Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

Margosa, ext. [cold-pressed oil of *Azadirachta indica* seeds without shells extracted with super-critical carbon dioxide]

EC Number: 283-644-7
CAS Number: 84696-25-3

CLH-O-0000001412-86-202/F

Adopted

9 March 2018

9 March 2018

CLH-O-0000001412-86-202/F

OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL

In accordance with Article 37 (4) of Regulation (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted an opinion on the proposal for harmonised classification and labelling (CLH) of:

Chemical name: **Margosa, ext. [cold-pressed oil of *Azadirachta indica* seeds without shells extracted with super-critical carbon dioxide]**

EC Number: **283-644-7**

CAS Number: **84696-25-3**

The proposal was submitted by **Germany** and received by RAC on **14 December 2016.**

In this opinion, all classification and labelling elements are given in accordance with the CLP Regulation.

PROCESS FOR ADOPTION OF THE OPINION

Germany has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **14 March 2017**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **28 April 2017**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Christine Hölzl**

Co-Rapporteur, appointed by RAC: **Pietro Paris**

The opinion takes into account the comments provided by MSCAs and concerned parties in accordance with Article 37(4) of the CLP Regulation and the comments received are compiled in Annex 2.

The RAC opinion on the proposed harmonised classification and labelling was adopted on **9 March 2018** by **consensus**.

Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)

	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATE	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard state-ment Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	No current Annex VI entry										
Dossier submitters proposal	TBD	margosa, ext. [cold-pressed oil of *Azadirachta indica* seeds without shells extracted with super-critical carbon dioxide]	283-644-7	84696-25-3	no classification	-	-	-	-	-	-
RAC opinion	TBD	margosa, ext. [cold-pressed oil of *Azadirachta indica* seeds without shells extracted with super-critical carbon dioxide]	283-644-7	84696-25-3	Aquatic Chronic 3	H412		H412			
Resulting Annex VI entry if agreed by COM	TBD	margosa, ext. [cold-pressed oil of *Azadirachta indica* seeds without shells extracted with super-critical carbon dioxide]	283-644-7	84696-25-3	Aquatic Chronic 3	H412		H412			

GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

Different botanical extracts made from *Azadirachta indica* (Synonym: Margosa, Neem) are used as biocidal active substances and are all covered by the same chemical numerical identifiers (EC: 283-644-7, CAS: 84696-25-3). According to the Guidance for identification and naming of substances under the REACH and CLP Regulations (May 2017, Version 2.1), such different extracts should receive different names. Due to different raw materials and extraction methods (e.g. methods using water or other organic solvents), the constituents vary substantially between different extracts. *Margosa CO_2-ext.* therefore is a substance with unknown or variable composition, complex reaction products or biological materials (UVCB) with unspecified molecular and structural formula.

The following opinion specifically covers Margosa, ext. of cold-pressed oil of *Azadirachta indica* seeds without shells extracted with super-critical carbon dioxide (hereinafter *Margosa CO_2-ext.*). *Margosa CO2-ext*. is a biocidal active substance approved for use as an insect repellent biocide (PT19). The total content of limonoids is 2.7 ± 0.4% including azadirachtin A (the active substance) derived from kernels. The content of azadirachtin A in *Margosa CO_2-ext.* is much lower than for other extracts, which indicates that the removal of shells in the manufacturing process has an important impact on the amount of azadirachtin A. The main constituents (> 90%) of *Margosa CO_2-ext.* are triglycerides of fatty acids (oleic, stearic and linoleic acid). Different batches of *Margosa CO_2-ext.* (including those used to perform (eco-)toxicological studies) were analysed and it is noted that the concentrations of individual constituents were very similar between them.

RAC evaluation of physical hazards

Summary of the Dossier Submitter's proposal

Margosa CO_2-ext. is not a flammable liquid. Furthermore, the classification for pyrophoric liquids is not considered applicable, as the substance is known to be stable in contact with air at room temperature for prolonged periods of time. The substance has no oxidising or explosive properties according to results of method A.21 (67/548 EEC, Annex V) and of method A.14 (92/69/EEC), respectively. Waiving arguments have been provided for the following hazard classes: flammable gases, oxidising gases, gases under pressure, flammable solids, pyrophoric solids/liquids, oxidising solids, flammable aerosols and self-heating substances

Overall, no classification was proposed by the dossier submitter (DS) for the physical hazards.

Comments received during public consultation

No comments were received on physical hazards.

Assessment and comparison with the classification criteria

The tests conducted according to the methods A.14 (explosive), A.21 (oxidising), and A.15 (auto-ignition) demonstrate that *Margosa CO_2-ext.* is not explosive, oxidising or auto-flammable. Moreover, *Margosa CO_2-ext.* comprises mainly fatty acids and limonoids, and none of the

constituents is known to be flammable in contact with water (nor show exothermic reaction under normal condition) indicating that *Margosa CO_2-ext.* is not highly flammable.

RAC agrees with the DS that **classification for physical hazards is not warranted**.

HUMAN HEALTH HAZARD EVALUATION

RAC evaluation of acute toxicity

Summary of the Dossier Submitter's proposal

The DS presented three studies performed in accordance with Good Laboratory Practices (GLP) and OECD Test Guidelines (TG) 423, 402, 403, respectively for acute oral, acute dermal and acute inhalation toxicity.

Oral toxicity

In a limit test, *Margosa CO_2-ext.* was administered by oral gavage to three adult Sprague-Dawley rats of each sex at a dose of 2000 mg/kg bw. No mortality or any other toxic reaction occurred. No abnormalities were found in the animals upon macroscopic post mortem examination 15 days after the treatment. There was no significant effect on body weight. The oral LD_{50} value of *Margosa CO_2-ext.* in rats was established as exceeding 2000 mg/kg bw.

