverse effects on colonies and especially on larvae. In this study, we investigated the effects of acute and chronic exposure to thymol on larvae reared *in vitro* with contaminated food and compared results to the theoretical larval exposure based on the amount of pollen and honey consumed by larvae during their development.
RESULTS: The laboratory assays reveal that, first, the 48 h-LD_{50} of thymol introduced into larval food is 0.044 mg/larva. Second, the 6 day-LC_{50} is 700 mg/kg food. A significant decrease of larval survival and mass occurred from 500 mg thymol/kg food ($P < 0.0001$). Finally, vitellogenin expression, which reached a maximum at the fifth instar larvae, is delayed for individuals exposed to 50 mg thymol/kg food ($P < 0.0006$). That is 10 times higher than the theoretical level of exposure.
CONCLUSION: Based on the level of thymol residue found in honey and pollen, these results suggest that the contamination of food by thymol represents no notable risk for the early-developing larvae.

Materials and methods

Test material

Name:	thymol crystals
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma
Active substance content:	99.5%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species:	Honey bees, *Apis mellifera*
Strain/clone:	Four Buckfast colonies (crossed with the *Apis mellifera mellifera* honey bee)
Age at study initiation:	Second-instar larvae
Source:	Honey bee colonies provided by the APIREM Company (Saint-Juery, France) and maintained in the Jean-François Champollion University Center. The queen bee was confined to a defined area on a comb containing empty cells for 30 hours to control the age of the brood. Half of the brood was used for investigation of the effects of toxicants on the parameters of development under laboratory conditions and the other half was used as a control.

Feeding during test:	Yes, royal jelly
Acclimation:	Not relevant

Test conditions

Test temperature:	Not reported
Relative humidity:	Not reported
Photoperiod:	Not reported
Light intensity:	Not reported

Test system

Study type:	Laboratory honey bee larval toxicity tests (i) single exposure and (ii) repeated exposure
Duration of study:	i) 48 hours ; ii) 6 days
Treatments:	1, 5, 50, 100, 500, 1000, 3000 mg thymol/kg food (with 1% acetone) (nominal)
Analytical determination of test concentrations: No	
Negative control included:	Yes, control (untreated) and acetone control
Positive control included:	Yes, 10 mg dimethoate/kg food
Parameters measured:	Mortality, body mass (gene expression and enzymatic biomarkers also assessed, but not discussed further here as not considered relevant).
Validity criteria:	Not reported

The *in vitro* rearing was conducted according to the procedure described by Aupinel *et al*. (2005) with a few modifications. Modifications included: only early second-instar larvae were grafted. The royal jelly in the diet was furnished by the Chanteclair miellerie (Chanlencon, France), larvae were grafted using a picking instead of a paintbrush and sodium dichloroisocyanurate (Micropur forte®; Katadyn, Kemptthal,Switzerland) was used instead of methyl benzethonium chloride. Before each experiment, the required amount of diet including different concentrations of thymol, as described below, was prepared the day before the experiment and stored at 4 °C. Briefly, seven stock solutions of thymol (0.1, 0.5, 5, 10, 50, 100, 300 mg/mL) were prepared by dissolving thymol crystals (99.5%; Sigma, Saint-Quentin Fallavier, France) in acetone and diluted in feeding mixture to obtain a final concentration of 1, 5, 50, 100, 500, 1000, 3000 mg of thymol/kg food (with 1% acetone). Second-instar larvae were divided into experimental groups of 30 individuals grafted in plastic queen cups and inserted in 48-well cell culture plates. To control the effect of thymol vapours on larvae survival, control plates containing only larvae fed with untreated food and acetone treated food were incubated in separate thymol-free hermetic Plexiglas desiccators.

The 48 hour-LD_{50} was determined from three independent trials by feeding second-instar larvae with 0, 0.0011, 0.0022, 0.011, 0.022 and 0.066 mg of thymol per larva. The chronic toxicity (6 day-LC_{50}) of thymol was tested in larvae feeding until the fifth-instar larvae using the following concentration gradient: 0, 1, 5, 50, 100, 500, 1000 and 3000 mg/kg food. The individuals were monitored daily under a stereomicroscope. Each day, larval survival was recorded and dead individuals were removed. Dead larvae were recognised by immobility of their stigmata, and dead prepupae and pupae by occasional black or white sub-dermal necrotic stains or visible wilting.

Statistical analyses were performed using the open source statistic software R version 2.11.1 (r-project.org). Survival analyses were performed using the Cox proportional hazards regression model included in the R package 'survival'. The model applied to analyse effects of treatment on mortality include thymol, dimethoate and thymol vapours as covariates and trial as frailty. A post hoc analysis with the replicates background of test larvae as a random factor was performed using a G-rho family of tests in order to determine the lowest concentration inducing a significant mortality (survdiff function package MASS). A generalised linear model providing a binomial–logit logistic regression model was fitted on survival data of a 48 h thymol exposed second-instar larvae. The fitting of this model was adjusted using the Pearson χ^2 test. The lethal dose 50 (LD_{50}) value was estimated using the function dose.p of the 'MASS' package and the 95% confidence interval was calculated using the standard error. The same procedure was applied to estimate the 6 day-LC_{50}.

Results

The acute toxicity was assessed for second instar larvae 48 hours after feeding with a single dose of toxicant. The reference toxicant dimethoate induced a very highly significant decrease of larval survival (P<0.0001). The Cox proportional hazard regression model indicated a highly significant effect of thymol on survival of larvae (P<0.0001). The generalised linear mixed model approach showed a thymol 48 hour-LD_{50} of 0.044 mg/larva (nominal) with 95% confidence interval of 0.038–0.050 mg/larva (n=540) that corresponds to a 48 hour-LC_{50} of 2010 mg/kg food (nominal).

Chronic toxicity was assessed during the 6-day feeding period of larval development by monitoring survival and body mass. The reference toxicant dimethoate induced a very highly significant decrease of larval survival (P<0.0001) without affecting the larvae body mass (P=0.35). Results showed a highly significant effect of thymol on larval survival during this period (P<0.0001). The post hoc comparison tests showed a dose–effect from 100 to 3000 mg/kg food (0.017–0.528 mg/larva). The generalised linear mixed model approach showed a thymol 6 day-LC_{50} of 703 mg/kg (n=540) with 95% confidence interval of 600–829 mg/kg food. Following the feeding period, a highly significant decrease of prepupal and pupal survival was observed for individuals previously exposed to 500 and 1000 mg thymol/kg food (P<0.0001). Body mass of larvae was assessed over the feeding period as the endpoint for chronic toxicity. A highly significant decrease of larval mass occurred at the day 6 of exposure to 500 and 1000 mg thymol/kg food (P<0.0001). In addition, the daily observation revealed that, in the plastic queen cells containing the smallest larvae, food remained at the end of the feeding period.

Assessment and conclusion

Reliability assessment
For full details and justification, please refer to Document KCA 9.4.2/02.
Proposed category: 2 reliable with restrictions
No analytical verification is included (as standardly required for honey bee larval toxicity tests). (Test duration also only up to 6 days, whereas a 22-day test up to adult emergence is now routinely required for use in the risk assessment).

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).

Apis mellifera: 48-hour (single dose) larval toxicity LD_{50} = 0.044 mg thymol/larva with 95% confidence interval of 0.038–0.050 mg thymol/larva that corresponds to a 48 hour-LC_{50} of 2010 mg thymol/kg food (based on nominal concentrations).

Apis mellifera: 6-day (repeated exposure) LC_{50} = 703 mg thymol/kg food with 95% confidence interval of 600–829 mg thymol/kg food (based on nominal concentrations).

Data point:	CA 9.6.3.4/20 [CA 8.3.1]
Report author	Colin, T., Meikle, W.G., Paten, A.M., Barron, A.B.
Report year	2019
Report title	Long-term dynamics of honey bee colonies following exposure to chemical stress
Report No	-
Document No	Internal reference: Study 234 Science of the Total Environment, Volume 677, Pages 660–670 (2019)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)

Pesticide residues have been linked to reduced bee health and increased honey bee colony failure. Most research to date has investigated the role of pesticides on individual honey bees, and it is still unclear how trace levels of pesticides change colony viability and productivity over seasonal time scales. To address this question we exposed standard bee colonies to chemical stressors known to have negative effects on individual bees, and measured the productivity of bee colonies across a whole year in two environments: near Tucson Arizona and Sydney Australia. We exposed hives to a trace amount of the neonicotinoid imidacloprid and to the acaricide thymol, and measured capped brood, bee and honey production, as well as the temperature and foraging force of the colonies. The effect of imidacloprid on colony dynamics differed between the two environments. In Tucson we recorded a positive effect of imidacloprid treatment on bee and brood numbers. Thymol was associated with short-term negative effects on bee numbers at both locations, and may have affected colony survival at one location. The overall benefits of thymol for the colonies were unclear. We conclude that long-term and colony-level measures of the effects of agrochemicals are needed to properly understand risks to bees.

Materials and methods

Test material

Name:	Apiguard®
Formulation type:	Thymol based gel
Source and lot/batch no.:	Not reported
Active substance content:	250 g/kg of thymol in the gel
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species:	honey bee (*Apis mellifera*)
Strain/clone:	Not reported
Age at study initiation:	Not reported
Weight/length/height at study initiation:	not reported
Source:	**Site 1:** 28 colonies of European honey bees (*Apis mellifera*) were established at a site in the Santa Rita Experimental Range, south of Tucson, AZ, from packages containing 1 kg of bees each..Colonies were stocked with Cordovan-Italian queens (C.F. Koehnen & Sons, Glenn, CA, USA) and housed in painted standard hives referred to as 10-framewooden "Langstroth" deep boxes fitted with American migratory wooden lids (Mann Lake Ltd., Hackensack, MN, USA). **Site 2:** 20 colonies of European honey bees (*Apis mellifera*) were established on the campus of Macquarie University, north of Sydney, NSW, Australia, from packages containing about 1.8 kg of bees. Colonies were stocked with Golden Italian queens (Australian Queen Bee Exporters Pty. Ltd., Blayney, NSW) and housed in painted, standard hives referred to as 8-frame wooden "Langstroth" deep boxes, fitted with Australian migratory lids (vents were closed with tapes for the length of the experiment).
Feeding during test:	No.
Acclimation:	Bees were fed with 400 g of pollen patty and 4 L of 50:50 water:sugar syrup on establishment. **Site 1:** The experiment was started on the 13th of June and treatments started on 11th of July 2016. **Site 2:** The experiment started on the 22nd of December 2016 and the treatments on the 20th of January 2017.

Test conditions

Test temperature:	Temperature data for the Sydney Macquarie Campus site were obtained from the Automatic Weather Station of Department of Environmental Sciences of Macquarie University with a 15 min interval and averaged per

	day, daily rainfall data were obtained from the Australian Bureau of Meteorology. Daily temperature and rainfall data for the Tucson Santa Rita Experimental Range site were obtained from the AmeriFlux US-SRM Santa Rita Mesquite station in Tucson.
Photoperiod:	Not reported
Light intensity:	Not reported
Test system	
Study type:	Field trial in which honey bee colonies were treated with the *Varroa*-treatment formulation "Apiguard®"
Duration of study:	4 weeks exposure; duration of study: one year
Treatments:	50 g of Apiguard (12.5 g thymol) in the beginning for 2 weeks, renewed with 50 g of Apiguard (12.5 g of thymol) for another 2 weeks' period.

Analytical determination of test concentrations: Yes, in wax and honey

Negative control included:	Yes, the control syrup solution was prepared from sucrose and distilled water.
Positive control included:	No
Parameters measured:	Behaviour
Validity criteria:	Not reported

This study recorded a full year of worker and brood populations, food stores and daily weight dynamics of standard honey bee colonies. Two locations were chosen, Tucson, Arizona (US) and Sydney (Australia). Three treatments were given, one using imidacloprid, one with imidacloprid + Apiguard and one with Apiguard only. Reported results in this summary are for thymol only.

All statistical analyses were conducted under R v3.4.3.

Results

Following treatment in Tucson, thymol was found in the wax in amounts at least 1587 times larger than in the pre-treatment samples for the groups treated with Apiguard as well as in honey, and only traces of thymol were found in the wax and honey of the control and imidacloprid groups.

Apiguard had a negative impact on the colonies in the short term, but its effect on the long term differed between the two locations. In Sydney Apiguard had short-term effects on the brood during treatment. As a possible delayed consequence of this effect on brood, bee populations were lower in Apiguard-treated hives in the first weeks following the treatment. Two months after the end of the treatment, as the cohort of workers that grew during treatment was expected to start foraging, honey stores increased more slowly in Apiguard treated hives than in control hives and remained low until the end of the experiment. In Tucson, hives were left undisturbed during the treatment and the effect of Apiguard on brood during this period was not measured. In the first month following the end of the treatment bee populations were lower in hives treated with Apiguard than in the control group. Unlike Sydney, there was no long-term effect of the Apiguard exposure on the amount of honey stored. This could reflect that in Tucson hives benefited from a reduction in parasite load possibly increasing colony-level metabolic efficiency. It was noted, however, that four out of the six colonies that died in Tucson had been treated with Apiguard. It was previously suggested that Apiguard treatments may not always benefit the colonies, possibly by directly affecting or causing the removal of healthy and these results suggest that a re-evaluation of the benefits of miticide treatments at the level of the colony could benefit the beekeeping industry.

In conclusion, Apiguard was associated with short-term negative effects on bee numbers at both locations, and may have affected colony survival at one location. The overall benefits of Apiguard for the colonies were unclear.

Assessment and conclusion

Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 2 reliable with restrictions

Study tested a slow-release gel formulation containing thymol, but exact exposure of thymol to bees is unknown (and no analytical verification of test item included).

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).

In a field trial, honey bee colonies in Arizona and Australia were treated with the anti-*Varroa* treatment "Apiguard®". Apiguard is a slow-release gel, added in aluminium trays to the honey bee hive; each tray contains 12.5 g of thymol. In this study, one Apiguard tray (containing 12.5 g thymol) was introduced into the hive for 2 weeks, followed by a second tray that was also removed after 2 weeks. Apiguard treatment was associated with short-term negative effects on bee numbers at both locations and may have affected colony survival at one location. The overall benefits of Apiguard for the colonies were unclear.

