CLH report

Proposal for Harmonised Classification and Labelling

**Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2**

Substance Name:Margosa, ext.

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

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

Index Number: **-**

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Part A.

Part A.

1 PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

1.1 Substance

Table 1: Substance identity

Substance name:	***Margosa ext.***
EC number:	***283-644-7***
CAS number:	***84696-25-3***
Annex VI Index number:	**-**
Degree of purity:	***100% w/w***
Impurities:	***confidential***

1.2 Harmonised classification and labelling proposal

Table 2: The current Annex VI entry and the proposed harmonised classification

	CLP Regulation
Current entry in Annex VI, CLP Regulation	-
Current proposal for consideration by RAC	Repr. 2; H361d Skin Sens. 1; H317 Aquatic Chronic 1; H410 M-Factor 10
Resulting harmonised classification (future entry in Annex VI,CLP Regulation)	Repr. 2; H361d Skin Sens. 1; H317 Aquatic Chronic 1; H410 M-Factor 10

1.3 Proposed harmonised classification and labelling based on CLP Regulation

Table 3: Proposed classification according to the CLP Regulation

CLP Annex I ref	Hazard class	Proposed classification	Proposed SCLs and/or M-factors	Current classification[1)]	Reason for no classification [2)]
2.1.	Explosives				
2.2.	Flammable gases				
2.3.	Flammable aerosols				
2.4.	Oxidising gases				
2.5.	Gases under pressure				
2.6.	Flammable liquids				
2.7.	Flammable solids				
2.8.	Self-reactive substances and mixtures				
2.9.	Pyrophoric liquids				
2.10.	Pyrophoric solids				
2.11.	Self-heating substances and mixtures				
2.12.	Substances and mixtures which in contact with water emit flammable gases				
2.13.	Oxidising liquids				
2.14.	Oxidising solids				
2.15.	Organic peroxides				
2.16.	Substance and mixtures corrosive to metals				
3.1.	Acute toxicity - oral				conclusive but not sufficient for classification
	Acute toxicity - dermal				conclusive but not sufficient for classification
	Acute toxicity - inhalation				conclusive but not sufficient for classification
3.2.	Skin corrosion / irritation				conclusive but not sufficient for classification
3.3.	Serious eye damage / eye irritation				conclusive but not sufficient for classification
3.4.	Respiratory sensitisation				data lacking
3.4.	Skin sensitisation	Skin Sens. 1; H317			
3.5.	Germ cell mutagenicity				conclusive but not sufficient for classification

3.6.	Carcinogenicity				data lacking
3.7.	Reproductive toxicity	Repr. 2; H361d			
3.8.	Specific target organ toxicity –single exposure				conclusive but not sufficient for classification
3.9.	Specific target organ toxicity – repeated exposure				conclusive but not sufficient for classification
3.10.	Aspiration hazard				data lacking
4.1.	Hazardous to the aquatic environment	Aquatic Chronic 1; H410	M-Factor 10	-	
5.1.	Hazardous to the ozone layer				

[1)]Including specific concentration limits (SCLs) and M-factors
[2)] Data lacking, inconclusive, or conclusive but not sufficient for classification

Table 4: Proposed labellingbased according to the CLP Regulation

	Labelling	**Wording**
Pictograms	GHS07 GHS08 GHS09	
Signal Word	Warning	
Hazard statements	H361d H317 H410	Suspected of damaging the unborn child May cause an allergic skin reaction Very toxic to aquatic life with long lasting effects
Suppl. Hazard statements	-	-
Precautionary statements	(102) P260 P273 P281 P302 + P352 P308 + P313 P363 P391 P405 P501	(Keep out of reach of children) Do not breathe dust/fume Avoid release to the environment Use personal protective equipment as required IF ON SKIN: Wash with plenty of soap and water IF exposed or concerned: Get medical advice/ attention Wash contaminated clothing before reuse Collect spillage Store locked up Dispose of contents/container to …

Proposed notes assigned to an entry: -

2 BACKGROUND TO THE CLH PROPOSAL

2.1 Short summary of the scientific justification for the CLH proposal

Considering the reported findings in the relevant toxicological studies, a classification of the technical material as skin sensitiser (Skin Sens. 1; H317) and as developmental toxicant (Repr. 2; H361d) is proposed. For the other toxicological hazards, either the data were conclusive but not sufficient for classification or the relevant data were lacking.

2.2 Current harmonised classification and labelling

Not yet listed

3 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

Margosa, ext. is an active substance in the meaning of Directive 98/8/EC and therefore subject to harmonised classification and labelling (Regulation (EC) No 1272/2008 article 36.2).

Part B.

SCIENTIFIC EVALUATION OF THE DATA

1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 5: Substance identity

EC number:	283-644-7
EC name:	Margosa, ext.
CAS number (EC inventory):	84696-25-3
CAS number:	84696-25-3
CAS name:	Margosa, ext.
IUPAC name:	margosa extract from the kernels of Azadirachta indica extracted with water and further processed with organic solvents
CLP Annex VI Index number:	-
Molecular formula:	Not available since substance is an UVCB substance
Molecular weight range:	Not available since substance is an UVCB substance

Structural formula:

Not available since substance is an UVCB substance

1.2 Composition of the substance

For confidential information please refer to confidential Annex

Table 6: Constituents (non-confidential information)

Constituent	Typical concentration	Concentration range	Remarks
confidential			

Table 7: Impurities (non-confidential information)

Impurity	Typical concentration	Concentration range	Remarks
Aflatoxines B1 (main compound), B2, G1, G2	Sum < 100 µg/kg		

Table 8: Additives (non-confidential information)

Additive	Function	Typical concentration	Concentration range	Remarks

1.2.1 Composition of test material

NeemAzal technical. Purity 100% Neem seed kernel extract

1.3 Physico-chemical properties

Table 9: Summary of physico - chemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
State of the substance at 20°C and 101,3 kPa	Margosa extract technical is a pale yellow to light brownish powder with garlic like odour (purity 100% margosa extract) azadirachtin A is a white odourless powder.	Kleeberg, 1994a/b	
Melting/freezing point	Margosa extract partially liquifies above 120 °C and decomposes above 200 °C (purity 100% margosa extract)	Werle, 1995	
Boiling point	The boiling point of margosa extract cannot be observed since decomposition occurs already during melting.		
Relative density	D^{20}_{4} = 1.340 at 20 °C (purity 100% margosa extract)	Thom, 2007	
Vapour pressure	No test conducted (extraction mixture). Based on the calculated vapour pressure of 3.6·10-13 Pa for Azadirachtin A the vapour pressure of the extraction mixture should be << 10^{-5}Pa.		
Surface tension	Test not applicable because no saturated test solution with the same ratio of components as in margosa extract could be produced.		
Water solubility	Test not conducted (extraction mixture) solubility of azadirachtin A: 2.9 g/L at 20 °C	Troß, 1995b	
Partition coefficient n-octanol/water	Test not applicable (extraction mixture)		Margosa extract was used in this study, but only the partition coefficients for Azadirachtin A, B, and H could be determined based on the analytical quantitation of the three solutes in either phase.
Flash point	The flash point is only relevant to liquids		
Flammability Flammability upon ignition (solids, gases) –	Preliminary test: The burning time for the	Franke, 2005a	

EU-Method A.10 Flammability in contact with water Pyrophoric properties	distance of 200 mm was 5 minutes and 47 seconds (347 s). The test item is not a flammable solid sense of REGULATION (EC) No 1272/2008. The study does not need to be conducted because the experience in production or handling shows that the substance does not react with water, e.g. the substance is manufactured with water or washed with water. The classification procedure needs not to be applied because the substance is known to be stable into contact with air at room temperature for prolonged periods of time (days)	 BAM 2.2 (2012) BAM 2.2 (2012)	
Explosive properties	The heat of decomposition was below 500 J/g. (DSC) The test substance has no explosive properties.	Smeykal, 2002	
Self-ignition temperature for solids - EU-Method A.16	No self-ignition temperature was observed up to the melting point.	Franke, 2005b	
Oxidising properties – EU-Method A.17	The maximum burning rate of the mixture of the test item and cellulose (0.82 mm/s) is lower than the maximum burning rate of the reference mixture of cellulose and barium nitrate (1.05 mm/s). Due to this, the test item has no oxidizing properties.	Franke, 2005d	
Granulometry			
Stability in organic solvents and identity of relevant degradation products	Solvent used is sesame oil; stability tests suggest the active substance to be acceptably stable		
Dissociation constant	Test not required (extraction mixture)		
Viscosity			

2 MANUFACTURE AND USES

2.1 Manufacture

The active substance margosa extract is an extract derived from ground seed kernels of the tropical neem tree *Azadirachta indica* using the manufacturing method developed by the applicant.

3 CLASSIFICATION FOR PHYSICO-CHEMICAL PROPERTIES

The substance is not classified for physico-chemical endpoints.

4 HUMAN HEALTH HAZARD ASSESSMENT

In total, three technical extracts were submitted for the evaluation as the pesticide active ingredient "azadirachtin". The notifiers named their extracts "NeemAzal", Fortune Aza" or "NPI720"/"ATI 720". A fourth notifier (IAB) did not submit any toxicological data; hence, this latter extract is not covered by this CLH dossier.

One technical extract was submitted for the evaluation as the biocide active ingredient "margosa extract (product type 18)". The extracts named "margosa extract (product type 18)" and NeemAzal under these two procedures are produced by the same company, the applicant/notifier is the same and the submitted toxicological data/information is the same. A further extract was notified as biocide active ingredient (initially under product type 19) by another company, which is not covered by this CLH dossier and therefore, no data/information from that dossier is included.

Experts for identity of chemical substances were of the opinion that Azadirachtin and margosa extract are distinct substances in the meaning of REACH and CLP regulations, hence the German CA decided that two separate CLH dossies need to be prepared. Even though the identity of "azadirachtin" or "margosa extract (product type 18)" and the data available / needed for their evaluation are distinct[1], it was decided to have identical toxicological chapters in the CLH dossiers for both substances. This was mainly based on the evaluation of toxicological similarity of the extracts (see below).

The terms Azadirachtin and Margosa extract are used as synonyms within the context of this report.

The technical extracts evaluated in this report are extracts of seed kernels of neem tree. Constituents of kernels differ can from the constituents of other parts of neem tree (e.g., leaves, flowers, stem bark) qualitatively and quantitatively. Additionally, the extraction process (e.g., pre-processing, solvent, temperature, clean up) has a great impact on the constitution of the technical extract. It is difficult to compare the results of published literature studies with the results of the studies that were submitted for the PPP/BPD evaluation, as they were most often conducted with different test compounds. Furthermore, only few constituents of neem tree are identified.

The extracts under evaluation consist of several components, e.g., Azadirachtin A, Azadirachtin B, Nimbin or Salannin, of which Azadirachtin A has the highest abundance. Finally, both in the PPP and the BPD procedure, the whole extracts were considered the toxicologically relevant substance because no toxicological data were available to demonstrate that certain components were responsible for the observed toxicological effects.

Aflatoxins might be present in the extracts; being relevant impurities, maximum levels were defined for them.

The chemical compositions of the three extracts evaluated under the PPP procedure are distinct (*c.f.* confidential annex). During an expert consultation in the PPP procedure, the similarity of the toxicological properties of the extracts was discussed. The findings observed (including the dose

[1]In fact, for the evaluation of "margosa extract (product type 18)" the studies performed with NeemAzal would be sufficient; the studies performed with Fortune Aza and ATI 720 would not be needed for the evaluation of that substance (besides limitations in the studies on long-term toxicity/carcinogenicity and no developmental toxicity study in rabbits, the data set of NeemAzal is rather complete).

levels they occurred at) in the available studies on acute toxicity, short-term toxicity, genotoxicity/mutagenicity and developmental toxicity were compared. The participants concluded that "the NeemAzal and Fortune Aza extracts appear to be toxicologically equivalent. The ATI 720 extract has a number of data gaps and therefore a conclusion cannot be drawn with regard to toxicological equivalence" (cited from the meeting minutes). Since then, some more studies with ATI-720 have been submitted by the applicant to support the assessment of equivalence, which are included in this CLH report. The rapporteur concluded – taking into account these new data – that the extract ATI-720 should be considered toxicologically equivalent with NeemAzal and Fortune Aza (this latter evaluation was recently distributed for commenting).

Margosa extract was discussed during an expert consultation in the BPD procedure (technical meeting III 2010) and in general the evaluation by the rapporteur was agreed with.

Short summaries of the available information/data are included in this section. Longer (robust) study summaries are included in section 9. They were extracted from the documentation submitted for the EU PPP procedure (i.e. draft assessment report (2007), additional report (2009) and addendum 7 (2013)). In certain cases, waiving arguments or argumentations only relevant for the PPP procedure were removed. The assessments prepared for the PPP or BPD procedures are attached to the technical dossier.

No information was provided by risk management whether registration dossiers are available nor were such dossiers made available for the preparation of this CLH report. Therefore, no information was included in this CLH dossier which was taken from a registration dossier for Azadirachtin or margosa extract. ECHA indicated during accordance check, that no REACH registration dossiers were available at that time.

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

4.1.1 Non-human information

No studies submitted by the applicants

4.1.2 Human information

No studies submitted by the applicants

4.1.3 Summary and discussion on toxicokinetics

No studies were available on absorption, distribution, metabolism and excretion. Such studies require radioactive labelled compounds to allow the sensitive detection and identification of parent compound and metabolites. Azadirachtin technical is a mixture of several different limonoids and other compounds extracted from the seed kernels of the Neem tree. It is therefore not feasible to perform a metabolism study with azadirachtin technical. It is furthermore also not possible to perform such a study for its analytically leading compound azadirachtin A due to the unavailability of chemically synthesised and radioactively labelled azadirachtin A, since it can be obtained by extraction and cleanup of the seed kernels of the Neem tree only. [Note: in open literature a total

synthesis of azadirachtin A was described (reviewed in Jauch, 2008). However, having an overall recovery of 0.00015%, it is considered of no practical use.] Therefore, it is not possible to obtain radioactive labelled material and it was accepted, that no studies on metabolism and toxicokinetics were submitted.

No information was available on the products of mammalian metabolism. From *in vitro* experiments it was evident that mammalian metabolism resulted in reduced cytotoxicity.

In vitro studies indicated that azadirachtin was hydrolysed in aqueous media also at neutral pH values. Therefore, it was conceivable that ester groups were hydrolysed in mammalian body.

4.2 Acute toxicity

4.2.1 Non-human information

4.2.1.1 Acute toxicity: oral

No mortalities were observed in all studies but that of Moorthy (1993, TOX9750130) with 20% dead rats in the high dose group. Clinical signs of toxicity (such as piloerection, pallor of the extremities, dullness, reduced activity) were seen, but resolved within a few days.

Table 10: Summary of acute oral toxicity

Animal species & strain	Number of animals per dose level	Doses, route of administration, vehicle	LD50 (mg/kg bw) Test compound	Reference year Method
Rat, Hsd/Ola:Sprague-Dawley (CD)	5 M & 5 F	5000 mg/kg bw, gavage, distilled water (10 mL/kg bw)	> 5000 NeemAzal	McRae, 1997 TOX9700502 OECD TG 401
Rat, Wistar	5 M & 5 F	0, 1190, 2380, 4760 mg/kg bw gavage DMSO (20 mL/kg bw)	> 4760 NeemAzal (20% mortality in high dose group)	Moorthy, 1993 TOX9750130 Similar to OECD TG 401
Mouse, Swiss albino	5 M & 5 F	0, 1190, 2380, 3365 mg/kg bw gavage DMSO (15 mL/kg bw)	> 3365 NeemAzal	Moorthy, 1993 TOX2006-592 Similar to OECD TG 401
Rat, Hsd/Ola:Sprague-Dawley (CD)	5 M & 5 F	5000 mg/kg bw, gavage, distilled water (10 mL/kg bw)	> 5000 Fortune Aza	McRae, 1997 TOX2005-2362 OECD TG 401
Rat, CD	5 M & 5 F	5000 mg/kg bw, gavage, 1% carboxymethyl cellulose	> 5000 NPI 720	Furedi-Machacek, 1990 TOX2005-2357 OECD TG 401

4.2.1.2 Acute toxicity: inhalation

No mortalities were observed in all studies but that one of Jackson (1997, TOX2005-2373) with one dead female in the treated group. Clinical signs of toxicity were seen during exposure (hunched posture, partial closed or red eyes, wetness around mouth) and after exposure (wet fur around snout and jaws, exaggerated respiratory movements, wheezing, rales, mouth breathing), but resolved within a few days. One male treated with Fortune Aza had dark subpleural foci on all lobes of the lung and the deceased female showed severe congestion of the lungs and gas filled stomach.

Table 11 Summary of acute inhalation toxicity

Animal species & strain	Number of animals per dose level	Doses, route of administration, vehicle	LC_{50} (mg/L) Test compound	Reference year Method
Rat, Sprague-Dawley	5 M & 5 F	0.72 mg/L air (4 h), whole body	> 0.72 (highest attainable conc.) NeemAzal	Jackson, 1997 TOX9750135 OECD TG 403
Rat, Sprague-Dawley	5 M & 5 F	2.45mg/L air (4 h), whole body	>2.45 (highest attainable conc.) Fortune Aza (1 F died)	Jackson, 1997 TOX2005-2373 OECD TG 403
Rat, Sprague-Dawley	5 M & 5 F	2.41mg/L air (4 h), whole body	>2.41 (highest attainable conc.) NPI-720-F (formulation)	Aranyi, 1990 TOX2005-2371 OECD TG 403

4.2.1.3 Acute toxicity: dermal

No mortalities were observed in all studies. No clinical signs of toxicity were seen. In the study with NPI 720, dermal reactions (oedema, erythema, eschra) were observed, but resolved within a few days.

Table 12 Summary of acute dermal toxicity

Animal species & strain	Number of animals per dose level	Doses, route of administration, vehicle	LD_{50} (mg/kg bw) Test compound	Reference year Method
Rat, Hsd/Ola:Sprague-Dawley (CD)	5 M & 5 F	2000 mg/kg bw, dermal (24 h), water moistened	> 2000 NeemAzal	Mc Rae, 1997 TOX9700503 OECD TG 402
Rat, Hsd/Ola:Sprague-Dawley (CD)	5 M & 5 F	2000 mg/kg bw, dermal (24 h), water moistened	> 2000 Fortune Aza	Mc Rae, 1997 TOX2005-2370 OECD TG 402
Rabbit, New Zealand albino	5 M & 5 F	2000 mg/kg bw, dermal (24 h), water moistened	> 2000 NPI 720	Furedi-Machacek, 1990 TOX2005-2364 OECD TG 402

4.2.1.4 Acute toxicity: other routes

No studies with application via other routes were available.

4.2.2 Human information

No studies submitted by the applicants

4.2.3 Summary and discussion of acute toxicity

The three tested technical extracts were of low acute toxicity following oral, dermal or inhalative exposure. Single rats died after inhalation or gavage administration of azadirachtin technical. No further mortalities or signs of toxicity were observed in rats upon treatment with single doses via either route.

4.2.4 Comparison with criteria

Table 13 present the relevant CLP criteria. LD50/LC50 values after oral, dermal or inhalative administration were above the threshold levels leading to a classification.

Table 13: CLP criteria for classification for acute toxicity

CLP criteria
Cat 4 (H302): $300 < LD_{50} \leq 2000$ mg/kg (oral) Cat. 3 (H301): $50 < LD_{50} \leq 300$ mg/kg (oral) Cat. 2 (H300): $5 < LD_{50} \leq 50$ mg/kg (oral) Cat. 1 (H300): $LD_{50} \leq 5$ mg/kg (oral)
Cat. 4 (H332): $10.0 < LC_{50} \leq 20.0$ mg/L(vapours) $1.0 < LC_{50} \leq 5.0$ (dusts and mists) Cat. 3 (H331): $2.0 < LC_{50} \leq 10.0$ mg/L (vapours) $0.5 < LC_{50} \leq 1.0$ (dusts and mists) Cat. 2 (H330): $0.5 < LC_{50} \leq 2.0$ mg/L (vapours) $0.05 < LC_{50} \leq 0.5$ (dusts and mists) Cat. 1 (H330): $LC_{50} \leq 0.5$ mg/L(vapours) $LC_{50} \leq 0.05$ (dusts and mists)

Cat. 4 (H312): $1000 < LD_{50} \leq 2000$ mg/kg (dermal) Cat. 3 (H311): $200 < LD_{50} \leq 1000$ mg/kg (dermal) Cat. 2 (H310): $50 < LD_{50} \leq 200$ mg/kg (dermal) Cat. 1 (H310): $LD_{50} \leq 50$ mg/kg (dermal)

4.2.5 Conclusions on classification and labelling

In summary and based on the submitted data, azadirachtin did not meet the criteria to be classified for oral, dermal or inhalative toxicity according to the criteria in CLP regulation.

4.3 Specific target organ toxicity – single exposure (STOT SE)

4.3.1 Summary and discussion of Specific target organ toxicity – single exposure

Transient clinical signs of toxicity were seen in animals treated with single doses of the test materials.

4.3.2 Comparison with criteria

Table 14 Classification criteria for Categories 1 and 2 of specific target organ toxicity-single exposure (C: guidance value)

CLP criteria	
Category 1 (H370) Oral (rat): C ≤ 300 mg/kg bw Dermal (rat or rabbit): C ≤ 1000 mg/kg bw Inhalative (rat, dust/mist/fume): ≤ 1 mg/L/4 h	Substances that have produced significant toxicity in humans or that, on the basis of evidence from studies in experimental animals, can be presumed to have the potential to produce significant toxicity in humans following single exposure - reliable and good quality evidence from human cases or epidemiological studies; or - observations from appropriate studies in experimental animals in which significant and/or severe toxic effects of relevance to human health were produced at generally low exposure concentrations.
Category 2 (H371) Oral (rat): 2000 ≥ C > 300 mg/kg bw Dermal (rat or rabbit): 2000 ≥ C > 1000 mg/kg bw	Substances that, on the basis of evidence from studies in experimental animals can be presumed to have the potential to be harmful to human health following single exposure - observations from appropriate studies in experimental animals in which significant toxic effects, of relevance to human health, were produced at generally moderate exposure concentrations.

Inhalative (rat, dust/mist/fume): 5 ≥ C > 1 mg/L/4 h	
Category 3 (H335/H336) Guidance values do not apply (mainly based on human data)	Transient target organ effects This category only includes narcotic effects and respiratory tract irritation. These are target organ effects for which a substance does not meet the criteria to be classified in Categories 1 or 2 indicated above. These are effects which adversely alter human function for a short duration after exposure and from which humans may recover in a reasonable period without leaving significant alteration of structure or function.

4.3.3 Conclusions on classification and labelling

Considering that the observed non-lethal effects reported after acute exposure were transient andwere not of considerably adverse nature with no significant impact on health, no classification with STOT-SE is proposed.

4.4 Irritation

4.4.1 Skin irritation

4.4.1.1 Non-human information

Very slight erythema (score: 1) were seen in animals treated with NeemAzal, but not in animals treated with the other compounds. Erythema had resolved with in one day. No signs of systemic toxicity were reported.

Table 15: Summary of skin irritation

Animal species & strain	Number of animals	Doses	Result	Reference Method
Rabbit, New Zealand albino	6 M	0.5 g (4 h)	Not irritating NeemAzal	Parcell, 1996 TOX9700505 OECD TG 404
Rabbit, New Zealand albino	6 M	0.5 g (4 h)	Not irritating Fortune Aza	Parcell, 1997 TOX2005-2378 OECD TG 404
Rabbit, New Zealand albino	3 M & 3 F	0.5 g (4 h)	Not irritating NPI 720	Furedi-Machacek, 1990 TOX2005-2375 OECD TG 404

4.4.1.2 Human information

No studies submitted by the applicants

4.4.1.3 Summary and discussion of skin irritation

Azadirachtin technical extracts exhibited no irritating potential to skin.

4.4.1.4 Comparison with criteria

Table 16: CLP criteria

CLP criteria
Irritating to skin (Category 2, H315): at least in 2/3 tested animal a positive response of: Mean value of ≥ 2.3 - ≤ 4.0 for erythema/eschar or for oedema

Highest score observed in skin irritation studies was 1 for erythema.

As the results did not meet the criteria laid down in CLP regulation classification and labelling for skin irritation is not needed.

4.4.1.5 Conclusions on classification and labelling

In summary and based on the submitted data, azadirachtin did not meet the criteria to be classified for skin irritation/corrosion according to the criteria in CLP regulation.

4.4.2 Eye irritation

4.4.2.1 Non-human information

Dulling of cornea, discharge and redness of conjunctiva were seen 1 h after instillation of test compounds. Effects declined with time and were absent within one or two days. Signs of eye irritation were less severe than the criteria for classification would require.

Table 17: Summary of eye irritation

Animal species & strain	Number of animals	Doses	Result*	Reference Method
Rabbit, New Zealand albino	5 M & 1 F	70 mg	Not irritating Cornea opacity: 0.0 / 0.0 / 0.0 Iris: 0.0 / 0.0 / 0.0 Redness of conjunctivae: 1.0 / 0.3 / 0.2 Chemosis: 0.7 / 0.3 / 0.0 NeemAzal	Parcell, 1996 TOX9700506 OECD TG 405
Rabbit, New Zealand albino	1 M & 5 F	64 mg	Not irritating Cornea opacity: 0.0 / 0.0 / 0.0 Iris: 0.0 / 0.0 / 0.0 Redness of conjunctivae: 0.7 / 0.0 / 0.0 Chemosis: 0.0 / 0.0 / 0.0 Fortune Aza	Parcell, 1997 TOX2005-2382 OECD TG 405
Rabbit, New Zealand albino	4 M & 2 F	100 mg	Not irritating Cornea opacity: 0.2 / 0.0 / 0.0 Iris: 0.0 / 0.0 / 0.0 Redness of conjunctivae: 1.3 / 0.0 / 0.0 Chemosis: 1.3 / 0.2 / 0.0 NPI 720	Furedi-Machacek, 1990 TOX2005-2379 OECD TG 405

**, mean scores at the reading times (24 h / 48 h / 72 h)*

4.4.2.2 Human information

No studies submitted by the applicants

4.4.2.3 Summary and discussion of eye irritation

Azadirachtin technical extracts exhibited very slight and reversible irritating potential to eye.

4.4.2.4 Comparison with criteria

Azadirachtin technical extracts exhibited very slight and reversible irritating potential to eye. The severity of findings did not reach the critical thresholds to be classified as eye irritant.

Table 18: CLP criteria

CLP criteria
Irritating to eyes (Category 2, H319): at least in 2/3 tested animal a positive response of: corneal opacity: ≥ 1 and/or iritis: ≥ 1 and/or conjunctival redness: ≥ 2 and/or conjunctival oedema (chemosis): ≥ 2

4.4.2.5 Conclusions on classification and labelling

In summary and based on the submitted data, azadirachtin did not meet the criteria to be classified for eye irritation/corrosion according to the criteria in CLP regulation.