In addition, information on human poisoning incidents following oral ingestion of "Margosa Oil" are available. The information is, however, of limited relevance since the composition of the respective "Margosa oil" is to a large extent unknown, and different starting material with different extraction procedures have been used. "Margosa oil" or "Neem oil" is used as a traditional medicine in Asia and Africa for the treatment of various diseases (e.g. common cold, deworming). Vomiting, drowsiness, convulsions, metabolic acidosis, and encephalopathy are among the reported signs of poisoning. Most of the cases were reported from the use of unrefined and not standardised home remedies lacking any quality control and containing unknown quantities of toxic substances (genuine to the seeds or other parts of the neem tree). For details see the Background Document (Tables 29 and 30, pp. 41-43).

Inhalation toxicity

In an acute inhalation toxicity study, groups of adult Sprague-Dawley rats (5/sex) were exposed by nose-only inhalation to an aerosol of *Margosa CO_2-ext.* for 4 hours at an actual concentration of 5.15 mg/L air which was the highest achievable concentration, limited by the nature of the test substance. The mass median aerodynamic diameter (MMAD) in the particulate aerosol was 8.75 µm and the concentration of particles with a respirable size was found to be only 0.82 mg/L. Under the conditions of this experiment *Margosa CO_2-ext.* caused no mortality. Toxicological symptoms could not be observed during a 14 day observation period. Post mortem examination did not show any macroscopic organ changes. The 4 hour inhalation LC_{50} of *Margosa CO_2-ext.* for male and female rats exceeded 0.82 mg/L air (the respirable fraction).

Dermal toxicity

In an acute dermal toxicity limit study, five adult Sprague-Dawley rats of each sex were exposed to *Margosa CO_2-ext.* by the dermal route. Test material was applied for 24 hours to 10% of each animal's body surface (30 cm^2) at a dose of 2000 mg/kg bw. Animals were observed for the following 15 days. No mortality occurred. No clinical signs of systemic toxicity were noted. The mean body weight gain during the observation period was within the range expected for rats

used in this type of study. No abnormalities were found at macroscopic post mortem examination of the animals. The dermal LD_{50} of *Margosa CO_2-ext.* in rats was >2000 mg/kg bw.

The DS concluded that *Margosa CO_2-ext.* did not warrant classification for acute oral, dermal or inahalation toxicity.

Comments received during public consultation

One comment from a Member State Competent Authority (MS) referred to the oral dose applied in the acute toxicity study (2000 mg/kg bw) that according to that MS cannot directly be used to conclude on a lack of poisoning in humans at this dose. The DS agreed to the comment and highlighted that medical observational data on workers support that *Margosa CO_2-ext.* has no potential for acute toxicity.

Assessment and comparison with the classification criteria

Oral

Classification is required where the LD_{50} is $\leqslant$ 2000 mg/kg bw based on results from animal studies. The acute oral LD_{50} for *Margosa CO_2-ext.* is > 2000 mg/kg bw in the rat and thus does not require classification.

Beside the acute oral toxicity study, the DS summarised data from human poisoning incidents. Indeed, "Margosa Oil" is used as a traditional medicine in Asia and Africa to cure various diseases (e.g. cold, deworming). In the background document (pp. 41-43, Tables 29 and 30) these poisoning incidents are described and show that the accidental ingestion of "Margosa Oil" may lead to signs of vomiting, drowsiness, convulsions, metabolic acidosis, and encephalopathy.

RAC notes that no specific human poisoning case is reported for *Margosa CO_2-ext.* (covered by the present classification proposal). Since varying extractions methods, starting material (e.g. seeds, bark) and impurities clearly lead to different compositions, the poisoning incidents summarised by the DS do not constitute relevant information to be taken into account for classification purposes.

Inhalation

Classification is required where the LC_{50} value of $\leqslant$ 5 mg/L (dusts and mists). The highest achievable concentration was 5.15 mg/L air. The concentration of particles with a respirable size was found to be only 0.82 mg/L. This concentration did not cause mortality in rats and no toxicological symptoms were observed during a 14 day observation period. Thus, the 4h LC_{50} (dust/solid aerosols) to rats for *Margosa CO_2-ext.* is > 0.82 mg/l, which is reported to be the maximum technically achievable concentration.

Dermal

Classification is required where the LD_{50} is $\leqslant$2000 mg/kg bw. The LD_{50} in rats was > 2000 mg/kg bw.

Conclusion

RAC agrees with the DS that **no classification is warranted for acute oral, dermal or inhalation toxicity**.

RAC evaluation of specific target organ toxicity – single exposure (STOT SE)

Summary of the Dossier Submitter's proposal

The DS did not propose to classify *Margosa CO_2-ext*. as STOT SE 1 or 2 considering that non-lethal adverse effects were not reported after acute exposure. In addition, based on the submitted data, the DS concluded that *Margosa CO_2-ext.* does not meet the criteria to be classified as STOT SE 3 for respiratory tract irritant or narcotic effects.

Comments received during public consultation

No comment was received during public consultation.

Assessment and comparison with the classification criteria

No signs of organ toxic effects were observed in the acute oral, dermal or inhalation toxicity studies with rats exposed to *Margosa CO_2-ext*. The animal data submitted did not provide evidence for respiratory tract irritation or narcotic effects.

Information on human poisoning incidents following oral ingestion of "Margosa Oil" are considered by RAC as of limited relevance as explained in the section above. Besides the human poisoning, data from medical observations on workers involved in the production of the Margosa extract were negative over a three-year observation period.

Based on this information RAC agrees with DS that **no classification is warranted for STOT SE**.