Data point:	CA 9.6.3.4/21 [CA 8.6]
Report author	Araniti,F., Miras-Moreno, B., Lucini, L., Landi, M., Abenavoli, M.R.
Report year	2020
Report title	Metabolomic, proteomic and physiological insights into the potential mode of action of thymol, a phytotoxic natural monoterpenoid phenol
Report No	-
Document No	Internal reference: Study 239 Plant Physiology and Biochemistry, Volume 153, Pages 141-153 (2020)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)

Thymol is a natural phenolic monoterpene widely produced by different species belonging to the *Labiateae* family. Although the thymol phytotoxicity is well known, the knowledge of its potential toxic mechanism is still limited. In this regard, the model species *Arabidopsis thaliana* was treated for 16 days by sub-irrigation with 300 µM of thymol. The results confirmed the high phytotoxic potential of this phenolic compound, which caused a reduction in plant growth and development. Thymol induced a water status alteration accompanied by an increase in ABA content and stomatal closure. Furthermore, leaves appeared necrotic in the margins and their temperature rinsed. The increase in H_2O_2 content suggested an oxidative stress experienced by treated plants. Both metabolomic and proteomic analysis confirmed this hypothesis showing a strong increase in osmoprotectants content, such as galactinol and proline, and a significant up-accumulation of proteins involved in ROS detoxification. Furthermore, the down-accumulation of proteins and pigments involved in the photosynthetic machinery, the increase in light sensitivity and the lower PSII efficiency well indicated a reduction in photosynthetic activity. Overall, we can postulate that thymol-induced phytotoxicity could be related to a combined osmotic and oxidative stress that resulted in reduced plant development.

Materials and methods

Test material

Name:	Thymol
Formulation type:	Not relevant
Source and lot/batch no.:	Not reported
Active substance content:	Not reported
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species:	*Arabidopsis thaliana* L. (Heyn.)
Strain/clone:	ecotype Columbia (Col-0)
Age at study initiation:	seedling just emerged (test 1) and 3 weeks old adult plants (test 2)
Weight/length/height at study initiation:	Not reported
Source:	Not reported
Feeding during test:	Test 1: Petri dishes containing agar medium (0.8%) enriched with macro and micronutrients (Murashige – Skoog basal salt; Sigma-Aldrich) and sucrose (1%) (test 1) Test 2: Pots (6 × 6 × 8 cm) containing Perlite (Agrilit 3), previously sterilized and moistened with half-strength Hoagland nutrient solution
Acclimation:	Test 1: Sown plates were placed, in a vertical position, in a growth chamber, at 22 °C ± 2 °C, 8/16 h, light (90 mol/m²/s)/ darkness photoperiod, and 55% relative humidity. Test 2: Transplanted seedlings were allowed to establish for 7 days and during which they were sub-irrigated every other day with a same nutrient solution (Hoagland nutrient solution).

Test conditions

Test temperature:	22 °C ± 2 °C
Relative humidity:	55%
Photoperiod:	8/16 h light/darkness
Light intensity:	90 mol/m/s
Test system	
Study type:	Phytotoxicity test
Duration of study:	14 days for just emerged seedlings, 16 days for 3 weeks old adult plants
Treatments:	seedling treatment: 0, 50, 100, 200, 400, 800 and 1200 µM thymol (nominal) Adult plants: 0 µM (control) and 300 µM (nominal), application through sub-irrigation (25 mL/pot) every day

Analytical determination of test concentrations: No

Negative control included:	Yes
Positive control included:	No
Parameters measured:	Growth
Validity criteria:	Not reported

The present paper investigates the phytotoxic effects of thymol and its mode of action on adult plants of *Arabidopsis thaliana.*

The ED_{50} value was evaluated by a nonlinear regression model using a log-logistic function

Results

In the seedlings test, thymol severely affected shoot growth of *A. thaliana* in a dose dependent manner. In particular, fresh weight reduction was significantly evident already at 100 µM (25%). Such reduction increased along with the increasing of thymol concentrations reaching 81% and 93% with 800 µM and 1200 µM treatments, respectively.

Thymol treatment significantly altered all the morpho-physiological parameters evaluated. In particular both fresh and dry weight pointed out a 47% and 33% reduction, respectively. On the other hand, DW/FW (dry weight/fresh weight) ratio significantly increased (25%). At the same, plant growth inhibition was accompanied by a reduction of the leaf number, already observed after 4 days of treatment, a decrease of the leaf area (36%) and the RWC (69%), and an increase of the Ψπ (16%) (Ψπ is characterized by negative value, so an increase of 16% means that the Ψπ in plants treated was a 16% more negative than control).

The results strongly indicated that thymol caused an alteration of the plant water status that induced a cascade effect that started with ABA signaling and accumulation, and consequently a stomatal closure followed by a reduction of carbon absorption and metabolization.

LD_{50} value for seedlings of *A. thaliana* was reported to be 289.2 µM thymol (based on nominal concentrations).

Assessment and conclusion

Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 2 reliable with restrictions
Test item purity not given and no analytical verification.

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).
Arabidopsis thaliana L.: 14d-LD_{50} = 289.2 µM thymol (based on nominal concentrations) for seedlings.

Data point:	CA 9.6.3.4/22 [CA 8.2.1]
Report author	Bianchini, A.E.; Garlet, Q.I.; da Cunha, J.A., Bandeira Junior, G.; Brusque, I.C.M.; Salbeg, J.; Heinzmann, B.M.; Baldisserotto, B.
Report year	2017
Report title	Monoterpenoids (thymol, carvacrol and S-(+)-linalool) with anesthetic activity in silver catfish (*Rhamdia quelen*): evaluation of acetylcholinesterase and GABAergic activity
Report No	-
Document No	Internal reference: Study 245 Brazilian Journal of Medical and Biological Research, Volume 50, Pages 1-8 (2017)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	No

Abstract (copied from original literature)
This study evaluated the anaesthetic potential of thymol and carvacrol, and their influence on acetylcholinesterase (AChE) activity in the muscle and brain of silver catfish (*Rhamdia quelen*). The AChE activity of S-(+)-linalool was also evaluated. We subsequently assessed the effects of thymol and S-(+)-linalool on the GABAergic system. Fish were exposed to thymol and carvacrol (25, 50, 75, and 100 mg/L) to evaluate time for anaesthesia and recovery. Both compounds induced sedation at 25 mg/L and anaesthesia with 50–100 mg/L. However, fish exposed to carvacrol presented strong muscle contractions and mortality. AChE activity was increased in the brain of fish at 50 mg/L carvacrol and 100 mg/L thymol, and decreased in the muscle at 100 mg/L carvacrol. S-(+)-linalool did not alter AChE activity. Anaesthesia with thymol was reversed by exposure to picrotoxin (GABAA antagonist), similar to the positive control propofol, but was not reversed by flumazenil (antagonist of benzodiazepine binding site), as observed for the positive control diazepam. Picrotoxin did not reverse the effect of S-(+)-linalool. Thymol exposure at 50 mg/L is more suitable than carvacrol for anaesthesia in silver catfish, because this concentration did not cause any mortality or interference with AChE activity. Thymol interacted with GABAA receptors, but not with the GABAA/benzodiazepine site. In contrast, S-(+)-linalool did not act in GABAA receptors in silver catfish.

Materials and methods

Test material

Name:	Thymol
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma-Aldrich, Brazil
Active substance content:	≥ 99.0%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species:	Silver catfish (*Rhamdia quelen*)
Strain/clone:	Not reported
Age at study initiation:	juveniles
Weight/length at study initiation:	12.84±0.34 g and 10.5±0.22 cm
Source:	fish farm in Cruz Alta (Southern Brazil)
Feeding during test:	No
Acclimation:	1 week in continuously aerated 250 L tanks at a temperature 19.76± 0.11°C, pH 7.15±0.12, dissolved oxygen 6.86±0.33 mg/L, and total ammonia nitrogen 0.12±0.02 mg N/L). Animals were fed to satiation with commercial feed (42% crude protein, Supra-Alisul Alimentos®, Brazil) once per day, and fasted for 24 hours before the experiments.

Test conditions

Hardness:	Not reported
Test temperature:	19.76± 0.11°C
pH:	7.15±0.12
Dissolved oxygen:	6.86±0.33 mg/L
Conductivity:	Not reported
Photoperiod:	Not reported
Light intensity:	Not reported

Test system

Study type:	Acute toxicity test with maximum 30 minute exposure
Duration of study:	Exposure period for a maximum of 30 min, 48 hours mortality assessment
Treatments:	25, 50, 75, and 100 mg thymol/L (nominal)
Analytical determination of test concentrations: No	
Negative control included:	No
Positive control included:	No
Parameters measured:	Mortality
Validity criteria:	Not reported

Silver catfish juveniles (n=6 for each concentration) were individually exposed to concentrations of thymol in an aquarium filled with 1 L of water. Evaluation was carried out until the animals reached the anesthesia stage, or after a maximum period of 30 min. Survival of the animals was assessed 48 hours after the experiment.

Results

No mortality has been seen following the use of thymol as an anaesthetic up to the highest concentration of 100 mg thymol/L.

Assessment and conclusion

Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable since the analytical verification of the test concentrations was not reported and the exposure is very short compared to current guidelines for acute toxicity test

Assessment and conclusion by applicant:

The study is not acceptable (not reliable).

Silver catfish (*Rhamdia quelen*) following a maximum exposure period of 30 minutes at 100 mg thymol/L (nominal), there was no mortality observed.

Data point:	CA 9.6.3.4/23 [CA 8.2.6]
Report author	de Saravia, S.G.G., Rastelli, S.E., Blustein, G., Viera, M.R.
Report year	2018
Report title	Natural compounds as potential algaecides for waterborne paints
Report No	-
Document No	Internal reference: Study 248 J. Coat. Technol. Res., Volume 15, Pages 1191–1200 (2018)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Not reliable

Abstract (copied from original literature)
To eliminate and prevent the formation of phototrophic biofilms on painted walls, chemicals are added to paints. These chemicals, in general, also have toxic effects on the environment. Plants synthesize secondary metabolites which have antimicrobial properties and that may be a sustainable alternative to traditional biocides. In this work, algal species isolated from biofilms formed on deteriorated painted surfaces were taxonomically identified. Observations with the optical microscope revealed the presence of mixed biofilms composed of algae belonging to the Cyanophyta, Chlorophyta, and Streptophyta divisions. The algaecide effect of natural compounds of vegetal origin such as anisole, eugenol, guaiacol, and thymol was evaluated by the microatmosphere technique. Growth inhibition of phototrophic organisms was obtained with thymol in all the concentrations used. Eugenol and guaiacol showed a total growth inhibition at the highest concentration assayed (300 µmol/cm^2) and a decrease in growth at 40 µmol/cm^2, while anisole did not exert any inhibitory action at the assayed concentrations. Due to these results, eugenol, thymol, and guaiacol were incorporated in an exterior waterborne paint at a concentration of 2%. Samples painted with these formulations were not colonized by algae, indicating that these compounds could be used as biocides in paint formulations.

Materials and methods

Test material 1

Name:	Eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma-Aldrich, USA
Active substance content:	Not reported
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test material 2

Name:	Thymol
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma-Aldrich, USA
Active substance content:	Not reported
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species:	algae belonging to the Cyanophyta, Chlorophyta, and Streptophyta divisions
Strain/clone:	Not reported

Age at study initiation: 7-day old algae culture
Weight/length/height at study initiation: Not relevant
Source: flaking paint from two surfaces showing colonization and biodeterioration signs
Acclimation: The scrapped material was transferred to BG11 broth medium for maintenance of the algae communities. To isolate single microalgal species from the samples, standard plating methods were used: 200 µL of the cultures was seeded in BG11 agar plates, incubated under controlled photoperiod conditions (16-/8-h light/darkness) and 25ºC. Different algal cells were picked from the plates and seeded in new plates for isolation.

Test conditions

Test temperature: 25ºC
Photoperiod: 16-/8-h light/darkness
Light intensity: Not reported
Test system
Study type: Chronic toxicity test
Duration of study: 28 days
Treatments: 0, 20, 40, 300 µmol/cm^2 for eugenol (nominal)
5 to 300 µmol/cm^2 for thymol (nominal)
Analytical determination of test concentrations: No
Negative control included: Yes (control + solvent control)
Positive control included: No
Parameters measured: growth
Validity criteria: Not reported
Considering that the compounds of interest are volatile substances, the microatmosphere test was used to evaluate their algaecide activity. For this, 200 µL of a 7-day-old algae culture containing 1.2 x 10^5 algae/mL was inoculated uniformly in a Petri dish with BG11 agar. The number of cells was determined using a Neubauer haemocytometer. A sterile disk of filter paper (13 mm) was placed on the center of the inverted Petri dish lid and loaded with 50 µL of the pure tested compound. The Petri dish was then hermitically sealed and incubated for 28 days. The total growth inhibition has been reported. All the tests were performed in duplicate.

Results
Thymol was observed to be the most effective algaecide, exerting a total inhibition of algae growth in all the concentrations assessed from 5 to 300 µmol thymol/cm^2.
Eugenol showed a total growth inhibition at the highest concentration assayed (300 µmol eugenol/cm^2), while lower concentrations (20 and 40 µmol eugenol/cm^2) allowed a low algal growth.
Assessment and conclusion
Reliability assessment
For full details and justification, please refer to Document KCA 9.4.2/02.
Proposed category: 3 not reliable.
There is no analytical verification of the concentrations tested and test item purity not reported.

Assessment and conclusion by applicant:

The study is not acceptable (not reliable).

Thymol was observed to be the most effective algaecide, exerting a total inhibition of algae growth in all the nominal concentrations assessed from 5 to 300 µmol thymol/cm^2.
Eugenol showed a total growth inhibition at the highest nominal concentration assayed (300 µmol eugenol/cm^2), while lower concentrations (20 and 40 µmol eugenol/cm^2) allowed a low algal growth.

Data point:	CA 9.6.3.4/24 [CA 8.6]
Report author **Report year**	Nasrollahi, P., Razavi, S.M., Ghasemian, A,, Zahri, S. 2017
Report title	Physiological and Biochemical Responses of Lettuce to Thymol, as Allelochemical
Report No	-
Document No	Internal reference: Study 265 Russian Journal of Plant Physiology, Volume 65, Pages 598–603 (2017)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)
Nowadays, allelopathic investigations have focused to identify action mechanisms of this compound on target organisms in natural habitats. It may tend to introduce new natural herbicides and pesticides for replacing the synthetic ones. In the present study, the allelopathic effects of thymol on lettuce (*Lactuca sativa* L.) as a model plant were investigated in physiological and biochemical aspects. At the first stage, the effect of different concentrations of thymol on some growth parameters, including seed germination, radicle and plumule growth were evaluated to determine the optimum concentration for the continuation of our experiment. Then, the lettuce seedlings were cultured in pots containing peat and were irrigated with Hoagland nutrient solution supplemented with thymol at the concentration of 50 and 100 µg/mL. The effect of these treatments on physiological, biochemical aspects of the plant were studied following the plant growth. The results showed whereas, the shoot fresh and dry weights and photosynthetic rate in the treated group were decreased significantly compared to the control group at $P \leq 0.05$, photochemical efficiency of photosystem II, total protein concentration, proline content and the activity of some antioxidant enzymes such as polyphenol oxidase, ascorbate peroxidase and catalase were increased in treated plants than control ones. However, there was no significantly change in chlorophyll content in treated group than control. It can be concluded that the thymol as an allelochemical caused some physiological and biochemical responses in the lettuce which are much similar to induced responses under abiotic stress. Indeed, it induces a kind of stress named as allelochemical stress.