4.4.3 Respiratory tract irritation

No specific studies (conducted in non-humans or humans) concerning respiratory tract irritation were available. In the acute inhalation studies in rats, findings relating to changes in respiratory pattern were transient and of low severity. Neither histopathological findings nor practical observations in humans are available. In summary and based on the submitted data, azadirachtin did not meet the criteria to be classified as respiratory tract irritant.

4.5 Corrosivity

No specific studies regarding corrosion were submitted. Corrosion was not seen in the studies for dermal or eye irritation. Hence, no classification for corrosion of skin or eye was needed. Please compare also section 4.4 (Irritation).

4.6 Sensitisation

4.6.1 Skin sensititsation

4.6.1.1 Non-human information

NeemAzal and Fortune Aza were tested according to the protocol of Magnusson & Kligman, whereas NPI 720 was tested according to Buehler, i.e. without adjuvant. Fortune Aza, NeemAzal, and NPI 720 showed sensitising potential upon skin contact.

Table 19: Summary of skin sensitisation

Animal species & strain	Number of animals	Doses	Result	Reference Method
Guinea pig, Dunkin Hartley albino	20 M treated 10 control	Intradermal: 5% (w/v) in acetone/alembicol Dermal: 80% in acetone	Sensitising (M&K) [all animals sensitised] NeemAzal	Allan & Coleman, 1997 TOX9700507 OECD TG 406
Guinea pig, Dunkin Hartley albino	20 M treated 10 control	Intradermal: 0.5% (w/v) in acetone/alembicol Dermal: 60% in alembicol	Sensitising (M&K) [all animals sensitised] Fortune Aza	Allan & Coleman, 1997 TOX2005-2384 OECD TG 406
Guinea pig, Hartley albino	10 M treated 10 control	Dermal: 25% (w/v) in ethanol	Sensitising (Buehler) [2/10 animals sensitised] NPI 720	Sherwood, 1990 TOX2005-2383 OECD TG 406

Slight irritation was observed in all animals after intradermal application of NeemAzal or solvent (Allan & Coleman, 1997 TOX9700507). Necrosis was recorded in sites receiving Freund's complete adjuvant. One day before dermal application, skin was treated with a 10% solution of SDS in petrolatum. Slight erythema were observed after topical application of test compound or vehicle in treated or control animals, respectively. On challenge, no skin reactions were observed in control animals. In contrast, all animals of treatment group showed slight to well defined oedema and erythema upon challenge with NeemAzal solutions (40 and 80% in acetone). Hence, NeemAzal showed sensitising properties by skin contact.

Slight irritation was observed in all animals after intradermal application of Fortune Aza or solvent (Allan & Coleman, 1997 TOX2005-2384). Necrosis was recorded in sites receiving Freund's complete adjuvant. One day before dermal application, skin was treated with a 10% solution of SDS in petrolatum. Moderate erythema was observed in test animals following topical application with test compound; slight erythema was seen in control animals. All animals of the treatment group showed well defined oedema upon challenge with Fortune Aza solutions (30 and 60% in alembicol). In control animals, no erythema or oedema were observed. Therefore, Fortune Aza showed sensitising properties by skin contact.

Treatment with NPI 720 for induction led to slight to well defined erythema. Positive erythema reactions (i. e., a score greater/equal to 2) were observed in two of ten treated Guinea pigs but not in any of the controls during the challenge phase of this study.

Deficiencies of this study were: (1) no data on the latest reliability check performed by the laboratory, (2) only 10 animals (instead of 20). According to the criteria laid down in CLP regulation, a test (non-adjuvant test method) with more than 15% positive animals is considered positive. 2/10 animals, i.e. 20%, showed positive response to challenge. Moreover, the Buehler test

is not as rigorous as the Magnusson & Kligman assay, where the other extracts were found to be sensitising. Therefore, NPI 720 is considered to be a skin sensitiser.

4.6.1.2 Human information

No studies submitted by the applicants

4.6.1.3 Summary and discussion of skin sensitisation

Fortune Aza, NeemAzal, and NPI 720 showed sensitising potential by skin contact.

4.6.1.4 Comparison with criteria

Table 20 presents the toxicological results in comparison with CLP criteria.

Table 20: Results of skin sensitisation tests in comparison with CLP criteria

Toxicological result	CLP criteria
NeemAzal: 20/20 animals positive 5% intra dermal induction concentration Fortune Aza: 20/20 animals positive 0.5% intra dermal induction concentration	Guinea pig maximisation test Category 1A (H317): ≥ 30% responding at ≤ 0.1% intradermal induction dose or ≥ 60% responding at > 0.1% to ≤ 1% intradermal induction dose Category 1B (H317): ≥ 30% to < 60% responding at > 0,1% to ≤ 1% intradermal induction dose or ≥ 30% responding at > 1% intradermal induction dose
NPI 720: 2/10 animals positive 25% topical induction concentration	Buehler assay Category 1A (H317): ≥ 15% responding at ≤ 0.2% topical induction dose or ≥ 60% responding at > 0.2% to ≤ 20% topical induction dose Category 1B (H317): ≥ 15% to < 60% responding at > 0.2% to ≤ 20% topical induction dose or ≥ 15% responding at > 20% topical induction dose

Results with NeemAzal and NPI 720 lead to a classification in category 1B, whereas results with Fortune Aza lead to category 1A. Considering the contradictory categories, it is proposed to place azadirachtin into category 1 (without sub categories).

4.6.1.5 Conclusions on classification and labelling

In summary and based on the submitted data, azadirachtin did meet the criteria laid down in CLP regulation (as amended) to be classified with Skin sensitisation category 1 (H317 - May cause an allergic skin reaction)

4.6.2 Respiratory sensitisation

No data/information (from non-humans or humans) was submitted that would allow an evaluation of sensitising properties for the respiratory tract.

4.7 Specific target organ toxicity (CLP Regulation) – repeated exposure (STOT RE)

4.7.1 Non-human information

Studies in rats with repeated oral administration of test compound were available. Neither studies with other species, nor studies with other routes of administration were submitted.

4.7.1.1 Repeated dose toxicity: oral

Rats were treated with repeated doses of the different azadirachtin technical extracts. Toxicity of NeemAzal was assessed in a range of 14 to 90 daily doses. Fortune Aza was tested in 28-d and 90-d studies. ATI 720 was only tested in a 90-d study.

Clear evidence of toxicity was observed in the 28-d study with NeemAzal (Waterson, 1997, TOX9700508) in rats receiving dose levels of 3200, 8000 or 20000 ppm. Upon histopathological examination, all treated animals showed signs of substance effects in the thyroid (follicular epithelial hypertrophy) and the liver (periportal hepatocyte eosinophilia with clumping). Bodyweight gain was reduced in animals with dietary dose levels of 20000 and 8000 ppm. In animals receiving 20000 ppm, hepatocyte hypertrophy was noted. A NOAEL could not be established, the LOAEL was the lowest dose tested of 300 mg/kg bw/d (3200 ppm).

After treatment of rats for 90 d with 6400 ppm of NeemAzal in feed (achieved dose 490 and 525 mg/kg bw/d for males and females, respectively), evidence of hepatotoxicity (in both sexes: organ weight increase, hepatocyte hypertrophy; in females only: periportal fat deposition, (minimally) increased blood protein levels) was observed (Waterson, 1997, TOX9700509). Furthermore, effects on haematology (females: higher mean platelet values, (slightly) reduced thrombotest values; males: prolonged blood coagulation (APTT), prolonged thrombotest-values) and thyroid (increased relative weight, slight increase of incidence of follicular epithelial hypertrophy) were seen. At 1600 ppm (achieved dose 123 and 135 mg NeemAzal/kg bw/d for males and females, respectively) increased incidence and severity of periportal fat deposition was noted in females only, while slightly increased total protein levels were noted for both sexes and prolonged APTT values for males only. At 400 ppm (achieved dose 32 and 36 mg/kg bw/d for males and females, respectively) and 100 ppm (achieved dose 8 and 9 mg/kg bw/d for males and females, respectively) no signs of toxicity were observed. The NOAEL in this study was 32 mg/kg bw/d (400 ppm).

Fortune Aza was fed to rats during a period of 28 d (Waterson & Dawe, 1997, TOX2005-2385) in dose levels of 4000, 8000 or 16000 ppm. Clear evidence of toxicity was observed at the 16000 and 8000 ppm dose levels, where reduced bodyweight gain was noted for both sexes, reduced feed intakes were also observed at these levels. Various macroscopic findings in these two dose groups were considered to be a result of the effect on bodyweight (reduction in adipose tissue, small prostate glands, small ovaries and uteri). Clinical signs included piloerection in three males and one female of the high dose group. At 4000 ppm bodyweight was affected only during the first four days of the study. However, dose-related changes were noted in liver weights of both sexes, adrenal and ovary weights in females. In the absence of histological examination, these findings account as adverse effects. A NOAEL could not be established, the LOAEL was the lowest dose tested of 400 mg/kg bw/d (4000 ppm).

Following treatment of rats with Fortune Aza for 90 d (Waterson & Dawe, 1997, TOX2005-2386) in dose levels of 100, 400, 1600 or 6400 ppm, A wide range of signs of toxicity were observed in the 6400 ppm dose group, including hepatotoxicity (bile duct hyperplasia; hepatocyte hypertrophy, weight increase), effects on reproductive organs (organ weights in females decreased, decreased number of corpora lutea; endometrial atrophy in uterus, marked atrophy in testes seminiferous tubular) and sciatic nerve degeneration (Table 22). Furthermore, low food intake (81% and 77% of control in males and females, respectively) and low bodyweight gain (66% and 60% of control in males and females, respectively) were observed. At 1600 ppm (corresponding to 140 and 180 mg/kg bw/d for males and females, respectively) effects on liver (same effects as in 6400 ppm dose group) and on ovaries (slightly reduced weight, reduced number of corpora lutea) were noted. At 400 ppm (corresponding to 33 and 40 mg/kg bw/d for males and females, respectively) increased bodyweight adjusted liver weights in females were noted. As the effect on liver weight was not supported by histological findings, this dose level was considered the NOAEL.

Administration of ATI-720 (Johnson, 1994, TOX2005-2388) at a high dietary level (10000 ppm, corresponding to 585 mg and 680 mg/kg bw/d for males and females, respectively) over a period of 90 d resulted in several toxicological effects related to the test compound, including hepatotoxicity (organ weight increased, γGT), altered haematologic parameters (MCV and MCH decreased, RBC count increased, in females haemoglobin and haematocrit decreased), and hair loss. Decreased palatability of the test diet resulted in decreased feed intake, and, consequently, decreased bodyweight gain and bodyweight were observed in both sexes. Both, absolute and relative liver weights in females were significantly increased also in the mid dose group (2500 ppm, corresponding to 145 mg and 180 mg/kg bw/d for males and females, respectively). Additionally, γGT was increased in females of this dose level. No treatment related histopathological changes were observed in any of the treatment groups. Based on these observations the NOAEL was 500 ppm for females (corresponding to 35 mg/kg bw/d) and 2500 ppm (145 mg/kg bw/d) for males.

Table 21: Summary of oral RDT

Animal species & strain	Number of animals	Doses, vehicle, duration	Result	Reference Test compound Method
Rat, CD	5 M & 5 F	20000, 50000 ppm (equivalent to 2000, 5000 mg/kg bw/d) Feed 2-wk	LOAEL: 20000 ppm (2000 mg/kg bw/d) bw ↓; feed intake (50000ppm) ↓	Waterson & Hawkins, 1995 TOX9750142 NeemAzal OECD TG: n.a. (only data on bodyweight, food consumption, daily observations)
Rat, Crt: CD (SD) BR	5 M & 5 F	0, 3200, 8000, 20000 ppm (0, 320, 770, 1850 mg/kg bw/d in males; 0, 300, 790, 1750 mg/kg bw/d in females) Feed 4-wk	LOAEL: 300 mg/kg bw/d (3200 ppm) <u>All dose levels:</u> hepato toxicity (periportal hepatocyte eosinophilia with clumping), thyroid toxicity (follicular epithelial hypertrophy) <u>20000 ppm:</u> hepatocyte hypertrophy; bw gain ↓ <u>8000 ppm:</u> bw gain ↓ in females	Waterson, 1997 TOX9700508 NeemAzal OECD TG 407

Animal species & strain	Number of animals	Doses, vehicle, duration	Result	Reference Test compound Method
Rat, Crt: CD BR	10 M & 10 F	0, 100, 400, 1600, 6400 ppm (0, 8, 32, 123, 490 mg/kg bw/d in males; 0, 9, 36, 135, 525 mg/kg bw/d in females) Feed 90-d	NOAEL: 32 mg/kg bw/d (400 ppm) 6400 ppm: liver (wt ↑; hepatocyte hypertrophy, periportal fat deposition, blood protein levels ↑), thyroid (rel. wt ↑; follicular epithelial hypertrophy) 1600 ppm: liver (periportal fat deposition in females), haematology / clinical chemistry (total protein ↑, prolonged APTT)	Waterson, 1997 TOX9700509 NeemAzal OECD TG 408
Rat, Crt: CD (SD) BR	5 M & 5 F	0, 4000, 8000, 16000 ppm (0, 400, 780, 1420 mg/kg bw/d in males; 0, 400, 880, 1420 mg/kg bw/d in females) Feed 28-d	LOAEL: 400 mg/kg bw/d (4000 ppm) 8000, 16000 ppm: bw gain and feed intake ↓; clinical signs (16000 only) 4000 ppm: initial bw gain ↓; organ wt (liver ↑; females only: adrenals ↓, ovaries ↓)	Waterson & Dawe, 1997 TOX2005-2385 Fortune Aza OECD TG 407 *(no histopathology)*
Rat, Crt: CD (SD) BR	10 M & 10 F	0, 100, 400, 1600, 6400 ppm (0, 8.5, 33, 140, 520 mg/kg bw/d in males; 0, 11, 40, 180, 550 mg/kg bw/d in females) Feed 90-d	NOAEL: 33 mg/kg bw/d (400 ppm) 6400 ppm: liver (wt ↑, bile duct hyperplasia, hepatocyte hypertrophy), ovary (wt ↓, no. of corpora lutea ↓), sciatic nerve (fiber degeneration), bw gain and food intake ↓ 1600 ppm: liver (wt ↑, bile duct hyperplasia, hepatocyte hypertrophy), ovary (wt slightly ↓, no. of corpora lutea ↓) 400 ppm: liver wt ↑ but without histological findings	Waterson & Dawe, 1997 TOX2005-2386 Fortune Aza OECD TG 408
Rat, Sprague Dawley	10 M & 10 F	0, 500, 2500, 10000 ppm (0, 30, 145, 585 mg/kg bw/d in males; 0, 35, 180, 680 mg/kg bw/d in females) Feed 90-d	NOAEL: 35 mg/kg bw/d (500 ppm) in females 145 mg/kg bw/d (2500 ppm) in males 10000ppm: liver (wt ↑, γGT ↑), haematology (MCV ↓, MCH ↓), bw gain ↓ 2500 ppm (females only): liver (wt ↑, γGT ↑)	Johnson, 1994 TOX2005-2388 ATI 720 OECD TG 408 *(no urinalysis)*

Table 22: Microscopical findings in the rat 90-d study with Fortune Aza (Waterson & Dawe, 1997, TOX2005-2386)

Dose level (ppm)			Male					Female				
			0	100	400	1600	6400	0	100	400	1600	6400
Liver	Number of organs examined		10	10	10	10	10	10	10	10	10	10
	Hepatocyte hyper-trophy – periportal	Minimal	0	0	0	0	2	0	0	0	0	0
	Bile duct hyperplasia	Total	0	0	0	8**	10**	0	0	0	0	10**
		Trace	0	0	0	8**	0	0	0	0	0	10**
		Minimal	0	0	0	0	10**	0	0	0	0	0
	Hepatocyte cytoplasmic eosinophilia with clumping – periportal	Total	0	0	0	9**	10**	0	0	0	0	10**
		Trace	0	0	0	9**	0	0	0	0	0	6**
		Minimal	0	0	0	0	10**	0	0	0	0	4*
Thyroid	Number of organs examined		10	10	10	10	10	10	10	10	10	10
	Follicular epithelial hypertrophy	Trace	0	0	0	0	3	0	0	0	0	4*
Ovaries	Number of animals examined							10	10	10	10	10
	Absent corpora lutea							0	0	0	1	0
	Apparent decreased numbers of corpora lutea							1	0	1	1	10**
	Group mean number of corpora lutea§							36	39	38	28	21
Uterus	Number of organs examined							10	10	10	10	10
	Endometrial atrophy							0	0	0	0	6**
Testes	Number of organs examined		10	10	10	10	10					
	Seminiferous tubular atrophy	Total	0	0	1	1	2					
		Trace	0	0	1	0	0					
		Moderate	0	0	0	1	0					
		Marked	0	0	0	0	2					
Epididymi-des	Number of organs examined		10	10	10	10	10					
	Absence of spermatozoa		0	0	0	0	1					
	Decreased spermatozoa	Marked	0	0	0	0	1					
	Abnormal spermatids in ducts	Moderate	0	0	0	1	0					
	Ductal epithelial vacuolisation	Trace	0	0	0	0	1					
Sciatic nerve	Number of organs examined		10	10	10	10	10	10	10	10	10	10
	Nerve fiber degeneration	Total	4	5	5	4	8	1	2	4	3	7**
		Trace	4	4	5	3	5	1	2	3	3	2
		Minimal	0	1	0	1	3	0	0	0	0	5*
		Moderate	0	0	0	0	0	0	0	1	0	0

Fisher's Exact Test: *p <0.05; ** p <0.01 §: Statistical analysis not performed

4.7.1.2 Repeated dose toxicity: inhalation

No studies with repeated dose inhalative administration were available.

4.7.1.3 Repeated dose toxicity: dermal

No studies with repeated dose dermal administration were available.

4.7.1.4 Repeated dose toxicity: other routes

No studies with repeated dose administration via other routes were available.

4.7.1.5 Human information

No studies submitted by the applicants

4.7.1.6 Other relevant information

No studies with other mammalian species were submitted. There was no indication for toxic effects from feeding studies published in open literature conducted in various farm animals (cows, calves, and bulls, buffalo calves, growing pigs, sheep) with water-washed Neem seed kernel cake (typical contents were between 0.1 and 1 g AzaA/kg) (studies summarised by the notifiers: Anonymous, 2002, TOX2005-2335; Pfau, 2005, TOX2005-2389). No signs of toxicity regarding a diverse spectrum of parameters tested were reported upon admixing up to 45% water-washed Neem seed kernel cake to the regular concentrate mixture. Such feeding studies in farm animals were conducted for up to twelve months and no adverse effects were noted. Parameters were milk production in cows, sperm quality in bulls, growth rate in piglets, and cattle, meat characteristics. Also red and white cell counts as well as haemoglobin and liver enzymes were unaffected.

Unfortunately, the available data allow only a very rough estimate of the amount of azadirachtin to which the farm animals were exposed. According to the applicant, the highest concentration of neem extract in the diet of goats receiving 25% "water washed neem seed kernel cake" (WWNSKC) as protein concentrate mixture was 375 ppm. Growing calves were fed a concentrate mixture containing 45% water-washed Neem seed kernel cake, based on the azadirachtin A content, this was equivalent of a dietary dose of approx. 675 ppm NeemAzal. Using standard conversion factors for goats and cattle to adjust dietary concentrations to a mean daily intake per kg bodyweight, assuming a fraction of one third of the protein concentrate mixture in the total diet and taking into account the variability in azadirachtin A content in the extracts and other neem products, a mean daily dose of azadirachtin A in the range of 3-9 mg/kg bw (equivalent to 9-27 mg NeemAzal/kg bw) may be calculated. This would be in the same order of magnitude as the NOAEL in the subchronic study in rats and is much lower than doses that produced adverse effects in those experiments.

4.7.1.7 Summary and discussion of repeated dose toxicity

Effects seen in repeated-dose studies had NOAELs in the range of approx. 30 mg/kg bw/d with a LOAEL of approx. 120-180 mg/kg bw/d. Effects were seen predominantly in liver. Thyroid follicular epithelium hypertrophy was seen in the study with NeemAzal (Waterson, 1997, TOX9700508) at a dose level of 6400 ppm (achieved dose 490 and 525 mg/kg bw/d for males and females, respectively); no studies were submitted to explore if this effect was secondary to liver enzyme induction, which might be indicated by liver weight increase.

Concerning the sciatic nerve fibre degeneration seen in the high dose group (550 mg/kg bw/d in females) treated with Fortune Aza, no similar findings were observed in any other study (nerve fibres were also assessed in 90-d studies in rats with NeemAzal and ATI-720, in the 2-yr study in rats with NeemAzal and 18-mo study in mice with NeemAzal-F5%). Even though, studies with FOB were not available, regular observance of the animals for abnormal clinical signs did not cause concern of neurotoxicity.

Additionally, in rats treated with 6400 ppm Fortune Aza effects on the ovaries were observed: decrease of organ weight and reduction of number of corpora lutea. In lower extent, these effects were also seen in 1600 ppm group animals. The reason for the weight decrease was not further evaluated. Effects at 6400 ppm might be associated with the marked decrease of bodyweight gain.

4.7.1.8 Summary and discussion of repeated dose toxicity findings relevant for classification as STOT RE according to CLP Regulation

Severe effects (such as sciatic nerve fibre degeneration) were seen in a 90-d rat study in rats with Fortune Aza. However, the effect level was above the guidance value for classification.

4.7.1.9 Comparison with criteria of repeated dose toxicity findings relevant for classification as STOT RE

Table 23 presents the CLP criteria for classification.

Table 23: criteria of specific target organ toxicity – repeated exposure

CLP criteria
Category 1 (H372): Substances that have produced significant toxicity in humans or that, on the basis of evidence from studies in experimental animals, can be presumed to have the potential to produce significant toxicity in humans following repeated exposure. Substances are classified in Category 1 for target organ toxicity (repeat exposure) on the basis of: reliable and good quality evidence from human cases or epidemiological studies; or observations from appropriate studies in experimental animals in which significant and/or severe toxic effects, of relevance to human health, were produced at generally low exposure concentrations. Equivalent guidance values for 28-day and 90-day studies: Oral, rat: 28-day: ≤ 30 mg/kg bw/d 90-day: ≤ 10 mg/kg bw/d
Category 2 (H373): Substances that, on the basis of evidence from studies in experimental animals can be presumed to have the potential to be harmful to human health following repeated exposure. Substances are classified in category 2 for target organ toxicity (repeat exposure) on the basis of observations from appropriate studies in experimental animals in which significant toxic effects, of relevance to human health, were produced at generally moderate exposure concentrations. Guidance dose/concentration values are provided below (see 3.9.2.9) in order to help in classification. In exceptional cases human evidence can also be used to place a substance in Category 2. Equivalent guidance values for 28-day and 90-day studies: Oral, rat: 28-day: ≤ 300 mg/kg bw/d 90-day: ≤ 100 mg/kg bw/d

No severe findings were observed in rats at dose levels below the respective guidance values. Hence, it is proposed not to classify for STOT-RE.

4.7.1.10 Conclusions on classification and labelling of repeated dose toxicity findings relevant for classification as STOT RE

Classification for effects seen in repeated-dose studies was considered not necessary.

4.8 Germ cell mutagenicity (Mutagenicity)

4.8.1 Non-human information

4.8.1.1 In vitro data

The results of the submitted tests did not show a potential to induce gene mutations under the test conditions used. All extracts showed clastogenic activity in cytotoxic concentrations in chromosomal aberration test in cultured human lymphocytes.

In the chromosomal aberration study with NeemAzal (Stien, 2006, TOX2006-739), cytotoxicity (lower mitotic index) was observed in concentrations of 2500 µg/mL and above; in these concentrations, test compound was observed to precipitate. Significantly increased CA rate was observed at 5000 µg/mL without metabolic activation (4 h exposure). The aberration rates in the other incubations were within the range of incubations with solvent or within the range of historical control incubations.

In the study with Neem seed extract (Stien, 2006, TOX2006-463), lower mitotic index was observed in concentrations of 250 µg/mL after 4-h exposure (with and without metabolic activation). In the experiment with 24 h exposure, cytotoxicity was observed at concentrations of 125 µg/mL. (Significantly) increased aberration rates were observed at a concentration of 500 µg/mL in the experiments with the shorter exposure time. In the experiment with 24 h of incubation, this was observed at 125 µg/mL. In all these cases, the report pointed out that there were not enough (i.e., 100) metaphases available to be evaluated.

In the study with azadirachtin tech. (Stien, 2006, TOX2006-464), lower mitotic index was observed in concentrations of 125 or 250 µg/mL after 4h exposure (with and without metabolic activation, respectively). In the experiment with 24 h exposure, cytotoxicity was observed at concentrations of 125 µg/mL. Significantly increased aberration rates were observed at a concentration of 500 µg/mL in the experiments with the shorter exposure time (with and without metabolic activation). In all these cases, the report pointed out that there were not enough (i.e. 100) metaphases available to be evaluated.

Table 24: Summary of in vitro mutagenicity

Test system	Test object	Concentration	Results Test compound	Reference Method
Ames test	Salmonella typhimurium TA98, TA100, TA1535, TA1537, TA1538	50-5000µg/plate	Non mutagenic (+/- S9) **NeemAzal**	Jones & Gant, 1997 TOX9700511 OECD TG 471
	Salmonella typhimurium TA98, TA100, TA1535, TA1537, TA1538	50-5000µg/plate	Non mutagenic (+/- S9) **Fortune Aza**	Jones & Gant, 1997 TOX2005-2393 OECD TG 471
	Salmonella typhimurium TA98, TA100, TA1535, TA1537, TA1538	50-5000µg/plate	Non mutagenic (+/- S9) **NPI 720**	Barbera, 1990 TOX2005-2392 OECD TG 471
CA	Cultured human lymphocytes	312.5-5000 µg/mL	Clastogenic (- S9), non-clastogenic (+ S9) **NeemAzal**	Stien, 2006 TOX2006-739 OECD TG 473
	Cultured human lymphocytes	15.6-1000 µg/mL	Clastogenic (+/- S9) **Azadirachtin techn. (SIPCAM)**	Stien, 2006 TOX2006-464 OECD TG 473
	Cultured human lymphocytes	15.6-500 µg/mL	Clastogenic (+/- S9) **Neem seed extract (MITSUI)**	Stien, 2006 TOX2006-463 OECD TG 473
HPRT gene mutation	CHO cells	(25)200-1250 µg/mL	Non mutagenic (+/- S9) **NeemAzal**	Adams & Kirkpatrick, 1997 TOX9700512 OECD TG 476
	CHO cells	5-750 µg/mL	Non mutagenic (+/- S9) **Fortune Aza**	Adams & Ransome, 1997 TOX2005-2395 OECD TG 476
	V79 cells	9.77-1250 µg/mL	Non mutagenic (+/- S9) **Azatin technical***	Flügge, 2011 ASB2012-6693 OECD TG 476

*, the study with "Azatin technical" was submitted by the notifier of the technical material "ATI 720"

4.8.1.2 In vivo data

The tested extracts did not induce micronucleated polychromatic erythrocytes, when tested in mouse micronucleus assay. Ratio of polychromatic to normochromatic erythrocytes was decreased in mice treated with Fortune Aza, indicating that the test compound had reached bone marrow, whereas there was no influence on the ratio of polychromatic to normochromatic erythrocytes in mice treated with NeemAzal or Azatin. The top dose in the study with Azatin was limited by toxicity observed in the range-finding study.