RAC evaluation of skin corrosion/irritation

Summary of the Dossier Submitter's proposal

The DS provided results from a dermal irritation study conducted according to OECD TG 404. Three male Himalayan rabbits were exposed via the dermal route to 0.5 mL of *Margosa CO_2-ext.* each. The test material was applied for 4 hours to the clipped skin of the back, using a semi-occlusive dressing. No symptoms of systemic toxicity were found and no mortality occurred. Exposure to *Margosa CO_2-ext.* did not result in any skin reactions. Based on these results, *Margosa CO_2-ext.* is not regarded as a skin irritant.

Furthermore, additional repeated dose toxicity studies conducted via the dermal route are summarised by the DS.

In a 28-day study, rats were exposed dermally to *Margosa CO_2-ext*. at dose levels of 100, 500, 1000 mg/kg bw/d (semi-occlusive). A dose-dependent slight to severe erythema with and without desquamation was observed transiently for about 3-4 days, but resolved spontaneously despite continuing treatment.

In a pre-natal developmental toxicity study (PNDT), rabbits were exposed dermally to *Margosa CO_2-ext.* at dose levels of 0, 50, 200, 800 mg/kg bw/d (semi-occlusive). The effects were also dose-dependent and continued to be present for the duration of the study at the two highest doses (200 and 800 mg/kg bw/d). After the first application of 800 mg/kg bw/d (corresponding to 4,5 mg/cm^2) *Margosa CO_2-ext.* to female rabbits, only one of a total of 20 females showed

very slight erythema. The DS did not consider these effects as sufficient for classification and labelling as a skin irritant.

In addition, labelling as EUH066 (repeated exposure may cause skin dryness or cracking) was not relevant according to the DS because the observed effects were not characterised as dryness of the skin. *Margosa CO_2-ext.* has a high content of fatty acids, therefore dryness of the skin is not expected.

In summary and based on the submitted data, the DS concluded that *Margosa CO_2-ext.* does not meet the criteria for skin irritation/corrosion according to the criteria of the CLP Regulation. These repeated dose toxicity studies will be further discussed in the section on repeated dose toxicity.

Comments received during public consultation

No comment was received during public consultation.

Assessment and comparison with the classification criteria

Skin irritation means the production of reversible damage to the skin following the application of a test substance for up to 4 hours.

In a standard skin irritation assay in which rabbit skin was exposed to 0.5 mL *Margosa CO_2-ext.* for 4 hours, no skin reaction was observed. In the repeated dose toxicity studies the irritation effects became apparent after repeated application. Skin irritation findings in dermal rat studies were transient despite of continuing treatment. In rabbits, the effect were dose-dependent and continued to be present for the duration of the study. After first application of 800 mg/kg bw (corresponding to 4.5 mg/cm^2) only one out of 20 females showed very slight erythema, which is not considered to be sufficient for classification.

Therefore, RAC concurs with the DS that *Margosa CO_2-ext.* **does not warrant classification for skin irritation**.

RAC evaluation of serious eye damage/irritation

Summary of the Dossier Submitter's proposal

The eye irritation potential of *Margosa CO_2-ext.* was tested in a standard guideline study (OECD TG 405) in which 0.1 mL of *Margosa CO_2-ext.* was instilled into the conjunctival sac of the right eyes of three adult male Himalayan rabbits. The test substance did not cause clinical signs or mortality but resulted in a transient grade 1 corneal opacity in two out of three animals 1 h after application and had resolved within 24 hours. Based on these results, *Margosa CO_2-ext.* is not regarded as an eye irritant.

Margosa CO_2-ext. exhibited very slight and reversible irritating potential to the eye. According to the study reports, the severity of findings did not reach the critical thresholds to be classified as eye irritant according to the DS.

Comments received during public consultation

No comment was received during public consultation.

Assessment and comparison with the classification criteria

A substance should be classified for reversible eye effects (Category 2) if, in at least two of three tested animals, a positive response is observed of corneal opacity ≥1 and/or iritis ≥1 and/or conjunctival redness ≥2 and/or conjunctival oedema ≥2; calculated as mean score following grading at 24, 48 and 72 hours and which are fully reversible.

The findings of the eye irritation study demonstrate that two of three tested animals showed grade 1 corneal opacity 1 hour after application. These effects resolved after 24 hours. Therefore, *Margosa CO_2-ext.* exhibits very slight and reversible irritation potential. However, the criteria to classify *Margosa CO_2-ext.* for eye damaging/irritating effects are not met.

RAC concurs with the DS´s proposal that *Margosa CO_2-ext.* **does not require classification for serious eye damage or for eye irritation**.

RAC evaluation of skin sensitisation

Summary of the Dossier Submitter's proposal

In a test for dermal sensitisation according to Magnusson and Kligman (OECD TG 406), 20 young adult female albino guinea pigs were intradermally injected with 50% (w/v; vehicle: coconut oil) of *Margosa CO_2-ext.* with Freund's Complete Adjuvant and dermally exposed to 50% (w/v, vehicle coconut oil) *Margosa CO_2-ext*. Ten control animals were treated similarly, but with vehicle alone. Two weeks after the epidermal application, all animals were challenged with 50% *Margosa CO_2-ext.* in coconut oil. In this study, *Margosa CO_2-ext.* produced no evidence of skin sensitisation. The DS did not propose to classify *Margosa CO_2-ext*. for skin sensitisation.

Comments received during public consultation

No comments received.

Assessment and comparison with the classification criteria

No signs of sensitisation were seen in the Magnusson and Kligman study according to TG 406. The doses applied were in accordance with OECD TG 406, i.e. the concentration of *Margosa CO_2-ext*. used for induction exposure caused moderate skin irritation and the challenge exposure was a non-irritant dose.

Since no signs of sensitisation were observed, RAC agrees with the DS that **no classification for skin sensitisation is warranted**.