Materials and methods

Test material

Name: Thymol
Formulation type: Not relevant
Source and lot/batch no.: Not reported
Active substance content: Not reported
Expiry date of lot/batch: Not reported
Storage conditions: Not reported

Test organism

Species: Lettuce, *Lactuca sativa* L.
Strain/clone: Not reported
Age at study initiation: seeds and seedlings
Weight/length/height at study initiation: Not reported
Source: Not reported
Feeding during test: Not reported
Acclimation: All of seeds were firstly disinfected with sodium hypochlorite 1% and then washed with distilled water and placed in Petri dishes that previously autoclaved for sterilizing at 121°C for 20 min.

Test conditions

Test temperature:	25ºC
Photoperiod:	daylight
Light intensity:	6000 lux
Test system	
Study type:	Seed germination and plant cultivation
Duration of study:	7 days for seed germination test and 37 days for plant cultivation
Treatments:	1, 10, 50, 100 and 1000 µg thymol/mL (nominal) for seed germination test 50 and 100 µg thymol/mL (nominal) for plant cultivation
Analytical determination of test concentrations: No	
Negative control included:	Yes (distilled water for seed germination test, Hoagland solution for plant cultivation)
Positive control included:	No
Parameters measured:	Growth (germination percent, radicle and plumule lengths for seed germination test; shoot fresh weight and fresh and dry weight of roots for plant cultivation)
Validity criteria:	Not reported

The effect of different concentrations of thymol on germination of lettuce was evaluated in 4 replicates. Within each Petri dish, 25 seeds were placed on Whatman paper and 3 mL of thymol solution were added with certain concentrations. Petri dishes were incubated at 25°C and the daily counting of germinated seeds was done for seven days. At the end of this period, the germination percent was calculated and the radicle and plumule length of the seedlings in each Petri dish was measured using a millimeter ruler.
Seedlings were cultivated in pots containing peat and irrigated with Hoagland solution supplemented with 50 and 100 µg thymol/mL. The pots were transferred to a germinator condition for 37 days under daylight of 6000 lux and the relative humidity of 80%. Control group of plants were irrigated daily with Hoagland solution. Plants were grown reach up to 7-leave stage and then were harvested. Following the harvest, shoot fresh weight was determined as well as the fresh and dry weight of roots.
Experiment was fulfilled in a randomized block design with four replications. All statistical analysis was done using SPSS 14 software. The comparison of means was evaluated by Duncan test at $P \leq 0.05$

Results
The results of growth parameters evaluation indicated that thymol entirely inhibited lettuce seed germination in the concentration of 1000 µg thymol/mL. However, radicle and plumule growth of lettuce seedlings were reduced in concentrations higher than 1 and 10 µg/mL, respectively. In this way, concentrations of 50 and 100 µg/mL (interstitial concentrations) were selected as the optimum concentration for continuing the test. Results showed that in treated groups at 50 and 100 µg thymol/mL, thymol induced a significant reduction in fresh and dry weight of shoots at $P \leq 0.05$. Dry weights of shoots decreased 20 and 80% than control in plant treated with 50 and 100 µg thymol/mL, respectively.
Assessment and conclusion
Reliability assessment
For full details and justification, please refer to Document KCA 9.4.2/02.
Proposed category: 2 reliable with restrictions
Source and purity of test item not reported, and no analytical verification of test concentrations.

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).

Lettuce, *Lactuca sativa* L.: Results showed that in treated groups at nominally 50 and 100 µg thymol/mL, thymol induced a significant reduction in fresh and dry weight of shoots at $P \leq 0.05$. Dry weights of shoots decreased 20 and 80% than control in plant treated with 50 and 100 µg thymol/mL, respectively.

Data point:	CA 9.6.3.4/25 [CA 8.6]
Report author	Pinheiro, P.F., Costa, A.V., Alves, T.A., Galter, I.N., Pinheiro, C.A., Pereira, A.F., Oliveira, C.M.R., Fontes, M.M.P.
Report year	2015
Report title	Phytotoxicity and Cytotoxicity of Essential Oil from Leaves of *Plectranthus amboinicus*, Carvacrol, and Thymol in Plant Bioassays
Report No	-
Document No	Internal reference: Study 269 Journal Agricultural and Food Chemistry, Volume 63, Pages 8981–8990 (2015)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)
The essential oil of *Plectranthus amboinicus* and its chemotypes, carvacrol and thymol, were evaluated on the germination and root and aerial growth of *Lactuca sativa* and *Sorghum bicolor* and in acting on the cell cycle of meristematic root cells of *L. sativa*. The main component found in the oil by analysis in gas chromatography–mass spectrometry and gas chromatography flame ionization detection was carvacrol (88.61% in area). At a concentration of 0.120% (w/v), the oil and its chemotypes retarded or inhibited the germination and decreased root and aerial growth in monocot and dicot species used in the bioassays. In addition, all substances caused changes in the cell cycle of the meristematic cells of *L. sativa*, with chromosomal alterations occurring from the 0.015% (w/v) concentration. The essential oil of *P. amboinicus*, carvacrol, and thymol have potential for use as bioherbicides.

Materials and methods

Test material 1

Name: Thymol
Formulation type: Not relevant
Source and lot/batch no.: Sigma Aldrich
Active substance content: Not reported
Expiry date of lot/batch: Not reported
Storage conditions: Not reported

Test organism

Species: Lettuce, *Lactuca sativa* and sorghum, *Sorghum bicolor*
Strain/clone: Not reported
Age at study initiation: seeds
Weight/length/height at study initiation: not reported
Source: *Lactuca sativa* seeds purchased at Feltrin sementes (Farroupilhas Brazil), and *Sorghum bicolor* seeds purchased from LG Sementes (Goianesia, Brazil)
Feeding during test: Not relevant
Acclimation: Not reported

Test conditions

Test temperature: 24 ± 2ºC
Photoperiod: Not reported
Light intensity: Not reported
Test system
Study type: Phytotoxicity test (germination test)
Duration of study: 48 hours
Treatments: 0.015, 0.030, 0.060, 0.120 % w thymol/v (nominal)
Analytical determination of test concentrations: No
Negative control included: Yes (water and dichloromethane (used as solvent))
Positive control included: Yes (glyphosate and boral)
Parameters measured: Growth (germination of seeds, root length and aerial growth)
Validity criteria: Not reported
This study aimed to investigate the effects of thymol on the germination and root growth of *L. sativa* (dicotyledons) and *S. bicolor* (monocotyledons). For the germination tests, 2 mL of each solution were added to Petri dishes wit 9 cm diameter containing filter paper. Twenty-five seeds were used for each treatment, with 5 replicates per treatment. The number of germinated seeds was evaluated from 8 to 48 hours, at 8 hours intervals. The percentage of germinated seeds and the root length were obtained after 48 hours. The aerial parts of the plantlets were measured after 120 hours to determine the aerial growth.
For statistical analysis of qualitative factors (Dunnett, 5% significance) used the Genes program, and the quantitative factors (regression) used the Sisvar program.
Results
In all treatments, the solutions with higher concentrations had the most significant effects in the referenced tests. The percentage of germinated seeds for *S. biocolor* in all treatments at the concentrations of 0.060% and 0.120% were lower than the negative control, although germination occurred in all tests. For *L. sativa*, only the concentrations at 0.120% thymol significantly differ from the positive control treatment.

Results are presented in the table below.

Concentration thymol (% m/v)	*L. sativa*			*S. bicolor*		
	Germinated seeds (%)	**Root length (cm)**	**Aerial growth (cm)**	**Germinated seeds (%)**	**Root length (cm)**	**Aerial growth (cm)**
Water	91.2	91.2	7.87	96.8	7.38	4.91
Dichloromethane (solvent)	89.6	89.6	2.15	84.0	7.26	4.97
0.015	89.6	3.78	0.34	89.6	3.01*	2.64
0.030	82.4	2.68	0.18	90.4	2.57	2.94
0.060	70.4	0*	0	86.4	2.05	2.87
0.120	0*	7.7*	2.69	54.4	1.49	1.06*

* significantly different from control ($p<0.05$)
Assessment and conclusion
Reliability assessment
For full details and justification, please refer to Document KCA 9.4.2/02.
Proposed category: 2 reliable with restrictions
Test item purity not reported and no analytical verification.

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).

Lettuce, *Lactuca sativa* and sorghum, *Sorghum bicolor*
The percentage of germinated seeds for sorghum, *Sorghum bicolor*, in all treatments at the nominal concentrations of 0.060% and 0.120% w thymol/v were lower than the negative control, although germination occurred in all tests. For lettuce, *Lactuca sativa*, only the concentrations at 0.120% w thymol/v were significantly different from the control treatment.

Data point:	CA 9.6.3.4/26 [CA 8.3.1]
Report author	Abd-El-Megeed, S.M.
Report year	2013
Report title	Queen honey bee characteristics and their relation to colony performance
Report No	-
Document No	Internal reference: Study 277 Ain-Shams Univ., Faculty of Agriculture Egypt (2013)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Cannot be assessed as full text not available

Abstract (copied from original literature; Full text was not available)
The bee colonies used in the present work were headed with open mated local Carnica queen *Apis mellifera carnica*. Percentage queen success and weight of newly emerged queen showed that queens reared in the months of spring was significantly higher to that in autumn. Also queen honeybees weighed more in spring than those reared in autumn i.e. 181 and 168 mg respectively. Grafting with 12 or 24 hour old larvae increased the percentage of queen acceptance than when grafting was conducted with 36 or 48 hour old larvae. Honeybee colonies supplemented with a diet made of pollen and sugar at a ratio of 1: 1 plus 5 % thymol increased queen success in the spring and autumn 90.4% and 77.4 % respectively. This diet also resulted in a highest mean weight of newly emerged virgin queens. Also the use of new wax was superior to the use of previously utilized wax in improving the specification of queen honeybee. A colony comprising 9 combs was superior than colonies comprising 6 or 3 combs / colony in increasing queen success (89.6 %) as well as higher weight in produced queens (184 mg). Percentage of queen honeybees reared by young nurse honeybee workers was 81.0% and their weight 173 mg which was significantly higher than those reared by older field honeybee workers. Under conditions of the present work the Italian honeybee race was found to be superior to the Carniolan race in producing higher specification of honeybee queens. The number of ovarioles volume of spermatheca was determined; these two factors were reflected in the number of brood cells produced by the queen honeybee. Highest number of brood cells was recorded in the spring season in the 4 h brood cycle in a colony supplement by a diet made with pollen sugar cake plus thymol and when queen grafting was carried out by 12 or 24 hour old larvae. Furthermore strong colonies comprising 9 combs / colony showed high number of brood cells to that in moderate or weak colonies. The biometrics of somebody appendages was determined (e.g. antennal flagellum length and width of fore and hind wings number of hooks on the costa of the hind wing length of the femur tibia and tarsus of the hind leg length of the 3. d 4. h and 7. h abdominal segment as well as sting). Results showed that their measurement or dimensions was always higher in honeybee queens emerging in spring and from the grafting of 12 hr old larvae. SDS-P AGE electrophoresis was conducted on 4. h instar worker larvae and queen larvae grafted from 12 and 48 hour old larvae. Four monomorphic protein bands were evident their molecular weights were 66.778 46.630 39.938 and 33.374 KDa. In the 4. h instar worker larva a protein band in a molecular weight of 55.02 KDa was absent but was exhibited in queen larvae. Queen larvae grafted by 12 hour old larva exhibited a unique protein band in a molecular weight of 91.353 KDa. However these queen larvae lacked a protein in the lowest molecular weight 16.216 KDa. This protein was obvious in larvae of worker and also in queen larvae grafted by younger 48 hour old 1arva. Electrophoretic banding patterns of total protein for adult honeybee worker and queen emerging from grafting of either 12 or 48 hour old larvae showed six protein bands ranging.in the molecular weights 128.03 to 29.429 KDa. Three monomorphic bands were exhibited their molecular weights were 89.89 37.43 and 29.43 KDa. These mentioned three proteins were the only ones observed in worker honeybee. A queen honeybee emerging from grafting of 12 hour old larva displayed all six protein bands in the molecular weights of 128.033 89.89 58.0 47.45 37.43 and 29.43 KDa. The first band was unique in this queen as it was not detected in a queen emerging from grafting of 48 hour old larva and also in adult honeybee worker.

Assessment and conclusion by applicant:
The study cannot be fully assessed as full text not available.

Data point:	CA 9.6.3.4/27 [CA 8.6]
Report author	Dufau Mattos, B., da Silva, L.R., Rodrigues de Souza, I., Esteves Magalhaes, W. L., Morais Leme, D.
Report year	2019
Report title	Slow delivery of biocide from nanostructured, microscaled, particles reduces its phytoxicity: A model investigation
Report No	-
Document No	Internal reference: Study 300 Journal of Hazardous Materials Volume 367, Pages 513-519 (2019)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)
Nano-engineered delivery systems have emerged as possible solutions for more efficient pest management in agriculture. Likewise for nanostructured drug delivery systems (DDS) in medicine, the use of biocide delivery systems (BDS) brought concerns on their toxicology on non-targeted organisms. Plants, for instance, are the foundation of the ecosystem, acting as primary actor in the food chain and is associated with the whole biodiversity, being strictly related to human health. This is a very important consideration to fully understand the benefits of using delivery systems for crop protection and production. Herein, a biocide delivery system was prepared by loading nanostructured, microscaled, biogenic silica particles with thymol, a known phytotoxicant. The resulting system contains 120 mg of thymol per gram of silica and displays slow release features. The *Allium cepa* bioassay was chosen to demonstrate how the toxicity and cellular damages induced by thymol can be significantly reduced through a slow, controlled, release strategy. The lower mobility of the reference particles associated with slow-delivery features reduced the toxicity and cellular damages caused by thymol in the plant genetic model.