Table 25 Summary of in vivo mutagenicity

Test system	Method	Route of administration	Dose levels	Result Test compound	Reference Method
Mice, CD-1	Micronucleus test, bone marrow	Gavage (1% methyl cellulose)	0, 1250, 2500, 5000 mg/kg bw	Non genotoxic **NeemAzal**	Proudlock et al., 1997 TOX9700513 OECD TG 474
Mice, CD-1	Micronucleus test, bone marrow	Gavage (1% methyl cellulose)	0, 1250, 2500, 5000 mg/kg bw	Non genotoxic **Fortune Aza**	Proudlock et al., 1997 TOX2005-2399 OECD TG 474
Mice, NMRI	Micronucleus test, bone marrow	Gavage (0.8% hydroxypropylmethyl cellulose)	250, 500, 1000 mg/kg bw	Non genotoxic **Azatin technical***	Flügge, 2011 ASB2011-14529 OECD TG 474

*, the study with "Azatin technical" was submitted by the notifier of the technical material "ATI 720"

4.8.2 Human information

No studies submitted by the applicants

4.8.3 Other relevant information

No other relevant information available.

4.8.4 Summary and discussion of mutagenicity

The three azadirachtin technical extracts were tested in a battery of in vitro and in vivo genotoxicity assays, measuring different mutagenicity endpoints like gene mutations in bacterial and mammalian cells, and chromosomal mutations in vitro and in vivo.

The results of all the tests did not show a potential to induce gene mutations of the azadirachtin technical extracts under the test conditions used. However, all extracts showed clastogenic activity in cytotoxic concentrations in chromosomal aberration test in cultured human lymphocytes. The tested extracts did not show genotoxic potential in an *in vivo* micronucleus test in mice.

4.8.5 Comparison with criteria

Following criteria for classification for gem cell mutagens are given in CLP regulation:

CLP regulation
The classification in Category 1A is based on positive evidence from human epidemiological studies. Substances to be regarded as if they induce heritable mutations in the germ cells of humans. The classification in Category 1B is based on: — positive result(s) from in vivo heritable germ cell mutagenicity tests in mammals; or — positive result(s) from in vivo somatic cell mutagenicity tests in mammals, in combination with some evidence that the substance has potential to cause mutations to germ cells. It is possible to derive this supporting evidence from mutagenicity/genotoxicity tests in germ cells in vivo, or by demonstrating the ability of the substance or its metabolite(s) to interact with the genetic material of germ cells; or — positive results from tests showing mutagenic effects in the germ cells of humans, without demonstration of

transmission to progeny; for example, an increase in the frequency of aneuploidy in sperm cells of exposed people.

The classification in Category 2 is based on:
— positive evidence obtained from experiments in mammals and/or in some cases from in vitro experiments, obtained from:
— somatic cell mutagenicity tests in vivo, in mammals; or
— other in vivo somatic cell genotoxicity tests which are supported by positive results from in vitro mutagenicity assays.
Note: Substances which are positive in in vitro mammalian mutagenicity assays, and which also show chemical structure activity relationship to known germ cell mutagens, shall be considered for classification as Category 2 mutagens.

No human data are available, hence a classification in category 1A is not possible. Neither vivo heritable germ cell mutagenicity tests nor positive results from in vivo somatic cell mutagenicity tests in mammals are available; hence a classification in 1B is not possible. In some vitro studies (clastogenicity) were positive, others (Ames, HPRT) and the respective in vivo studies showed a negative outcome, hence a classification in category 2 is considered not necessary.

4.8.6 Conclusions on classification and labelling

No classification for mutagenicity was considered necessary, as criteria laid down in CLP regulation were not met.

4.9 Carcinogenicity

4.9.1 Non-human information

4.9.1.1 Carcinogenicity: oral

In a two year carcinogenicity study in rats (Kumar, 2000, TOX2001-170), NeemAzal technical was dosed up to 448 mg/kg bw in males or 635 mg/kg bw/d in females (6400 ppm in feed). No test substance related carcinogenic effect was seen in this study. Gross and histopathologic findings were considered incidental and typical of the rat strain employed. No effects were found, thus the high dose level was considered the NOAEL. Deficiencies in the study design of this study concerning chronic toxicity (urinalysis not performed; haematology and clinical chemistry performed only after 6 and 12 and at necropsy with limited parameters assessed) can be put aside with information of subchronic and carcinogenicity studies (*urinalysis:* histopathological investigation of kidneys and blood urea nitrogen concentration in this long-term study and urinalysis in 90-d study did not indicate nephrotoxicity; *haematology/clinical chemistry:* full macro- and microscopic pathological investigation showed no adverse findings (all findings were considered incidental and typical for the rat strain employed) and full clinical chemistry analysis was performed in 90-d study and showed only few modified parameters which were not investigated in this long-term study [MCV, MCHC, globulin]). In conclusion and considering the information requirements for pesticides and biocides, the list of parameters examined in this study was not fully complete as compared to requirements of OECD guidelines 452 and 453. It however appears unlikely that toxicologically relevant adverse changes with respect to these parameters have been overlooked by these omissions.

The results of this study are not in agreement with the results of the 90-d feeding studies in rats. In the subchronic studies findings were hepatotoxicity, follicular epithelial hypertrophy, and prolonged coagulation time. One explanation for these distinctions might be the use of different rat strains (Wistar rats in carcinogenicity and reproductive study, Crl: CD BR rats in subchronic studies).

This study was discussed during an expert consultation of the PPP procedure: "The validity of the study was questioned, especially as no effects were seen at the highest dose tested (approx. 400 and 500mg/kg bw/day in males and 560 and 700 mg/kg bw/day in females). In the 90-dstudy effects were observed at 32 mg/kg bw/day. […] Strong doubts were raised about the validity of the long term study: - Uncertainties over the specification of material tested; - No control animals developed tumours (and no hypertrophy) after two years. The doubts raised for this study mean that there is no reliable long term information on long term toxicity for azadirachtin (the mouse study was deemed unacceptable because only a 5% Azadirachtin formulation was used). It was questioned whether the effects seen in the 90-d study be adaptive? No conclusion on long term toxicity and/or carcinogenicity can be drawn due to the limited information available" (cited from the meeting minutes).

We were informed by UK GLP authority that the testing facility was not part of its GLP monitoring program.

The mouse carcinogenicity study (Moorthy, 1996, TOX9700523) with the formulation NeemAzal-F 5% (contains approx. 20% NeemAzal and 80% polyethylene oxide) showed no carcinogenic potential and also no treatment related histopathological findings were noted (highest dose tested: 63 mg/kg bw/d in males, 72 mg/kg bw/d in females (1000 ppm)). Gross and histopathologic findings were considered incidental and typical of the mouse strain employed. No effects were found, thus the high dose level was considered the NOAEL. Notifier proposed a correction factor of 5 to calculate NeemAzal dose levels from NeemAzal-F5% dose levels, leading to an estimated NOAEL of 12.6 mg/kg bw/d.

No studies were submitted that were conducted with Fortune Aza or ATI 720.

Table 26: Summary of oral carcinogenicity

Animal species & strain	Number of animals	Doses, vehicle, duration	Result Test compound	Reference Method
Rat, Wistar	50 M & 50 F	0, 400, 1600, 6400 ppm (0, 29, 114, 448 mg/kg bw/d in males; 0, 38, 167, 635 mg/kg bw/d in females) Feed 105-wk	NOAEL: 448 mg/kg bw/d (6400 ppm) No toxic effects reported No carcinogenic effects reported NeemAzal	Kumar, 2000 TOX2001-170 Similar OECD TG 451 (clinical chemistry performed)
Mouse, Swiss albino	50 M & 50 F	0, 100, 300, 1000 ppm (0, 6.6, 18.4, 63 mg/kg bw/d in males; 0, 7.0, 21, 72 mg/kg bw/d in females) Feed 18-mo	NOAEL: 63 mg/kg bw/d (1000 ppm) No toxic effects reported No carcinogenic effects reported NeemAzal-F 5% (formulation)	Moorthy, 1996 TOX9700523 Similar OECD TG 451 (feed analysis not performed, clinical signs not reported)

4.9.1.2 Carcinogenicity: inhalation

No information concerning carcinogenicity after inhalative administration available.

4.9.1.3 Carcinogenicity: dermal

No information concerning carcinogenicity after dermal administration available.

4.9.2 Human information

No information concerning carcinogenicity in humans available.

4.9.3 Other relevant information

No other relevant information available.

4.9.4 Summary and discussion of carcinogenicity

Based on this information, NeemAzal did not induce tumours in rats. However, the limitations of the available studies need to be taken into account.

4.9.5 Comparison with criteria

Table 27 presents CLP criteria.

Table 27: Criteria for classification

CLP regulation
A substance is classified in Category 1 (known or presumed human carcinogens) for carcinogenicity on the basis of epidemiological and/or animal data. A substance may be further distinguished as: Category 1A, known to have carcinogenic potential for humans, classification is largely based on human evidence, or Category 1B, presumed to have carcinogenic potential for humans, classification is largely based on animal evidence. The classification in Category 1A and 1B is based on strength of evidence together with additional considerations (see section 3.6.2.2). Such evidence may be derived from: — human studies that establish a causal relationship between human exposure to a substance and the development of cancer (known human carcinogen); or — animal experiments for which there is sufficient (1) evidence to demonstrate animal carcinogenicity (presumed human carcinogen). In addition, on a case-by-case basis, scientific judgement may warrant a decision of presumed human carcinogenicity derived from studies showing limited evidence of carcinogenicity in humans together with limited evidence of carcinogenicity in experimental animals. The placing of a substance in Category 2 (suspected human carcinogens) is done on the basis of evidence obtained from human and/or animal studies, but which is not sufficiently convincing to place the substance in Category 1A or 1B, based on strength of evidence together with additional considerations (see section 3.6.2.2). Such evidence may be derived either from limited (1) evidence of carcinogenicity in human studies or from limited evidence of carcinogenicity in animal studies. […] 3.6.2.2.3. Strength of evidence involves the enumeration of tumours in human and animal studies and determination of their level of statistical significance. Sufficient human evidence demonstrates causality between human exposure and the development of cancer, whereas sufficient evidence in animals shows a causal relationship between the substance and an increased incidence of tumours. Limited evidence in humans is demonstrated by a positive association between exposure and cancer, but a causal relationship cannot be stated. Limited evidence in animals is provided when data suggest a carcinogenic effect, but are less than sufficient. The terms 'sufficient' and 'limited' have been used here as they have been defined by the International Agency for Research on Cancer (IARC) and read as follows: (a) Carcinogenicity in humans The evidence relevant to carcinogenicity from studies in humans is classified into one of the following categories: — sufficient evidence of carcinogenicity: a causal relationship has been established between exposure to the agent and human cancer. That is, a positive relationship has been observed between the exposure and cancer in studies in which chance, bias and confounding could be ruled out with reasonable confidence; — limited evidence of carcinogenicity: a positive association has been observed between exposure to the agent and cancer for which a causal interpretation is considered to be credible, but chance, bias or confounding could not be ruled out with reasonable confidence. (b) Carcinogenicity in experimental animals Carcinogenicity in experimental animals can be evaluated using conventional bioassays, bioassays that employ genetically modified animals, and other in-vivo bioassays that focus on one or more of the critical stages of carcinogenesis. In the absence of data from conventional long-term bioassays or from assays with neoplasia as the end-point, consistently positive results in several models that address several stages in the multistage process of carcinogenesis should be considered in evaluating the degree of evidence of carcinogenicity in experimental animals. The evidence relevant to carcinogenicity in experimental animals is classified into one of the following categories: — sufficient evidence of carcinogenicity: a causal relationship has been established between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in (a) two or more species of animals or (b) two or more independent studies in one species carried out at different times or in different laboratories or under different protocols. An increased incidence of tumours in both sexes of a single species in a well-conducted study, ideally conducted under Good Laboratory Practices, can also provide sufficient evidence. A single study in one species and sex might be considered to provide sufficient evidence of carcinogenicity when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour or age at onset, or when there are strong findings of tumours at multiple sites; — limited evidence of carcinogenicity: the data suggest a carcinogenic effect but are limited for making a definitive evaluation because, e.g. (a) the evidence of carcinogenicity is restricted to a single experiment; (b) there are unresolved questions regarding the adequacy of the design, conduct or interpretation of the studies; (c) the agent increases the

incidence only of benign neoplasms or lesions of uncertain neoplastic potential; or (d) the evidence of carcinogenicity is restricted to studies that demonstrate only promoting activity in a narrow range of tissues or organs.
3.6.2.2.4. Additional considerations (as part of the weight of evidence approach (see 1.1.1)). Beyond the determination of the strength of evidence for carcinogenicity, a number of other factors need to be considered that influence the overall likelihood that a substance poses a carcinogenic hazard in humans. The full list of factors that influence this determination would be very lengthy, but some of the more important ones are considered here.
3.6.2.2.5. The factors can be viewed as either increasing or decreasing the level of concern for human carcinogenicity. The relative emphasis accorded to each factor depends upon the amount and coherence of evidence bearing on each. Generally there is a requirement for more complete information to decrease than to increase the level of concern. Additional considerations should be used in evaluating the tumour findings and the other factors in a case-by-case manner.
3.6.2.2.6. Some important factors which may be taken into consideration, when assessing the overall level of concern are:
(a) tumour type and background incidence;
(b) multi-site responses;
(c) progression of lesions to malignancy;
(d) reduced tumour latency;
(e) whether responses are in single or both sexes;
(f) whether responses are in a single species or several species;
(g) structural similarity to a substance(s) for which there is good evidence of carcinogenicity;
(h) routes of exposure;
(i) comparison of absorption, distribution, metabolism and excretion between test animals and humans;
(j) the possibility of a confounding effect of excessive toxicity at test doses;
(k) mode of action and its relevance for humans, such as cytotoxicity with growth stimulation, mitogenesis, immunosuppression, mutagenicity.
Mutagenicity: it is recognised that genetic events are central in the overall process of cancer development. Therefore evidence of mutagenic activity in vivo may indicate that a substance has a potential for carcinogenic effects.

There are no relevant data from epidemiological studies submitted by the notifier, hence no classification with Cat 1A according to CLP regulation is proposed.

Considering the limitations of the studies regarding carcinogenicity with NeemAzal (as discussed during an expert consultation of the PPP procedure), no sufficient data seem to be available to allow a robust evaluation.

No studies were submitted that were conducted with Fortune Aza or ATI 720.

4.9.6 Conclusions on classification and labelling

Data lacking to allow a firm conclusion.

4.10 Toxicity for reproduction

4.10.1 Effects on fertility

4.10.1.1 Non-human information

In the two generation reproduction study NeemAzal technical (Ramamoorthy, 2000, TOX2001-173) had no impact on clinical signs, bodyweight, feed consumption and gross (and microscopic) pathology of parental animals (highest dose tested: 50.7 mg/kg bw/d in males, 59.6 mg/kg bw/d in females (750 ppm)). Treatment with NeemAzal technical had no influence on reproduction or the development of the offspring.

In another (not acceptable) two generation reproduction study (Mani, 1996, TOX9700522) with the formulation NeemAzal-F 5%, increased relative weights of ovaries and spleen in maternal rats were noted in all treatment groups (appr. 13-333 mg/kg bw/d (200-5000 ppm)). Additionally, mean bodyweights in intermediate and high dose animals were reduced. The formulation had no effect on reproduction or developmental parameters.

A third (not acceptable) one generation reproductive toxicity study (Ramamoorthy, 2000, TOX2001-171) could not be taken into account due to deficiencies in the study design and the study report.

Table 28: Summary of effects on fertility

Animal species & strain	Number of animals	Doses, vehicle, duration	Result Test compound	Reference
Rat, Wistar	10 M & 20 F	0, 250, 500, 750 ppm (0, 16.8, 34, 50.7 mg/kg bw/d in males; 0, 19.9, 38.9, 59.6 mg/kg bw/d in females) Feed 2-gen. study	Parental: No effects on parents NOAEL: 50 mg/kg bw/d (750 ppm) Reproductive: No effects on reproduction NOAEL: 50 mg/kg bw/d (750 ppm) Developmental: No effects on offspring NOAEL: 50 mg/kg bw/d (750 ppm) NeemAzal	Ramamoorthy, 2000 TOX2001-173 Similar OECD TG 416 (no data on feed analysis, time to fertilisation not reported)
Rat, Charles Foster	10 M & 20 F	0, 200, 1000, 5000 ppm (equivalent to 0, 13, 67, 333 mg/kg bw/d) Feed 2-gen. study	Parental: spleen, ovary wt ↑, bw ↓ LOAEL: appr. 13 mg/kg bw/d (200 ppm) Developmental: No effects on offspring NOAEL: appr. 333 mg/kg bw/d (5000 ppm) Reproductive:No effects on reproduction NOAEL: appr. 333 mg/kg bw/d (5000 ppm) NeemAzal F 5% (formulation)	Mani, 1996 TOX9700522 Similar OECD TG 416 (no data on feed analysis, time to fertilisation and duration of gestation not reported)

No studies were submitted that were conducted with Fortune Aza or ATI 720.

4.10.1.2 Human information

No studies submitted by the applicants

4.10.2 Developmental toxicity

4.10.2.1 Non-human information

The results of the available studies are summarised in Table 29.

Table 29: Summary for developmental toxicity

Reference	Protocol Species	Doses	Maternal effects Test compound	Developmental effects
Myers & Dawe, 1997 TOX9700510	OECD 414 (only 10 F per dose group, only external morphology examination) Rat, Crl:CD BR VAF/plus	0, 100 ,300, 1000 mg/kg bw/d	300, 1000 mg/kg bw/d: Bw ↓, feed intake (only 1000) ↓, post-dosage salivation NOAEL: 100 mg/kg bw/d NeemAzal	No effects on foetuses NOAEL: 1000 mg/kg bw/d
Myers &	OECD 414	0, 50, 225,	1000 mg/kg bw/d:	255 mg/kg bw/d:

Dawe, 1997 TOX9700514	Rat, Crl:CD BR VAF/plus	1000 mg/kg bw/d	Bw ↓, feed intake ↓, post-dosage salivation NOAEL: 225 mg/kg bw/d NeemAzal	Malformations (cf. Table 30), supernumerary ribs (only 1000) NOAEL: 50 mg/kg bw/d
Waterson, 1997 TOX2005-2400	OECD 414 (only 10 F per dose group, only external morphology examination) Rat, Crl:CD BR VAF/plus	0, 100 ,300, 1000 mg/kg bw/d	<u>1000 mg/kg bw/d:</u> Bw ↓, feed intake ↓ NOAEL: 300 mg/kg bw/d Fortune Aza	No effects on foetuses NOAEL: 1000 mg/kg bw/d
Waterson, 1997 TOX2005-2401	OECD 414 Rat, Crl:CD BR VAF/plus	0, 100 ,300, 1000 mg/kg bw/d	<u>1000 mg/kg bw/d:</u> Bw ↓, feed intake ↓ NOAEL: 300 mg/kg bw/d Fortune Aza	No effects on foetuses NOAEL: 1000 mg/kg bw/d
Ryan, 1994 TOX2005-2402	OECD 414 Rabbit, New Zealand white	0, 20, 100, 500 mg/kg bw/d	<u>100, 500 mg/kg bw/d:</u> Bw ↓, feed intake ↓ NOAEL: 20 mg/kg bw/d ATI 720	<u>500 mg/kg bw/d:</u> No. of dead foetuses ↑, malformations ↑ (cf. text) NOAEL: 100 mg/kg bw/d

Treatment of pregnant rats with high (and intermediate) doses of <u>NeemAzal</u> technical (≥ 300 mg/kg bw/d) induced signs of toxicity (reduced bodyweight gain, lower feed intake and higher water consumption). In a preliminary study (Myers & Dawe, 1997, TOX9700510) no effects on foetuses were observed (up to 1000 mg/kg bw/d), whereas in the main study (Myers & Dawe, 1997, TOX9700514) an increase of the incidence of malformations (interventricular septal defects, malrotated heart; *c.f.* Table 30) were observed in litters of high and intermediate dose groups (1000 and 225 mg/kg bw/d) and increase of the incidence of supernumerary ribs in litters of high dose groups.

The developmental toxicity studies were discussed during an expert consultation of the PPP procedure. For the main study with NeemAzal, it was agreed to set the NOAELs for maternal and developmental effects at 225 mg/kg bw/d based on bodyweight effects or 14th ribs, respectively.

In the rat developmental study with NeemAzal, litter 63 (of mid dose group) and litters 80, 84, 88 (of high dose group) showed malformations associated with heart. Variations associated with heart were seen in litters 65, 68, 74 (of mid dose group) and litters 85, 98 (of high dose group).

The notifier argues that malformations were seen only at maternally toxic doses and were not relevant because they were induced by high maternal toxicity. In the mid dose group, initial (GD 6-8) bodyweight gain (8.5 g vs. 10.4 g in controls) was slightly reduced and the initial (GD 6-7) feed intake (24 g vs. 26 g in controls) was significantly reduced. However, bodyweight was comparable to control group and later on, bodyweight gain and feed intake were comparable to controls. Hence, the DS did not consider the findings observed in mid dose group as adverse (and established the NOAEL at the mid dose level). In high dose dams, initial (GD 6-8) bodyweight gain (6.1 g vs. 10.4 g in controls), the initial (GD 6-7) feed intake (23 g vs. 26 g in controls) were significantly reduced and water intake was significantly increased.

In the mid dose group only one litter was affected with heart-associated malformations. Indeed (as argued by the notifier), in case this finding had been observed in isolation it probably would have been dismissed as incidental, however, in the high dose group the same and further heart-associated malformations were detected. Therefore, the findings observed in mid dose group were considered as dose-related and adverse. This evaluation is in line with the evaluation by the study director: "*Of the remaining 2 malformed foetuses, it was noted that one showed interventricular septal defect. A further 3 foetuses (3 further litters affected) showed small interventricular septal defect (classified*

as a visceral anomaly). The combined incidence of interventricular septal defect (4 foetuses (4 litters affected)) was comparable to that observed at 1000 mg/kg/day and, as such, the possibility that this isolated finding may be attributable to treatment cannot be discounted."

Table 30: Foetal (litter) incidences of selected findings (Myers & Dawe, 1997 TOX9700514)

Observation		**Dose level (mg/kg bw/d)**			
		0	**50**	**225**	**1000**
Number of foetus (litters) examined:		305 (23)	323 (23)	306 (23)	308 (23)
Visceral findings					
Thoracic (malformations)	Malformed systemic/pulmonary arteries	0 (0)	0 (0)	0 (0)	1 (1)
	Atrial septal defect with narrow pulmonary vein	0 (0)	0 (0)	0 (0)	1 (1)
	Interventricular septal defect	0 (0)	0 (0)	1 (1)	2 (2)
	Malrotated heart	0 (0)	0 (0)	1 (1)	1 (1)
	Duplicated inferior vena cava	0 (0)	0 (0)	0 (0)	2 (2)
Thoracic (anomalies)	Anomalous cervicothoracic arteries	1 (1)	0 (0)	0 (0)	0 (0)
	Interventricular septal defect (small)	0 (0)	1 (1)	3 (3)	2 (2)

Gavage of Fortune Aza technical to groups of pregnant rats (Waterson, 1997, TOX2005-2400 and TOX2005-2401) led to reduction of bodyweight gain and lower feed consumption in the high dose group (1000 mg/kg bw/d). Treatment had no effect on foetuses (highest dose tested 1000 mg/kg bw/d).

Pregnant rabbits (Ryan, 1994, TOX2005-2402) showed signs of toxicity (scant faeces, bloody urine, reduced bodyweight gain and feed consumption) during treatment with NPI 720 technical in high and intermediate doses (500 and 100 mg/kg bw/d). The number of viable litters and of live foetuses per dam were reduced, whereas the number of in utero deaths was elevated in the high dose group (500 mg/kg bw/d). Consistent with the low foetal weight in the high dose group, foetuses had domed shaped heads. Additional gross external foetal malformations (*c.f.*,

Table 31), consisting of intestines and liver outside body, umbilical hernia with exposed intestines, clubbed feet/forelimbs, absence of forelimbs (abrachia) or forelimbs digits, and absence of eyelids, were seen only in the high dose group. Significant signs of developmental toxicity were observed in the high dose group only and may be related to maternal toxicity. No effects on litter size and development were observed in the mid dose and low dose group (100 and 20 mg/kg bw/d).
Considering the high level of toxicity observed in top dose group, the low number of available litters and the low mean litter size of 0.9 live foetuses per litter (compared to 8.4 in the control group), the findings reported for the top dose group contribute only to a minor extent to the evaluation of possible teratogenic properties of the test material. It seems that the dose level of 500 mg/kg bw/d was too high (compared to test guideline requirements), when taking into account the extent of foetotoxicity.

Table 31: Foetal malformations (foetuses / litters) in rabbits (Ryan, 1994 TOX2007-2402) (for details, *c.f.*, Table 140 and Table 141)

	Dose level (mg/kg bw/d)			
	0	**20**	**100**	**500**
Gross and visceral malformations				
Number examined	118 / 13	120 / 14	112 / 12	14 / 5
Incidence	1 / 1 (1 / 8%)	1 / 1 (1 / 7%)	1 / 1 (1 / 8%)	5 / 4 (36 / 80%)
Cephalic malformations				
Number examined	40 / 13	40 / 14	37 / 12	5 / 5
Incidence	14 / 10 (35 / 77%)	9 / 6 (23 / 43%)	8 / 4 (22 / 33%)	3 / 3 (60 / 60%)
Skeletal malformations				
Number examined	118 / 13	120 / 14	112 / 12	14 / 5
Incidence	2 / 2 (2 / 15%)	0 / 0 (0 / 0%)	2 / 2 (2 / 17%)	1 / 1 (7 / 20%)

4.10.2.2 Human information

Purified neem oil was used in first clinical trials as intravaginal/-uterineal used contraceptive (Talwar *et al.*, 1995, TOX2006-3053, 1997, TOX2006-3054).

4.10.3 Other relevant information

Various extracts or oil of different parts of neem tree were reported in literature to induce reproductive toxic effect. An aqueous leaves extract was reported to reduce fertility in male mice (Deshpande et al., 1980, TOX2006-3046; Sadre et al., 1984, TOX2006-3049), whereas a methanolic seed kernel extract had no impact on fertility (Krause & Adami, 1984, TOX2006-3047). *In vitro* treatment of spermatozoe with neem seed kernel oil had spermatocidal effects (Sinha, Riar, Bardhan et al., 1984, TOX2006-3051). Intrauterine application of the oil in various species prevented gravity (Tewari et al., 1986, TOX2006-3055; Lal et al., 1986, TOX2006-3048; Talwar et al., 1997, TOX2006-3054). Furthermore, female rats showed reduced implantation rates and increased resorption rates after intravaginal, oral, or subcutaneous application (Sinha, Riar, Tiwary et al., 1984, TOX2006-3052; Tewari et al., 1986, TOX2006-3055; Lal et al., 1986, TOX2006-3048). Abortus was seen in female baboons after oral intake of neem oil (Talwar et al., 1997, TOX2006-3054).