RAC evaluation of specific target organ toxicity– repeated exposure (STOT RE)

Summary of the Dossier Submitter's proposal

The DS summarised two oral feeding studies (28- and 90-day studies) and two dermal toxicity studies (28-day study, prenatal development toxicity study).

Oral

The only altered observation in a 28 day rat feeding study with *Margosa CO_2-ext.* (dose levels: males: 0, 102, 520, 1047 mg/kg bw/d, females: 0,96, 481, 992 mg/kg bw/d) was a slight increase of relative liver weight in males at the mid and high dose levels and in females at the top dose. This effect was not considered adverse by the DS because the increases in liver weight were ≤10% in males and ≤15 % in females and histopathologic correlates were lacking. Moreover, the organ weight increase was reversible within a two-week recovery period.

In the 90 day rat feeding study with *Margosa CO_2-ext.* (dose levels: males: 0, 145, 436, 962 mg/kg bw, females: 0, 147, 442, 979 mg/kg bw), the top dose induced an increase in liver weight in males and females, without any histopathological correlates, which was reversible within the 4 week recovery period. Since liver weight increases were ≥15% in both sexes, the effect was considered adverse by the DS.

However, the DS concluded that these findings did not constitute significant organ damage in line with the CLP criteria since they were not observed in rats at dose levels within the respective guidance values for STOT RE 2 i.e. 30 < C ⩽ 300 mg/kg bw/d (28-day study) or 10 < C ⩽ 100 mg/kg bw/d (90-day study).

Dermal

In the 28 day rat study with *Margosa CO_2-ext.* (dose levels: 100, 500, 1000 mg/kg bw/d, semi-occlusive exposure, 6 hours per day, 7 days per week, no vehicle), no systemic effects were observed. Slight to well-defined erythema with or without desquamation were observed in all males and females exposed to 500 mg/kg bw/day towards the end of the first week of application (days 5-7). In the highest dose groups receiving 1000 mg/kg bw/day incidence and time of appearance were similar to the mid-dose group (days 5-8), but the grading ranged from slight to severe. In the table below the skin effects (6 hours after application) are summarised. The skin irritating effects disappeared during the second week of dosing and no further changes became apparent after that time point.

Table: Number of affected animals at day 5-11 six hours after bandage removal (28 d rat study)

Day	Sex	Finding	Dose group mg/kg bw/d			
			0	100	500	1000
5	M F	Erythema	0	1*slight	5*slight 5*slight	2*slight, 3*well-defined 2*slight
	M F	Desquamation	-	-	3 1	5 1
6	M F	Erythema	-	-	4*slight 4*slight	4*well defined, 1*moderate to severe 2*slight, 1*well defined
	M F	Desquamation	-	-	4 2	5 1
7	M F	Erythema	-	-	2*slight 3*slight	4*slight, 1*well-defined 2*slight
	M F	Desquamation	-	-	2 2	5 1
8	M F	Erythema	-	-	-	3*slight 3*slight

Day	Sex	Finding	Dose group mg/kg bw/d			
			0	100	500	1000
	M F	Desquamation	-	-	-	3 1
9	M F	Desquamation			-	2
10	M F	Desquamation			-	2
11	M F	Desquamation			-	2

*Skin reaction (slight, well defined or moderate to severe)

Local skin irritating effects were also observed in a prenatal toxicity study in rabbits in all dose groups (0, 50, 200, 800 mg/kg bw/d, semi-occlusive exposure, 6 hours per day, no vehicle used, 10% of body surface, on day 26-28 post mating) and are considered by DS as adverse from a dose level of 200 mg/kg bw/d onwards. Irritation scores in the lowest dose (50 mg/kg bw/d) were low with only a few females affected. The number of females with irritation and the observed scores for irritation and oedema were clearly below the classification criteria for skin irritation. Therefore, the slight irritating effects in the lowest dose group (50 mg/kg bw/d) were not regarded as adverse. Moderate local skin effects with persistent erythema and oedema were observed after application of 200 mg/kg bw/d *Margosa CO_2-ext.* at the end of the study. Very slight erythema/oedema appeared on day 2 and 5 of treatment in one female whilst on day 16, erythema (with an average score of 1.90) were evident in all animals. Further skin changes in a few animals in consequence of treatment were desquamation, fissuration and scabs. At the highest concentration (800 mg/kg bw/d) very slight erythema appeared after the first application in one female. Persistent erythema (average score: 2.56) and oedema (average score: 2.71) were evident in all females from day 16 onwards. With prolonged treatment erythema and oedema became therefore more severe in individual females. These effects were accompanied by desquamation, fissuration and scabs. The macroscopic examination at terminal sacrifice revealed a dose related increase of red coloration and scabs in a few animals.

Table: Local skin irritation observation in prenatal developmental toxicity study in female rabbits after dermal application

Day of treatment	Finding	Dose groups (mg/kg bw/day)			
		0	50	200	800
8	Erythema: Score (Incidence %)	0	0.66 (52.9)	1.14 (85.7)	2.00 (100)
	Oedema: Score (Incidence %)	0	0.14 (14.3)	0.49 (35.7)	1.83 (91.4)
16	Erythema: Score (Incidence %)	0	1.04 (88.6)	1.90 (100)	2.56 (100)
	Oedema: Score (Incidence %)	0	0.53 (41.4)	1.64 (91.4)	2.71 (100)
23	Erythema: Score (Incidence %)	0	0.99 (84.3)	1.86 (96.9)	2.66 (100)
	Oedema: Score (Incidence %)	0	0.37 (31.4)	1.49 (95.4)	2.81 (100)

The DS regarded the severity and duration of the irritation in rats and rabbits as being not sufficient for classification as STOT RE for dermal exposure. Irritant effects observed in the highest dose group were above the concentration required for STOT RE 2 according to the CLP Criteria (highest dose group: 800 mg/kg bw/d, guidance value for STOT RE 2: 60 < C ≤ 600 mg/kg bw/d for a 28-day study).