Materials and methods

Test material

Name: thymol
Formulation type: Not relevant
Source and lot/batch no.: Not reported
Active substance content: Not reported
Expiry date of lot/batch: Not reported
Storage conditions: Not reported

Test organism

Species: *Allium cepa*
Strain/clone: “Baia Periform” onion
Age at study initiation: Not relevant
Weight/length/height at study initiation: seeds

Source: Not relevant
Feeding during test: Not relevant
Acclimation: Not relevant

Test conditions

Photoperiod: Not reported
Light intensity: Not reported
Test system
Study type: Seed germination and root elongation toxicity tests
Duration of study: 4 days
Treatments: control 1, control 2, 7, 14, 28, 56 and 112 mg thymol/L (nominal)
Analytical determination of test concentrations: No
Negative control included: Yes (ultrapure water and DMSO)
Positive control included: Yes (Zinc sulfate heptahydrate)
Parameters measured: index of germination (%) and the average root length (cm)
Validity criteria: Not reported
The target systems at given concentrations were prepared in polystyrene Petri dishes covered with a nylon net where the seeds were placed on (100 seeds/plate). Each test solution was replaced by fresh ones every 24 hours to ensure good dispersion, and bioavailability.
A. cepa seeds were exposed to five concentrations of the target nanoparticles, biocides and controls during four days. The index of germination (%) and the average root length (cm) were acquired from 100 seeds of each plate. Three plates were used in each essay, resulting in 300 samples. Toxicity was expressed as half-maximal effective concentration (EC_{50}) in seed germination or root growth inhibition.
Results
Pure thymol acted as a phytotoxicant, inhibiting both seed germination (EC_{50} of 57.14 mg/L) and root growth (EC_{50} of 14.72 mg/L). Thymol, on the other hand, had a strong allelopathic effect on the *A. cepa*, decreasing seed germination rates and affecting normal root development.
Assessment and conclusion
Reliability assessment
For full details and justification, please refer to Document KCA 9.4.2/02.
Proposed category: 2 reliable with restrictions
Test item purity not reported and no analytical verification.

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).
Allium cepa: EC_{50} (seedling germination) = 57.14 mg thymol/L and EC_{50} (root growth) = 14.72 mg thymol/L based on nominal concentrations

Data point:	CA 9.6.3.4/28 [CA 8.2.2]
Report author	Aanyu, M., Betancor, M. B., Monroig, O.
Report year	2020
Report title	The effects of combined phytogenics on growth and nutritional physiology of Nile tilapia *Oreochromis niloticus*
Report No	-
Document No	Internal reference: Study 315 Aquaculture Volume 519, 734867 (2020)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	No

Abstract (copied from original literature)
This study investigated whether dietary supplementation of phytogenic compounds limonene and thymol had synergistic or additive effects on growth and selected nutritional physiology pathways in Nile tilapia. A 63-day feeding experiment was conducted using fish of 1.5 ± 0.0 g (± standard error) fed on a commercial diet coated with either 0 ppm limonene and thymol (control), 400 ppm limonene (L), 500 ppm thymol (T), or a combination of 400 ppm limonene and 500 ppm thymol (LT). Final fish weight (FW) was significantly improved to similar extents by diet LT (16.7 ± 0.3 g) and L (16.6 ± 0.4 g). Dietary thymol alone and the control did not enhance FW (15.0 ± 0.4 g and 13.7 ± 0.4 g respectively). Dietary thymol had shown a strong tendency to improve somatic growth (P = 0.052). The analysed candidate genes involved in the pathways of nutrient digestion, absorption and transport (*muc*), lipid metabolism (*lpl*), antioxidant enzymes (*cat*) and somatotropic axis growth (*igf-I*) were also up-regulated to similar extents in Nile tilapia by diet L and LT (P < 0.05), above the regulation observed with the diet supplemented exclusively with thymol. This suggests lack of synergistic or additive effects on growth and nutritional physiology pathways when limonene and thymol are supplied in the diet.

Materials and methods

Test material

Name:	thymol
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma Aldrich
Active substance content:	95 %
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species:	Nile tilapia, *Oreochromis niloticus*
Strain/clone:	Not reported
Age at study initiation:	Juvenile
Weight/length/height at study initiation:	1.54 g / 0.1 cm
Source:	ARDC fish farm.
Feeding during test:	Yes, the fish were fed the experimental diets twice a day to apparent satiation.
Acclimation:	Each tank had a water holding capacity of 60 L, in a flow through system with a flow rate of –2 L/min.

Test conditions

Hardness:	Not reported
Test temperature:	23.3 - 24.3°C (rearing tanks information)
pH:	6.8 ± 0.3 (rearing tanks information)
Dissolved oxygen:	6.6 ± 0.6 mg O_2/L (rearing tanks information)
Conductivity:	Not reported
Photoperiod:	12 hours light/12 hours' dark
Light intensity:	Not reported

Test system

Study type:	Chronic toxicity test
Duration of study:	63 days
Treatments:	500 ppm thymol (nominal)
Analytical determination of test concentrations:	No
Negative control included:	Yes
Positive control included:	No
Parameters measured:	Mortality/growth
Validity criteria:	Not reported

A standard commercial feed for juvenile Nile tilapia produced at the ARDC was supplemented with thymol (95% purity) using concentrations found to have growth-promoting potential in Nile tilapia. The diet included: 0

ppm thymol (Control) and 500 ppm thymol (T). Thirty eight (38) fish were stocked in each of the 16 experimental tanks. Each experimental diet was tested in quadruplicate tanks and the treatments were distributed randomly. The feed intake was recorded.
The data were analysed using the Statistical Package for the Social Sciences (SPSS) version 19 (Chicago, USA).
Results
The diet supplemented exclusively with thymol (T) did not significantly improve the final weight of the fish. There were no significant differences in the final survival of the fish among treatments.
Assessment and conclusion
Reliability assessment
For full details and justification, please refer to Document KCA 9.4.2/02.
Proposed category: 3 not reliable
No analytical verification of thymol in fish diet

Assessment and conclusion by applicant:

The study is considered as not reliable

Oreochromis niloticus: the fish diet supplemented with nominally 500 ppm thymol did not affect the survival or growth of fish after 63 days.

Data point:	CA 9.6.3.4/29 [CA 8.2.2]
Report author	Amer, S.A., Metwally, A.E., Ahmed, S.A.A.
Report year	2018
Report title	The influence of dietary supplementation of cinnamaldehyde and thymol on the growth performance, immunity and antioxidant status of monosex Nile tilapia fingerlings (*Oreochromis niloticus)*
Report No	-
Document No	Internal reference: Study 327 Egyptian Journal of Aquatic Research Volume 44, Pages 251-256 (2018)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	No

Abstract (copied from original literature)
This study investigated the effects of two essential oils (Eos), cinnamaldehyde and thymol on the growth performance, antioxidant status of the meat and the immunity of Nile tilapia fingerlings, *Oreochromis niloticus*. A total of 375 *O. niloticus* (10.2 ± 0.06 g) were randomly assigned to 5 dietary treatments (3 replicates for each). Diet 1 was the control with no additives. Diets 2 and 3 were supplemented with 1 and 2 ml cinnamaldehyde /kg diet (CINN1 or CINN2), respectively. While diets 4 and 5 were supplemented with 1 and 2 ml thymol /kg diet (THYM1 or THYM2), respectively. Fish feeding was done by hand until satiation 3 times daily for 75 days. The results showed that dietary supplementation with 1 ml thymol/ kg diet increased the growth performance significantly than other groups. Dietary supplementation with cinnamaldehyde or thymol significantly reduced ($P \leq 0.05$) the malondialdehyde (MDA) formation and increased glutathione reductase (GR) in the muscle, and increased lysozyme activity, IgM, IgG levels and catalase activity in the serum thus improved the antioxidant protective capacities and the immune status of the fish. Furthermore, thymol supplementation can stimulate more growth and immune responses than cinnamaldehyde as essential oils in Nile tilapia nutrition.
Materials and methods

Test material 1

Name: thymol
Formulation type: Not relevant

Source and lot/batch no.: Oxford Laboratory Company, Mumbai India
Active substance content: 99%
Expiry date of lot/batch: Not reported
Storage conditions: Not reported

Test organism

Species: Nile tilapia, *Oreochromis niloticus*
Strain/clone: Not reported
Age at study initiation: fingerling fish
Weight/length/height at study initiation: 10.2 ± 0.06 g
Source: fish hatchery
Feeding during test: Yes
Acclimation: Fish were submitted to 14 days adaptation period before the beginning of the experiment. Fish feeding was done by hand until satiation 3 times daily for 75 days. Fish were individually weighed every 2 weeks following a 14-h starvation period.

Test conditions

Hardness: Not reported
Test temperature: 24 ± 2 ºC
pH: 8 ± 1
Dissolved oxygen: 6 ± 1 mg O_2/L
Conductivity: Not reported
Photoperiod: 12 hours: 12 hours, light / dark
Light intensity: Not reported
Test system
Study type: Chronic dietary toxicity test
Duration of study: 75 days.
Treatments: 1 and 2 mL thymol/kg diet (nominal)
Analytical determination of test concentrations: No
Negative control included: Yes
Positive control included: No
Parameters measured: Mortality/growth
Validity criteria: Not reported

A total of 375 *O. niloticus* were randomly assigned to 5 dietary treatments in 3 replicates. Diet 1 was the control diet with no additives. Diets 2 and 3 were supplemented with 1 and 2 mL cinnamaldehyde/kg diet (CINN1 or CINN2), respectively. While diets 4 and 5 were supplemented with 1 and 2 mL thymol/kg diet (THYM1 or THYM2), respectively. Thymol and Cinnamaldehyde were mixed with fish oil then mixed with other feed ingredients. Fish were randomly assigned to the 5 groups at 25 fingerlings per group and in triplicates. The body weight (BW), body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) were recorded every 2 weeks and at the end ofthe experimental period. One way ANOVA was used to determine the effects of supplementing the diets of Nile tilapia fingerlings.

Results

In the period from 0 to 15 day, there were significant increases ($P \leq 0.05$) in body weight, body weight gain and significant decreases ($P \leq 0.05$) in feed conversion ratio in fish fed 1 and 2 mL thymol/kg diet. From 16 to 30 day, there was a significant increase ($P \leq 0.05$) in body weight in thymol supplemented diets. The period from 31 to 45 day there was a significant increase ($P \leq 0.05$) in body weight only in fish fed 1 mL thymol/kg diet. From 46 to 60 day, there was a significant increase ($P \leq 0.05$) in body weight, body weight gain in 1 mL thymol/kg diet while groups of fish fed 2 mL thymol/kg diet increased in body weight and body weight gain numerically. The food conversion ratio was decreased significantly ($P \leq 0.05$) in 1 and 2 mL thymol/kg diet. In the period from 61 to 75 day, the body weight was increased significantly ($P \leq 0.05$) in the group fed 1 mL thymol/kg diet while it was increased numerically in the group fed 2 mL thymol/kg diet. The food conversion ratio was not affected in all groups. The results of the overall performance revealed significant increase ($P \leq 0.05$) in the final body weight (BW), total body weight gain (TBWG), daily body weight gain (DBWG), protein efficiency ratio

(PER) and specific growth rate (SGR%) in the group of fish fed 1 mL thymol/kg diet than other groups. While, the groups fed 1 mL thymol/kg diet showed numerical increase in body weight, total weight gain and daily weight gain over the control group. There were no significant differences (P > 0.05) in feed intake by fish in the control group and in other groups. However, the group of fish fed 1 mL thymol/kg diet converted their feed so well that the feed conversion ratio was significantly better than in the control group. The results of the present study have established that dietary supplementation of thymol improved the growth performance of the Nile tilapia, and also it can improve the antioxidant status and the immunity of Nile tilapia. No mortality was recorded during the experimental period.

Assessment and conclusion

Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

No analytical verification of thymol in fish diet.

Assessment and conclusion by applicant:

The study is considered as not reliable.

Oreochromis niloticus (Nile tilapia fish) the results of the present study showed that dietary supplementation of thymol at 1 and 2 mL thymol/kg diet (nominal) for 75 days improved the growth performance, the antioxidant status and the immunity of the Nile tilapia fingerlings.

No mortality was recorded during the experimental period.

Data point:	CA 9.6.3.4/30 [CA 8.3.1]
Report author	Colin, T., Plath, J.A., Klein, S., Vine, P., Devaud, J-M., Lihoreau, M., Meikle, W.G., Barron, A.B.
Report year	2020
Report title	The miticide thymol in combination with trace levels of the neonicotinoid imidacloprid reduces visual learning performance in honey bees (*Apis mellifera*)
Report No	-
Document No	Internal reference: Study 329 Apidologie volume 51, Pages 499–509 (2020)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)

Despite growing concerns over the impacts of agricultural pesticides on honey bee health, miticides (a group of pesticides used within hives to kill bee parasites) have received little attention. We know very little about how miticides might affect bee cognition, particularly in interaction with other known stressors, such as crop insecticides. Visual learning is essential for foraging bees to find their way to flowers, recognize them, and fly back to the nest. Using a standardized aversive visual conditioning assay, we tested how field exposure to three pesticides affects visual learning in European honey bees (*Apis mellifera*). Our pesticides were two commonmiticides, thymol in the commercial formulation Apiguard® and tau-fluvalinate in the formulation Apistan® and one neonicotinoid, imidacloprid. We found no effect of miticides alone, nor of field-relevant doses of imidacloprid alone, but bees exposed to both thymol and imidacloprid showed reduced performance in the visual learning assay.

Materials and methods

Test material

Name:	Apiguard®
Formulation type:	Slow-release gel
Source and lot/batch no.:	Vita Europe Ltd
Active substance content:	25% thymol (12.5 g thymol in each Apiguard® aluminium tray)
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species:	*Apis mellifera*, honey bees.
Strain/clone:	Golden Italian strain.
Source:	Sourced from the Macquarie University Research Apiary, Australia. Forager bees were collected when leaving the hive, using a hand-held bee vacuum device.
Feeding during test:	Not relevant
Acclimation:	Not relevant

Test system

Study type:	Visual learning assay in the laboratory following field exposure in which honey bee colonies were treated with the *Varroa*-treatment formulation "Apiguard®"
Duration of study:	Exposure of 4 weeks (see below)
Treatments:	One Apiguard® gel container was placed on top of the top-box frames of each of the treated colonies for 2 weeks and renewed after 2 weeks for another 2 weeks. Apiguard containers were thus present in the hive for a total duration of 4 weeks

Analytical determination of test concentrations: No

Negative control included:	Yes
Positive control included:	No
Parameters measured:	Behaviour (visual learning)
Validity criteria:	Not reported

Results

During the test, there was no significant difference in the visual learning performances or speed of movement between the control group and the Apiguard-treated group.

Assessment and conclusion

Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 2 reliable with restrictions

Study tested a slow-release gel formulation containing thymol, but exact exposure of thymol to bees is unknown (and no analytical verification included).

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).