4.10.4 Summary and discussion of reproductive toxicity

For the evaluation of **effects on fertility or reproduction**, findings in single-dose (e.g. histopathology of testes [however not done for the azadirachtin technical extracts]), short-term, long-term, multi-generation and one-generation studies can be used. All azadirachtin technical extracts (evaluated in this report) were evaluated in short-term studies in rats. Additionally, Neem-Azal was evaluated in a long-term as well as a 2-generation and a 1-generation study.

In the 28-d, 90-d and long-term studies in rats with NeemAzal, no findings on sex organs were reported in the study reports. No effects on fertility or reproduction were observed in the submitted 1-generation (considered not acceptable) or 2-generation (considered acceptable) toxicity studies with NeemAzal. Dose levels in the 2-generation study were calculated as mean of the compound intake in weeks 0, 5, 10 and 15 (Pfau, 2009, 1863427). Therefore, compound intake was based only on the intake during the pre-mating period.

In the 28-d study in rats with Fortune Aza,findings on sex organs were reported in the study report (ovary weight ↓). In the 90-d study, reduced number of corpora lutea (one animal was reported with apparent decreased number of corpora lutea (which is comparable with the incidence reported for control females) and one with absent corpora lutea) and slightly reduced ovary weights were observed at 1600 ppm. At 6400 ppm, uteri (small, lower weight and endometrial atrophy), ovaries (lower weights, reduced number of corpora lutea) and testes (seminiferous tubular atrophy) exhibited findings. Compared to the control groups, animals treated with 6400 ppm had a bodyweight gain of 60-66% and a feed intake of 77-81%. Effects at 6400 ppm might be associated with the marked decrease of bodyweight gain. No long-term or multi-generation studies performed with Fortune Aza were submitted.

In the 90-d study in rats with ATI 720, findings on sex organs (relative testes weight ↑) were reported. However, absolute testes weight was unchanged, therefore, this finding was considered to be not adverse. No long-term or multi-generation studies performed with ATI 720 were submitted.

In reports from open literature, various findings with respect to fertility or reproduction are described. However, in the literature reports different test compounds (other extraction methods, other starting materials, etc.) were used when compared to the technical extracts used for PPP. There seem to be some differences in properties, when comparing different preparations of different parts of neem tree (e.g., flower, leaves, seed kernel). In the available reproductive toxicity study, no effects on fertility were observed.

This argumentation was supported by the participants of an expert consultation in the PPP procedure.

Considering the findings seen in the **developmental toxicity** study in rats performed with NeemAzal (interventricular septal defects, malrotated heart, supernumerary ribs) and the study in rabbits performed with ATI-720 (high post implantation loss, various foetal malformations, low foetal weight, in utero deaths), the effects were seen at or around doses where maternal toxicity could be observed. Additionally, the incidences in the rat study were increased only slightly and the possibility of non-specific causes such as general toxicity could not be excluded.

Considering that the effects described in sections 4.10.2.2 and 4.10.3 were seen after administration of extracts prepared from neem seed kernels or neem leaves which were not identical to the azadirachtin technical extracts evaluated here, it is considered appropriate that these effects are not used for classification and labelling of NeemAzal, Fortune Aza and ATI 720.

This argumentation was supported by the participants of an expert consultation in the PPP procedure.

4.10.5 Comparison with criteria

Table 32 and Table 33 present the CLP criteria.

Adverse effects on sexual function and fertility:

Table 32: Classification criteria concerning adverse effects on sexual function and fertility

CLP criteria
Category 1A: Known human reproductive toxicant Category 1B: Presumed human reproductive toxicant largely based on data from animal studies - clear evidence of an adverse effect on sexual function and fertility in the absence of other toxic effects, or - the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects Category 2: Suspected human reproductive toxicant - some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on sexual function and fertility and - where the evidence is not sufficiently convincing to place the substance in Category 1 (deficiencies in the study). - the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects

In the submitted multigeneration study, under the conditions of the study, no findings with relevance for a classification for adverse effects on sexual function and fertility were reported up to the highest dose tested.

There are no epidemiological data to evaluate effects on fertility, hence azadirachtin cannot be placed in category 1A (CLP).

Only in repeat-dose studies with FortuneAza pathological indications for adverse effects on fertility (ovary weight, corpora lutea count, and uterus effects) were reported mainly in animals of high dose levels. Overall, there was no consistent picture of effects induced by the three extracts. Therefore, no classification for effects on fertility/reproduction is proposed.

Adverse effects on development:

Table 33: Classification criteria concerning adverse effects on development

CLP criteria
Category 1A: Known human reproductive toxicant Category 1B: Presumed human reproductive toxicant largely based on data from animal studies - clear evidence of an adverse effect on development in the absence of other toxic effects, or - the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects Category 2: Suspected human reproductive toxicant - some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on development and - the evidence is not sufficiently convincing to place the substance in Category 1 (deficiencies in the study). - the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects

There are no appropriate epidemiological studies available on developmental effects in humans. Hence, classification with Category 1A according CLP regulation is not possible.

The prenatal developmental toxicity was investigated in rats and rabbits complying with international test guidelines and GLP.

Considering the findings seen in the developmental toxicity study in rats performed with NeemAzal (interventricular septal defects, malrotated heart, supernumerary ribs), the effects were seen at or around doses, where maternal toxicity could be observed. Additionally, the incidences in the rat study were increased only slightly and the possibility of non-specific causes such as general toxicity could not be excluded.

Taking into account the high level of toxicity observed in rabbits of the top dose group, the low number of available litters and the low mean litter size of 0.9 live foetuses per litter (compared to 8.4 in the control group), the findings reported for the top dose group contribute only to a minor extent to the evaluation of possible teratogenic properties of the test material.

Considering that the effects described in sections 4.10.2.2 and 4.10.3 were seen after administration of extracts prepared from neem seed kernels or neem leaves which were not identical to the azadirachtin technical extracts evaluated here, it is considered appropriate that these effects are not used for classification and labelling of NeemAzal, Fortune Aza and ATI 720.

This argumentation was supported by the participants of an expert consultation in the PPP procedure.

According to regulation (EC) No 1272/2008 major manifestations of developmental toxicity include death of the developing organism, structural abnormality, altered growth, and functional deficiency.

ECHA's Guidance on the application of the CLP criteria (Version 3.0 November 2012, Section 3.7.2.2.1.1, p. 325) cites the CLP regulation: "3.7.2.4.3 Classification shall not automatically be discounted for substances that produce developmental toxicity only in association with maternal toxicity, even if a specific maternally-mediated mechanism has been demonstrated. In such a case, classification in Category 2 may be considered more appropriate than Category 1. …".

No information is available to judge whether the observed effects on (rat) offspring have to be regarded as secondary non-specific consequences of maternal toxicity.

In summary, classification in Category 2 (H361d, CLP criteria) is considered appropriate.

The notifiers considered a classification as a developmental toxicant as not necessary, because in their opinion, effects on foetuses occurred in the presence of maternal toxicity only. Hence, the effects were deemed as secondary non-specific consequences of maternal toxicity which would not warrant classification.

During an expert consultation in the PPP procedure, it was discussed whether classification with R63 should be proposed: "There was a feeling that R63 was not appropriate based on the dataset available and incidences seen in the rat studies. […] Experts voted on the classification issue and a majority agreed to not propose any classification" (cited from the meeting minutes). This recommendation was based mainly on the low incidences observed in the developmental toxicity study in rats with NeemAzal.

Adverse effects on lactation:

No data are available to judge whether there are specific effects on or via lactation (H362). Under the conditions of the 2-generation study, no effects on any investigated parameter were reported up to the highest dose tested.

4.10.6 Conclusions on classification and labelling

Regarding effects on fertility, the data are considered conclusive but not sufficient to trigger classification for such effects.

Regarding developmental toxicity, classification in Category 2 (H361d, CLP criteria) is considered appropriate.

No data are available to judge whether there are specific effects on or via lactation (H362).

4.11 Other effects

4.11.1 Non-human information

4.11.1.1 Neurotoxicity

A 21-d study on repeated-dose delayed neurotoxicity in chicken was conducted (Chandrasekaran, 1998, TOX1999-226) with a 21-d post-dosing recovery period. After gavage of NeemAzal technical (up to 1000 mg/kg bw/d), neither neurotoxicological nor other effects were observed. Deficiencies in the study design were that neuropathy target esterase was not measured and that only 3 animals per dose group were used.

Azadirachtin technical is not known to contain organophosphorous structures; therefore, no additional studies on delayed neurotoxicity were necessary.

No neurotoxicity studies in rats were submitted that were conducted with any of the extracts.

4.11.1.2 Immunotoxicity

No studies were submitted that were conducted with any of the extracts.

4.11.1.3 Specific investigations: other studies

No studies were submitted that were conducted with any of the extracts.

4.11.1.4 Human information

Routine medical observation (general [e.g., fever, weakness, sweating] and special signs [*gastro intestinal:* e.g., nausea, vomiting; *neuromuscular:* e.g., headache, dizziness; *cardio respiratory:* e.g., nasal discharge, cough, tachycardia; *eye:* e.g., ophthalmic examination, double vision; *psychological:* e.g., temperament, nervousness] of toxicity, vital signs [e.g., blood pressure, pulse, respiratory rate], blood chemistry, haematology) of workers exposed to neem extracts did not show adverse health effect (Venkataram, 2002-2004, TOX2005-2337, TOX2005-2338, TOX2005-2339; Kumar, 2005, TOX2005-2403; Mahesh, 2005, TOX-2404).

There were reports in open literature about intoxications (and deaths) of infants after intake of neem oil as medication (estimated intake: 5-50 mL). Initial clinical signs included vomiting, convulsion, and at later stages metabolic acidosis with coma. Post-mortem examination revealed histological liver damage, such as lipid infiltration in hepatocytes, damage of mitochondria, and sometimes encephalopathy (Sundaravalli et al., 1982, TOX2006-3064; Sinniah et al., 1981, TOX2006-3062; Sinniah et al., 1982, TOX2006-3061). In some reports relatively high case numbers are given, e.g. more than 60 (supposed or verified) intoxications of children with neem oil within 5 yr in one hospital in Madras/India (Sinniah et al., 1981, TOX2006-3062). Neem oil is a common treatment in southern Asia, therefore, the incidence of cases with such severe adverse effects can not be judged. Clinical signs, occurrence in children following often an infection, and pathology results are similar to Reye-syndrome, which occurs rarely, but most times after virus infections (influenza, chicken pox) and subsequent treatment with certain drugs (e.g., acetyl salicylic acid) (Sinniah & Baskaran, 1981, TOX2006-3060; Beers & Berkow, 1999, TOX2006-3056; Gerok, 1996, TOX2006-3058). A Reye-like syndrome was induced by treatment of rats and mice with neem oil. In contrast to

humans, however, microsomal liver enzymes were not decreased, and brain oedema did not occur (Sinniah et al., 1985, TOX2006-3063).

The toxic substance and the mode of action were unknown. Therefore, the observed effects could not be attributed to any single constituent of neem oil.

Neem oil and azadirachtin technical extracts are both generated from neem seed (kernels). Neem oil is generated out of crushed kernels by pressing or by extraction with hexane. One of the azadirachtin technical extracts (NeemAzal) is generated by extraction with polar protic and aprotic solvents and precipitation with a non-polar solvent. For another extract (SIPCAM), the kernel press cake (i.e., without oil) is extracted with polar protic and polar aprotic solvents and precipitated with non-polar aprotic solvent. The third extract (ATI-720) is generated by extraction with polar aprotic solvent and precipitation with unpolar solvent followed by further physical clean-up. Chemical composition of the extracts was described by the notifiers, but the composition of neem oil is unknown up to a great extent. Lipids/fatty acids (total fatty acid content: 10-90% (wt/wt)), azadirachtin (between "not detectable" up to 2323 ppm), nimbin (between "not detectable" up to 18132 ppm) and salannin (between "not detectable" up to 47150 ppm) have been described in neem oil (Kumar & Parmar, 1996). Therefore, even though neem oil and azadirachtin technical extracts have – in part – the same constituents, it is unknown if the observed effects on human and rat livers were caused by these known compounds. Hence, it is proposed not to use the results derived from other extracts than azadirachtin technical extracts for classification and labelling.

4.11.2 Summary and discussion

No relevant information on the extracts NeemAzal, Fortune Aza or ATI-720 was submitted.

4.11.3 Comparison with criteria

No data available to allow a comparison

4.11.4 Conclusions on classification and labelling

Data lacking.

5 ENVIRONMENTAL HAZARD ASSESSMENT

Margosa extract is currently not legally classified related to the environmental hazards. The aquatic effect studies that are relevant for classification are presented in the following.

5.1 Degradation

Table 34: Summary of relevant information on degradation

Method	Results	Remarks	Reference
OECD 301 D	5.6% after 28 d	Not readily biodegradable	Werle (1998), report no. 97 50 40 787
OECD 301 F	36.8 – 48.2% after 35 d	Not readily biodegradable	Hund, K. (1998a), report no. TRF-001/3-15
OECD 301 F	49.1% after 47 d	Not readily biodegradable	Hund, K. (1999a), report no. TRF-001/3-15/1
OECD 111	Half life at 12 °C: pH 4 = 112.7 d pH 7 = 40.9 d pH 8 = 8.2 d	hydrolytic degradation, increasing with temperature and pH	Troβ, R. (1996a), report no. TM 1195.15 and Troß, R. (1997), report no. LP 97.04

5.1.1 Stability

It has to be noted that for the available stability studies margosa extract (30% azadirachtin A) was the test substance and azadirachtin A was used as lead substance since it is the major component (30 - 34%) of margosa extract.

Table 35: Hydrolytic degradation

Method / Guideline	pH	Temperature [°C]	Initial TS concentration, C_0 & [mol/L x 10^{-4}]	Reaction rate constant, K_h [1/h]	Half-life, DT_{50} [h]	Coefficient of correlation, r_2	Reference
OECD 111	4	30	0.82	0.00271	256	0.9174	Troβ, R. (1996a), report no. TM 1195.15
	7		0.78	0.00610	114	0.9927	
	8		1.12	0.03027	23	0.9987	
	4	40	1.24	0.01061	65	0.9604	
	7		1.24	0.02376	29	0.9986	
	8		1.22	0.12891	5	0.9963	
	4	40	1.16	0.01244	56	0.9749	
	7		1.13	0.02201	31	0.9945	
	8		1.09	0.12636	5	0.9993	
Method / Guideline	**pH**	**Temperature [°C]**	**Initial TS concentration, C_0 & [mol/L x 10^{-4}]**	**Reaction rate constant, K_h [1/h]**	**Half-life, DT_{50} [d]**	**Coefficient of correlation, r_2**	**Reference**
OECD 111 (Mathematical Calculation)	4	18		0.00042	68.8		Troß, R. (1997), report no. LP 97.04
	7			0.00111	26.1		
	8			0.00472	6.1		
	4	20		0.00058	49.9		
	7			0.00148	19.5		
	8			0.00651	4.4		
	4	22		0.00079	36.4		
	7			0.00198	14.6		
	8			0.0892	3.2		
	4	12		$2.563*10^{-4}$	112.7		
	7			$7.056*10^{-4}$	40.9		
	8			$3.503*10^{-3}$	8.2		

& concentrations refer to azadirachtin A, i.e. the major component (ca. 30% of TS) of the test substance margosa extract

In the first study, the hydrolysis of azadirachtin A as function of the pH was tested by two temperatures, 30 °C and 40 °C. The hydrolytic stability of azadirachtin A is strongly pH-dependent as indicated by a significant increase in the rate of degradation with increasing pH. At high water temperatures of 30 to 40 °C, azadirachtin A has a rapid half-life time of 5 to 23 hours in slightly alkaline conditions at pH 8 to ca. 2 ¼ to 10 days in acidic conditions at pH 4.

In the second study no materials were used, the study involves a mathematical calculation. The experimental determination of the reaction rate for the hydrolysis of Azadirachtin A was been conducted at two temperatures (30 and 40 °C, refer to the first study). These reaction rate values were extrapolated for other temperatures (18, 20 and 22 °C) with the help of the "Arrhenius equation": $\ln k = \ln A - E_a/RT$.

The extrapolation of the test results to the average outdoor temperature in the EU of 285.15 K using the Arrhenius equation yields a half-life of 112.7, 40.9 and 8.2 days at pH 4, 7 and 8, respectively. Hydrolysis products are not detectable due to the technical limitations with regard to radiolabelling of the test substance and synthesis of reference substances.

Azadirachtin A undergoes hydrolytic degradation, the rate of degradation is pH and temperature dependant, it increases with increasing pH and temperature.

Table 36: Photolysis in water

Method / Guideline	Initial TS concentration, C_0 [&] [mol/L x 10^{-6}]	Total recovery of test substance [% of applied a.s.]	Photolysis rate constant (k^c_p)	Direct photolysis sunlight rate constant (k_{pE})	Reaction quantum yield (Φ^c_E)	Half-life ($t_{1/2E}$)	Reference
OECD Draft (part A) "Direct Phototrans-formation", 1990	9.1	test conducted with unlabelled TS, therefore no balance established	not given	not given	5.55 x 10^{-4}	not determined	Werle, H. (1995), report no. 95 50 40 827 B and Werle, H. (1999), report no. 995040 819 (calcu-lation)

[&] concentration refer to azadirachtin A, i.e. the major component (ca. 30% of TS) of the test substance margosa extract

Aqueous photolytic half-lives for margosa extract were calculated based on the quantum yield and UV/VIS data from the direct phototransformation study in water of margosa extract and parameters included in the computer model "ABIWAS" (initial a.s. concentration: 10^{-5} mol/L; water body: 100 m^2 surface, 0.1 m depth; degradation only via direct photolysis; spectral photon irradiance latitude 55°N; January scenario: 2 °C, 8.0-hour day; July scenario: 20 °C, 16.1-hour day).

The half-life times for January were estimated to be:
Minimum: 26.5 days; Normal: 1.8 months; Maximum: 7.2 months.

The half-life times for July were estimated to be:
Minimum: 3.8 days; Normal: 5.5 days; Maximum: 19.2 days.

Table 37: Phototransformation in air

Method / Guideline	Time-dependent OH-radical concentration [OH radicals cm^{-3}]	Overall reaction rate constant k [cm^3 x $molecule^{-1}$ x s^{-1}]	Half-life [h]	Reference
Model calculation using estimation method by AOPWIN version 1.88	24-h average 5.0 x 10^5	227.03 x 10^{-12}	1.696	Mueller, M. (1999), report no. notgiven

Degradation of organic compounds in the atmosphere is mainly based on the reaction with hydroxyl radical. For this reaction, the rate constant can be determined by AOP. Together with an assumed hydroxyl radical concentration in the atmosphere, an estimate of the atmospheric half life is possible. The calculated half-life for azadirachtin A is 1.696 h (equivalent to 0.071 d).

With regard to this estimated valuefor azadirachtin A, long-term transport and accumulation in air are not to be expected.

Furthermore, the tendency of azadirachtins, the major components of margosa extract, to enter the atmosphere is considered to be low taking into account both the vapour pressure of these compounds (3.6 x 10^{-13} Pa) and the Henry's Law Constant (2.4 x 10^{-14} Pa m^3/mol).

5.1.2 Biodegradation

5.1.2.1 Biodegradation estimation

No estimation of biodegradation was conducted.

5.1.2.2 Screening tests

Table 38: Ready biodegradability

Method/ Guideline	Test para-meter	Inoculum	Test conditions			Test substance conc.	Degradation		Reference
		Type	Tempera ture	pH	Toxicity control		Incub. period	Degree [%]	
OECD 301 D[1]	oxygen con-sumption	activated sludge	19.6°C to 22.9°C	Not specified in report	NeemAzal (at 5.4 mg /L), 5.046 mg sodium acetate/L	1.8, 3.6 & 5.4 mg margosa extract (a.s.)/L	28 days	5.6	Werle (1998), report no. 97 50 40 787
OECD 301 F[1]	oxygen con-sumption	activated sludge	22 ± 1°C	At the end of the test (35 d), pH of solutions with test substance ranged from 6.0 to 8.5	NeemAzal (at 100 mg/L), sodium benzoate (at 100 mg/L)	100 mg margosa extract (a.s.)./L	35 days	36.8 (23.7 at the end of the 10-day window)	Hund, K. (1998a), report no. TRF-001/3-15
		activated sludge & aqueous soil extract with soil micro-organisms				100 m g margosa extract (a.s.)/L	35 days	48.2 (36 at the end of the 10-day window)	
OECD 301 F[1]	oxygen con-sumption	activated sludge & aqueous soil extract with soil micro-organisms	22 ±1°C	At the end of the test (47 d), pH of solutions with test substance ranged from 3.16 to 3.67	NeemAzal (at 100 mg/L), sodium benzoate (at 100 mg/L)	100 mg margosa extract (a.s.)/L (dissolved in DMSO)	47 days	49.1 (28.1 at the end of the 10-day window)	Hund, K. (1999a), report no. TRF-001/3-15/1

[1] Test on ready biodegradability according to OECD criteria

The biodegradability of the active substance margosa extract (34% azadirachtin A) was investigated in a total of three tests on ready biodegradability. In all tests the degradation was followed by the determination of oxygen consumption. The inoculum was not pre-adapted to the test substance and no additional substrate was used.

One test was conducted according to OECD guideline 301 D using activated slugde as inoculum. The % biological degradation, as calculated from the biological oxygen demand (BOD)/COD ratio

never increased above 10 % during the course of the test and was 5.6 % at the end of the test. Therefore margosa extract was shown to be not readily biodegradable within 28 days.

In a second test conducted according to OECD guideline 301 F, ready biodegradability was investigated using two different kinds of inoculum, activated sludge and a mixture of activated sludge and aqueous soil extract containing soil microorganisms. The results of this test confirmed margosa extract as not being readily biodegradable with 36.8% and 48.2% degradation within 35 days. At the end of the 10-day window the a.s. was degraded to 23.7% and 36%, respectively.

The third test was conducted according to OECD guideline 301 F and investigated ready biodegradability of margosa extract using a mixture of activated sludge and aqueous soil extract containing soil microorganisms. As in both other tests, the result of 49.1% degradation within 47 days and 28.1% at the end of the 10-day window demonstrated margosa extract to be not readily biodegradable.

5.1.2.3 Simulation tests

The technical active substance margosa extract consists of a complex mixture of related triterpenoids extracted from the seed kernels of the neem tree *Azadirachta indica A. Juss.*using a specific procedure. Taking into consideration the origin of the extract from higher plants and the biosynthetic pathway leading to these triterpenoids, radiolabelling of the main components of the active substance is not feasible since it is not possible to synthesise margosa extract chemically. A way to synthesise the major individual component of the active substance, azadirachtin,was published in 2007 (S. Ley et al., Angewandte Chemie, 119, 40, 7773-7776), therefore even synthesis of the lead substances was not possible up to the time-point of dossier submission by the applicant in 2006.

In view of this dilemma, the major individual component of margosa extract, i.e., azadirachtin A, which accounts for about one third of the total mass of the extract, was chosen as the lead substance for describing the behaviour of margosa extract in the environment.

Since data on ready biodegradability are available for margosa extract and thus classification of the active substance margosa extract is based on these data, results from simulation studies conducted with azadirachtin A are not described in this report. Furthermore, only literature data on degradation behaviour in water-sediment-systems for the compound azadirachtin A were presented, which were only be regarded as additional information. Information on simulation studies conducted with azadirachtin can be found in the CLH report of azadirachtin.

5.1.3 Summary and discussion of degradation

Margosa extract has shown a biodegradation of 5.6% in 28 days, 36.8% and 48.2% in 35 days and 49.1% in 47 days in tests according to OECD guidelines 301 D and F and has therefore to be regarded as not readily biodegradable.

The extrapolation of the hydrolysis stabilisation test results to the average outdoor temperature in the EU (285.15 K) yields a half-life of 112.7, 40.9 and 8.2 days at pH 4, 7 and 8, respectively. Therefore, margosa extract is expected to undergo hydrolysis degradation under natural conditions. Hydrolysis products are not detectable due to the technical limitations with regard to radiolabelling of the test substance and synthesis of reference substances.

5.2 Environmental distribution

5.2.1 Adsorption/Desorption

The adsorption/desorption-study was conducted with margosa extract (34% azadirachtin A) as test substance and azadirachtin A was used as lead substance since it is the major component (30-34% of margosa extract.

Table 39: Adsorption/desorption screening test

Method / Guideline	Tested soils/ Classifi-cation	Adsorbed a.s. [&] [%]	K_a [1]	K_{aOC} [2]	K_d [3]	K_{dOC} [4]	K_a / K_d [5]	Degradation products		Reference
								Name	[%] of a.s.	
OECD 106	Speyer 2.1/ sand	7.55	0.405	65.4	n.d.	--	n.a.	none	--	Troβ, R. (1996b), report no. TM 995.12
	Speyer 2.2/ loamy sand	8.70	0.479	20.6	n.d.	--	n.a.	none	--	
	Speyer 2.3/ loamy sand	6.95	0.373	30.6	n.d.	--	n.a.	none	--	

[1] K_a = Adsorption coefficient; [2] K_{aOC} = Adsorption coefficient based on organic carbon content; [3]K_d = Desorption coefficient; [4] K_{dOC} = Desorption coefficient based on organic carbon content; [5] K_a / K_d = Adsorption / Desorption distribution coefficient

[&] concentration refer to azadirachtin A, i.e. the major component (ca. 30% of TS) of the test substance margosa extract; n.d. = not determined due to the low adsorption (< 10%); n.a. = not applicable

The adsorption properties of azadirachtin A were investigated in three soils of two different soil types (sand, sandy loam) in the study of Troß (1996b). The resulting K_{OC} values were in the range of 20.6 mL/g in loamy sand to 65.4 mL/g in sand. With regard to the low K_{OC} values in the tested soils, azadirachtin A is slightly adsorbed to soil, indicating a high to moderate potential mobility in soil.

5.2.2 Volatilisation

The tendency of azadirachtins, the major components of margosa extract, to enter the atmosphere is considered to be low taking into account the low vapour pressure of these compounds (3.6×10^{-13} Pa) and the Henry's Law Constant (2.4×10^{-14} Pa m^3/mol).

5.2.3 Mobility

The column leaching study was conducted with margosa extract as test substance and azadirachtin A was used as head substance since it is the major componentof margosa extract.