As no signs of toxicity were observed in addition to skin irritation, classification for STOT RE for the dermal route was considered not justified by the DS.

Comments received during public consultation

No comments received during public consultation.

Assessment and comparison with the classification criteria

In the two oral repeated dose toxicity studies in rats, the only findings described are changes in liver weight. In the 28 day study the liver weight change (males: ≤10%, females: 13%, relative) was only observed at levels far above the guidance values for STOT RE 2 classification. This observation was reversible (14 day recovery group) and without histopathological correlates. In the 90 day repeated dose toxicity study liver weight change was induced in the top dose of approximatively 960 mg/kg bw/d (above 15%). The changes were reversible within the 4 week recovery period and no histopathological correlates were observed. Although, the effects are considered as adverse they occurred only far above the guidance values for STOT RE 2 classification (see Table).

Table: Overview of main findings in repeated dose toxicity studies and comparison with guidance values

Study	Observed effect	Effect level, mg/kg bw/day	Guidance values (STOT RE 2), oral rat mg/kg bw/day
28 day, rat, oral	Increased liver weight: M: ≤10% F: 13%	436, 1047 992	30 < C ≤ 300
90 day, rat, oral	Increased liver weight: M/F: absolute 13.5% M: relative 14.6 % F: relative 18.1 %	~ 960	10 < C ≤ 100

M; males, F; females

In the 28 day dermal toxicity study (OECD TG 410) dose-dependent skin irritating effects have been observed at days 5-11, the effects were not apparent during the second week of dosing and no further changes were observed after that time point. The most pronounced effects were observed at day 6 at a dose level of 1000 mg/kg bw, in which 8 out of 10 animals were affected (slight (n=2), well defined (n=5) to moderate to severe skin reactions (n=1)) accompanied with desquamation (n=6).

Skin irritation effects have been also observed in a prenatal developmental toxicity study (OECD 414), in which pregnant rabbits were exposed to 0, 50, 200, 800 mg/kg bw/d (semi-occlusive exposure, 6 hours per day, no vehicle used) from GD 6-28. For the severity of damage the responses are evaluated according to the Draize score ranking from '0' ('no response') up to '4' ('severe response'). Most pronounced effects were observed on day 16 and on day 23 of application at the highest dose group (800 mg/kg bw/d). The erythema and oedema score at day

16 and 26 was 2.56 and 2.71 (incidence 100), and 2.66 and 2.81 (incidence 100), respectively. The erythema and oedema score at the dose level of 200 mg/kg bw/d was 1.9 and 1.64 at day 16, and 1.86 and 1.49 at day 23, indicating that observed effect does not worsen during the last week of exposure.

RAC agrees with the DS that the skin irritating effects observed in the repeated dose toxicity studies carried out with rats (28d study) and rabbits (prenatal developmental toxicity study) are considered dose dependent, however the effects were less severe or did not worsen at the end of the studies. The severity of the observed effects, which are pronounced at the highest dose levels (800 mg/kg bw/d (rabbit) to 1000 mg/kg bw/d (rat)) do not warrant classification for STOT RE effects.

Therefore, RAC concurs with the DS submitter that the adverse skin irritating effects observed are not severe enough.

RAC agrees with the DS that based on the observations described in the oral and dermal repeated dose toxicity studies **no classification for STOT RE is warranted**.

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

In vitro data

Margosa CO_2-ext. was tested in different *in vitro* assays (reverse bacterial mutation assay, Mammalian chromosome aberration/ cell gene mutation test and erythrocyte MN test).

In five strains of *Salmonella typhimurium* (Ames Test) with or without metabolic activation *Margosa CO_2-ext.* did not induce mutations at concentrations up to 5000 µg/plate.

In Chinese hamster lung fibroblast cells (V79 cells) a slightly increased incidence of structural chromosomal aberrations at the highest concentration of 5000 µl/mL in the presence of metabolic activation was detected. In a second experiment a slight increase in the aberration frequency was observed for the early sampling time only but not at the late sampling time. The changes observed were not dose related, i.e. were only observed at the highest concentration tested, where cytotoxicity was observed. Nevertheless, the results with metabolic activation were regarded as positive due to statistical significance.

In a gene mutation assay in Chinese hamster V79 cells *in vitro* (V79/HPRT), a significant increase in mutant frequency occurred at two experimental points at an intermediate concentration level (1.1 µl/mL) in the 1st experiment with metabolic activation and in the 2nd experiment without metabolic activation. Since these increases in either the presence or absence of metabolic activation occurred only in one of the two independent experiments (i.e., the effect was not reproducible) and due to the absence of dose-response, the observed increases were considered coincidental and therefore regarded as negative. In conclusion, the HPRT test result was considered negative for *Margosa CO_2-ext*.

In vivo data

Margosa CO_2-ext. was tested in an *in vivo* mammalian erythrocyte micronucleus test (OECD TG 474). *Margosa CO_2-ext.* was not genotoxic in the *in vivo* micronucleus test in mice exposed at dose levels up to and including 2000 mg/kg. At the two tested sampling times no increase of micronucleated polychromatic erythrocytes (PCE) was observed. The positive control cyclophosphamide induced significant increases in micronucleated PCEs.

In conclusion, based on the results of *in vitro* and *in vivo* genotoxicity tests, including adequate positive and negative study controls, it is considered unlikely that *Margosa CO_2-ext.* poses a genotoxic risk to humans.

Comments received during public consultation

No comments received during public consultation.

Assessment and comparison with the classification criteria

There are no human data available for *Margosa CO_2-ext*.