In a field trial, honey bee colonies in Australia were treated with the anti-*Varroa* treatment "Apiguard®". Apiguard is a slow-release gel, added in aluminium trays to the honey bee hive; each tray contains 12.5 g of thymol. In this study, Apiguard trays (containing 12.5 g thymol each) were administered by introducing one tray on top of the brood frames for 2 weeks, following by a replacement tray for a further 2 weeks. Following this treatment, foragers were collected from the hive entrance and in a laboratory assay there was no significant difference in the visual learning performances or speed of movement between the control group and the Apiguard-treated group.

Data point:	CA 9.6.3.4/31 [CA 8.2.1]
Report author	Yousefi, M., Hoseini, S. M., Vatnikov, Y. A., Nikishov, A. A., Kulikov, E. V.
Report year	2018
Report title	Thymol as a new anesthetic in common carp (*Cyprinus carpio*): Efficacy and physiological effects in comparison with eugenol
Report No	-
Document No	Internal reference: Study 335 Aquaculture Volume 495, Pages 376-383 (2018)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	No

Abstract (copied from original literature)
The aim of the present study was to investigate thymol anesthetic efficiency and biochemical effects in common carp in comparison to eugenol. In the first experiment, time of induction of and recovery from anesthesia were recorded in the fish anesthetized with either eugenol or thymol at the concentrations of 6.25, 12.5, 25, 50, 75, 100, 125, 150 and 200 mg / L (ten fish per concentration). In the second experiment, stress responses, oxidative stress and biochemical effects of eugenol and thymol were investigated in the fish after short-term (5 min; 43 mg / L eugenol or 52 mg / L thymol) anesthesia and long-term (3 h; 10 or 20 mg / L of either of the anesthetics) anesthetic exposure. Eugenol anesthetized the fish within 600–90 s (with recovery time of 190–380 s) at the concentrations of 25–150 mg / L. Thymol induced anesthesia within 850–60 s (with recovery time of 210–1200 s) at the concentrations of 25–200 mg / L. Eugenol resulted in a significant increase in plasma aspartate transaminase (AST), lactate dehydrogenase (LDH), malondialdehyde (MDA) and phosphate levels compared to thymol after the short-term anesthesia. After the long-term exposure, eugenol led to significant elevations in cortisol, glucose, lactate, AST, LDH, total antioxidant capacity (TAC), MDA, superoxide dismutase (SOD) and phosphate and decrease in catalase (CAT) compared to thymol. In conclusion, thymol is an efficient anesthetic in carp allowing for rapid sampling as well as surgery. In addition, thymol has fewer side effects compared to eugenol in the case of induction of stress, oxidative stress and tissue damage, thus is recommended for carp anesthesia.

Materials and methods

Test material 1

Name: eugenol
Formulation type: Not relevant
Source and lot/batch no.: Aldrich, Milwaukee, USA
Active substance content: 99%
Expiry date of lot/batch: Not reported
Storage conditions: Not reported

Test material 2

Name: thymol
Formulation type: Not relevant
Source and lot/batch no.: Sigma, St. Louis, USA
Active substance content: 98.5%
Expiry date of lot/batch: Not reported
Storage conditions: Not reported

Test organism

Species: common carp, *Cyprinus carpio*
Strain/clone: Not reported
Age at study initiation: Not reported
Weight/length/height at study initiation: 110 ± 5.65 g
Source: Not reported
Feeding during test: No
Acclimation: 10 days during which they were fed with commercial feed.

Test conditions

Hardness: Not reported
Test temperature: 25.3 ± 1.25 ºC
pH: 7.58 ± 0.45
Dissolved oxygen: 6.32 ± 0.87 mg O_2/L
Conductivity: Not reported
Photoperiod: Not reported
Light intensity: Not reported

Test system
Study type: Anaesthetic effect
Duration of study: Approximately 10-30 minutes
Treatments: 6.25, 12.5, 25, 50, 75, 100, 125, 150 and 200 mg thymol/L; 6.25, 12.5, 25, 50, 75, 100, 125, 150 and 200 mg eugenol/L
Analytical determination of test concentrations: No
Negative control included: No
Positive control included: No
Parameters measured: Mortality/behaviour
Validity criteria: Not reported
Fish were exposed to nominally 6.25, 12.5, 25, 50, 75, 100, 125, 150 and 200 mg/L anesthetics eugenol and thymol (10 fish of each tank per concentration) to record induction and recovery time. Anesthetic and recovery chambers were 10-L tanks equipped with aeration. The fish were individually caught from the holding tanks and placed into the anesthetic chamber. The time to reach stages 3 and 4 anesthesia were recorded and then the fish was caught and placed into the recovery tank to record recovery time.

Results

Both eugenol and thymol were not able to induce any anesthesia stages in the fish at the nominal concentration of 6.25 mg/L. Thymol at the nominal concentration of 12.5 mg/L induced stage 3 anesthesia; however, eugenol failed to induce anesthesia at this concentration. Eugenol induced stage 4 anesthesia within 600–90 s (with recovery time of 190–380 s) at the nominal concentrations of 25–150 mg/L; however, there was no significant difference in induction between the nominal concentrations 125 and 150 mg/L. Thymol induced stage 4 anesthesia within 850–60 s (with recovery time of 210–1200 s) at the nominal concentrations of 25–200 mg/L. There were relationships between the anesthetics' concentrations and induction time, and between the anesthetics' concentrations and recovery time. In general, induction time of the anesthetics was similar (except at the concentration 50 mg / L); however, the thymol-anesthetized fish recovered within longer period compared to eugenol. Eugenol failed to anesthetize the fish in <90 s, but thymol induced anesthesia in the fish within 60 s at the nominal concentration of 200 mg/L.

Assessment and conclusion

Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.
Proposed category: 3 not reliable
Analytical verification of test concentrations was not reported.

Assessment and conclusion by applicant:

The study is not acceptable (not reliable).

At nominal concentrations of 25 – 200 mg thymol/L, *Cyprinus carpio* (common carp) reached anaesthesia after approximately 1 to 15 minutes, and recovered after approximately 3 to 20 minutes.
At nominal concentrations of 25 – 150 mg eugenol/L, *Cyprinus carpio* (common carp) reached anaesthesia after approximately 1.5 to 10 minutes, and recovered after approximately 3 to 6 minutes.
No mortality was reported.

Data point:	CA 9.6.3.4/32 [CA 8.6]
Report author	De Assis Alves, T., Fontes Pinheiro, P., Praça-Fontes, M. M., Andrade-Vieira, L. F., Barelo Correa, K., de Assis Alves, T, Aparecida da Cruz, F., *et al*.
Report year	2018
Report title	Toxicity of thymol, carvacrol and their respective phenoxyacetic acids in *Lactuca sativa* and *Sorghum* bicolor
Report No	-
Document No	Internal reference: Study 363 Industrial Crops & Products Volume 114, Pages 59-67 (2018)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)
Natural and semisynthetic compounds may be useful to obtain novel herbicides. Thymol and carvacrol, which belong to the class of phenols, are examples of molecules found in the nature that present phytotoxic activity. Thus, we aimed to synthesize phenoxyacetic acids from thymol and carvacrol and to use them in plant bioassays. The thymoxyacetic and carvacroxyacetic acids were obtained from thymol and carvacrol, respectively, in the presence of sodium chloroacetate, under reflux, followed by acidification. These compounds were tested against *Lactuca sativa* and *Sorghum bicolor* initial seed development and their effects were tested on *L. sativa* and *S. bicolor* DNA. We also evaluated the performance of these compounds in the mitotic cycle of meristematic cells of L. sativa roots. The synthesized compounds were characterized by mass spectrometry and nuclear magnetic resonance spectroscopy (NMR) of 1H and 13C. The compounds were tested in different concentrations regarding the biological assays: 0.0, 0.375, 0.750, 1.50 and 3.0 mmol / L. After the analysis of the comet assay, carvacrol and carvacroxyacetic acid showed the most damaging effects on *L. sativa* and *S. bicolor* DNA. To corroborate the results found for these two compounds, we carried out an analysis with ISSR to verify the molecular damages in the two tested plants. We concluded that carvacrol, thymol, carvacroxyacetic and thymoxyacetic acids presented phytotoxic and cytotoxic activities. Carvacrol and the carvacroxyacetic acid also showed genotoxic action at the concentration of 3.0 mmol /L. The toxic effect of the thymoxyacetic acid was similar to that of the commercial herbicide 2,4-D, that is, more pronounced in *L. sativa* (eudicot) than in *S. bicolor* (monocot).

Materials and methods

Test material 1

Name:	Thymol
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma Aldrich
Active substance content:	Not reported
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species: Commercial seeds of the eudicot *L. sativa* L. (lettuce) var. Mônica (Feltrin) and of the monocot *S. bicolor* L. (sorghum) var. Moench (BR seeds)
Strain/clone: Not reported
Age at study initiation: Not relevant
Weight/length/height at study initiation: Not reported
Source: shops for agricultural products
Feeding during test: Not relevant
Acclimation: Not relevant

Test conditions

Test temperature: 25 ± 2 ºC
Photoperiod: 12 hours: 12 hours
Light intensity: Not reported
Test system
Study type: germination and toxicity test
Duration of study: 120 hours
Treatments: 0.375, 0.75, 1.5 and 3 mmol thymol/L (nominal)
Analytical determination of test concentrations: No
Negative control included: Yes (water and acetone)
Positive control included: Yes (2, 4–D)
Parameters measured: growth/ germination
Validity criteria: Not reported
Five Petri dishes containing 25 seeds each were used per treatment. Four nominal concentrations of thymol 0.375, 0.75, 1.5 and 3 mmol thymol/L were assessed. The macroscopic assays evaluated the percentage of germination (%G), germination speed index (GSI), root growth (RG) and shoot length (SL).
Results
Thymol exhibited phytotoxicity at different concentrations, as reflected on inhibited germination and growth of monocot and dicot seeds. Growth inhibition is seen in the two plant models with thymol at concentrations of 3 mmol thymol/L. In *L. sativa* inhibition was approximately 93% and in *S. bicolor* inhibition was 58% (compared with the negative control). Effect on root growth and shoot length were also observed at 3 mmol thymol/L.
Assessment and conclusion
Reliability assessment
For full details and justification, please refer to Document KCA 9.4.2/02.
Proposed category: 2 reliable with restrictions
Analytical verification of test concentrations was not reported. Purity of thymol not reported.

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).
L. sativa (lettuce) inhibition 93% germination at 3 mmol thymol/L, based on nominal concentrations
S. bicolor (sorghum) inhibition 58% germination at 3 mmol thymol/L, based on nominal concentrations

Data point:	CA 9.6.3.4/33 [CA 8.3.1]
Report author	Colin, T., Forster, C.C., Westacott, J., Wu, X., Meikle, W.G., Barron, A.B.
Report year	2021
Report title	Effects of late miticide treatments on foraging and colony productivity of European honey bees (*Apis mellifera)*
Report No	-
Document No	Internal reference: Study 18 complementary Apidologie (2021), ISSN: 1297 - 9678
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)
Chemical miticides are used routinely in honey bee colonies worldwide as treatment for the parasitic mite *Varroa destructor*, but there have been very few long-term colony-level field studies of the impacts of miticides on the bees themselves. Lab-based studies with individual bees or bees in small groups have highlighted many negative effects of miticides on bee behaviour and physiology; hence, there is an urgent need to better understand the consequences of miticides on honey bee colonies in an apicultural setting. Here we compared effects of commercial treatments of the miticides tau-fluvalinate and thymol, and controls, on honey bee colonies and bee foraging behaviour over five months, from autumn through winter in Sydney, Australia. Since *V. destructor* does not occur in Sydney, in this study, we could isolate the direct effects of the miticides from indirect effects resulting from reduced mite load. We found the autumn treatment of either miticide caused no significant change in bee adult or brood population or size of food stores. The average temperature in the thymol group differed from the temperature in the control group and was lower during winter. Neither miticide reduced bee longevity. Tau-fluvalinate caused bees to start foraging earlier in life and perform shorter trips, but no other effects on foraging behaviour were documented. To conclude, in Sydney, Australia, limited negative effects of autumn thymol or tau-fluvalinate treatments were observed on bees or bee colony performance.

Materials and methods

Test material 1

Name:	Apiguard®
Formulation type:	Slow release gel
Source and lot/batch no.:	Vita Europe Ltd; Not reported
Active substance content:	thymol (12.5 g) [mixed with 37.5 g of a slow-release gelatine (2-isopropyl-5-methylphenol]
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species:	Honey bees
Strain/clone:	Golden Italian
Source:	Colonies located at Macquarie University research apiary.
Feeding during test:	Yes. Colonies were fed with 6 L of syrup and 1 kg of pollen patty during the first month after establishment, after which no more food was given. Pollen patty was composed of pollen, sucrose, glucose, fructose, water in

	the ratios 1:1:0.75:0.75:1. Pollen was obtained as honeybee "feedback" pollen pellets from Hornsby Beekeeping Supplies (Hornsby, NSW). The frame feeder was then removed and an additional 4 frames with printed wax comb were added to each hives.
Acclimation:	25 hives were established on the campus of Macquarie University (33° 46′ 06.6″ S 151° 06′ 43.8″ E, Macquarie Park, NSW, Australia). Colonies were established from bee packages containing 1.5 kg of bees sourced from the Macquarie University research apiary. Honey bees were housed in standard wooden "deep Langstroth" 8- frames hives of a volume of about 35.5 L with Australian "migratory" lids (equipped with vents that were shut during treatment). In starting condition, colonies were given four frames of printed wax comb and a frame feeder.

Test conditions

Test temperature:	35 ºC
Photoperiod:	Not reported
Light intensity:	Not reported
Test system	
Study type:	Field trial in which honey bee colonies were treated with the *Varroa*-treatment formulation "Apiguard®"
Duration of study:	5 months
Treatments:	Apiguard® trays (containing 12.5 g thymol each) were administered as per the manufacturer's instructions by introducing one tray on top of the frames of each hive, under the lid. This was left in the hive for two weeks, then replaced with a second tray for a further two weeks.
Analytical determination of test concentrations: No.	
Negative control included:	Yes (six control colonies received no treatment)
Positive control included:	No
Parameters measured:	Mortality/behaviour
Validity criteria:	Not reported

Hives were randomly assigned to three treatment groups. Seven hives were assigned to a thymol treatment group and six to a control group (initially seven: one hive was removed following storm damage). The position of the hives was randomised across the apiary. Nine other hives were present in the apiary but located at least 10 m apart from the hives in this experiment.
Colonies assigned to the thymol group were treated with Apiguard® (Vita Europe Ltd.), which contains 12.5 g of thymol mixed with 37.5 g of slow-release gelatine (2-isopropyl-5-methylphenol) in a small aluminium tray. The six control colonies received no treatment.
Effects on foraging behaviour, colony productivity and survival were addressed.
Statistical analyses were conducted in R v 3.6.2.