Table 40: Column leaching study

Method/ Guideline	Soils / Classification	OC	pH	Design	Application rate	Residues in leachate [% of applied Aza A]	Reference
BBA Part IV, 4-2	Speyer 2.1/ sand	0.62	5.9	glass columns, 65 mm i.d.; 30 cm soil depth of water- saturated soil; 200 mm rain within 2 d	33 mL of 10% aq. solution of NeemAzal-T/S eqv. to 32.8 mg azadirachtin A	90.4	Troβ, R. (1995), report no. TM 995.11
	Speyer 2.2/ loamy sand	2.32	5.6			55.1	
	Speyer 2.3/ loamy sand	1.22	6.4			42.1	

i.d. = inner diameter

The high mobility of azadirachtin A in soil as already indicated by the low K_{OC} is confirmed under the stringent conditions of the laboratory column leaching test, i.e., highly exaggerated concentration of substance applied to soil, maximum water saturation of soils at test start, watering with 200 mm rain within two days following test substance application. However, contamination of groundwater by azadirachtin A under actual use conditions seems to be unlikely taking into account its short degradation half-life in soil.

5.3 Aquatic Bioaccumulation

Table 41: Summary of relevant information on aquatic bioaccumulation

Method	Results	Remarks	Reference
Estimation	BCF : 2.5 (Azadirachtin B) BCF 1.38 (Azadirachtin A)	Low potential for bioaccumulation	-

5.3.1 Aquatic bioaccumulation

5.3.1.1 Bioaccumulation estimation

Table 42: Determination of aquatic bioaccumulation

Basis for estimation	log K_{OW} (measured)	Estimated BCF for fish (freshwater) on wet weight basis	Estimated BCF for fish eating bird/predator	Reference
Standard equation (74), TGD on Risk Assessment (2003), Part II, chapter 3.8.3.2	1.29 (Azadirachtin B)	2.5	- -	- -
	0.99 (Azadirachtin A)	1.38	-	-

Determination of log K_{ow} for Margosa extracts is technically not feasible. Therefore, the bioaccumulation potential was estimated on the basis of the log Kow of selected azadirachtins.

5.3.1.2 Measured bioaccumulation data

No data available.

5.3.2 Summary and discussion of aquatic bioaccumulation

The calculated BCF_{fish} values of 2.5 (azadirachtin B) and 1.38 (azadirachtin A) indicate a low potential for aquatic bioaccumulation of the main components of margosa extract.

Furthermore, no other indicators point to an intrinsic potential for bioconcentration; for instance, the surface tension is 56.4 mN/m and thus lies above the trigger value of ≤ 50 mN/m.

5.4 Aquatic toxicity

Table 43: Summary of relevant information on aquatic toxicity

Method	Results	Remarks	Reference
OECD 203: Short-term toxicity to rainbow trout	96h-LC50 = 4.14 mg a.s./L	Study performed with the product NeemAzal-T/S	Grunert, B. (1996) report no. 94 50 41 389 C
OECD 202: Daphnia magna	48h-EC50 = 9.69 mg a.s/L		Schmitz, A. (1999) report no. TRF-001/4-21
OECD 219: *Chironomus riparius* emergence and development test	28d-NOEC = 0.0075 mg a.s/L		Gonsior, G. (2008a) report no. 2007/1356/01-ASCr
OECD 219: *Chironomus riparius* emergence and development test	28d-NOEC = 0.006 mg a.s./L	Study performed with the product NeemAzal-T/S	Gonsior, G. (2008b) report no. 2007/1355/01-ASCr

5.4.1 Fish

5.4.1.1 Short-term toxicity to fish

Table 44: Short-term toxicity to fish

Guideline / Test method	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]			Remarks	Reference
			design	duration	LC_0	LC_{50}	LC_{100}		
OECD 203 (1992)	Rainbow trout (*Onco-rhynchus mykiss*)	Mortality	Semi-static (48-h intervals)	96 h	0.9	4.14	8.5	effect values based on geometric mean of the measured concentrations at t=0 and t=48 h test substance: Neem/Azal-T/S, containing 4 % margosa extract	Grunert, B. (1996) report no. 94 50 41 389 C

The acute toxicity of margosa extract to rainbow trout was extrapolated from a semi-static test with the product NeemAzal-T/S. The test was conducted according to OECD No. 203 (1992). Each test system comprised ten fish in a volume of 30 L tap water. Five test substance concentrations (50-800 mg/L NeemAzal-T/S) and a control were established. The test organisms were transferred to fresh medium after 48 h. Analytical determination of the leading component azadirachtin A was performed at test start and after 48 h (before renewal of test solution). It is assumed that the mean

measured concentration for the first phase of the test is also representative for the second phase (48-96 h). Therefore, the effect values are based on the geometric mean of the measured concentrations at test start and after 48 h.

5.4.1.2 Long-term toxicity to fish

Table 45: Long-term toxicity to fish

Method / Guideline	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]		Remarks	Reference
			design	duration	NOEC	LOEC		
OECD 204	Rainbow trout, *Oncorhynchus mykiss*	Mortality, growth	Flow-through	28 d	1.9	4.4	Study performed with product NeemAzal-T/S, effect values related to active substance Margosa (content in product 4%)	Schmitz A. (1999) Report no TRF-002/4-17
OECD 210	Zebra fish, *Danio rerio*	Hatching and survival rate, length and weight (FI-, FII-generation); daily egg production and fertilisation rate (FI-generation)	Flow-through	174 d	2.0	6.4	Not valid, as survival of fertilized eggs in contol was < 70%	Schmitz, A. (2000) report no. TRF-001/4-60

A long-term fish test is available for the product NeemAzal-T/S. The study was performed according to OECD 204, however the study design is rather comparable to OECD 215 and therefore acceptable as long-term study. Test species was *Oncorhynchus mykiss*. Six test substance concentrations in the range of 4.7 to 150 mg/L NeemAzal-T/S as well as a control were prepared. 10 fish per concentration were exposed in a flow-through system over 28 days. Analytical monitoring of the test substance concentration was performed two times per week using azadirachtin A as leading compound (1% content in NeemAzal-T/S). The mean measured concentrations were in the range of 3.9 to 147.5 mg NeemAzal-T/S/L. A 28d-NOEC for mortaliy of 63.6 mg NeemAzal-T/S/L was found (based on mean measured concentrations). This corresponds to a NOEC related to the active substance margosa extract of 1.9 mg/L. No significant effects on growth rate or other sublethal parameters were found. Although the study was performed with the formulated product instead of the active substance, it is considered as adequate for the effects assessment of the active substance. According to the available data on the two formulation additives, the ecotoxicity of the b.p. is expected to be associated with the a.s. rather than to any of those additives.

In a further study, the chronic toxicity of margosa extract (purity 29.9 % Azadirachtin A) to zebra fish, *Danio rerio*, was investigated under flow-through conditions according to OECD No. 210 (1992). Four test substance treatments (nominal 0.20, 0.63, 2.00 and 6.40 mg a.s./L) and one blank control were set up at test start with each two replicates each containing 100 fertilized eggs in 12 L test medium. Survival and growth (body weight, length) of larvae was recorded on day 37. On day 38, juvenile fish were transferred to chambers with 25 L volume. On day 50, the number of fish per replicate was impartially equated to 50 and on day 84, when sexual development was finished,

number of fish was further reduced to 24 per replicate (sex ratio 2:1 male:female). Reproduction of F1 generation was evaluated between days 91 and 118. On day 135, 100 fertilised eggs of each replicate were transferred to 12 L test medium, and survival and growth of fry (F2) was determined after another 38 days. Nominal concentrations were satisfactory maintained up to and including the reproduction phase, but significantly lower than nominal during the second (F2) early life stage phase. No statistically significant difference between any test substance treatment and the control was found during the entire test period for any test parameter using average values of both replicates for the statistics. However, in one replicate of the 6.4 mg a.s./L treatment group, survival of fry of F1 was clearly decreased indicating a threshold for survival of fry at this concentration level. Although there was no similar finding with the F2 generation, this is not considered to disqualify the indication of a toxic effect in the F1 due to the significant decrease in the test substance concentrations during the second ELS phase. Therefore, the NOEC is established at 2.0 mg a.s./L. However, as the average survival of the control was only 56.6% after 37 d, the study is not valid and cannot be used for the further effects assessment.

5.4.2 Aquatic invertebrates

5.4.2.1 Short-term toxicity to aquatic invertebrates

Table 46: Short-term toxicity to invertebrates

Method / Guideline	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]			Remarks	Reference
			design	duration	EC_0	EC_{50}	EC_{100}		
OECD 202, Pt. I	*Daphnia magna*	Immobility	static	48 hours	2.00	9.69	>26.34	effect values based on initial measured conc.	Schmitz, A. (1999) report no. TRF-001/4-21

The acute toxicity of margosa extract (purity 33.4 % Azadirachtin A) to *Daphnia magna* was determined in a static test according to OECD No. 202 (1984). Five neonates (< 24 h) were held in 60 mL glass beakers containing 25 mL test medium and four replicate test systems were set up per treatment group. Six test substance concentrations (nominal: 2.5, 5.0, 10, 20, 40 and 80 mg a.s./L) were prepared adding the same volume of appropriate stock solutions in acetone to the test medium (≤ 0.01%). A blank and a vehicle control were tested in addition. Concentrations of the test substance were measured at 0 and 48 h using azadirachtin A as lead substance. The measured concentrations were lower than nominal at 0 h and increasing by 48 h in the medium and higher treatments (probably due to inhomogeneous mixing at start of the test). Therefore, as a worst-case approach, the toxicity values are calculated based on measured initial concentrations. Immobility of test organisms, determined at 24 and 48 h, was increasing with time showing a concentration-effect relationship (90% at the highest treatment level). Despite the analytical peculiarities, the test is considered acceptable and the toxicity data are reliable.

5.4.2.2 Long-term toxicity to aquatic invertebrates

Table 47: Long-term toxicity to invertebrates

Method / Guideline	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]		Remarks	Reference
			design	duration	NOEC	LOEC		
OECD 202, Pt. II	*Daphnia magna*	Reproduction & mortality	semi-static	21 days	1.84	>1.84	Effect values based on mean measured conc.	Schmitz, A. (1999) report no. TRF-001/4-21
OECD 202, Pt. II	*Daphnia magna*	Reproduction & mortality	semi-static	21 days	0.1	0.22	Study performed with product NeemAzal-T/S, effect values related to active substance Margosa extract (content in product 4%)	Schmitz A. (1999) Report no. TRF-002/4-21

The chronic toxicity of margosa extract (purity 33.4 % Azadirachtin A) to *Daphnia magna* was determined in a semi-static test according to OECD No. 202, Pt. II (1984). Ten daphnids per treatment level were individually confined in 60 mL glass beakers containing 50 mL test medium. The concentration of the test substance in the medium varied by more than ± 20%, therefore, the toxicity values were based on mean measured concentrations of 0.10, 0.21, 0.42, 0.90 and 1.84 mg a.s./L. Mortality of adult daphnids, appearance of first young and number of young daphnids were regularly checked. There was no statistically significant difference for any test parameter between any treatment level and the blank control. Accordingly, the NOEC was established as 1.84 mg a.s./L. The test is considered acceptable and the toxicity data are reliable.

In a second reproduction study with *Daphnia magna*, the chronic toxicity of the formulated product NeemAzal-T/S was examined. 10 daphnids per concentration were individually exposed in a semi-static system to 6 test substance concentrations in the range of 3.125 to 100 mg NeemAzal-T/S/L. Analytical monitoring of the test substance concentration was performed in fresh and old medium at each medium change using azadirachtin A as leading compound (1% content in NeemAzal-T/S). The mean measured concentrations were in the range of 1.7 to 62.5 mg NeemAzal-T/S/L. A 21d-NOEC for reproduction of 3.4 mg NeemAzal-T/S/L was found (based on mean measured concentrations). This corresponds to a NOEC related to the active substance margosa extract of 0.102 mg/L. Although the study was performed with the formulated product instead of the active substance, it is considered as adequate for the effects assessment of the active substance. According to the available data on the two formulation additives, the ecotoxicity of the b.p. is expected to be associated with the a.s. rather than to any of those additives.

5.4.3 Algae and aquatic plants

Table 48: Toxicity to algae

Method / Guideline	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]			Remarks	Reference
			design	duration	E_rC_{10}	E_bC_{50}[1]	E_rC_{50}[2]		
OECD 201	*Scenedesmus subspicatus* (green alga)	Cell density, biomass, growth rate	static	72 h	332	482	1041	Effect values based on nominal concentration; no exponential growth during the whole test duration	Wenzel, A. (2002) report no. TRF-001/4-30

The toxicity of margosa extract (purity 35 % Azadirachtin A) to the green alga *Scenedesmus subspicatus* was determined in a static test according to OECD No. 201 (1984). At the start of the test, alga inoculum of 10^4 cells/mL was introduced in a volume of 100 mL test medium in a 250 mL glass flask. Three replicate flasks were set up per treatment group and maintained under continuous light and shaking. The nominal test concentrations were 0, 10, 50, 100, 500 and 1000 mg a.s./L. Both azadirachtin A and azadirachtin B were measured at test start and end. As azadirachtin A was not stable in the test system (degradation by 96%), azadirachtin B was used as leading compound and was found to be stable also after 72 h. The concentration of azadirachtin B was > 120% of nominal and the concentration of azadirachtin A at test start was in the range of 85-113%. As it is unclear which azadirachtin is responsible for the effects, the effect values are based on nominal concentrations.

Clear adverse effects on the growth of algae were found at the two highest treatment levels in comparison with the control. The 72h-E_rC_{50} was calculated as 1041 and the respective E_bC_{50} was 482 mg a.s./L., The 72h-E_rC_{10} was calculated as 332 mg a.s./L. The contol cultures did not follow exponential growth during the whole test duration. Instead, a lag phase was observed for the first 24 h. As exponential growth is a prerequisite for growth rate evaluation, the test is formally not valid. However, as algae are clearly the least sensitive of the tested aquatic organisms, the test is regarded as acceptable for the effects assessment.

5.4.4 Other aquatic organisms(including sediment)

Table 49: Long-term toxicity to Chironomid larvae

Method / Guideline	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]		Remarks	Reference
			design	duration	NOEC	LOEC		
OECD 219	*Chironomus riparius*	Emergence, development rate	static	28 days	0.0184 (nominal conc.) 0.0075 (mean measured conc.)	0.0368 (nominal)		Gonsior, G. (2008a) report no. 2007/1356/01-ASCr
OECD 219	*Chironomus riparius*	Emergence, development rate	static	28 days	0.018 (nominal conc.) 0.006 (mean measured conc.)	0.036 (nominal)	Study performed with product NeemAzal-T/S, effect values related to active substance Margosa extract	Gonsior, G. (2008b) report no. 2007/1355/01-ASCr

The long-term toxicity of margosa extract (purity 34 % Azadirachtin A) to *Chironomus riparius* was examined according to OECD 219. Chironomid larvae were exposed to 0.0023, 0.0046, 0.00919, 0.0184, 0.0368, 0.0735, 0.147 and 0.294 mg a.i./L in a static water-sediment system for a period of 28 days. Four replicate test vessels were prepared for each test substance treatment group and for a blank control group. Additional 18 vessels were prepared for chemical analyses of the test item. During the experimental phase the larvae were fed daily with 1 mg fish food per larvae.

Based on the nominal concentrations, the 28-day EC_{50} for emergence was determined to be 0.0329 mg/L. The number of emerged midges in the test item treatments did not show a significant difference to the control at the nominal concentration up to and including 0.0184 mg/L. The time course of emergence, represented by the development rate, did not show a significant difference to the control at the nominal concentration up to and including 0.0368 mg/L. The overall NOEC was estimated to be 0.0184 mg/L and the overall LOEC was estimated to be 0.0368 mg/L.

Samples taken from the water phase, the pore water and the sediment of 0.0184 and 0.294 mg/L test vessels and of the control vessels were analysed at day 0, 7 and 28. The analytical measurements after 7 and 28 days showed a degradation of the test substance below the limit of quantification (LOQ) of 0.00625 mg/L for water and pore water and 0.0156 mg/kg for sediment. In the sediment the margosa extract concentrations did not exceed the LOQ during the whole study. Consequently the chironomids were not exposed to the nominal concentrations over the whole time. Therefore the mean of the NOEC based on nominal concentrations and the ½ LOQ (for water and pore water, because no test substance was found in the sediment) was calculated. The NOEC based on the geometric mean concentration was calculated to be 0.0075 mg/L.

In a further study the toxicity of the formulated product NeemAzal-T/S (purity 1 % Azadirachtin A) to *Chironomus riparius* was studied. Chironomid larvae were exposed to nominal concentrations of 0.0717, 0.143, 0.287, 0.573, 1.15, 2.29, 4.59 and 9.17 mg NeemAzal-T/S/L and an untreated control in using the same test design as described above.

Based on the nominal concentrations, the 28-day EC_{50} for emergence was determined to be 1.15 mg NeemAzal-T/S/L. The number of emerged midges in the test item treatments did not show a significant difference to the control at the nominal concentration up to and including 0.573 mg NeemAzal T/S/L. The time course of emergence, represented by the development rate, did not show a significant difference to the control at the nominal concentration up to and including 1.15mg/L. The overall NOEC was estimated to be 0.573 mg NeemAzal T/S/L and the overall LOEC was estimated to be 1.15 mg NeemAzal T/S/L.

Samples of the overlying water, pore water and the sediment were taken 1 hour, 7 days and 28 days after application for the concentrations 0.573 and 9.17 mg NeemAzal T/S/L and for the control. The analytical measurements after 7 and 28 days showed a degradation of the test substance below the limit of quantification (LOQ) of 0.183 mg NeemAzal-T/S/L for water and pore water and 0.475 mg/kg for sediment. In the sediment the NeemAzal-T/S concentrations did not exceed the LOQ during the study (measured on day 0, 7 and 28). Consequently the chironomids were not exposed to the nominal concentrations over the whole time. Therefore the mean of the NOEC based on measured concentration at test start and the ½ LOQ (for water and pore water, because no test substance was found in the sediment) was calculated. The NOEC based on the geometric mean concentration was calculated to be 0.2 mg NeemAzal-T/S/L. This corresponds to a NOEC related to the active substance margosa extract of 0.006 mg/L.

The results from both studies related to marogsa extract are in good agreement.

5.5 Comparison with criteria for environmental hazards (sections 5.1 – 5.4)

5.1 → Degradation: not rapidly degradable;

→ Hydrolysis: hydrolytical degradable

According "Guidance on the application of the CLP criteria" hydrolysis might be considered for classification only when the longest half-life determined with the pH-range 4-9 is shorter than 16 days and if the hydrolysis products do not fulfil the criteria for classification as hazardous to the aquatic environment. Because the longest half-life for margosa is 112.7 days, hydrolysis will not be considered.

5.2 →Adsorption/desorption: not relevant for classification and labelling

Volatilisation: not relevant for classification and labelling
According to "Guidance on the application of the CLP criteria", volatilization only represents removal of a chemical from the water phase, and not degradation, the Henry's Law constant cannot be used for assessment of degradation in relation to aquatic hazard classification of substances.

Mobility: not relevant for classification and labelling

5.3 → Aquatic Bioaccumulation: log K_{ow}< 4 (low bioaccumulation potential)

5.4 → Aquatic Toxicity: not acutely toxic (EC/LC_{50}> 1 mg/L), but toxic to aquatic life with long lasting effects (NOEC < 0.1 mg/L)

The lowest long-term effect value (28d-NOEC = 0.006 mg a.s./L) was found for the midge larvae *Chironomus riparius* in a water-sediment study according to OECD 219 (spiked water). Although this is not a standard test system for classification, the use of this value is

justified by the insecticidal mode of action of the substance as well as by the fact that exposure of the test organisms was predominantly via the water phase.

5.6 Conclusions on classification and labelling for environmental hazards (sections 5.1 – 5.4)

The effect level for acute category 1 with $EC_{50} \leq 1$ mg a.s./L is not reached for margosa extract. The lowest acute value is the 96h-LC50 of 4.14 mg a.s./L from an acute toxicity test with rainbow trout.

In two long-term toxicity studies with *Chironomus riparius* NOEC values of 0.006 and 0.0075 mg a.s./L were found, which triggers the environmental classification for chronic toxicity for not rapidly degradable substances.

According to CLP-Regulation the substance is classified as Aquatic Chronic 1 (H410).

M-Factor: The chronic M-Factor is 10 based on the NOEC from test with Chironomus of 0.006 mg a.s./L for a not rapidly degradable substance (i.e. $0.001 < NOEC \leq 0.01$mg/L).

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McRae LA	1997	NeemAzal technical - Acute oral toxicity to the rat. PROJECTID.: EIP 6/950799/AC unpublished TOX9700502	TRF
McRae LA	1997	Fortune Aza technical acute oral toxicity to the rat FBT 6/951815/AC unpublished TOX2005-2362	SIP
McRae LA	1997	NeemAzal technical - Acute dermal toxicity to the rat. PROJECTID.: EIP 7/950800/AC unpublished TOX9700503	TRF
McRae LA	1997	Fortune Aza technical - Acute dermal toxicity to the rat FBT 7/951816/AC unpublished TOX2005-2370	SIP
Moorthy MV	1993	Acute oral toxicity of neemazal technical in rat. REP. NO.: 1744 ! PROJ. NO.: 05-021-93 unpublished TOX9750130	TRF
Moorthy MV	1993	Acute oral toxicity of Neemazal technical in mice <none> unpublished TOX2006-592	TRF

Author(s)	Year	Title source (where different from company) report no. published or not BBA registration number	Owner
Moorthy MV	1996	Carcinogenicity study of NeemAzal-F 5% in mice. 1544/JRF/TOX/96 unpublished TOX9700523	TRF
Murli, H.	1992	Dose rangefinding and mutagenicity test on Neem concentrate TGAI in an in vivo mammalian mutagenicity assay 15032-0-455 ! 15032-0-459PO - GLP: Y, published: N 1863424 /	MTA
Myers DP, Dawe IS	1997	NeemAzal technical - A preliminary study of developmental toxicity in rats. PROJECTID.: EIP 1/951879 unpublished TOX9700510	TRF
Myers DP, Dawe IS	1997	NeemAzal technical - A study of developmental toxicity in rats (gavage administration). PROJECTID.: EIP 2/952493 unpublished TOX9700514	TRF
Parcell BI	1996	NeemAzal technical - Skin irritation to the rabbit. PROJECTID.: EIP 8/950822/SE unpublished TOX9700505	TRF
Parcell BI	1996	NeemAzal technical - Eye irritation to the rabbit. PROJECTID.: EIP 9/950823/SE unpublished TOX9700506	TRF
Parcell BI	1997	Fortune Aza technical - Skin irritation to the rabbit FBT 8/951939/SE unpublished TOX2005-2378	SIP
Parcell BI	1997	Fortune Aza technical - Eye irritation to the rabbit FBT 9/952651/SE unpublished TOX2005-2382	SIP
Pfau W	2005	Statement on subchronic toxicity study in a second mammal with Azadirachtin 161266-A2-050303-1 unpublished TOX2005-2389	SIP/ MIT
Pfau, W.	2009	Evaluation of the reproductive toxicity of Azadirachtin 379234-A2-050601-01 - GLP: N, published: N 1863427 /	TAF

Author(s)	Year	Title source (where different from company) report no. published or not BBA registration number	Owner
Pfau, W.	2012	Toxicological and metabolism studies on the active substance - Tier 2, IIA-5 Date: March 2012 Report number: MII / Sec. 3 BfR report number: ASB2012-6696	TAF
Proudlock R J, Statham J, Howard WR, Dawe IS	1997	Fortune Aza technical Mouse micronucleus test FBT 13/952782 unpublished TOX2005-2399	SIP
Proudlock RJ, Statham J, Howard WR, Dawe IS	1997	NeemAzal technical - Mouse micronucleus test. PROJECTID.: EIP 13/952782 unpublished TOX9700513	TRF
Ramamoorthy S	2000	Evaluation of toxicity of NeemAzal technical to reproductive process in Wistar rats - segment IV - toxicity to two generation reproductive process. 4826 unpublished TOX2001-173	TRF
Ryan B	1994	A developmental toxicity study of orally administered ATI-720 in rabbits L 08424 Study No2b unpublished TOX2005-2402	MIT
Sadre NL, Deshpande VY, Mendulkar KN, Nandal, DH	1984	Male antifertility activity of Azadirachta indica in different species. In: Schmutterer & Ascher (eds.): Natural pesticides from the neem tree (Azadiracha indica A. Juss) and other tropical plants: Proceedings of the 2nd Internat. Neem Conference, Rauischholzhausen/FRG. Schriftenreihe der GTZ (No. 161) Eschborn. Published TOX2006-3049	
Scott, R.H., O'Brien, K., Roberts, L., Mordue, W., Mordue Luntz, J.	1999	Extracellular and intracellular actions of azadirachtin on the electrophysiological properties of cultured rat DRG neurones Comparative Biochemistry and Physiology, Part C Pharmacology, Toxicology and Endocrinology 123, 85-93 GLP: O, published: Y 1893615 /	-
Sherwood R	1990	Dermal sensitization study of NPI 720 in guinea pigs using the modified Buehler method L 08257 Study No 1 unpublished TOX2005-2383	MIT
Singh, U.P., Singh, D.P.	2002	Neem in human and plant disease therapy Journal of herbal pharmacotherapy 2, 13-28 GLP: O, published: Y 1893672 /	-

Author(s)	Year	Title source (where different from company) report no. published or not BBA registration number	Owner
Sinha KC, Riar SS, Bardhan J, Thomas P, KainAK, Jain RK	1984	Anti-implantation effect of neem oil. Indian J Med Res (80) 708-710. Published TOX2006-3051	
Sinha KC, Riar SS, Tiwary RS, DhawanAK, Bardhan J, Thomas P, KainAK, Jain RK	1984	Neem oil as a vaginal contraceptive. Indian J Med Res (79) 131-136. Published TOX2006-3052	
Sinniah D, Baskaran G	1981	Margosa oil poisoning as a cause of Reye's syndrome. Lancet (317) 487-489. Published TOX2006-3060	
Sinniah D, Baskaran G, Looi LM, Leong KL	1982	Reye-like syndrome due to margosa oil poisoning: report of a case with postmortem findings. Am J Gastroenterol (77) 158-161. Published TOX2006-3061	
Sinniah D, Baskaran G, Vijayalakshmi B, Sundaravelli N	1981	Margosa oil poisoning in India and Malaysia. Trans R Soc Trop Med Hyg (75) 903-904. Published TOX2006-3062	
Sinniah D, Schwartz PH, Mitchell RA, Arcinue EL	1985	Investigation of an animal model of a Reye-like syndrome caused by Margosa oil. Pediatr Res (19) 1346-1355. Published TOX2006-3063	
Stien J	2006	In Vitro assessment of the clastogenic activity of Neemazal in cultured human peripheral lymphocytes 19026/1/05 unpublished TOX2006-739	TRF
Stien J	2006	In vitro assessment of the clastogenic activity of Azadirachtin (A+B) in cultured human peripheral lymphocytes 19026/3/05 unpublished TOX2006-464	SIP
Stien J	2006	In Vitro assessment of the clastogenic activity of Neem Seed extract in cultured human peripheral lymphocytes 19026/2/05 unpublished TOX2006-463	MIT

Author(s)	Year	Title source (where different from company) report no. published or not BBA registration number	Owner
Strang, R.H.C.	2009	Opinion on the feasibility of sufficient isotopically-labelled Azadirachtin A GLP: N, published: N 1863421 /	TRF
Sundaravalli N, Raju BB, Krishnamoorthy KA	1982	Neem oil poisoning. Indian J Pediatr (49) 357-359. Published TOX2006-3064	
Talwar GP, Pal R, Singh O, Garg S, Taluja V, Upadhyay SN, Gopalan S, Jain V, Kaur J, Sehgal S	1995	Safety of intrauterine administration of purified neem seed oil (Praneem Vilci) in women & effect of its co-administration with the heterospecies dimer birth control vaccine on antibody response to human chorionic gonadotropin. Indian J Med Res (102) 66-70. Published TOX2006-3053	
Talwar GP, Raghuvanshi P, Misra R, Mukherjee S, Shah S	1997	Plant immunomodulators for termination of unwanted pregnancy and for contraception and reproductive health. Immunol Cell Biol (75) 190-192. Published TOX2006-3054	
Tewari RK, Mathor R, Prakash AO	1986	Post-coital antifertility effect of neem oil in female albino rats. IRCS Med Sci (14) 1005-1006. Published TOX2006-3055	
Venkataram TV	2002	Employees health record 2001 unpublished TOX2005-2337	TRF
Venkataram TV	2003	Employees health record 2002 unpublished TOX2005-2338	TRF
Venkataram TV	2004	Employees health record 2003 unpublished TOX2005-2339	TRF
Waterson L, Hawkins A	1995	Neemazal technical - 2 week palatability study in the rat. BDP/18 unpublished TOX9750142	TRF
Waterson LA	1997	NeemAzal technical - Toxicity study in rats by dietary administration for 4 weeks. PROJECTID.: EIP 3/960397 unpublished TOX9700508	TRF
Waterson LA	1997	NeemAzal technical - Toxicity study in rats by dietary administration for 13 weeks. PROJECTID.: EIP 4/963100 unpublished TOX9700509	TRF

Author(s)	Year	Title source (where different from company) report no. published or not BBA registration number	Owner
Waterson LA	1997	Fortune Aza technical - A preliminary study of the developmental toxicity in rats FBT 1/952837 unpublished TOX2005-2400	SIP
Waterson LA	1997	Fortune Aza technical - A study of the developmental toxicity in rats FBT 2/960340 unpublished TOX2005-2401	SIP
Waterson LA, Dawe IS	1997	Fortune Aza technical Toxicity study in rats by dietary administration for 4 weeks FBT 3/961630 unpublished TOX2005-2385	SIP
Waterson LA, Dawe IS	1997	Fortune Aza technical Toxicity study in rats by dietary administration for 13 weeks FBT 4/962744 unpublished TOX2005-2386	SIP

8 ANNEXES

confidential Annex

9 SUMMARY OF STUDIES RELATING TO HUMAN HEALTH HAZARD ASSESSMENT

The following evaluations were extracted from the documentation submitted for the EU PPP procedure (i.e., draft assessment report (2007), additional report (2009) and addendum 7 (2013)). In certain cases, waiving arguments or argumentations only relevant for the PPP procedure were removed.