A slight increased incidence of structural chromosomal aberration were detected in V97 lung fibroblast cells at the highest concentration (5000 µl/mL) in the presence of metabolic activation, however the changes are not considered dose dependent and occurred at concentrations were cytotoxicity was observed. The increased mutant frequency at intermediate concentration levels (1st experiment with and 2nd experiment without metabolic activation) were not reproducible and are considered coincidental. The *in vitro* tests were negative.

The *in vivo* mammalian micronucleus test did not indicate any genotoxic potential at dose levels up to 2000 mg/kg bw.

RAC concurs with the DS that **no classification for germ cell mutagenic effects is warranted.**

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

No chronic or carcinogenicity study is available for *Margosa CO_2-ext.* No human information has been submitted by the applicant. Therefore no classification is proposed by the DS due to data lacking.

Comments received during public consultation

A comment has been submitted by MS. The author clarified that in the 90 day repeated dose toxicity study adverse effects at the highest dose (liver weight change) were detected and that the statement of the DS in the background document that no adverse effects have been observed is not appropriate. DS clarified the contradiction and agreed to MS comment.

Assessment and comparison with the classification criteria

RAC concurs with the DS that **classification for carcinogenicity is not warranted due to the absence of relevant data**.

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

Margosa CO_2-ext. was tested in a dermal prenatal developmental toxicity study (PNDT; OECD TG 414). No generation toxicity study has been conducted.

After dermal application of the test substance (dose levels: 0, 50, 200, 800 mg/kg bw/d) to pregnant rabbits, local skin irritation occurred in all dose groups and was considered adverse from 200 mg/kg bw/d onwards (see sections above).

Furthermore, a slight, dose related tendency towards reduction of maternal body weight gain was observed. Net body weight loss (body weight at necropsy minus gravid uterus weight and minus body weight at Day 0) was observed in mid and high dose females without statistical significance. Therefore the reduced body weight gain is not considered biologically relevant and is not regarded as an adverse effect.

No embryo- or foetotoxicity was apparent. Small foetuses in all groups, including the control, were found mostly in litters of larger size and it appears that the higher proportion of such litters, rather than the treatment, contributed to the slightly increased number of small foetuses in the high dose group. Thus, the maternal and the developmental NOAEL is 800 mg/kg bw/d.

A prenatal toxicity study in rodents has not been submitted. Whether the rat or the rabbit is the more sensitive species in developmental toxicity studies depends on the test substance, its toxicokinetics and mode of action and cannot be generalised. In the case of *Margosa CO_2-ext.* it appears that adult rabbits (pregnant rabbits) are slightly more sensitive to the local effects of repeated dermal exposure than rats. On the basis of the submitted data sensitivity towards systemic effects appears to be comparable between the rabbit and the rat. No adverse effects were observed in reproductive organs in repeat dose toxicity studies.

In the PNDT rabbit study, no findings in offspring relevant for a possible classification for developmental effects were reported. Overall, the data were considered conclusive by the DS but not sufficient to trigger classification for such effects.

Reproductive toxicity on sexual function and fertility cannot be addressed due to absence of data. No data are available to judge whether there are specific effects on or via lactation.

Comments received during public consultation

One MS commented that "anti-fertility (contraceptive and abortive)" effects of oils and extracts are reported in studies with various mammalian species including humans and if relevant the data should be more deeply described and discussed. The DS clarified that those results are not applicable to *Margosa CO_2-ext.*, since adverse effects are reported in particular for oral intake of large amounts of neem preparations with unknown compositions.

Assessment and comparison with the classification criteria

According to the CLP Regulation, reproductive toxicity includes adverse effects on sexual function and fertility in adult males and females, developmental toxicity in the offspring as well as effects on or via lactation.

RAC concurs with the DS that reproductive effects on sexual function and fertility cannot be addressed due to absence of data. The observed antifertility effects of oils and extracts in studies with various mammals species (including) humans cannot be considered for classification purpose. RAC agrees with DS's conclusion that no data are available to judge whether there are specific effects on or via lactation.

RAC agrees that there are no signs of developmental toxicity effects in the PNDT study with rabbits. The observed small changes of foetus weights can be regarded as not substance related. No further data have been submitted. The developmental toxicity cannot be concluded due to limited data available.

RAC supports the conclusion from the DS that classification for reproductive toxicity cannot be assessed due to the absence of suitable data for sexual function and fertility, development and lactation.

ENVIRONMENTAL HAZARD EVALUATION

RAC evaluation of aquatic hazards (acute and chronic)

Summary of the Dossier Submitter's proposal

The DS initially proposed no classification as hazardous to the aquatic environment. However, following the public consultation, the DS changed their position to consider *Margosa CO_2-ext.* as not rapidly degradable which, in combination with aquatic toxicity tests, results in a classification proposal as Aquatic Chronic 3 – H412.

Degradation

Three hydrolysis studies were run on the purified limonoid constituents azadirachtin, nimbin and salannin at different pH and temperature. The susceptibility of the limonoids to hydrolysis under standard outdoor conditions (12°C, pH = 7) decreases from azadirachtin (DT_{50} = 363.9 h) and nimbin (DT_{50} = 1783.2 h) to salannin (DT_{50} = 22063.2 h). The hydrolysis of azadirachtin, performed with a method based on basic principles of OECD TG 111 and EC C.7, is pH-dependent as indicated by a significant increase in the rate of degradation with increasing pH (recalculated half-lives at 12°C: 1731 h at pH=5; 364 h at pH=7; 76 h at pH=8). Also the hydrolysis of nimbin and salannin, both carried out according to OECD TG 111 and EC C.7, are influenced by pH (for nimbin recalculated half-lives at 12°C: 1481 h at pH=5, 1783 h at pH=7, 1995 h at pH=9; for salannin recalculated half-lives at 12°C: 16577 h at pH=5, 22063 h at pH=7, 6649 h at pH=9). The DS concludes that hydrolysis might contribute to the degradation of azadirachtin and nimbin under environmental conditions, whereas hydrolysis processes are negligible for salannin.