Results

There were no significant differences in number of capped cells, number of adult bees, or mass of food stores between treatment groups in any of the three hive evaluations.
The daily weight gains in colonies treated with thymol differed significantly from the daily weight gains in control colonies and this effect varied with time. They were lower at the end of the treatment and in the following summer but not during spring. There were no differences in trip duration in orientation flights, and no difference in foraging trip duration between thymol treated bees and controls. However, bees exposed to thymol were found to perform more lifetime foraging trips than bees in the control group.
Mortality rate was not significantly different between the control group and thymol treatment.
No significant differences in brood amounts were observed between treatment groups throughout the experiment, but the thymol group had lower brood amounts in the first evaluation following treatment, which was the period over which the temperature data were analysed. This might have contributed to a reduction of the average temperature overwinter in the thymol group.
Summer thymol treatment was not found to significantly reduce average temperature overwinter but affected brood amounts in bee colonies temporarily. Summer thymol treatment is not associated with increased overwinter mortality, unlike late thymol application which was previously associated with higher winter mortality. An effect of thymol on brood amounts shortly before winter may not leave enough time for the hives to recover, which could explain lower average temperatures during winter.

Assessment and conclusion
Reliability assessment
For full details and justification, please refer to Document KCA 9.4.2/02.
Proposed category: 2 reliable with restrictions
Study tested a slow-release gel formulation containing thymol, but exact exposure of thymol to bees is unknown (and no analytical verification included).

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).

In a field trial, honey bee colonies in Australia were treated with the anti-*Varroa* treatment "Apiguard®". Apiguard is a slow-release gel, added in aluminium trays to the honey bee hive; each tray contains 12.5 g of thymol. In this study, Apiguard trays (containing 12.5 g thymol each) were administered as per the manufacturer's instructions by introducing one tray on top of the frames. Trays were replaced after 2 weeks. The second tray was also removed after 2 weeks.
No adverse effects of miticide treatment Apiguard® (thymol) on brood and bee numbers at dose of 12.5 g of thymol mixed with 37.5 g of a slow-release gelatine.

Data point:	CA 9.6.3.4/34 [CA 8.2.1]
Report author	Aydin, B., Orhan, N.
Report year	2021
Report title	Effects of thymol and carvacrol anesthesia on the electrocardiographic and behavioral responses of the doctor fish *Garra rufa*
Report No	-
Document No	Internal reference: Study 19 complementary Aquaculture volume 533, 736134 (2021)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	No

Abstract (copied from original literature)
The present study examined the anesthetic efficiency and electrocardiographic and behavioral responses of the doctor fish *Garra rufa* anesthetized with thymol and carvacrol. Fish were subjected to anesthetic baths in five different concentrations of the thymol (25, 50, 75, 100, and 150 mg/L) and carvacrol (25, 50, 75, 100, and 150 µL/L). Induction and recovery times were recorded for each fish separately, and 10 fish were used per anaesthetic concentration. Video tracking and processing were performed for each anesthetic to obtain changes in behavioral parameters between anaesthetic concentrations. Thereafter, a different experiment was established for cardiographic evaluations, and necessary electrocardiographic (ECG) records were obtained in 5 fish. At the end of the experiments, all tested thymol and carvacrol concentrations effectively induced anesthesia in *G. rufa*. Video tracking analyses demonstrated that *G. rufa* did not exhibit any changes in behavioral parameters when submitted to 25–50 mg/L concentrations of thymol compared to anesthetic-free water. However, movement distance and mobility significantly increased with increasing carvacrol concentrations. We found that high concentrations of thymol (150 mg/L) and carvacrol (100 and 150 µL/L) caused avoidance behaviors in *G. rufa*. ECG recordings showed that heart rate decreased, and PR and QT intervals were prolonged with increased thymol concentrations. Thymol concentrations did not affect PQ intervals. No significant changes in ECG parameters were observed with carvacrol. These results demonstrated that concentrations of 25 and 50 mg/L thymol and 50 µL/L carvacrol can be used for general aquaculture procedures, and 100 mg/L thymol and 75 µL/L carvacrol can be used as rapid anesthesia in *G. rufa*.

Materials and methods

Test material 1

Name:	Thymol
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma-Aldrich Corporation, St. Louis, MO, USA
Active substance content:	99%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species:	*Garra rufa*, doctor fish.
Strain/clone:	Not reported
Age at study initiation:	Not reported
Weight at study initiation:	1.8 ± 0.2 g
Source:	Not reported.
Feeding during test:	No
Acclimation:	Prior to experiments, the fish were hand-fed twice (09.00 a.m. and 16.00 p m.) daily until apparent satiation using commercial feed (41% crude protein, 7% crude lipid) for 21 days for acclimatization. Feeding stopped 24 h before the experiments.

Test conditions

Hardness:	Not reported
Test temperature:	25.0 ± 1.0 ◦C,
pH:	7.5 ± 0.2
Dissolved oxygen:	8.1 ± 0.3 mg/L
Conductivity:	Not reported
Photoperiod:	12:00 h light: 12:00 h darkness
Light intensity:	Not reported

Test system

Study type:	Anaesthesia test
Duration of study:	10 minutes (exposure period of approximately 1 to 3 minutes)
Treatments:	25, 50, 75, 100, and 150 mg thymol/L (nominal)

Analytical determination of test concentrations: No

Negative control included:	Yes
Positive control included:	No
Parameters measured:	Mortality/behaviour
Validity criteria:	Not reported

A total of 110 fish were used to assess anaesthesia, recovery times and behavioural analysis. The fish were exposed to 25, 50, 75, 100, and 150 mg thymol/L. Each fish (n = 10 each anaesthetic concentration) was individually transferred into a continuously aerated plastic container (30 × 30 × 30 cm; length*width*height) (3 L of water) containing the test aesthetic concentration in water. After anaesthesia fish were transferred to anaesthetic-free and continuously aerated plastic containers (40 × 40 × 40 cm; length*width*height) (5 L of water) to evaluate the recovery time from the test anesthetic concentration. The fish were considered recovery stage 1 when a partial recovery of equilibrium with partial recovery of swimming motion was observed. The fish were considered recovery stage 2 when normal swimming returned.

Results

Nominal concentrations of thymol at 25 – 150 mg thymol/L caused anesthesia in fish. The lowest effective anaesthetic concentration of thymol was 25 mg thymol/L. The time to reach induction (stage 1) and anaesthesia (complete lack of voluntary movement Stage 2) were 60.7 ± 3.5 and 202.2 ± 23.6 seconds; respectively. Following this short exposure period, the recovery time for thymol from stage 1 and 2 were 159.7 ± 27.9 and 268.8 ± 33.7 seconds, respectively.

The time to reach induction (stage 1) and anaesthesia (complete lack of voluntary movement Stage 2) with the highest effective anaesthetic concentration of thymol of 150 mg/L were 19.8 ± 2.9 and 46.9 ± 6.6 seconds

respectively. Following this short exposure period, the recovery time for thymol from stage 1 and 2 were 345.9 ± 36.8 and 508.2 ± 62.6 seconds, respectively.
The swimming behaviour of fish the first 1 minute after the release of the fish into the water containing thymol demonstrated no significant changes in movement distance and mobility between thymol concentrations (25–150 mg/L). However, there was a significant difference in mobility between anaesthetic-free water and 100–150 mg thymol/L. Significant differences were observed between anaesthetic-free water and 150 mg thymol/L concentration in all the behavioural parameters. The movement distance of fish anesthetized with thymol ranged from 557.9 ± 61.7 cm to 709.4 ± 95.2 cm.
The results of the present study determined that anaesthetic concentration had a significant effect on movement distance, swimming speed, and mobility of *G. rufa*. Low concentrations of thymol (25–75 mg/L) had no adverse effect on the behavioural parameters of *G. rufa.*

Assessment and conclusion
Reliability assessment
For full details and justification, please refer to Document KCA 9.4.2/02.
Proposed category: 3 not reliable
Analytical verification of test concentrations was not reported.

Assessment and conclusion by applicant:

The study is not acceptable (not reliable).

At nominal concentrations of 25 – 150 mg thymol/L, *Garra rufa* (doctor fish) reached anaesthesia after approximately 1 to 3 minutes, and recovered after approximately 3 to 5 minutes. After this short period of exposure, no mortality on *Garra rufa* (doctor fish) at 150 mg thymol/L during the anesthetic exposure (approximately 3 minutes).

At nominal concentrations of 25–75 mg thymol/L, no adverse effects on the behavioural parameters of *G. rufa* (swimming behavoir) were observed.

Data point:	CA 9.6.3.4/35 [CA 8.3.1]
Report author	Sabahi, Q., Morfin, N., Emsen, B., Gashout, H.A., Kelly, P.G., Otto, S., Merrill, A. R., Guzman-Novoa, E.
Report year	2020
Report title	Evaluation of Dry and Wet Formulations of Oxalic acid, Thymol, and Oregano Oil for Varroa Mite (Acari: Varroidae) Control in Honey Bee (Hymenoptera: Apidae) Colonies
Report No	-
Document No	Internal reference: Study 20 complementary Journal of Economic Entomology, Volume 113(6), Pages 2588–2594 (2020)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)
The efficacy and safety of dry and wet formulations of three non-synthetic compounds, oxalic acid (OA), thymol (T), and oregano oil (OO), for the control of *Varroa destructor* Anderson and Trueman infestations in honey bee (*Apis mellifera* Linnaeus) colonies were determined. The treatments were OA in dust, OA diluted in glycerine solvent embedded in a towel, T in dust, T in glycerin solvent and towel, OO in dry microcapsules, OO in

glycerin solvent and towel, and the control. The treatments were applied weekly for 4 wk during the fall season. The rates of acaricide efficacy, weekly mite fall, bee mortality, colony survivorship, and strength, were determined for each of the treatments. All formulations, with the exemption of OO microcapsules, were effective at controlling infestations of *V. destructor*. The most effective formulations were T dust (96.6%), T glycerin (92.4%), and OA glycerin (79%). More than 85% of the mites were killed during the first 2 wk of treatment with T formulations, compared to less than 30% for the OA glycerin formulation. The lowest efficacy rate was for OO microcapsules (21.3%), and the only treatment that significantly increased bee mortality was OA glycerin. The rates of winter colony survival and honey bee populations were related to the varroacidal efficacy of the formulations. The implications of these findings are discussed.

Materials and methods

Test material 1

Name: Thymol
Formulation type: Finely powdered (dust)
Source and lot/batch no.: Sigma-Aldrich
Active substance content: Not reported
Expiry date of lot/batch: Not reported
Storage conditions: Not reported

Test organism

Species: honey bee (*Apis mellifera)*
Strain/clone: Colonies headed by Buckfast queens
Age at study initiation: Not reported
Weight/length/height at study initiation: Not reported
Source: Colonies maintained at the University of Guelph Honey Bee Research Center, Canada. Not reported
Feeding during test: Not reported
Acclimation: Not reported

Test conditions

Test temperature: Not reported
Photoperiod: Not reported
Light intensity: Not reported
Test system
Study type: Field trial in which honey bee colonies were treated with thymol. Duration of study: 4 weeks (weekly treatment)
Treatments: 30 g formulation (0.2 g thymol per g of total powder)
Analytical determination of test concentrations: No
Negative control included: Yes
Positive control included: No
Parameters measured: Mortality
Validity criteria: Not reported

Forty-two honey bee colonies headed by Buckfast queens housed in Langstroth hives that were similar in mite infestation rates and bee populations were selected for the experiments. The hives were fitted with 3 mm screened bottom boards to insert sticky paper sheets underneath them to capture falling varroa mites. Six colonies were randomly assigned to each of seven treatments. The thymol treatment 1 was prepared as a dust with 0.2 g thymol per g of total powder and mixed with confectionary sugar**.** In total, 30 g of formulation was applied to each colony on a piece of newspaper (25 × 10 cm), which was placed on top of the frames of the brood chamber. Treatment 3, T dust, was prepared as per Emsen et al. (2007), like treatment 1**.**The experimental trials began in mid-September, 2018, by treating the colonies weekly during a 4-wk period. Data on bee mortality were log transformed prior to analysis by ANOVA. When significance was found, the means were separated by least significant difference tests. Descriptive statistics of colony survival and strength were obtained

during the spring following the treatments. The SPSS v.22 software was used for the data analysis with the significance level set at $P < 0.05$ ($\alpha = 0.05$).

Results

After exposure of thymol dust (T dust treatment), the highest rate of winter colony survival was for the T dust treatment. The survivorship was 100%. Meanwhile, the % survivorship was 83% in the control. No significant differences with control were observed.

The populations of the experimental colonies after the winter (the mean colony strength) varied from 6.3 ± 1.26 and 3.8 ± 1.08 in the thymol treatment and in the control respectively.

No adverse effects were observed at concentrations 0.2 g thymol per g of total powder (30 g of powder mixed with confectionary sugar applied weekly to the hives) after 4 weeks in hives.

Assessment and conclusion

Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 2 reliable with restrictions

Test conditions not reported. Study tested exposure to thymol dust, but exact exposure of thymol to bees is unknown (and no analytical verification included).

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).

No adverse effects were observed at concentrations 0.2 g thymol per g of total powder (30 g of powder mixed with confectionary sugar applied weekly to the hives) after 4 weeks in hives.