9.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

No toxicokinetic studies available.

The notifiers submitted a position paper:

Reference: IIA 5.1.1 / 02

Report: Strang, R.H.C. (2009)

Opinion on the feasibility of sufficient isotopically-labelled azadirachtin A; Report No: none; Date: 06/05/09

In order to obtain meaningful data from *in vivo* metabolism and toxicokinetic studies at relevant dose levels, the employment of ^{14}C-labelled test material is inevitable. Because of the complexity of the chemical structure it is not possible to synthesise ^{14}C-labelled azadirachtin A. Although most recently the synthesis of azadirachtin A has been accomplished, the synthetic procedure consisted of over 70 steps with an overall yield of 0.00015%. Radiolabelled synthesis is normally even more complicated and, thus, practically impossible.

It is possible to synthesise azadirachtin A with a labelled acetyl group (C3 position) or tigloyl group (C1 position). However, these will be most probably lost during initial metabolic steps.

Comment by RMS:

Certainly, data on metabolites would be interesting and probably helpful, but they were not provided by the notifiers.

Indeed, it is possible to radiolabel azadirachtin A at the C1 or C3 position (see above), however, this would provide little new information: it is known or at least expected that ester groups are cleaved during metabolism, which would lead to a non-labelled remaining molecule. What would be needed is a compound that is (radio-) labelled at a position which is metabolically stable.

At the time the DAR was drafted, a total synthesis was not available, which has changed since then (reviewed by Jauch, 2008). It should be noted that a total synthesis with an overall yield of 0.00015% (Jauch, 2008) is of no practical use (this yield means: for each 1 g of azadirachtin A synthesised, 660 kg (!) of educts are needed). In addition, all other components of the technical

extracts would not be labelled. In our understanding, the notified active substance was neem kernel extract containing and erroneously named azadirachtin and not the pure chemical substance azadirachtin A.

In theory it would be possible to perform metabolism studies with non-labelled material and using instrumental analytical methods (e.g., LC-MS or GC-MS) to detect and quantify the metabolites. However, they would be highly complicated to interpret due to the complex nature/composition of the technical extracts even if the analytical methods for all compounds and their (potential) metabolites were available.

9.2 Acute toxicity

9.2.1 Non-human information

9.2.1.1 Acute toxicity: oral

Studies performed with NeemAzal

Reference:	TRF IIA 5.2.1 / 01
Report:	McRae, L. A. (1997) NeemAzal technical acute oral toxicity to the rat Huntingdon Life Sciences Ltd, Huntingdon, EnglandEIP 6/950799/AC ; TOX9700502
Guidelines:	EPA Pesticide Assessment Guideline 152-10 (1984) Corresponding to OECD Guideline 401 (1987), EEC Directive 92/69/EEC B.1
Deviations:	None
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be acceptable.

Material and Methods:

The test substance, NeemAzal technical (batch no.: IV, purity: 36% azadirachtin A), was administered by oral gavage to five overnight fastened Hsd/Ola:Sprague-Dawley(CD) rats (animals provided by Harlan Orlac, England) of each sex at a dose of 5000 mg/kg bw. The compound was dissolved in distilled water (10 mL/kg bw). Animals were observed for gross toxicity, behavioural changes and/or mortality at periodic intervals on the day of dosing (day 1) and twice daily, thereafter, until day 15. Bodyweights were determined on day 1 (pre-administration), day 8 and day 15. All animals were subjected to macroscopic gross examination consisting of opening the abdominal and thoracic cavities.

Findings:

No mortality occurred. Piloerection and pallor of the extremities were seen in all animals and were the only clinical signs observed. Recovery was complete on day 2. Slightly low bodyweight gains were recorded for four females on day 8 with a similar trend noted for one female on day 15. All other animals achieved satisfactory bodyweight gains throughout the study.

No abnormalities were found in the animals upon macroscopic post mortem examination 15 days after the treatment.

Conclusions:

The oral LD_{50} value of NeemAzal technical in rats was established as exceeding 5000 mg/kg bw.

Reference:	TRF IIA 5.2.1 / 02
Report:	Moorthy, M. V. (1993) Acute oral toxicity of NeemAzal technical in the rat Fredrick Institute of Plant Protection, Pappadai, India Report No 1744 ; TOX9750130
Guidelines:	Not given (method similar to OECD 401)
Deviations:	Necropsy not performed, no presentation (summarising or individual) of data on clinical signs and bodyweight. Dosing volume (20 mL DMSO / kg bw) is considered high. Sex of dead animals not reported. Unclear identity of test compound.
GLP:	No Statement on quality assurance. The facility was inspected 1999 by UK GLP monitoring authority.
Acceptability:	The study is considered to be supplementary.

Material and Methods:

The test substance, NeemAzal technical ("Azadirachtin Technical 25%"), was administered by oral gavage to five albino wistar rats of each sex (animals provided by the animal house of Fredrick Institute of Plant Protection and Toxicology) at a dose of 0, 1190, 2380 or 4760 mg/kg bw (compound dissolved in DMSO, dosing of 20 mL/kg bw).

Findings:

At the highest dose, 20% mortality occurred.

Clinical signs (dullness and reduced activity) were reported within first 24 h after dosing, no clinical signs were noted during the following observation time up to 2 weeks.

Conclusion:

The oral LD_{50} value of NeemAzal technical in rats was established as exceeding 4760 mg/kg bw.

Reference:	TRF IIA 5.2.1 / 03
Report:	Moorthy, M. V. (1993) Acute oral toxicity of NeemAzal technical in mice Fredrick Institute of Plant Protection, Pappadai, India Report No 1749; TOX2006-592
Guidelines:	Not given
Deviations:	No data on bodyweight and incidence of clinical signs reported. Unclear identity of test compound.
GLP:	No Statement on quality assurance. The facility was inspected 1999 by UK GLP monitoring authority.
Acceptability:	The study is considered to be supplementary.

Material and Methods:

The test substance, NeemAzal technical ("Azadirachtin Technical 25%"), was administered by oral gavage to five Swiss albino mice of each sex (animals provided by the animal house of Fredrick Institute of Plant Protection and Toxicology) at a dose of 0, 1190, 2380 or 3365 mg/kg bw (compound dissolved in DMSO, dosing 15 mL/kg bw).

Findings:

No mortalities occurred.

Reduced locomotor activity was observed within 48 h after dosing. No further clinical signs were reported during the following observation time up to 2 weeks. The study report does not report any characteristic abnormalities related to the test compound which were observed during gross pathological examination of dosed animals.

Conclusion:

The oral LD_{50} value of NeemAzal technical in mice was established as exceeding 3365 mg/kg bw.

Studies performed with Fortune Aza

Refrence:	SIP IIA 5.2.1 / 03
Report:	McRae, L. A. (1997) Fortune Aza technical acute oral toxicity to the rat Huntingdon Life Sciences Ltd, Huntingdon, England FBT 6/951815/AC; TOX2005-2362
Guidelines:	EPA Pesticide Assessment Guideline 152-10 (1984) Corresponding to OECD Guideline 401 (1987), EEC Directive 92/69/EEC B.1
Deviations:	None
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be acceptable.

Material and Methods:

The test substance, Fortune Aza technical (batch no.: 0010195-0050195, purity: 8.5% azadirachtin A+B), was administered by oral gavage to five Hsd/Ola:Sprague-Dawley (CD) rats (animals provided by Harlan Orlac, England) of each sex at a dose of 5000 mg/kg bw. Animals were overnight fastened. The compound was dissolved in distilled water (10 mL/kg bw). Animals were observed for gross toxicity, behavioural changes and/or mortality at periodic intervals on the day of dosing (day 1) and twice daily, thereafter, until day 15. Bodyweights were determined on day 1 (pre-administration), day 8 and day 15. All animals were subjected to macroscopic gross examination consisting of opening the abdominal and thoracic cavities.

Findings:

No mortality occurred. Piloerection was observed in all rats within five minutes of dosing and hunched posture was noted in all animals. Wadding gait and increased salivation were observed in one female and two males showed increased salivation. Recovery was complete on day 4. Slightly low bodyweight gains were recorded for one male and three females on day 8. The mean bodyweight gain shown by the animals over the study period was considered to be similar to that expected of normal untreated animals of the same age and strain. No abnormalities were found in the animals upon macroscopic post mortem examination 15 days after the treatment. There was no effect on bodyweight at termination.

Conclusions:

The oral LD_{50} value of Fortune Aza technical in rats was established as exceeding 5000 mg/kg bw.

Studies performed with ATI 720

Reference:	MAS IIA 5.2.1 / 01
Report:	Furedi-Machacek, E. M. (1990) Acute oral toxicity study of NPI 720 in rats (limit-test) IIT Research Institute, Life Science Research, 10 West 35th Street, Chicago, Illinois, USA, Project No L 08270 Study No 1; TOX2005-2357
Guidelines:	EPA Pesticide Assessment Guideline 152-10 (1984) Corresponding to OECD Guideline 401 (1987), EEC Directive 92/69/EEC B.1
Deviations:	There are no data on purity, stability, identity or batch number of the test article given in the report (notifier claimed, that typical concentrations were in the range of 8.3-9.5% Aza A). The study did not include concentration analysis of the test article in the suspension used for dosing.
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be acceptable.

Material and Methods:

The test substance, NPI 720 in 1% carboxymethyl cellulose, was administered by oral gavage in a twosplit dose to five overnight fastened CD rats (animals provided by Charles River) of each sex at a dose of 5000 mg/kg bw. Animals were observed for gross toxicity, behavioural changes and mortality for up to 14 days. All animals were subjected to gross examination.

Findings:

No mortality occurred. Lethargy and hunched posture were seen in all animals and were the only clinical sign observed. Recovery was complete on day 2. No abnormalities were found in the animals upon macroscopic post mortem examination 15 days after the treatment. There was no effect on bodyweight.

Conclusion:

The oral LD_{50} value of NPI 720 in rats was established as exceeding 5000 mg/kg bw.

Reference:	MAS IIA 5.2.1 / 02
Report:	Mega, W. M. (1992) Oral toxicity assay of NPI-720, Azatin technical grade, batches in female rats, IIT Research Institute, Life Science Research, 10 West 35th Street, Chicago, Illinois, USA, Project No L 08367 Study No 3; TOX2005-2361
Guidelines:	EPA Pesticide Assessment Guideline 152-10 (1984) Corresponding to OECD Guideline 401 (1987), EEC Directive 92/69/EEC B.1
Deviations:	No analysis to confirm homogenicity, stability or concentration of the test substance or of the test substance-suspension were performed. Only female rats were included in study. Dosage volume of 25 mL/kg bw is to high. On day 4 after dosing animals were observed only once. Only one week observation period. Necropsy not performed.
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be supplementary.

Material and Methods:

Two different batches of NPI 720 (batch no.: 22212R3 Sublot B and 22213R3 Sublot A, purity: 10% azadirachtin) in 1% aqueous carboxymethyl cellulose, were administered by oral gavage in a split dose (2x) to five female CD Sprague Dawley rats (animals provided by Charles River) each at a total dose of 5000 mg/kg bw. The compound (suspension in 1% carboxymethylcellulose) was applied by gavage as a twosplit doses of 25 mL/kg bw each with approximately 4 hours between doses. Control group received the vehicle alone. Animals were observed for gross toxicity, behavioural changes and/or mortality at periodic intervals on the day of dosing (day 1) and twice daily, thereafter, until day 7. Bodyweights were determined on day 1 (pre-administration), and on day 8 (sacrifice).

Findings:

No mortality occurred. No signs of toxicity were observed. There was no effect on bodyweight.

Conclusions:

The oral LD_{50} value of two batches of NPI 720 to female rats was found to exceed 5000 mg/kg bodyweight.

In a dose rangefinding study for chromosomal aberrations *in vivo* mouse bone marrow cells with ATI-720 1/3 female died at a dose level of 5000 mg/kg bw (Murli, 1993, TOX2005-2363). Males and all animals in lower dose groups survived the three day observation period.

9.2.1.2 Acute toxicity: inhalation

Studies performed with NeemAzal

Reference:	TRF IIA 5.2.3 /01
Report:	Jackson, G. C. (1997) NeemAzal technical acute inhalation toxicity in rats 4-hour exposure. Huntingdon Life Sciences Limited, England Report-no. EIP 5/951566.; TOX9750135
Guidelines:	EPA FIFRA Guideline 152-12 (1984) OECD 403, limit test (1981)
Deviations:	None
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be acceptable.

Material and Methods:

In an acute inhalation toxicity study, groups of young adult Sprague Dawley rats (animals provided by Charles River, England; 5/sex) were exposed by the inhalation route (whole body) to an aerosol of NeemAzal technical (batch no.: IV, purity: 36% azadirachtin A) for 4 hours at an actual concentration of 0.72 mg/L air. Other groups were exposed to air only. Compound concentration in the air and particle size were determined. Animals were observed during exposure and for 14 days post exposure. Bodyweights, food and water consumption were recorded daily. All animals were necropsied and subjected to gross macroscopic examination.

Findings:

Measured compound concentration in the air was 0.72 mg/L, nominal concentration was 15.3 mg/L air. Analysis of the particle size distribution resulted in a mass median aerodynamic diameter of 3.5 µm (standard geometric deviation: 2.4). The respirable portion was determined at 78%. No mortalities occurred. Signs seen during exposure to NeemAzal technical included a partial closing of eyes and the adoption of a hunched posture. A deposition of test material on the fur was seen with all test animals during exposure. Control animals appeared and behaved normal. No signs of toxicity were reported during the observation period. A deposition of test material on the fur was

seen in all test rats only after exposure. From the next day on, all animals appeared normal. The bodyweight gains were within the range expected for rats used in this type of study. Food consumption was slightly reduced for one day in test rats following exposure to NeemAzal technical. Subsequently, it was similar to that of control animals. The post-mortem findings after euthanasia did not show any macroscopic organ changes.

Conclusions:

From the results with NeemAzal technical it is concluded that the four-hour inhalation LC_{50} in rats (whole body) is greater than 0.72 mg/L, i.e., the highest technically achievable concentration.

Studies performed with Fortune Aza

Reference:	SIP IIA 5.2.3 / 02
Report:	Jackson, G. C. (1997) Fortune Aza technical acute inhalation toxicity in rats (4-hour exposure) Huntingdon Life Sciences Limited, England Report-no. FBT 5/952698; TOX2005-2373
Guidelines:	EPA FIFRA Guideline 152-12 (1984) OECD 403, limit test (1981)
Deviations:	None
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be acceptable.

Material and Methods:

In an acute inhalation toxicity study, groups of young adult Sprague-Dawley (CD) rats (animals provided by Charles River, England; 5/sex) were exposed (whole body) by the inhalation route to an aerosol of Fortune Aza technical (batch no.: 0010195-0050195, purity: 8.5% azadirachtin A+B) for 4 hours at an actual concentration of 2.45 mg/L air (nominal concentration: 11.7 mg/L air). Other groups were exposed to air only. Compound concentration in the air and particle size were determined. Animals were observed during exposure and for 14 days post exposure. Bodyweights, food and water consumption were recorded daily. All animals were necropsied and subjected to gross macroscopic examination.

Findings:

Analysis of the particle size distribution resulted in a mass median aerodynamic diameter of 3.7 µm (standard geometric deviation: 2.28). The respirable portion (< 7 µm) was determined to account for 78.1%. Under the conditions of this experiment, Fortune Aza caused one death (female). Clinical

signs of toxicity during exposure included partially closed eyes and wetness around the mouth. Residues of test material on the fur, wet fur around the snout and jaws were reported during the observation period while exaggerated respiratory movements and clear discharge from the eyes were observed in females only. All surviving animals were normal in appearance and behaviour by day 2. There was a reduction in bodyweight gain on day 1 in males exposed to Fortune Aza technical. Otherwise, the bodyweight gain for test rats was similar to that of the control rats. Food consumption was reduced one day following exposure to Fortune Aza technical. Food consumption was normal from day 2 of the observation period. Macroscopic abnormalities seen in the deceased female included severe congestion of the lungs and a gas filled stomach. One male rat had dark subpleural foci on all lobes of the lung. No abnormalities were observed in the other animals.

Conclusions:

From the results with Fortune Aza technical it is concluded that the 4-h inhalation (whole body) LC_{50} Fortune Aza technical in rats is greater than 2.45 mg/L, i.e, the highest technically achievable concentration.

Studies performed with ATI 720

Reference:	MAS IIA 5.2.3 / 01
Report:	Aranyi, C. (1990) Acute inhalation toxicity study of NPI 720-F in rats IIT Research Institute, Life Science Research, 10 West 35th Street, Chicago, Illinois, USA Project No L 08270 Study No L06-1; TOX2005-2371
Guidelines:	EPA FIFRA Guideline 152-12 (1984) OECD 403, limit test (1981)
Deviations:	There were no data on purity (notifier was not able to provide further information), or stability of the test article given. A formulation was tested. The respirable proportion of the dose was not determined. Due to high viscosity of test article the limit concentration of 5 mg/L was not reached. Individual data for determination of aerosol particle size distribution were not reported.
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be supplementary.

Material and Methods:

In an acute inhalation toxicity study, groups of young adult Sprague Dawley rats (animals provided by Charles River, USA; 5/sex) were exposed by the inhalation route (whole body) to an aerosol of the formulation NPI-720-F (lot no.: 13, purity: not stated and the notifier was not able to provide

further information) for 4 hours at an actual concentration of 2.41 mg/L air. Animals were observed during exposure and for 14 days post exposure. Bodyweights, food and water consumption were recorded daily. All animals were necropsied and subjected to gross macroscopic examination. Compound concentration in the air and particle size were determined. Nominal concentration was calculated from the amount of NPI-720-F dispersed in the generator and the total air flow during the exposure.

Findings:

Mean concentration of NPI-720-F was determined: 2.41 mg/L, standard deviation 0.15 mg/L. Analysis of the particle size distribution resulted in a mass median aerodynamic diameter of MMAD = 1.51 µm (geometric standard deviation 1.83). No mortalities occurred. Observations included animals covered with test substance, redness around eyes and nose, salivation, nasal congestion, rales, wheezing, mouth breathing and wet/discoloured inguinal area. With the exception of one animal with discoloured inguinal fur, clinical signs had resolved at the end of the observation period. Bodyweight loss was observed in one female and four male rats on day 8. All rats gained weight during the second week. In one male only, bodyweight did not reach to the pre-study level. No treatment related anomalies were noted upon necropsy.

Conclusions:

From the results with NPI-720-F, it is concluded that the four-hour inhalation LC_{50} in rats is greater than 2.41 mg/L, the highest technically achievable concentration.

9.2.1.3 Acute toxicity: dermal

Studies performed with NeemAzal

Reference:	TRF IIA 5.2.2 / 01
Report:	Mc Rae, L. A (1997)
	NeemAzal technical Acute dermal toxicity to the rat
	Huntingdon Life Sciences Limited, England
	Report-no. EIP 7/950800/AC; published: no; TOX9700503
Guidelines:	EPA FIFRA Guideline 152-11 (1984)
	Corresponding to OECD 402, limit test (1987)
	EC Directive 92/69/EEC B.3
Deviations:	None
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be acceptable.

Material and Methods:

In an acute dermal toxicity study groups of adult Hsd/Ola:Sprague-Dawley (CD) rats (animals provided by Harlan Orlac, England; 5/sex) were exposed by the dermal route to NeemAzal technical (batch no.: IV, purity: 36% azadirachtin A). Water moistened test material was applied for 24 hours to 10% of each animal's body surface at a dose of 2000 mg/kg bw. Animals were observed for clinical signs at periodic intervals on the day of dosing and twice daily thereafter for the duration of the study. Mortality checks were conducted twice daily. Local dermal irritation at the treatment site was assessed daily using a numerical grading system (0 to 4 for erythrema / eschar formation and oedema formation). Individual bodyweights were measured and recorded on days 1, 8 and 15. On day 15 the animals were sacrificed and examined for gross pathological changes.

Findings:

No mortality occurred. No clinical signs of systemic toxicity were noted. Sites of application showed no irritation or other dermal changes. The mean bodyweight gain during the observation period was slightly low for all males and one female on day 8 with a similar trend noted for one male and four females on day 15. No abnormalities were found at macroscopic post mortem examination of the animals.

Conclusions:

The percutaneous LD_{50} of NeemAzal technical was found to be in excess of 2000 mg/kg bw.

Studies performed with Fortune Aza

Reference:	SIP IIA 5.2.2 / 02
Report:	Mc Rae, L. A (1997)
	Fortune Aza technical - Acute dermal toxicity to the rat
	Huntingdon Life Sciences Limited, England
	Report-no. FBT 7/951816/AC; TOX2005-2370
Guidelines:	EPA FIFRA Guideline 152-11 (1984)
	Corresponding to OECD 402, limit test (1987)
	EC Directive 92/69/EEC B.3
Deviations:	None
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be acceptable.

Material and Methods

In an acute dermal toxicity study groups of adult Hsd/Ola:Sprague-Dawley(CD) rats (animals provided by Harlan Orlac, England; 5/sex) were exposed by the dermal route to Fortune Aza technical (batch no.: 0010195-0050195, purity: 8.5% azadirachtin A+B). Water moistened test material was applied for 24 hours to 10% of each animal's body surface at a dose of 2000 mg/kg bw. Animals were observed for clinical signs at periodic intervals on the day of dosing and twice daily thereafter for the duration of the study. Mortality checks were conducted twice daily. Individual bodyweights were measured and recorded on days 1, 8 and 15. On day, 15 the animals were sacrificed and examined for gross pathological changes.

Findings:

No mortality occurred. No clinical signs of systemic toxicity or local irritation were noted. The mean bodyweight 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.

Conclusions:

The percutaneous LD_{50} of Fortune Aza technical was found to be in excess of 2000 mg/kg bw.

Studies performed with ATI 720

Refrence:	MAS IIA 5.2.2 / 01
Report:	Furedi-Machacek, E. M. (1990)
	Acute dermal toxicity study of NPI 720 in rabbits (limit-test)
	IIT Research Institute, Life Science Research, 10 West 35th Street, Chicago, Illinois, USA
	Project No L 08270 Study No 3; TOX2005-2364
Guidelines:	EPA FIFRA Guideline 152-11 (1984)
	Corresponding to OECD 402, limit test (1987)
	EC Directive 92/69/EEC B.3
Deviations:	There are no data on purity, stability, identity or batch number of the test article given in the report. Notifier stated, that the technical extracts had a typical Aza A content of 8.3-9.5% at that time.
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be acceptable.

Material and Methods:

In an acute dermal toxicity study groups of adult New Zealand albino rabbits (animals provided by Johnson Rabbit Ranch, USA; 5/sex) were exposed by the dermal route to NPI 720. Test material was applied for 24 hours to the clipped and moistened body surface at a dose of 2000 mg/kg bw. Animals were observed for clinical signs at periodic intervals on the day of dosing and once daily thereafter for the duration of the study. Mortality checks were conducted twice daily. Individual bodyweights were measured and recorded prior to dosing and on days 1, 8 and 15. On day 15 the animals were sacrificed and examined for gross pathological changes.

Findings:

No mortality occurred. Dermal responses included oedema, erythema and eschar that had resolved by day 8. The changes noted in bodyweight gain in males and females were within the range expected for rabbits used in this type of study. Two male rabbits suffered from diarrhea, which was considered incidental. No other clinical signs of systemic toxicity were reported. No treatment related abnormalities were found at macroscopic post mortem examination of the animals.

Conclusions:

The percutaneous LD_{50} of NPI 720 technical was found to exceed 2000 mg/kg bw.

9.2.1.4 Acute toxicity: other routes

No studies with application via other routes submitted by the applicants.

9.2.2 Human information

No studies submitted by the applicants.

9.2.3 Other relevant information

For purpose of national registration in Germany, Trifolio had submitted studies performed with the product NeemAzal-F-5%, which consists of 20% NeemAzal and 80% polyethylene oxide. Some of these studies were not submitted for preparation of this DAR. Due to its more critical toxicological and ecotoxicological properties compared to NeemAzal (and NeemAzal-T/S), attempts for registration of this product have not been continued further. Some of these data were published in open literature by BfR scientists (Niemann & Hilbig, 2000) and reported as follows: “Studies with NeemAzal-F-5% gave evidence of considerably increased acute oral toxicological properties, it induced high mortality in the higher dose groups, a broad spectrum of clinical signs of toxicity, and pathological findings in several organs”.