No photodegradation study in water was performed since the UV/VIS absorption spectrum of *Margosa CO_2-ext.* shows no significant absorption above 290 nm.

The ready biodegradability of *Margosa CO_2-ext.* was determined in a Closed Bottle Test according to OECD TG 301D and Directive 92/69/EEC using activated sludge as inoculum. In this test *Margosa CO_2-ext.* was degraded to 60.5% within 7 days and 73.5 % after 28 days. In summary, the DS considers *Margosa CO_2-ext.* as readily biodegradable, fulfilling the 10-day window criterion. It is therefore considered to be rapidly degradable for classification purposes.

In contrast to the initial assessment above resulting in no proposal for classification as hazardous to the aquatic environment, the DS in response to the comments received during PC (see below) revised their conclusion on the environmental classification. As a consequence and despite the total content of limonoids determined to be only 2.7 ± 0.4% w/w, the DS considered *Margosa CO_2-ext.* as not rapidly degradable for classification purposes based on the results of a ready biodegradability test (according to OECD TG 301F), conducted with the constituent azadirachtin demonstrating only 21.6% mineralisation in 28 days.

Bioaccumulation

No measured data on bioaccumulation is available but estimated BCF values calculated for the limonoid constituents azadirachtin, nimbin and salannin are included in the CLH report.

The bioconcentration factors in aquatic organisms (fish) were calculated using the BCFWIN Programme v2.15, 2000 by US_EPA. This QSAR estimation was conducted on the basis of measured log Kow values of the three limonoid constituents, ranging from 1.3 to 3.5. The obtained BCF-values (see table below) indicate a low potential of the three constituents of *Margosa CO_2-ext.* in aquatic organisms. All the resulting estimated BCF values were below 100 L/Kg wet weight and the DS concludes no concern for bioaccumulation.

Table: BCF-values for three constituents of Margosa CO_2-ext.

Method	Results	Remarks	Reference
QSAR estimation (BCFBAF) BCFWIN Programme v2.15, 2000 by US_EPA	BCF_{fish}= 3.35 L/Kg wwt Azadirachtin	Based on measured log Kow = 1.3	Fàbregas, 2006
QSAR estimation (BCFBAF) BCFWIN Programme v2.15, 2000 by US_EPA	BCF_{fish}= 44.3 L/Kg wwt Nimbin	Based on measured log Kow = 3.0	Fàbregas, 2006
QSAR estimation (BCFBAF) BCFWIN Programme v2.15, 2000 by US_EPA	BCF_{fish}=94.69 L/Kg wwt Salannin	Based on measured log Kow = 3.5	Fàbregas, 2006

Ecotoxicity

Short-term aquatic toxicity data are available for all three trophic levels, with long-term toxicity data only available for algae. A summary of the relevant information is provided in the following table (the key endpoints used in hazard classification are highlighted in bold). All studies were performed under (semi-)static conditions with results expressed in terms of mean measured concentrations (mmc).

Table: Summary of relevant information on aquatic toxicity

Method	Test organism	Endpoint	Toxicity values in mg a.s./L	Reference
OECD TG 203 (EU C.1) semi-static	*Oncorhynchus mykiss* (Rainbow trout)	96-h LC_{50} (mortality)	**11.2** (c.i.: 9.7 – 12.8 mg/L)	Anonymous, 2005a
OECD TG 202 (EU C.2) semi-static	*Daphnia magna*	48-h EC_{50} (immobilisation)	> 128	Stäbler, 2005b
OECD TG 201 (EU C.3) static	*Desmodesmus subspicatus*	72-h ErC_{50} 72-h NOErC (growth inhibition)	> 237 1.05	Dengler, 2005b

No lead substance is defined for this botanical extract and the effects assessment is mainly based on results for the whole extract. While it is not known which constituents mainly contribute to

the intended efficacy as a repellent, it can also be deduced that mainly the limonoids should be regarded as relevant for (potential) adverse effects on non-target organisms in the environment: The limonoids from the neem tree are known to act as anti-feeding and growth disruptor toward insects. Therefore the accompanying chemical analysis in the available effect studies is based on salannin as the limonoid with the highest proportion in *Margosa CO_2-ext.*. This also applies to recalculations to mean measured concentrations, where required.

One valid and reliable short-term toxicity study with rainbow trout (*O. mykiss*) is provided for *Margosa CO_2-ext.* in a 96-h semi-static test according to OECD TG 203. Six concentrations between 6.25 and 65.5 mg/L (nominal) were tested. Based on salannin, mean measured concentrations of the test substance were below 80 % of nominal. According to the results of the test, the LC_{50} of the test item (*Margosa CO_2-ext.)* after 96-h was determined to be 14.6 mg/L (nominal), equivalent to 11.2 mg/L mean measured concentration (95 % c.i.: 9.7 – 12.8 mg/L).

One valid and reliable short-term toxicity study with aquatic invertebrates is available for *Margosa extract* (100% purity of test material). A 48-h toxicity test was performed with *D. m*agna, according to OECD TG 202. Immobilisation was assessed at six concentrations tested between 10 and 189 mg a.s./L (nominal). The concentrations of salannin were 67.9 % of nominal values and were therefore recalculated to mean measured concentrations of the test substance. According to the test results, the 48-h EC_{50} was determined to be > 189 mg/L (nominal) equivalent to 128 mg/L mean measured concentrations.