Data point:	CA 9.6.3.4/36 [CA 8.3.1]
Report author	Borges Matos, W., Celestino Santos, A.C., Santana Lima, A.P., Rabelo Santana, E.D., Elias Silva, J., Fitzgerald Blank, A., Albano Araújo, A.P., Bacci, L.
Report year	2021
Report title	Potential source of ecofriendly insecticides: Essential oil induces avoidance and cause lower impairment on the activity of a stingless bee than organosynthetic insecticides, in laboratory
Report No	-
Document No	Internal reference: Study 22 complementary Ecotoxicology and Environmental Safety 209, 111764 (2021)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)

The negative effect of insecticides on bees has been reported as one of the factors associated with the decline in population of these pollinators. Thus, the aim of this study was to evaluate the response of the stingless bee *Nannotrigona* aff. *testaceicornis* (Lepeletier, 1836) to a promising source of new insecticide molecules obtained from *Lippia sidoides* (rosemary pepper) essential oil (EO) and its major compounds (thymol, *ρ*-cymene, and (*E*)-caryophyllene), comparing them to commercial insecticides (organosynthetic: imidacloprid, deltamethrin and semisynthetic: spinetoram). For this, stingless bees were exposed by contact with these compounds to evaluate the lethal and sublethal (locomotion and flight orientation) toxicity. The *L. sidoides* EO and its major compounds have low lethal toxicity to forager worker bees (*N.* aff. *testaceicornis*). The organosynthetics imidacloprid (LD_{50} = 0.00146 µg/bee) and deltamethrin (LD_{50} = 0.0096 µg/bee were about 209,589 and 31,875 times more toxic, respectively, than the least toxic natural compound, (*E*)-caryophyllene (LD_{50} = 306 µg/bee). Locomotion ability and flight orientation were little affected by spinetoram and by *L. sidoides* EO and its major compounds, however, were greatly reduced by the imidacloprid and deltamethrin insecticides. Besides shows low lethal and

sublethal toxicity, the bioinsecticides were also avoided by the forager bees. Individuals treated with the *L. sidoides* EO and thymol were avoided by the untreated bees. Therefore, the natural products studied here were promising due to their recognized effectiveness against pest insects and greater safety to bees *N.* aff. *testaceicornis*

Materials and methods

Test material 1

Name:	thymol
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma-Aldrich® (Steinheim, Germany)
Active substance content:	Not reported
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species:	native stingless bee, *Nannotrigona* aff. *testaceicornis*
Strain/clone:	Not reported
Age at study initiation:	Forager bees
Weight/length/height at study initiation:	Not relevant
Source:	Collected directly from the entrance of hives at Federal University of Sergipe (UFS), Sao Cristovao Campus, state of Sergipe (10◦55’47.6"S; 37◦06’14.7"W), Brazil.
Feeding during test:	No
Acclimation:	Not reportedThe captured bees were kept in 0.25-L plastic pots (10 bees/pot) sealed with organza, containing a piece of cotton soaked in a 50% (vol/vol) distilled water and honey (obtained from the nests of the colonies used) solution under controlled conditions (26 ± 2 ◦C and relative humidity of 70 ± 5%, constant scotophase) in biochemical oxygen demand (B.O.D.) incubator, until the bioassays were performed. All colonies used were free of visible signs of disease.

Test conditions

Test temperature:	26 ± 2 ◦C
Relative humidity:	70 ± 5%
Photoperiod:	Not reported
Light intensity:	Not reported
Test system	
Study type:	Acute contact toxicity test
Duration of study:	24 hours
Treatments:	6.6–85.8 µg thymol/bee (nominal)
Analytical determination of test concentrations:	No
Negative control included:	Yes
Positive control included:	No (but other insecticides also tested, such as deltamethrin)
Parameters measured:	Mortality/behaviour (locomotion and flight orientation)
Validity criteria:	Not reported

The bees were anesthetized at -8 °C for 50 seconds and treated by applying 0.5 µL of each dose/insecticide to the pronotum area, using a 10 µL microsyringe. Ten bees/colony/dose were used in each treatment. All colonies used were free of visible signs of disease. Mortality analysis was carried out 24 h after exposure to treatments, with intervals of 30 min. The treated bees were placed in Petri dishes (9 × 1.5 cm) lined with filter paper and covered with plastic film and kept under controlled conditions. Holes were made with entomological pins for air exchange in the covering the petri dishes. The effect of treatments on locomotion and flight orientation was

analysed by exposing the bees to a lethal dose capable of killing 20% of the population (LD_{20}), determined in the toxicity bioassays. The bioassay evaluations were performed twice, independently, using different individuals. The first time, bees were released in the arena at 3 h after being exposed to the treatments; the second time, bees was released at 24 h after exposure. At each exposure time, 10 bees/ colony were analysed, resulting in 80 bees per treatment/time. Probit analysis was performed on the mortality data using the SAS PROC PROBIT procedure (SAS Institute Inc. 2002) to obtain the dose-response curves. From these curves, LD_{20}, LD_{50}, and LD_{90} were determined, with their respective confidence intervals, at 95% probability.
Survival analysis was performed using Kaplan–Meier estimators by the Log-Rank test (Sigmaplot, version 11.0). From this analysis, survival curves and the LT_{50} were determined.
Analysis of variance, followed by the Dunnett test ($p < 0.05$) using the SAS PROC ANOVA procedure (SAS Institute Inc. 2002), was performed to verify whether the distances traveled by the bees in the locomotion and flight orientation in each treatment differ to control.

Results

LD_{50} value was reported to be 33.6 µg thymol/bee (C.I (28.9–38.3 µg thymol/bee) (based on nominal doses).
LD_{20} value was reported to be 12.7 µg thymol/bee (C.I (9.23–15.9 µg thymol/bee) (based on nominal concentrations).
LD_{90} value was reported to be 149 µg thymol/bee (C.I (120–202 µg thymol/bee) (based on nominal concentrations).
The bioassay indicated that sublethal doses of thymol did not affect locomotion ability in *N.* aff. *Testaceicornis.* Therefore, thymol has low lethal and sublethal toxicity to the stingless bee N. aff. testaceicornis

Assessment and conclusion

Reliability assessment
For full details and justification, please refer to Document KCA 9.4.2/02.
Proposed category: 2 reliable with restrictions
No validity criteria reported.

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).
Nannotrigona aff. testaceicornis (Brazilian stingless bee)
LD_{50} value was reported to be 33.6 µg thymol/bee (C.I (28.9–38.3 µg thymol/bee) (based on nominal concentrations).
LD_{20} value was reported to be 12.7 µg thymol/bee (C.I (9.23–15.9 µg thymol/bee) (based on nominal concentrations).

Data point:	CA 9.6.3.4/37 [CA 8.3.1]
Report author	Gashout, H.A., Guzman-Novoa, E., Goodwin, P.H.
Report year	2020
Report title	Synthetic and natural acaricides impair hygienic and foraging behaviors of honey bees
Report No	-
Document No	Internal reference: Study 23 complementary Apidologie, Volume 51, Pages 1155-1165 (2020)
Guidelines followed in study	-
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	Supporting information only

Abstract (copied from original literature)

Acaricides commonly used to control the honey bee parasitic mite, *Varroa destructor*, may also adversely affect bees. Sublethal LD_{05} doses of synthetic (tau-fluvalinate, amitraz, and coumaphos) and natural (thymol and formic acid) acaricides did not significantly reduce bee survivorship. However, compared to the ethanol solvent

control, hygienic behavior critical for pathogen and parasite control was significantly reduced with coumaphos, whereas both pollen and non-pollen foraging behaviors critical for resource acquisition were reduced with tau-fluvalinate, coumaphos, and formic acid. Thymol significantly reduced non-pollen foraging but also reduced the negative effects of ethanol on hygienic behavior. Amitraz did not affect hygienic and foraging behaviors relative to the solvent. Thymol and amitraz appeared to be the safest acaricides based on these tests.

Materials and methods

Test material 1

Name:	thymol
Formulation type:	Not relevant
Source and lot/batch no.:	Fisher Scientific Ltd. (Ottawa, ON, Canada)
Active substance content:	>98%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism

Species:	honey bee, *Apis mellifera*
Strain/clone:	Buckfast
Age at study initiation:	10 -14 day old for hygienic behavior and 19–26 days old for foraging behavior
Weight/length/height at study initiation:	not relevant
Source:	Guelph's Honey Bee Research Center in Ontario, Canada. To obtain worker bees of the same age, frames with emerging brood collected from source colonies were placed inside wooden emergence cages (50 × 7 × 25 cm) and incubated overnight at 32 ± 2 °C and 60 ± 5% RH
Feeding during test:	Not reported
Acclimation:	Not reported

Test conditions

Test temperature:	22–28 °C (hygienic behaviour)
Photoperiod:	Not reported
Light intensity:	Not reported
Test system	
Study type:	Survival and behaviour after acute topical/contact application of thymol
Duration of study:	2 weeks
Treatments:	5 µL thymol (4.509 µg/bee)
Analytical determination of test concentrations:	No
Negative control included:	Yes
Positive control included:	No
Parameters measured:	Mortality/behaviour
Validity criteria:	Not reported

Newly emerged bees were marked with enamel paint of different colors on their thoraces on the day they were obtained from the incubator, to identify cohorts of bees of the same age as well as the different treatments. Marked bees were randomly assigned to different treatments (acaricides, ethanol solvent, or non-treated). Each bee was individually treated with 5 µL of treatment solution, which was applied on the dorsal surface of the thorax using a micropipette (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). For acaricides, bees were treated with the 48 hpt (hours post treatment) LD_{05} thymol (4.509 µg/bee). Bees treated with 95% ethanol (solvent) and non-treated bees were used as controls. After exposure, the bees were introduced into three observation hives until they were 10– 14 days old for hygienic behavior assessments, or introduced into three Langstroth hives until they were 19–26 days old for foraging behavior assessments. In total, 850 bees were marked, treated, and introduced into each hive for each treatment group and controls.

The data on the percentage of surviving bees were square-root- arcsine transformed before factorial analysis of variance (ANOVA) to determine the effects of treatment and age on length of life. To analyse hygienic behavior, ratios between counts of hygienic events in 15 min and number of bees that were alive during observation days (based on the censuses of observation hives) were subjected to chi-square tests for comparisons between control untreated bees and the rest of the treatments. To analyse foraging behavior, ratios were obtained between the number of bees foraging per hour of observation and numbers of bees that were alive during observation days. Then, the ratios were log transformed before ANOVA and Fisher LSD analysis to separate means when significance was detected. Transformations were carried out to comply with the assumption of normality required for ANOVA. Statistical analyses were performed using the statistical software R (version 3.3.1) (R Core Team 2012).

Results

Effect of acaricides on adult bee survivorship

There were no significant differences between the treatments for percent survivorship of adult bees. The detrimental effects of the acaricides on hygienic and foraging behaviors were not due to differences in survivorship. None of the acaricides tested reduced the bees' lifespan in comparison with the control ethanol-treated or non-treated bees.

Hygienic behaviour observations

The ethanol control significantly decreased the frequency of hygienic events relative to the non-treated control, which affected the results for all the acaricide treatments as they had ethanol as the solvent. The thymol treatment resulted in significantly higher hygienic instances than the other acaricide treatments and the ethanol control, even though thymol was dissolved in ethanol. Thus, it appears that thymol reduced the negative effects of ethanol, although bees treated with thymol performed 13% less hygienic behavior instances than the control. The effects of ethanol could not be separated from the effects of the acaricides, but the combination of ethanol and thymol was the least detrimental.

Foraging behavior observations

Pollen foraging trips comprised approximately one-quarter of the total trips and non-pollen foraging trips comprised approximately three-quarters of the total trips. Thymol-treated bees did not differ significantly in total foraging trips from the controls. Thymol-treated bees were significant lower than the controls for non-pollen foraging trips. Therefore, thymol treatment had a negative effect only on non-pollen foraging trips. It might be that thymol increased this behavior, which could have caused the bees to search for more concentrated nectar, thus lengthening foraging trips and reducing the number of nectar foraging trips. Therefore, thymol could have a negative effect on sucrose collection, even though pollen collection was unaffected.

Assessment and conclusion

Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 2 reliable with restrictions

No positive control, no validity criteria, only one dose tested

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).

Acute contact treatment of thymol 4.509 µg/bee did not affect survivorship and hygienic behaviour. However, thymol had a negative effect only on non-pollen foraging trips Bees tend to search for more concentrated nectar than pollen foragers, lengthening foraging trips and reducing the number of nectar foraging trips.

Data point:	CA 9.6.3.4/38 [CA 8.2.1]
Report author	Vieira, R., Venancio, C., Félix, L.
Report year	2021
Report title	Teratogenic, Oxidative Stress and Behavioural Outcomes of Three Fungicides of Natural Origin (*Equisetum arvense, Mimosa tenuiflora*, Thymol) on Zebrafish (*Danio rerio*)
Report No	-
Document No	Internal reference: Study 24 complementary Toxics 9, 8 (2021)
Guidelines followed in study	OCED 236 with modifications
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	No

Abstract (copied from original literature)
The improper use of synthetic fungicides has raised public concerns related to environmental pollution and animal health. Over the years, plant-derived antifungals have been investigated as safer alternatives, although little scientific evidence of its neurodevelopmental effects exist. The main objective of this study was to explore the effects of three alternative natural extracts (*Equisetum arvense, Mimosa tenuiflora*, Thymol) with antifungal properties during the early development of zebrafish by evaluating different teratogenic, oxidative stress and behavioural outcomes. Following the determination of the 96 h LC_{50}, exposure to sublethal concentrations showed the safety profile of both *E. arvense* and *M. tenuiflora*. However, following 96 h exposure to thymol, increased lethality, pericardial oedema, yolk and eye deformations, and decreased body length were observed. The reduced and oxidized glutathione (GSH:GSSG) ratio was increased, and the glutathione-s-transferase activity in the group exposed to the highest thymol concentration. Overall, these results support a more reducing environment associated with possible effects at the cellular proliferation level. In addition, the disruption of behavioural states (fear- and anxiety-like disorders) were noted, pointing to alterations in the c-Jun N-terminal kinase developmental signalling pathway, although further studies are required to explore this rationale. Notwithstanding, the results provide direct evidence of the teratogenic effects of thymol, which might have consequences for non-target species.

Materials and methods

Test material 1

Name: thymol
Formulation type: Not relevant
Source and lot/batch no.: EMD Millipore (Oeiras, Portugal)
Active substance content: extra pure
Expiry date of lot/batch: Not reported
Storage conditions: Not reported

Test organism

Species: Zebrafish (*Danio rerio*)
Strain/clone: Wild-type AB strain
Age at study initiation: Embryo
Weight/length/height at study initiation: Not reported
Source: Adult wild-type AB strain zebrafish were maintained at the University of Trás-os-Montes and Alto Douro (Vila Real, Portugal). Embryos were collected by the natural spawning method by maintaining a 2:1 male to

	female ratio in cages overnight. Embryos were collected within 1 hour after the onset of the light cycle
Feeding during test:	No
Acclimation:	Fish were maintained under standard conditions at 28.0 ± 0.5 ºC with a 14 h light/10 h dark photoperiod in an open water system with continuous supply of aerated, dechlorinated, charcoal-filtered and UV-sterilized City of Vila Real tap water (pH 7.3–7.5). Fish were fed with a commercial diet (Sera, Heinsberg, Germany) supplemented with *Artemia* sp. nauplii twice a day.