Table 50 Acute toxicity data of the product NeemAzal-F-5% (Niemann & Hilbig, 2000) and of NeemAzal

Study, species	**Results**	
	NeemAzal-F-5%	**NeemAzal**
Acute oral LD_{50}, rat (mg/kg bw)	765	> 5000
Acute oral LD_{50}, mouse (mg/kg bw)	1570	> 3365
Acute dermal LD_{50}, rat (mg/kg bw)	> 5000	> 2000
Acute inhalation LD_{50}, rat (mg/L air, 4 h)	(*no study*)	> 0.72
Primary skin irritation	moderately irritating	not irritating
Primary eye irritation, rabbit	severe irritating	not irritating
Dermal sensitisation, guinea pig	(*no study*)	sensitising

Some endpoints were not covered with studies performed with the technical extract but with studies performed with NeemAzal-F-5%. Based on the comparison of acute toxicity results of NeemAzal and NeemAzal-F-5% (Table 50), we considered NeemAzal-F-5% the compound with the higher toxicity.

9.3 Specific target organ toxicity – single exposure (STOT SE)

All available single dose studies are summarised in section 9.2.

9.4 Irritation

9.4.1 Skin irritation

9.4.1.1 Non-human information

Studies performed with NeemAzal

Reference: TRF IIA 5.2.4 / 01

Report: Parcell, B. I. (1996)

NeemAzal technical Skin irritation to the rabbit

Huntingdon Life Sciences Limited, England

Report-no. EIP 8/950822/SE

published: no; TOX9700505

Guidelines: EPA FIFRA Guideline 152-14 (1984)

Corresponding to OECD 404

EC Directive 92/69/EEC B.4

Deviations: Sponsor’s signature is missing in report

GLP: Yes (certified laboratory)

Acceptability: The study is considered to be acceptable.

Material and Methods:

In a primary dermal irritation study, 6 adult male New Zealand white albino rabbits (animals provided by Interfauna, England) were exposed via the dermal route to 0.5 g of NeemAzal technical (batch no.: IV, purity: 36.6% azadirachtin A) each. The test material was applied for 4 hours to the clipped skin of one flank, using a moistened surgical gauze patch and semi-occlusive dressing. Observations were made 1, 24, 48 and 72 hours and 7 days after exposure.

Findings:

Exposure to NeemAzal resulted in very slight erythema in three animals only that had resolved by day 2. Oedema were not observed. No symptoms of systemic toxicity were found and no mortality occurred.

Conclusions:

NeemAzal technical was not irritating to rabbit skin.

Studies performed with Fortune Aza

Reference: SIP IIA 5.2.4 / 02

Report: Parcell, B. I. (1997)
Fortune Aza technical - Skin irritation to the rabbit
Huntingdon Life Sciences Limited, England
Report-no. FBT 8/951939/SE; TOX2005-2378

Guidelines: EPA FIFRA Guideline 152-14 (1984)
Corresponding to OECD 404

Deviations: None

GLP: Yes (certified laboratory)

Acceptability: The study is considered to be acceptable.

Material and Methods:

In a primary dermal irritation study, 6 adult male New Zealand white albino rabbits (animals provided by Froxfield, England) were exposed via the dermal route to 0.5 g of Fortune Aza technical (batch no.: 0010195 - 0050195, purity: 8.5% azadirachtin A+B) each. The test material

was applied for 4 hours to the clipped skin of one flank, using a moistened surgical gauze patch and semi-occlusive dressing. Observations were made 1, 24, 48 and 72 hours and 7 days after exposure.

Findings:

Exposure to Fortune Aza technical resulted in no erythema or oedema (all scores were zero). No symptoms of systemic toxicity were found and no mortality occurred.

Conclusions:

Fortune Aza technical was not irritating to rabbit skin.

Studies performed with ATI 720

Reference:	MAS IIA 5.2.4 / 01
Report:	Furedi-Machacek, E.M. (1990) Primary dermal irritation testing of NPI 720 in rabbits IIT Research Institute, Life Science Research, 10 West 35th Street, Chicago, Illinois, USA Project No L 08270 Study No 5; TOX2005-2375
Guidelines:	EPA FIFRA Guideline 152-14 (1984) Corresponding to OECD 404 EC Directive 92/69/EEC B.4
Deviations:	Individual bodyweight data not reported.
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be acceptable.

Material and Methods

In a primary dermal irritation study, six adult New Zealand albino rabbits (animals provided by Johnson Rabbit Ranch, USA, 3/sex) were exposed via the dermal route to NPI 720 (batch no.: 13, purity: 8.6% azadirachtin). The test material was applied for 4 hours to the clipped and moistened body surface at a dose of 500 mg per animal using a semi-occlusive dressing. Observations were made 1, 24, 48 and 72 hours after exposure. The descriptive criteria and scores of Draize were used.

Findings:

No mortality occurred. No dermal responses were observed. Scores of 0 were noted at all observation times with respect to oedema, erythema and eschar. No clinical signs of treatment related toxicity were noted.

Conclusions:

NPI 720 technical was found to be not irritating to the skin of rabbits.

9.4.1.2 Human information

No studies submitted by the applicants.

9.4.2 Eye irritation

9.4.2.1 Non-human information

Studies performed with NeemAzal

Reference:	TRF IIA 5.2.5 / 01
Report:	Parcell, B. I. (1996) NeemAzal technical Eye irritation to the rabbit Huntingdon Life Sciences Limited, England Report-no. EIP 9/950823/SE published: no; TOX9700506
Guidelines:	EPA FIFRA Guideline 152-13 (1984) Corresponds to OECD Guideline 405
Deviations:	Sponsor's signature missing on GLP compliance statement.
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be acceptable.

Material and Methods:

In a primary eye irritation study 70 mg of NeemAzal technical (batch no.: IV, purity: 36.6% azadirachtin A) was instilled into the conjunctival sac of one eye of 7 young adult New Zealand White albino rabbits (animals provided by Froxfield, England, and by Interfauna, England). After application, the eyes were not rinsed to remove the compound. Observations were done on mortality/viability, clinical signs of toxicity (at least once daily) and on eye irritation 1, 24, 48 and 72 hours and 4 and 7 days after instillation of the test substance. Ocular response was scored according to the criteria of Draize. In a screening study only one animal was treated with test compound and the eye rinsed with distilled water after 30 sec of exposure. One further animal was treated with the test substance to assess the severity of ocular reactions produced, prior to treating the five remaining animals.

Findings:

The test substance did not cause any acute systemic toxicological signs or mortality. No corneal damage or iridial inflammation was seen in the screening study. Minimal transient conjunctival irritation was seen accompanied by discharge with moistening of the lids and hairs for a considerable area around the eye at the 1 hour time point. One hour after exposure, dulling of the cornea was observed in one animal of the main study. No other corneal damage or iridial inflammation was seen. Diffuse crimson colouration of the conjunctivae was reported in two animals accompanied by considerable swelling with partial eversion of the eyelids and discharge with moistening of the lids and hairs, and considerable area around the eye. These effects persisted through day 2 in one and day 3 in the other animal. In the remaining animals mild conjunctival reactions were noted that were normal after 2 to 4 days.

Table 51: Ocular reactions of rabbit eyes after instillation with test compound (individual scores)

rabbit	**602 female (screening study)**				**523 female (pilot animal)**				**560 male**				**561 male**			
time	1 h	1 d	2 d	3 d	1 h	1 d	2 d	3 d	1 h	1 d	2 d	3 d	1 h	1 d	2 d	3 d
Cornea																
Density	0	0	0	0	0	0	0	0	D	0	0	0	0	0	0	0
Iris	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Conjunctiva																
Redness	1	1	0	0	1	0	0	0	1	1	0	0	1	1	0	0
Chemosis	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0
Discharge	3	0	0	0	3	0	0	0	1	0	0	0	2	0	0	0

screening study: one animal only; 30 sec after instillation with test substance the eye was rinsed with distilled water
pilot animal: only one animal treated
Cornea: D-dulling

Table 51: (continued)

rabbit	**562 male**				**644 male**				**645 male**				**Mean [b]**			
time	1 h	1 d	2 d	3 d	1 h	1 d	2 d	3 d	1 h	1 d	2 d	3 d	1 h	1 d	2 d	3 d
Cornea																
Density	0	0	0	0	0	0	0	0	0	0	0	0	0.0	0.0	0.0	0.0
Iris	0	0	0	0	0	0	0	0	0	0	0	0	0.0	0.0	0.0	0.0
Conjunctiva																
Redness	1	0	0	0	1	2[a]	1	0	1	2[a]	1	1	1.0	1.0	0.3	0.2
Chemosis	1	0	0	0	1	2	1	0	1	2	1	0	1.0	0.7	0.3	0.0
Discharge	2	0	0	0	3	2	1	0	2	2	1	0	2.2	0.7	0.3	0.0

[a)] sample residues in lower eyelid removed with cotton bud; [b)] mean of results of animals 523, 560, 561, 562, 644 and 645

Conclusions:

NeemAzal technical instilled into the rabbit eye produced a positive response in two of six treated rabbits inducing a dulling of the cornea and slight to well defined irritation. The eyes were normal by four days after instillation. NeemAzal technical was slightly irritating to the eye, no classification needed.

Studies performed with Fortune Aza

Reference:	SIP IIA 5.2.5 / 02
Report:	Parcell, B. I. (1997) Fortune Aza technical - Eye irritation to the rabbit Huntingdon Life Sciences Limited, England Report-no. FBT 9/952651/SE; TOX2005-2382
Guidelines:	EPA FIFRA Guideline 152-13 (1984) Corresponds to OECD Guideline 405
Deviations:	None
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be acceptable.

Material and Methods:

In a primary eye irritation study, 64 mg of Fortune Aza technical (batch no.: 0010195-0050195, purity: 8.5% azadirachtin A+B) was instilled into the conjunctival sac of one eye of each of 7 young adult New Zealand white albino rabbits (animals provided by Charles River, England, and by Froxfield, England). After application, the eyes were not rinsed to remove the compound. Observations were done on mortality/viability, clinical signs of toxicity (at least once daily) and on eye irritation 1, 24, 48 and 72 hours and 4 and 7 days after instillation of the test substance. Ocular response was scored according to the criteria of Draize. In a screening study, only one animal was treated with test compound and the eye rinsed with distilled water after 30 sec. of exposure. One further animal was treated with the test substance to assess the severity of ocular reactions produced, prior to treating the five remaining animals.

Findings:

The test substance did not cause any acute systemic toxicological signs or mortality. One hour after exposure, dulling of the cornea was observed in the animal of the screening study and in two further animals of the main study, this effect resolved within one day (Table 52). No iridial inflammation was observed. A diffuse crimson colouration of the conjunctivae was seen in all six animals of the main study one hour after instillation. This was accompanied in one animal by considerable swelling with partial eversion of the eyelids and in two animals by discharge with moistening of the lids and hairs either just adjacent to lids or for a considerable area around the eye. The eyes of all animals were normal one or two days after instillation.

Table 52: Ocular reactions of rabbit eyes after instillation with test compound (individual scores)

rabbit	**1295 female (screening study)**				**1297 female (pilot animal)**				**1298 female**				**1299 female**			
time	1 h	1 d	2 d	3 d	1 h	1 d	2 d	3 d	1 h	1 d	2 d	3 d	1 h	1 d	2 d	3 d
Cornea																
Density	D	0	0	0	D	0	0	0	0	0	0	0	0	0	0	0
Iris	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Conjunctiva																
Redness	2	1	0	0	2	0	0	0	2	1	0	0	2	0	0	0
Chemosis	1	0	0	0	2	0	0	0	1	0	0	0	1	0	0	0
Discharge	3	0	0	0	3	0	0	0	1	0	0	0	1	0	0	0

screening study: one animal only; 30 sec after instillation with test substance the eye was rinsed with distilled water
pilot animal: only one animal treated
Cornea: D-dulling

Table 52: (continued)

rabbit	**1300 female**				**1301 female**				**1364 male**				**Mean [a]**			
time	1 h	1 d	2 d	3 d	1 h	1 d	2 d	3 d	1 h	1 d	2 d	3 d	1 h	1 d	2 d	3 d
Cornea																
Density	0	0	0	0	D	0	0	0	0	0	0	0	0	0	0	0
Iris	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Conjunctiva																
Redness	2	1	0	0	2	1	0	0	2	1	0	0	2.0	0.7	0.0	0.0
Chemosis	1	0	0	0	1	0	0	0	1	0	0	0	1.2	0.0	0.0	0.0
Discharge	1	0	0	0	1	0	0	0	2	0	0	0	1.5	0.0	0.0	0.0

[a)] mean of results of animals 1297, 1298, 1299, 1300, 1301 and 1364

Conclusions:

Fortune Aza technical instilled into the rabbit eye produced a positive response in three of seven treated rabbits inducing a transient dulling of the cornea and slight to well defined irritation of the conjunctiva that rapidly resolved. Fortune Aza is slightly irritating to the rabbit eye, no classification needed.

Studies performed with ATI 720

Reference: MAS IIA 5.2.5 / 01

Report: Furedi-Machacek, E. M. (1990)

Primary eye irritation testing of NPI 720 in rabbits

IIT Research Institute, Life Science Research, 10 West 35th Street, Chicago, Illinois, USA

Project No L 08270 Study No 6; TOX2005-2379

Guidelines: EPA FIFRA Guideline 152-13

Corresponding to OECD 405

EC Directive 92/69/EEC B.4

Deviations: None

GLP: Yes (certified laboratory)

Acceptability: The study is considered to be acceptable.

Material and Methods:

In a primary eye irritation study 100 mg NPI 720 (batch no.: 13, purity: 8.6% azadirachtin) was instilled into the conjunctival sac of one eye of six adult New Zealand albino rabbits (animals provided by Johnson Rabbit Ranch, USA; three per sex). Observations were done on mortality, morbidity, physical appearance and behaviour (at least once daily) and on eye irritation 1, 24, 48 and 72 hours after instillation of the test substance. Ocular lesions were scored according to the criteria of Draize.

Findings:

The test substance did not cause any acute systemic toxicological signs or mortality.

One day after exposure mild opacity of the cornea was observed in one animal (Table 53). Discharge, chemosis and redness were observed one hour after instillation in most animals. The effects had resolved in all animals on day 2 with the exception of one female where mild swelling resolved on day 3.

Table 53: Ocular reactions

rabbit	**201 female**				**202 female**				**203 male**			
time	1 h	1 d	2 d	3 d	1 h	1 d	2 d	3 d	1 h	1 d	2 d	3 d
Cornea												
Density	0	1	0	0	0	0	0	0	0	0	0	0
Iris	0	0	0	0	0	0	0	0	1	0	0	0
Conjunctiva												
Redness	2	2	0	0	2	2	0	0	2	1	0	0
Chemosis	2	2	1	0	1	1	0	0	3	1	0	0
Discharge	3	0	0	0	2	0	0	0	3	0	0	0

Table 53: (continued)

rabbit	**204 male**				**205 male**				**206 male**				**Mean**			
time	1 h	1 d	2 d	3 d	1 h	1 d	2 d	3 d	1 h	1 d	2 d	3 d	1 h	1 d	2 d	3 d
Cornea																
Density	0	0	0	0	0	0	0	0	0	0	0	0	0.0	0.2	0.0	0.0
Iris	1	0	0	0	0	0	0	0	0	0	0	0	0.3	0.0	0.0	0.0
Conjunctiva																
Redness	2	1	0	0	2	1	0	0	2	1	0	0	2.0	1.3	0.0	0.0
Chemosis	3	1	0	0	2	2	0	1[a]	3	1	0	0	2.3	1.3	0.2	0.0
Discharge	3	0	0	0	1	0	0	0	3	0	0	0	2.5	0.0	0.0	0.0

a, considered traumatic (excluded from mean calculation)

Conclusions:

NPI-720 instilled into the rabbit eye produced as a transient response in all treated rabbits slight to well defined irritation that rapidly resolved. Based on these results, NPI-720 was found to be not irritating to the eye of rabbits.

9.4.2.2 Human information

No studies submitted by the applicants.

9.4.3 Respiratory tract irritation

9.4.3.1 Non-human information

No studies submitted by the applicants.

9.4.3.2 Human information

No studies submitted by the applicants.

9.5 Corrosivity

9.5.1 Non-human information

9.5.2 Human information

9.6 Sensitisation

9.6.1 Skin sensititsation

9.6.1.1 Non-human information

Studies performed with NeemAzal

Reference: TRF IIA 5.2.6 / 01

Report: Allan, S., Coleman, D. (1997)

NeemAzal technical Skin Sensitisation in the Guinea Pig

Huntingdon Life Sciences Limited, England

Report-no. EIP 10/950818/SS

	Published: no; TOX9700507
Guidelines:	EPA FIFRA Guideline 152-15
	Corresponds to OECD Guideline 406 (1992)
Deviations:	None
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be acceptable.

Material and Methods:

Test substance concentrations selected for the main study were based on the results of a preliminary study. In the main study, 20 young adult male Dunkin Hartley albino guinea pigs (animals provided by D. Hall, England) were intradermally injected with 5% (w/v) of NeemAzal technical (batch no.: IV, purity: 36.6% azadirachtin A) in 5% acetone in Alembicol (i.e., coconut oil), Freund's adjuvant, and a mixture of both. On day 6, the clipped scapular area between the injection sites was rubbed with 0.5 mL of 10% sodium lauryl sulfate in petrolatum. On day 7, the area was treated with 0.5 mL of a 80% test substance concentration in acetone for 48 hours. Ten control animals were similarly treated, but with vehicle alone. Two weeks after the epidermal application all animals were challenged with 80 and 40% NeemAzal in acetone. The dressing was removed after 24 hours exposure. The treated sites were assessed for challenge reactions 24 , 48 and 72 hours after removal of the dressing.

Findings:

Preliminary study:

Different concentrations were tested by intradermal injection (0.1 mL/site): 7.5%, 5%, 2.5%, 1.0%, 0.5%, 0.25%, and 0.1%. Dermal reactions were assessed 24 and 72 hours after treatment. The concentration of 5% w/v in 5% acetone in Alembicol D was the highest concentration tested that caused irritation but did not adversely affect the animals. Therefore this level was selected for the intradermal induction for the main study. Epidermal application was carried out in a concentration range from 30% to 80% in acetone for 24 h. Dermal reactions were assessed 0, 24 and 48 hours later. No signs of irritation were observed upon dermal application of up to 80% NeemAzal in acetone. Therefore, 10% sodium lauryl sulfate was employed 24 hours before the epidermal induction to provoke a mild inflammatory reaction.

Main study:

No mortality occurred and no symptoms of systemic toxicity were observed during main study. Bodyweights and bodyweight gain remained in the same range as controls.

Necrosis was recorded at sites receiving Freund's Complete Adjuvant in test and control animals. Slight irritation was seen in test animals at sites receiving NeemAzal technical 5% w/v in 5% acetone in Alembicol D and slight irritation was observed in control animals receiving vehicle

alone. Slight erythema was observed in test animals following topical application with NeemAzal technical (80% in acetone) and slight erythema was seen in the control animals. On challenge, no skin reactions were observed in control animals. In contrast, all animals of the treatment group showed slight to well defined oedema and erythema upon challenge for both 40 and 80% NeemAzal technical.

Table 54: Individual erythema and oedema scores after challenge

Freund's treated control animals:

Guinea-pig number	E = Erythema O = Oedema	Score					
		24 Hours		48 Hours		72 Hours	
		A	P	A	P	A	P
795	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
796	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
797	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
798	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
799	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
800	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
801	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
802	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
803	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
804	E	0	0	0	0	0	0
	O	0	0	0	0	0	0

A Anterior site, exposed to NeemAzal Technical, 80 % w/v in acetone
P Posterior site, exposed to NeemAzal Technical, 40% w/v in acetone

Test animals:

Guinea-pig number	E = Erythema O = Oedema	Score						Results Positive (+) Negative (-) Inconclusive (±)
		24 Hours		48 Hours		72 Hours		
		A	P	A	P	A	P	
805	E	2	L2	2	2	2	2	+
	O	1*	0	2*	1*	2*	1*	
806	E	L2	0	L2	0	L1	0	+
	O	0	0	0*	0	0*	0	
807	E	L2	2	2	2	Ø2	Ø2	+
	O	0	0	1*	0*	2	2	
808	E	2	2	2	2	Ø2	Ø2	+
	O	0	0	0*	0*	2	2	
809	E	L2	0	L2	0	L2	0	+
	O	0	0	0*	0	1*	0	
810	E	2	L2	Ø2	L2	Ø2	L1	+
	O	1	0	2	0	2	0*	
811	E	1	2	2	1	2	2	+
	O	0*	0	2*	1*	2*	1*	
812	E	2	1	2	2	Ø2	Ø2	+
	O	1	0	2*	1*	2	2	
813	E	2	L2	Ø2	ØL2	Ø2	Ø2	+
	O	0	0	2	2	2	1	
814	E	2	2	Ø2	Ø2	ØL2	ØL2	+
	O	1*	1	2	1	1	1	
815	E	L2	L2	ØL1	L1	1	1	+
	O	0	0	0	0	0*	0	
816	E	2	2	Ø2	Ø2	ØNP2	L1	+
	O	1	0	2	1	3	1*	
817	E	L2	0	2	0	2	L2	+
	O	0	0	0*	0	2*	1*	
818	E	2	L2	2	L2	Ø2	Ø2	+
	O	0	0	1*	0	1	1	
819	E	2	2	Ø2	Ø2	Ø2	Ø2	+
	O	2	1	2	2	2	2	
820	E	L2	L2	L2	L2	2	2	+
	O	0	0	0*	0*	2*	2*	
821	E	2	2	Ø2	Ø2	Ø2	Ø2	+
	O	1	1	2	2	2	2	
822	E	2	2	Ø2	Ø2	ØNP2	Ø2	+
	O	1	1	1	2	2	2	
823	E	L2	L2	ØL2	ØL2	Ø2	Ø2	+
	O	0	0	1	1	2	2	
824	E	2	2	2	2	Ø2	Ø2	+
	O	1	0	1*	1*	2	1	

L Localised dermal reaction (restricted to a small area of the challenge site)
NP Necrotic patch
* Dryness and sloughing of the epidermis
Ø Thickening, dryness and sloughing of the epidermis
A Anterior site, exposed to NeemAzal Technical, 80% w/v in acetone
P Posterior site, exposed to NeemAzal Technical, 40% w/v in acetone

Six tests with hexyl cinnamic aldehyde as positive reference substance (performed in December 1992 to January 1999) resulted in allergic reactions and have shown the sensitivity of the guinea pig strain used.

Conclusions:

The NeemAzal technical exhibited dermal sensitisation potential under the test conditions used. On the basis of this study NeemAzal technical has to be classified as a skin sensitiser.

Studies performed with Fortune Aza

Reference: SIP IIA 5.2.6 / 02

Report:	Allan, S., Coleman, D. (1997)
	Fortune Aza technical Skin Sensitisation in the Guinea Pig
	Huntingdon Life Sciences Limited, England
	Report-no. FBT 10/952234/SS; TOX2005-2384
Guidelines:	EPA FIFRA Guideline 152-15
	Corresponds to OECD Guideline 406 (1992)
Deviations:	None
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be acceptable.

Material and Methods:

Test substance concentrations selected for the main study were based on the results of a preliminary study. In the main study, 20 young adult male Dunkin Hartley albino guinea pigs (animals provided by D. Hall, England) were intradermally injected with 0.5% (w/v) of Fortune Aza technical (batch no.: 0010195-0050195, purity: 8.5% azadirachtin A+B) in Alembicol D (i.e., coconut oil), Freund's adjuvant, and a mixture of both. On day 6 the clipped scapular area between the injection sites was rubbed with 0.5 mL of 10% sodium lauryl sulfate in petrolatum. On day 7 the area was treated with 0.5 mL of a 60% Fortune Aza technical concentration for 48 hours using a Whatman No 3 paper covered with impermeable plastic tape and fixed with elastic adhesive bandage. Ten control animals were similarly treated, but with vehicle alone. For challenge on day 21 one flank of all animals was clipped and treated by epidermal application of 30% and 60% Fortune Aza technical in Alembicol D (0.2 mL each), using patch test plasters. The dressing was removed after 24 hours exposure and the skin cleaned of residual test substance and vehicle using water. The treated sites were assessed for challenge reactions 24, 48 and 72 hours after removal of the dressing.

Findings:

In a *preliminary study*, the following concentrations were tested by intradermal injection: 5%, 2.5%, 1.0%, 0.5%, 0.25%, and 0.1% in Alembicol D. Animals were pre-treated with an intradermal injection of Freund's complete adjuvant. The concentration of 0.5% w/v in Alembicol D was the highest concentration tested that caused irritation, but did not adversely affect the animals. Therefore this concentration was selected for intradermal induction for the main study. Epidermal application was carried out in a concentration range from 20% to 60%. No signs of irritation were observed upon dermal application of up to 60% Fortune Aza technical in Alembicol D. Therefore, 10% sodium lauryl sulfate was employed 24 hours before the epidermal induction to provoke a mild inflammatory reaction.

Main study:

No mortality occurred and no symptoms of systemic toxicity were observed. Bodyweights and bodyweight gain remained in the same range as controls. After intradermal injection with Freund's Complete Adjuvant necrosis was seen at injection sites in test and control animals. Slight irritation was seen in test animals at sites receiving Fortune Aza technical in Alembicol D and slight irritation was observed in control animals receiving Alembicol D. Moderate erythema was observed in test animals following topical application with Fortune Aza in Alembicol D. Slight erythema was seen in control animals. All animals of the treatment group showed well defined oedema upon challenge for both 30% and 60% Fortune Aza technical. Dermal reaction seen in all treated animals was more marked than those seen for the controls and was therefore considered a positive response.