One 72-h growth inhibition study with the green algae *D. subspicatus* was performed with *Margosa CO_2-ext.* The study, conducted in accordance with OECD TG 201 (1984 and 2002), covers both acute and long-term endpoints. Concentrations of salannin were below 80 % of nominal at the end of the study (between 24.5 – 87.5 %) and therefore concentrations of *Margosa CO_2-ext.* had to be recalculated to mean measured concentrations. After 72-h, a NOErC of 1.05 mg/L and an ErC_{50} > 237 mg/L was calculated based on growth rate and mean measured concentrations.

Based on the information above, the DS concluded not to classify *Margosa CO_2-ext.* as hazardous to the aquatic environment. In contrast to the initial assessment, the DS in response to the comments received during PC (see below) revised their conclusion on the environmental classification based on the results of a ready biodegradability test (according to OECD TG 301F), conducted with the constituent azadirachtin demonstrating only 21.6% mineralisation in 28 days, resulting in a proposed classification as Aquatic Chronic 3 – H412.

Comments received during public consultation

Two MSs provided public comments. One MS supported the initial proposal not to classify *Margosa CO_2-ext.* for the environment.

The other MS had specific comments and questions on the three following points:

- Further clarity on the total percentage of limonoids and individual key active constituents, because their concentrations could affect the overall environmental fate, toxicity and classification.
- Further clarification on the available information on the degradability of the key active constituents and the consequence on the conclusion on the rapid degradability of the substance according to CLP criteria.
- The applicability of the surrogate approach on the basis of the acute aquatic toxicity information for fish as the most acutely sensitive organism. In addition, consideration of

available aquatic toxicity data from other Margosa extracts was suggested, such as Chironomid data.

Finally, the MS also mentioned the use of mixture classification calculations to consider the likely contribution of the individual active constituents to the overall extract chronic aquatic toxicity, despite the uncertainties on their composition and degradability (see also section "Additional key elements" in the background document to this opinion).

Assessment and comparison with the classification criteria

Degradation

Based on the results of an OECD TG 301D test using *Margosa CO_2-ext.*, the substance was demonstrated to be readily biodegradable. However, the study does not allow to draw conclusions if and to what extent the constituents undergo degradation. Azadirachtin, one of the key active constituents of *Margosa CO_2-ext.*, is considered as not being readily biodegradable based on an OECD TG 301F test, a study that has been submitted within the biocidal approval process of Margosa ext. [from the kernels of *Azadirachta indica* extracted with water and further processed with organic solvents] for the use as insecticide (PT 18). For the other limonoids (nimbin and salannin), information on hydrolysis half-life values is available indicating that they are above the trigger of 16 days in the pH range 4-9.

According to the Guidance on the Application of the CLP criteria (version 4.1, June 2015), the biodegradation of a complex substance presents general interpretation problems where each constituent of the substance may behave differently. The guidance also states that a complex substance, such as UVCBs should be regarded as not rapidly degradable if the constituents that are notrapidly-degradable constitute a significant part of the substance, e.g. more than 20 %, or for a hazardous constituent an even lower content. Since no lower limit is given in the guidance, RAC supports the DS's proposal by applying the CLP cut off-values to trigger the consideration for classification (CLP, Annex I.1.1.2.2).

RAC notes that further supplemental information available within the BPC WG ENV documents supported the RAC opinion conclusion to consider *Margosa CO_2-ext.* as not rapidly degradable for classification purposes. In particular, calculations for ready biodegradability using QSAR (BIOWIN v4.10) are available for the limonoids azadirachtin (A and B), nimbin and salannin resulting in not being readily biodegradable for the relevant constituents.

On the basis of azadirachtin not being readily biodegradable and lack of data for the other limonoids, RAC considers *Margosa CO_2-ext.* as not rapidly degradable for classification purposes.

Bioaccumulation

No measured BCF_{fish} data is available. The measured log K_{OW} for the limonoids is below the CLP trigger value of ≥ 4. Therefore, RAC agrees with the DS's conclusion that the substance has a low bioaccumulation potential.

Aquatic toxicity

Acute aquatic hazard

No acute toxicity below the CLP trigger value of $L(E)C_{50}$ ≤ 1 mg/L was found. The lowest acute value is the 96-h LC_{50} of 11.2 mg/L (mmc) from an acute toxicity test with *O. mykiss.* Therefore *Margosa CO_2-ext.* does not fulfil the criteria and **no classification is proposed for acute aquatic hazards**.

Chronic aquatic hazard

With regard to chronic toxicity data, the NOErC = 1.05 mg/L for algae slightly exceeds the trigger for chronic hazard classification.

However, RAC agrees with the DS's revised assessment considering the substance as not rapidly degradable. As a consequence this **warrants classification as Aquatic Chronic 3 – H412** based on the fish 96-h LC_{50} of 11.2 mg/L (> 10 to ≤ 100 mg/L) for not rapidly degradable substances.

Additional references

European Food Safety Authority; Conclusion on the peer review of the pesticide risk assessment of the active substance azadirachtin. EFSA Journal 2011;9(3):1858. [76 pp.]. doi:10.2903/j.efsa.2011.1858. Available online: www.efsa.europa.eu/efsajournal.htm

Assessment Report, *Margosa Extract*, cold-pressed oil of *Azadirachta indica* seeds without shells extracted with super-critical carbon dioxide Product-type 19 (Repellents and attractants), 2017

Assessment Report, *Margosa Extract* Product-type 18 (Insecticides, Acaricides and Products to control other Arthropods), 2011

ANNEXES:

Annex 1 The Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH report prepared by the Dossier Submitter; the evaluation performed by RAC is contained in 'RAC boxes'.

Annex 2 Comments received on the CLH report, response to comments provided by the Dossier Submitter and RAC (excluding confidential information).