Test conditions

Hardness:	Not reported
Test temperature:	28 ºC
pH:	Not reported
Dissolved oxygen:	Not reported
Conductivity:	Not reported
Photoperiod:	14:10 h light-dark cycle
Light intensity:	Not reported
Test system	
Study type:	Fish embryo acute toxicity (FET) test
Duration of study:	96 hours (test 1), and up to 120 hours (96 hours exposure) for behavioural assays (test 2)
Treatments:	For determination of LC_{50}, concentrations not reported. For sublethal developmental toxicity test: 0.01, 0.1 and 1 mg thymol/L (nominal)
Analytical determination of test concentrations: No	
Negative control included:	Yes
Positive control included:	No
Parameters measured:	Mortality/ sub-lethal (developmental) effects / behaviour
Validity criteria:	Not reported

For the first test (determination of LC_{50}), embryos at 2 hours post fertilisation (hpf) were randomly distributed in 6-well culture plates (5 mL solution and 20 embryos per well) for triplicate exposure to seven test solutions (10-fold dilution from the stock solutions) for 96 h under the controlled conditions reported before. Embryo water was used as blank control and to prepare all test solutions. The exposure solutions were renewed every 24 h to keep the appropriate concentrations and water quality. The embryonic mortality was recorded daily and following correction for the percentage of mortality in the control group using Abbott's formula. The 96 h LC50 values were determined using the probit analysis.
In a second (sublethal toxicity) test, embryos were exposed to thymol nominal concentrations of 0.01, 0.1 and 1 mg thymol/L (approximately 1/200, 1/20 and ½ of the previously determined LC50 value). A blank control group (embryo water only) was also prepared and included in each plate. During the experimental period, the exposure solutions were replaced daily to maintain the appropriate concentration of the test compounds. The experiments were repeated independently five times. The zebrafish development (10 random animals removed from each group) was accompanied under a SMZ800 stereomicroscope with the cumulative mortality being assessed at 8, 24, 48, 72 and 98 hpf according to OECD Guidelines. Test No. 236: Fish Embryo Acute Toxicity. Lethal parameters such as failure of somites, eye and otolith development, missing heartbeat, and non-detached tail and head, were recorded at 24, 48, 72, and 98 hpf. Eleutheroembryo were further collected for subsequent biochemical analysis or washed three times with embryo medium and allowed to develop until 120 hpf for behavioural analysis. The zebrafish eleutheroembryo locomotor behaviour (exploratory open field test), the patterns of avoidance (in response to a bouncing ball stimulus) and anxiety-like behaviours (in the visual motor response test) were analysed 24 h after the end of the exposure, at 120 hpf, in a climatized dark room.
The statistical analyses were performed on the averaged values from each independent exposure using the GraphPad Prism software (Prism 8). The LC_{50} values were calculated using a variable slope model. The normality of data was controlled using Shapiro Wilk's test, and the homoscedasticity was checked with Brown-Forsythe's test. When data followed the normal distribution, differences among groups were assessed by one-way analysis of variance (ANOVA) followed by the Tukey multiple comparison test and data expressed as mean _ standard deviation (SD). When data followed a non-normal distribution, the data treatment was performed using the non-parametric Kruskal-Wallis analysis of variance followed by Dunn's test with a Bonferroni correction for multiple comparisons and data expressed as medians and interquartile range (25th; 75th

percentiles). The student's t test was used to evaluate differences for the aversive behavioural responses. A $p < 0.05$ was considered to be a statistically significant difference.

Results

The *Danio rerio* fish embryo 96 h LC_{50} and the 95% confidence limits were calculated as 2.35 mg/L (C.I. 0.78–5.55) for Thymol. Based on the calculated LC_{50}, three sub-lethal concentrations were selected for the subsequent experiments.

At 98 hpf, embryo development in the control groups was as expected with around 80% of the animals showing a normal development with mortalities of about 10%, and 6 to 10% malformed individuals. After 96 h exposure to thymol, the cumulative mortality increased significantly after exposure to 1 mg thymol/L in relation to the control group, while no significant differences were verified between the other groups (0.01 and 0.1 mg thymol/L). Malformed eleutheroembryos were noticed in treatment 1 mg thymol/L, showing a higher percentage in relation to the control group. The most evident malformations were related to the yolk and eye areas and to the overall body length of the eleutheroembryos.

Exposure for 96 h to thymol caused a decreased yolk (between 1 mg thymol/L and the control group and between 1 mg thymol/L and 0.1 mg thymol/L treatments). Thymol also caused an increased pericardial (between treatment 1 mg thymol/L and the control group), and a decreased eye (between treatment 1 mg thymol/L and the control group). In addition, exposure to treatments of 0.1 and 1 mg thymol/L caused a significant reduction on the body length of 98 h eleutheroembryos in relation to the control group.

The behavioural responses evaluated at 120 hpf after the 96-h exposure to thymol showed a reduced ability to escape the aversive stimulus spending the same amount of time in both halves of the well.

The individuals were also tested for anxiety-like behavioural changes using the light/dark test. Thymol exposure during initial zebra fish development induced no changes on the first light period, but exposure to T3 induced hyperactivity in relation to the control group in the first dark period. However, these differences disappeared in the second light and dark periods.

Assessment and conclusion

Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Dissolved oxygen, validity criteria and analytical verification of test concentrations were not reported.

Assessment and conclusion by applicant:

The study is not acceptable (not reliable).

Zebrafish (*Danio rerio*)

The 96 h acute embryo LC_{50} and the 95% confidence limits were calculated as 2.35 mg thymol/L (C.I. 0.78–5.55) (nominal).

Sublethal developmental effects (e.g. hatching rate, body lengthy, yolk area, heart area and eye area) and behavioural effects were also observed after 96 hours of exposure at nominal concentrations of 0.1 and 1 mg thymol/L, but no significant effects were observed at 0.01 mg thymol/L.

Data point:	CA 9.6.3.4/39 [CA 8.2.1]
Report author	Barbosa Pereira, I., de Sousa Carvalho, E.H., de Brito Rodrigues, L., Dufau Mattos, B., Esteves Magalhães, W. L., Morais Leme,D., Krawczyk-Santos, A.P., Fleury Taveira, S., Rodrigues de Oliveira, G.A.
Report year	2009
Report title	Thymol-Loaded biogenic silica nanoparticles in an aquatic environment: The impact of particle aggregation on ecotoxicity
Report No	-
Document No	Internal reference: Study 25 complementary Environmental Toxicology and Chemistry, Volume 40, Number 2, pages 333–341 (2021)
Guidelines followed in study	Protocols based on OECD 202 and OECD 236
Deviations from current test guideline	Yes (non-standard study type; see details in summary below)
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	No, not conducted under GLP
Acceptability/Reliability:	No

Abstract (copied from original literature)
Thymol, a monoterpene phenol, is used as a natural biocide. To circumvent its chemical instability, we propose use of thymol-loaded biogenic silica nanoparticles ($BSiO_2$#THY NPs); however, the toxicity of this system for aquatic organisms is unknown. Thus, the present study aimed to evaluate the toxicogenetic effects induced by thymol, $BSiO_2$ NP, and $BSiO_2$#THY on *Artemia salina* and zebrafish (*Danio rerio*) early life stages. We also investigated the impact of $BSiO_2$ aggregation in different exposure media (saline and freshwater). Based on the median lethal concentration at 48 h (48h LC_{50}), $BSiO_2$#THY (48h LC_{50} = 1.06 mg/L) presented similar toxic potential as thymol (48h LC_{50} = 1.03 mg/L) for *A. salina*, showing that $BSiO_2$ had no influence on $BSiO_2$#THY toxicity. Because $BSiO_2$ aggregated and sedimented faster in *A. salina* aqueous medium than in the other medium, this NP had lower interaction with this micro-crustacean. Thus, $BSiO_2$#THY toxicity for *A. salina* is probably due to the intrinsic toxicity of thymol. For zebrafish early life stages, $BSiO_2$#THY (96h LC_{50} = 13.13 mg/L) was more toxic than free thymol (96h LC_{50} = 25.60 mg/L); however, $BSiO_2$ NP has no toxicity for zebra fish early life stages. The lower aggregation of $BSiO_2$ in the freshwater medium compared to the saline medium may have enhanced thymol's availability for this aquatic organism. Also, $BSiO_2$#THY significantly induced sub-lethal effects as thymol, and both were genotoxic for zebrafish. In conclusion, although $BSiO_2$#THY still needs improvements to ensure its safety for freshwater ecosystems, $BSiO_2$ NP seems to be a safe nanocarrier for agriculture.

Materials and methods

Test material 1

Name:	thymol
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma-Aldrich
Active substance content:	≥98.5%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

Test organism 1

Species:	Brine shrimp, *Artemia salina*
Strain/clone:	Not reported
Age at study initiation:	Nauplii

Weight/length/height at study initiation: Not relevant
Source: Not reported (hatching dehydrated cysts)
Feeding during test: No
Acclimation: Maintained in artificially prepared seawater (3.5%) at 27 ± 1 °C under continuous light and aeration for 48 h

Test organism 2

Species: zebra fish, *Danio rerio*
Strain/clone: Not reported
Age at study initiation: embryo
Weight/length/height at study initiation: Not reported
Source: Not reported (commercial supplier)
Feeding during test: No food was provided during exposure
Acclimation: Adult organisms were fed daily with commercial dry flake food (TetraColor Flakes®) and live brine shrimp. On the day of the test, zebrafish eggs were collected approximately 30 min after natural mating, rinsed in water, and examined under a stereomicroscope (Leica S9i). Unfertilized or damaged eggs were discarded.

Test conditions 1 (Brine shrimp toxicity assay)

Hardness: Not reported
Test temperature: 27.1 ± 1 °C
pH: Not reported
Dissolved oxygen: Not reported
Salinity: 3.5 % commercial marine salt Ocean Reef® in deionized water
Conductivity: Not reported
Photoperiod: continuous light
Light intensity: Not reported

Test conditions 2 (Fish embryo acute toxicity test)

Hardness: Not reported
Test temperature: 26 ± 1 °C
pH: 7.5 ± 0.5
Dissolved oxygen: 8 ppm
Conductivity: 750 ± 50 µS/cm
Photoperiod: 12 hours light/darkness
Light intensity: Not reported

Test system 1 (Brine shrimp toxicity assay)
Study type: Acute toxicity test
Duration of study: 48 hours
Treatments: 0.63, 1.25, 2.50, 5.00, and 10.00 mg thymol/L (nominal)
Analytical determination of test concentrations: No
Negative control included: Yes (water and DMSO control and solvent control respectively).
Positive control included: Yes (sodium dodecyl sulfate salt, SDS)
Parameters measured: Mortality (immobility)
Validity criteria: Not reported

Test system 2 (Fish embryo acute toxicity test)
Study type: Embryo toxicity test
Duration of study: 96 hours
Treatments: 6.25, 12.50, 25.00, 50.00, and 100.00 mg thymol/L (nominal)
Analytical determination of test concentrations: No

Negative control included: Yes (water and DMSO control and solvent control respectively).
Positive control included: Yes (3,4-dichloroaniline)
Parameters measured: Mortality/development
Validity criteria: Not reported

Brine shrimp toxicity assay
For the test, 20 nauplii divided into 4 groups of 5 organisms each were exposed to nominally 0.63, 1.25, 2.50, 5.00, and 10.00 mg/L of thymol and control. Artificial seawater (3.5% of commercial marine salt Ocean Reef® in deionized water) was used as the negative control, sodium dodecyl sulfate salt (SDS) at 25 mg/L as the positive control, and dimethyl sulfoxide (DMSO) at 0.001% as the solvent control of thymol. Microplates were incubated in the dark in a climatic chamber at 27 ± 1 °C for 48 h. The number of dead nauplii in control and exposed groups was counted after 24 and 48 h. The percentage of mortality (immobility) was compared with the respective negative control. The number of dead nauplii in control and exposed groups was counted after 24 and 48 h. The percentage of mortality (immobility) was compared with the respective negative control.
Fish embryo acute toxicity test
Twenty fertilized eggs per concentration were distributed in a 24-well plate, filled with thymol at nominal concentrations of 6.25, 12.50, 25.00, 50.00, and 100.00 mg/L and controls. Maintenance water was used as the negative control, 3,4-dichloroaniline at 4.0 mg/L as the positive control, and DMSO at 0.001% as the solvent control. The test was carried out in triplicate in a climatized chamber at 26 ± 1 °C under 12-h light for 96 h. Neither food nor aeration was provided during exposure. Embryo development was assessed at 24, 48, 72, and 96 h post fertilization using a stereomicroscope (Leica S9i). Lethal (egg coagulation, no somite formation, non-detachment of the tail from the yolk sac, and no heart beating) and sub-lethal (effects on the eye and body pigmentation, absorption of the yolk sac, hatching rate, swimming bladder inflation, otolith, presence of edemas, spine and tail deformities) parameters were assessed as indicators of embryo development.

GraphPad Prism Software, Ver 5.0, was used for the statistical analysis of all assays performed. Comparisons between different experimental groups were performed with a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Each experimental value was compared with its corresponding control. Statistical significance was accepted at $p < 0.05$. Toxicity was expressed as median effective (EC_{50}) and lethal (LC_{50}) concentrations with 95% confidence limits. Also, tests yielding p values <0.05 were considered significant.

Results
Brine shrimp (*A. salina*) nauplii 48 h LC_{50} = 1.03 mg thymol/L (nominal)
Zebra fish (*D. rerio*) embryo 96 h LC_{50} = 25.60 mg thymol/L (nominal)
Thymol caused significant developmental abnormalities in zebrafish embryos and larvae, such as pericardial and yolk sac edemas, spine deformities, and delayed yolk sac absorption from 72 h of exposure. The relevant EC_{50} values after 72 hours and 96 hours are summarized below.
Summary of sub-lethal effects of thymol on developmental stages of zebrafish embryos after 72 and 96 h of exposure:

Endpoints	72 h EC_{50} ± SD (mg thymol/L)	96 h EC_{50} ± SD (mg thymol/L)
Hatching rate	15.70 ± 5.46	19.07 ± 4.66
Pericardial edema	7.78 ± 1.22	8.32 ± 3.23
Spine deformities	16.45 ± 7.46	13.50 ± 2.51
Yolk sac edema	16.28 ± 3.30	13.60 ± 1.58
Yolk sac absorption	10.75 ± 2.46	11.19 ± 2.19

Assessment and conclusion
Reliability assessment
For full details and justification, please refer to Document KCA 9.4.2/02.
Proposed category: 3 not reliable
Analytical verification of test concentrations was not reported. No validity criteria reported.

Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).

Brine shrimp (*A. salina*) nauplii 48 h LC_{50} = 1.03 mg thymol/L (nominal)
Zebra fish (*Danio rerio*) embryo 96 h LC_{50} = 25.60 mg thymol/L (nominal)
Zebra fish (*Danio rerio*) embryo 96 h EC_{50} values (based on developmental effects) of 8.32 – 19.07 mg thymol/L (nominal).