Table 55: Individual erythema and oedema scores after challenge

Freund's treated control animals:

Guinea-pig number	E = Erythema O = Oedema	Score					
		24 Hours		48 Hours		72 Hours	
		A	P	A	P	A	P
2615	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
2616	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
2617	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
2618	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
2619	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
2620	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
2621	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
2622	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
2623	E	0	0	0	0	0	0
	O	0	0	0	0	0	0
2624	E	0	0	0	0	0	0
	O	0	0	0	0	0	0

A Anterior site, exposed to FORTUNE AZA Technical, 60% w/v in Alembicol D
P Posterior site, exposed to FORTUNE AZA Technical, 30% w/v in Alembicol D

Test animals:

Guinea-pig number	E = Erythema O = Oedema	Score						Results Positive (+) Negative (-) Inconclusive (±)
		24 Hours		48 Hours		72 Hours		
		A	P	A	P	A	P	
2625	E	2	2	2	2	2	2	+
	O	0	0	0	0	0	0	
2626	E	L1	2	L1	2	1	2	+
	O	0	0	0	0	0*	0*	
2627	E	2	1	2	1	2	1	+
	O	0	0	0	0	0*	0	
2628	E	2	1	2	L1	2	2	+
	O	0	0	0	0	0*	0*	
2629	E	2	2	2	2	2	2	+
	O	1	1	1	1	1*	1*	
2630	E	1	L1	1	L1	1	L1	+
	O	0	0	0	0	0*	0	
2631	E	1	NE1	1	NE1	1	NE1	+
	O	0	0	0	0	0*	0*	
2632	E	2	2	2	2	2	2	+
	O	1	0	1	0	1*	0	
2633	E	L2	1	L2	L1	L2	L1	+
	O	0	0	0	0	0*	0*	
2634	E	2	2	2	2	2	2	+
	O	1	1	1	1	1	1	
2635	E	2	1	2	1	2	1	+
	O	0	0	0	0	0*	0*	
2636	E	2	1	2	1	2	1	+
	O	0	0	0	0	0	0	
2637	E	L1	0	L1	0	L1	0	+
	O	0	0	0	0	0	0	
2638	E	2	1	2	1	2	1	+
	O	0	0	0	0	0	0	
2639	E	2	1	2	1	2	1	+
	O	0	0	0	0	0	0	
2640	E	2	2	2	L2	2	L2	+
	O	1	0	1	0	1	0	
2641	E	2	NP2	2	NP2	2	NP2	+
	O	1	1	1	1	1	1	
2642	E	L2	1	L2	1	L2	1	+
	O	0	0	0	0	0	0	
2643	E	1	1	1	1	1	1	+
	O	0	0	0	0	0	1*	
2644	E	2	2	2	2	2	2	+
	O	1	1	1	1	1	1	

L Localised dermal reaction (restricted to a small area of the challenge site)
NP Necrotic patch
* Dryness and sloughing of the epidermis
A Anterior site, exposed to FORTUNE AZA Technical, 60% w/v in Alembicol D
P Posterior site, exposed to FORTUNE AZA Technical, 30% w/v in Alembicol D

Earlier tests with hexyl cinnamic aldehyde as positive reference substance (performed regularly) resulted in allergic reactions and had shown the sensitivity of the guinea pig strain used.

Conclusions:

In this study FortuneAza technical produced evidence of skin sensitisation (delayed contact hypersensitivity) in all twenty test animals. On the basis of this study Fortune Aza technical has to be classified as a skin sensitiser.

Studies performed with ATI 720

Reference:	MAS IIA 5.2.6 / 01
Report:	Sherwood, R. (1990) Dermal sensitisation study of NPI 720 in Guinea pigs using the modified Buehler method IIT Research Institute, Life Science Research, 10 West 35th Street, Chicago, Illinois, USA Project No L 08257 Study No 1; TOX2005-2383
Guidelines:	EPA FIFRA Guideline 152-15 Corresponds to OECD Guideline 406 (1981)
Deviations:	Only 10 animals tested. No summary of latest reliability check reported. Individual bodyweight data not reported.
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be supplementary.

Material and Methods:

Test substance concentrations selected for the main study were based on the results of a preliminary study. In the main study, 10 young adult male Hartley albino guinea pigs (animals provided by Murphy Breeding Laboratories, USA) were dermally treated with 25% (w/v) of NPI 720 (batch no.: 10; purity: 19.2% azadirachtin) in ethanol once per week for 6 hours during three consecutive weeks. Ten control animals were similarly treated, but with vehicle alone. Two weeks after the final dermal induction all animals were challenged with 0.5% NPI 720 in ethanol. Test sites were scored for erythema 24 and 48 h after the first induction and the challenge dose and scored according to Draize's method. All animals were observed daily for mortality or morbidity. Bodyweights were measured weekly. A two factor log-linear model was used to assess the effect of treatment and time of scoring on erythema reaction

Findings:

In a preliminary study, a concentration of 25% NPI-720 in ethanol (w/v) was identified as irritating and was subsequently applied in the induction phase. A concentration of 0.5% NPI-720 in ethanol (w/v) was identified as non-irritating and was used in the challenge phase of the study. No mortality occurred and no symptoms of systemic toxicity were observed. Bodyweights and bodyweight gain remained in the same range as controls. Treatment with NPI 720 for induction led to slight to well defined erythema. Positive erythema reactions (i. e., a score greater/equal to 2) were observed in two of ten treated guinea pigs but not in any of the controls during the challenge phase of this study (Table 56). The effect was statistically not significant (i.e., $p > 0.05$) and time of scoring was not a significant factor.

Table 56: Incidence of erythema scores after first induction and after challenge (number of animals with the individual score and ratio of these animals in percent)

	Time of scoring									
	24 h					48 h				
Score:	0	1	2	3	4	0	1	2	3	4
Induction 1										
Treated	0 (0%)	7 (70%)	3 (30%)	0 (0%)	0 (0%)	2 (20%)	4 (40%)	4 (40%)	0 (0%)	0 (0%)
Control	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Challenge										
Treated	1 (10%)	7 (70%)	2 (20%)	0 (0%)	0 (0%)	1 (10%)	9 (90%)	0 (0%)	0 (0%)	0 (0%)
Control	4 (40%)	6 (60%)	0 (0%)	0 (0%)	0 (0%)	6 (60%)	4 (40%)	0 (0%)	0 (0%)	0 (0%)

Table 57: Individual erythema scores after induction 1 and challenge

Treated Guinea Pigs					Control Guinea Pigs				
Animal Number	Induction 1 24 hrs.	Induction 1 48 hrs.	Challenge 1 24 hrs.	Challenge 1 48 hrs.	Animal Number	Induction 1 24 hrs.	Induction 1 48 hrs.	Challenge 1 24 hrs.	Challenge 1 48 hrs.
701	1	2	0	0	711	0[a]	0	1	1
702	1	2	1	1	712	0	0	1	1
703	1	0	1	1	713	0	0	1	1
704	1	1	1	1	714	0	0	1	0
705	2	2	1	1	715	0	0	0	0
706	1	1	1	1	716	0	0	0	0
707	2	2	2	1	717	0	0	1	1
708	2	1	2	1	718	0	0	0	0
709	1	0	1	1	719	0	0	1	0
710	1	1	1	1	720	0	0	0	0

[a] The control guinea pigs were not treated with test article at the Induction 1 time point.

As the effect was not statistically significant, the submitter considers NPI 720 as non-sensitising.

According to the criteria laid down in directive 67/548/EC (annex VI, section 3.2.7.2), a test (non-adjuvant test method) with more than 15% positive animals is considered positive. 2/10 animals, i.e. 20%, showed positive response to challenge. Additionally, the number of animals used was too low (10 instead of 20). Moreover, the Buehler test is not as rigorous as the Magnusson & Kligman assay, where the other extracts were found to be sensitising.

Therefore, NPI 720 is considered to be a skin sensitiser.

Conclusions:

The test substance NPI 720 did induce dermal sensitisation by repeated dermal exposure. On the basis of this study, NPI 720 is a skin sensitiser.

9.6.1.2 Human information

No studies submitted by the applicants.

9.6.2 Respiratory sensitisation

9.6.2.1 Non-human information

No studies submitted by the applicants.

9.6.2.2 Human information

No studies submitted by the applicants.

9.7 Specific target organ toxicity (CLP Regulation) – repeated exposure (STOT RE)

9.7.1 Non-human information

9.7.1.1 Repeated dose toxicity: oral

Studies performed with NeemAzal

Reference:	TRF IIA 5.3.1 / 02
Report:	Waterson, L. A., Hawkins, A. (1995) NeemAzal technical 2 week palatability study in the rat Huntingdon Life Sciences Limited, England Report-no. BDP/18 published: no; TOX9750142
Guidelines:	None; dose finding study
Deviations:	Batch number and purity of test compound not stated.
GLP:	No
Acceptability:	The study is considered to be supplementary.

Material and Methods:

In a dose finding palatability study, NeemAzal (batch number and purity not stated) was offered for 2 weeks to groups of 10 CD rats (origin of animals not stated; 5 of each sex) in the diet at concentrations corresponding to of 20000 and 50000 ppm of NeemAzal technical. Daily

observations were carried out on mortality, clinical signs; bodyweights and food consumption were noted twice weekly.

Findings and Conclusion:

Under the conditions of this 2-week rat-feeding study, no mortalities occurred. Bodyweight losses were noted for both sexes at 50000 and for females receiving 20000 ppm NeemAzal technical resulting mainly from initial bodyweight loss.

As compared to pre-treatment values, food intake was lower in the 50000 ppm group but similar in the 20000 ppm group. Therefore, 20000 ppm should be used as maximum dose in a further 4-week study.

Reference:	TRF IIA 5.3.1 / 01
Report:	Waterson, L.A. (1997) NeemAzal technical - toxicity study in rats by dietary administration for 4 weeks Huntingdon Life Sciences Limited, England Report-no. EIP 3/960397 published: no; TOX9700508
Guidelines:	OECD Guideline 407
Deviations:	None
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be acceptable.

Material and Methods:

NeemAzal technical (batch no.: VII, purity: 26.8 – 28.4% azadirachtin A) was offered for 4 weeks to groups of 10 Crl: CD (SD) BR rats (animals provided by Charles River, England; 5 of each sex) in the diet at concentrations corresponding to of 0; 3200; 8000 and 20000 ppm of NeemAzal technical (mean achieved doses of NeemAzal were 0; 322; 773 and 1844 mg/kg bw/d in males and 0; 301; 791 and 1747 mg/kg bw/d in females). Observations were carried out on mortality, clinical signs, bodyweights, and food consumption. Following the 4-week treatment period, all animals were sacrificed, weights were recorded for specific organs (adrenals, brain, epididymes, heart, kidneys, liver, ovaries, pituitary, spleen, testes, thyroid, uterus), detailed macroscopic and microscopic examinations (liver, and thyroids of all animals, ovaries, and uterus from females only, and adrenals from males only) were performed.

Statistics: Statistical analyseswere carried out separately for either sex. Data relating to food and water consumption were analysed on a cage basis, all other parameters were analysed using

individual animals as the basic experimental unit. Bodyweight gain, clinical pathology and organ weight data were analysed for heterogeneity of variance between treatments with Bartlett's test. Where significant heterogeneity (at the 1% level) was found a logarithmic transformation was tried to test for more stable variance. If no significant variance was detected a one-way analysis of variance was carried out. If significant heterogeneity of variance was present a Kruskal-Wallis-analysis of ranks was used.

Findings:

Concentration of azadirachtin in feed was determined chromatographically. Mean analytical results were within 4% of nominal concentrations. Under the conditions of this 4-week rat feeding study, no mortalities occurred and no clinical signs of toxicology were noted. During week 1, both sexes receiving the 20000 ppm dose showed weight loss (Table 58). Thereafter, weight gain improved in this high dose group but remained lower as compared to control. For females, weight gain was significantly lower in the first week in mid dose group and also transiently in the low dose group, but the latter finding was not related to dose.

Table 58: Bodyweight gain (g and percent of control group)

Dosage level	**Male**			**Female**		
	Day 1-4	**Day 4-8**	**Day 8-29**	**Day 1-4**	**Day 4-8**	**Day 8-29**
0	23 (100%)	37 (100%)	129 (100%)	12 (100%)	18 (100%)	54 (100%)
3200	23 (100%)	38 (103%)	113 (88%)	11 (92%)	5* (28%)	57 (106%)
8000	18 (78%)	34 (92%)	122 (95%)	5** (42%)	14* (78%)	50 (93%)
20000	0** (0%)	32 (86%)	87** (67%)	-3** (-25%)	12* (67%)	38* (70%)

* $p < 0.05$, ** $p < 0.01$

During week 1 both sexes receiving 20000 ppm and females receiving 3200 and 8000 ppm showed lower mean food intakes as compared to the controls. Thereafter, weekly food consumption improved but remained lower in high dose groups as compared to control. For males receiving low and mid dose diets food consumption was comparable with controls. No macroscopic observations were considered treatment related. For females of all doses increased bodyweight adjusted mean liver weights were noted. For males, elevated liver weights were observed in the two higher dose levels. Increased mean weights of the thyroid were noted for both sexes at all treatment levels. All males showed reduced mean weights of the adrenals, this was statistically significant at the highest dose only. There was no clear dose response relationship, no histopathological findings account for these differences, and adrenal weights in females were not affected. Reduced organ weights were noted for uteri and ovaries in the 20000 ppm group, a reduced mean uterus weight was noted at 8000 ppm, but these findings were not statistically significant and there was no effect observed upon histopathological examination. Reduced mean spleen weights were observed for both sexes at the highest dose. No further abnormalities were found at macroscopic post mortem examination of the animals.

Table 59: Mean organ weights in animals treated with NeemAzal

Males										
ppm	**Body-weight (g)**	**Liver (g)**	**Brain (g)**	**Thyroids (mg)**	**Pituitary (mg)**	**Spleen (g)**	**Heart (g)**	**Adrenals (mg)**	**Testes (g)**	**Epididymi-des (g)**
0	380	19.0	1.95	17.9	13.8	0.79	1.29	62.3	3.24	0.85
3200	362	19.2	1.90	20.1	13.4	0.71	1.23	51.4	3.19	0.84
8000	367	21.3*	1.98	24.7	13.5	0.77	1.25	52.5	3.40	0.90
20000	305	20.6**	1.93	22.9	14.1	0.62	1.08	49.3*	3.18	0.82
Females										
ppm	**Body-weight (g)**	**Liver (g)**	**Brain (g)**	**Thyroids (mg)**	**Pituitary (mg)**	**Spleen (g)**	**Heart (g)**	**Adrenals (mg)**	**Ovaries (mg)**	**Uterus (g)**
0	248	11.2	1.86	16.2	18.7	0.62	1.01	69.0	100.7	0.60
3200	232	12.6*	1.79	18.7	15.3	0.55	0.89	69.8	93.4	0.54
8000	232	13.6**	1.78	23.3*	16.7	0.57	0.88	70.5	93.3	0.42
20000	210	16.6**	1.74	24.2*	14.6	0.41	0.89	63.0	81.6	0.37

*p <0.05; ** p <0.01

Liver: In all animals receiving 20000 ppm and most animals (9/10) receiving 8000 ppm periportal hepatocyte eosinophilia with clumping was observed. Also in the lowest dose group, focal periportal hepatocyte eosinophilia with clumping was noted for all males and 2 females. These changes were dose-related in degree and extent. Minimal hepatocyte hypertrophy (generalised in females, periportal in males) was seen exclusively in rats receiving 20000 ppm.

Thyroid: Minimal or trace follicular epithelial hypertrophy was seen in the majority of all treated animals but only in a single male animal from the control group. While all treated females were affected, for males there was a dose-relation with 1; 2; 4 and 5 animals exhibiting follicular hypertrophy in the thyroids of the 0; 3200; 8000 and 20000 ppm treatment group.

Conclusions:

Clear evidence of toxicity was observed at the 20000 ppm dose level, where reduced bodyweight gain was noted for both sexes. Bodyweight gains were also lower for females at 8000 ppm dietary level of NeemAzal. Upon histopathological examination, all treated animals showed signs of substance effects in the thyroid and the liver. In all animals receiving 20000 ppm, hepatocyte hypertrophy was noted. Periportal hepatocyte eosinophilia with clumping was observed at all dose groups, extent and prevalence were dose-related. These findings are in accordance with observed changes in liver weights. Follicular epithelial hypertrophy (minimal or trace) was seen in the majority of all treated animals but only in a single male animal from the control group. While all treated females were affected, effects were dose related in males.

A NOAEL was not determinable. The LOAEL was the lowest dose level, 3200 ppm (males: 322 mg/kg bw/d; females: 301 mg/kg bw/d).

Reference: TRF IIA 5.3.2 / 01

Report:	Waterson, L. A. (1997) NeemAzal technical Toxicity study in rats by dietary administration for 13 weeks Huntingdon Life Sciences Limited, England Report-no. EIP 4/963100 published: no; TOX9700509
Guidelines:	EPA FIFRA 152-20 OECD Guideline 408
Deviations:	Test compound was used after expiring date. As concentration analysis of feed was done in weeks 1 and 11 of study, it is considered acceptable.
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be acceptable.

Material and Methods:

NeemAzal technical (batch no.: VII, purity: 26.8 – 28.4%azadirachtin) was offered for 13 weeks to groups of 20 Crl: CD BR rats (animals provided by Charles River Breeding Laboratories, England; 10 of each sex) in the diet at concentrations corresponding to of 0, 100, 400, 1600 and 6400 ppm of NeemAzal. Actual achieved mean intakes, based on food consumption were 8, 32, 123 and 490 mg/kg bw/d for males and 9, 36, 135, and 525 mg/kg bw/d for females. Animals were observed with respect to mortality, clinical signs; bodyweight and food consumption were recorded weekly, water consumption was recorded daily over a seven day period, blood samples were taken for haematology and biochemistry, samples of urine were obtained for the determination of specific parameters in the last week of treatment. Each animal was examined ophthalmoscopically at the beginning of the study and again all animals of the control group and the high dose group in week 13. Following the 13-week treatment period all animals were sacrificed. All animals were thoroughly examined visually and by palpation, numerous organs were dissected free of fat and weighed including adrenals, brain, epididymes, heart, kidneys, liver, ovaries, pituitary, prostate, seminal vesicles, spleen, testes, thyroid, uterus. Any macroscopically abnormal tissue wasexamined histopathologically, as well as adrenals, alimentary tract, aorta, brain, heart, lung, liver, lymph nodes, kidney, pancreas, salivary gland, sciatic nerve, sternum (for bone and marrow), thyroid, sciatic nerve, spleen, thymus, uterus, ovaries, urinary bladder, testes and epididymides from all rats of the control and high dose group. Lung, liver, thyroid and kidney also from the 100, 400, 1600 ppm groups. Statistical analyses were carried out separately for either sex. Data relating to food and water consumption were analysed on a cage basis, all other parameters were analysed using individual animals as the basic experimental unit. Bodyweight gain, food and water consumption, clinical pathology and organ weight data were analysed for heterogeneity of variance between treatments with Bartlett's test. Where significant heterogenity (at the 1% level) was found a logarithmic transformation was tried to test for more stable variance. If no significant variance was detected, a one-way analysis of variance was carried out. If significant heterogeneity of variance was present, a Kruskal-Wallis-analysis of ranks was used.

Findings:

Concentration of azadirachtin in feed was determined chromatographically. Mean compound concentration in feed was within 6% of nominal concentrations. No treatment related deaths were observed. One female animal of the 6400 ppm group died during scheduled blood sampling procedure in week 13. There were no macroscopic or microscopic findings related to treatment noted for this animal. Both sexes receiving 6400 ppm showed lower, albeit not statistically significant, weight gain as compared to the controls (Table 60). Reduced weight gain in the 100 ppm group (females) was considered incidental and no effects on bodyweight were observed in any of the other treatment groups as compared to control.

Table 60: Bodyweight gain (week 0 – 13)

	Male		Female	
Dosage level (ppm)	**Weight gain (g)**	**% of control**	**Weight gain (g)**	**% of control**
0	297	-	138	-
100	336	113	129	93
400	345	116	143	104
1600	340	114	136	99
6400	277	93	120	87

Females receiving the 6400 ppm diet showed slightly lower mean food intakes as compared to the controls (Table 61). The overall mean food intake during the treatment period for both sexes receiving 100, 400 and 1600 ppm were similar to controls. Water consumption was marginally lower for males receiving 6400 ppm. No effects were observed for females or any other treatment group.

Table 61: Average food consumption and NeemAzal intake

	Male		Female	
Dosage level (ppm)	**Mean food intake (g/animal/day)**	**Mean compound intake (mg/kg bw/day)**	**Mean food intake (g/animal/day)**	**Mean compound intake (mg/kg bw/day)**
0	28.3	0.0	22.8	0
100	31.3	7.7	23.9	9.4
400	31.4	31.6	22.9	35.7
1600	30.1	123	21.5	135
6400	27.4	487	20.3	525

There were no findings noted at ophthalmoscopic examination in week 13. No effects on urine output volumes, specific gravity and protein values and pH-values were observed. For male rats statistically significant elevated red blood cell counts for the 400 ppm, 1600 ppm and 6400 ppm and lower mean corpuscular values (MCV) were noted for the 1600 ppm and 6400 ppm dose groups (Table 62). Females of the 6400 ppm treatment group had significantly reduced packed cell volume (PCV), MCV and reduced platelet count values. MCHC values were elevated for the 1600 ppm and 6400 ppm dose groups. The coagulation parameter TT was prolonged for males but reduced for females of the highest dose group, while APTT was dose-related prolonged for 400, 1600 and 6400 ppm males. These effects were statistically significant but marginal at 400 ppm. The effects seen at 400 ppm were considered to be toxicologically not relevant, as they were only marginal.

Table 62: Data on haematological parameters

Males						
Dose (ppm)	**TT (s)**	**APTT (s)**	**RBC (10^{12}/L)**	**MCHC (g/dL)**	**MCV (fL)**	**PCV (%)**
0	25	19.2	8.95	32.8	53.8	48.1
100	26	20.4	9.01	33.3	53.6	48.2
400	26	21.0*	9.39*	33.1	52.6	49.4
1600	27	22.1**	9.30*	33.0	52.2*	48.5
6400	30**	24.1**	9.21*	33.1	52.2*	48.1
Females						
Dose (ppm)	**TT (s)**	**APTT (s)**	**RBC (10^{12}/L)**	**MCHC (g/dL)**	**MCV (fL)**	**PCV (%)**
0	20	16.4	8.31	33.4	56.3	46.8
100	20	16.8	8.41	33.6	55.4	46.5
400	21	16.2	8.27	33.4	55.2	45.7
1600	20	15.8	8.31	33.9*	55.1	45.7
6400	19*	15.6	8.44	34.4**	53.1**	44.8**

*p <0.05; ** p <0.01

Elevated globulin concentrations in the blood were noted for both sexes of the 6400 and 1600 ppm dose groups, and total protein levels were significantly increased for females at the highest dose only, but for males at 400, 1600 and 6400 ppm (Table 63). No further differences in biochemical parameters were considered of toxicological relevance. The significantly elevated total protein levels at 400 ppm in males were considered to be not relevant.

Table 63: Biochemical parameters at week 13

Dose	**0 ppm**	**100 ppm**	**400 ppm**	**1600 ppm**	**6400 ppm**
Globulin (g/dL)					
Male	3.8	3.8	4.0	4.1**	4.1**
Female	3.7	3.8	3.7	3.9*	4.0**
Total serum protein (g/dL)					
Male	6.5	6.7	6.7*	6.8**	6.9**
Female	6.9	7.0	6.9	7.1	7.3**

*p <0.05; ** p <0.01

No findings were reported during macroscopic examination.

For both sexes receiving 6400 ppm, increased bodyweight adjusted mean liver weights were noted (Table 64). Elevated bodyweight adjusted mean brain weights were noted in all treated males with the exception of the 100 ppm group but there was no dose response. Females receiving 1600 or 6400 ppm also showed higher, but not statistically significant, bodyweight-adjusted thyroid weights, in comparison with controls. No further abnormalities were found at macroscopic post mortem examination of the animals.

Table 64: Organ weights –bodyweight adjusted means

Males										
Dose (ppm)	**Body-weight (g)**	**Liver (g)**	**Brain (g)**	**Thyroids (mg)**	**Pituitary (mg)**	**Spleen (g)**	**Heart (g)**	**Adrenals (mg)**	**Testes[§] (g)**	**Epididymi-des (g)**
0	476	20.6	2.03	21.5	14.0	0.87	1.55	56.5	3.54	1.28
100	523	18.3	2.02	21.1	13.4	0.79	1.57	56.7	3.83	1.20
400	524	20.6	2.11*	20.7	13.1	0.85	1.52	62.2	3.61	1.29
1600	521	20.0	2.10*	22.5	14.4	0.85	1.55	60.1	3.51	1.26
6400	458	23.0*	2.11*	21.7	13.3	0.83	1.56	57.5	3.48	1.30
Females										
Dose (ppm)	**Body-weight (g)**	**Liver (g)**	**Brain (g)**	**Thyroids (mg)**	**Pituitary (mg)**	**Spleen (g)**	**Heart (g)**	**Adrenals (mg)**	**Ovaries[§] (g)**	**Uterus (g)**
0	301	11.1	1.93	16.9	18.2	0.55	1.03	66.7	81.8	0.55
100	291	10.1	1.90	16.0	18.3	0.56	1.01	65.2	81.0	0.65
400	301	11.1	1.92	16.7	17.1	0.62	1.03	72.2	83.4	0.63
1600	298	11.9	1.89	19.7	18.6	0.59	1.04	73.2	80.4	0.57
6400	282	14.5*	1.88	19.7	17.0	0.59	1.06	74.9	84.9	0.55

§: unadjusted means; *: p <0.05; **: p <0.01

Liver: In both sexes significantly increased incidence of generalised hepatocyte hypertrophy was noted in animals receiving 6400 ppm. Periportal fat deposition was significantly more frequent and more pronounced in female rats receiving 6400 ppm and 1600 ppm as compared to controls.

Table 65: Microscopic hepatic observations

Dose			**0 ppm**	**100 ppm**	**400 ppm**	**1600 ppm**	**6400 ppm**
Males	Number of livers examined		10	10	10	10	10
	Hepatocyte hypertrophy	Centrilobular	1	3	2	3	1
		Generalised	0	0	0	0	9**
Females	Number of livers examined		10	10	10	10	10+
	Hepatocyte hypertrophy	Centrilobular	2	2	3	5	3
		Generalised	0	0	0	0	4*
	Periportal fat deposition	Marked	0	0	0	0	1
		Moderate	0	0	1	5*	4*
		Minimal	4	3	6	5	5
		Total	4	3	7	10**	10**

Fisher's Exact Test: *p <0.05; ** p <0.01
+: includes the decedent female

Thyroid: In the 6400 ppm dosage group moderate follicular epithelial hypertrophy was seen in 3 females while minimal effects were noted for one female of the control and 400 ppm group and 2 females of the 1600 ppm group.

Table 66: Incidence of follicular cell hypertrophy in female rats.

Dose			**0 ppm**	**100 ppm**	**400 ppm**	**1600 ppm**	**6400 ppm**
Females	Number of thyroids examined		10	10	10	10	10+
	Follicular cell hypertrophy	Moderate	0	0	0	0	3
		Minimal	1	0	1	2	0
		Total	1	0	1	2	3

+: includes the decedent female

Conclusions:

At 6400 ppm (achieved dose 490 and 525 mg NeemAzal/kg bw/d, for males and females, respectively) clear evidence of hepatotoxicity was observed in both sexes (increased relative liver weight, generalised hepatocyte hypertrophy, in females: periportal fat disposition, (minimally) increased blood protein levels). In animals maintained on the 6400 ppm diet haematological effects were observed (females: higher mean platelet values, (slightly) reduced thrombotest values; males: prolonged blood coagulation (APTT), prolonged thrombotest-values). Increased mean bodyweight adjusted thyroid weight and also a slight increase in the incidence of follicular epithelial hypertrophy were observed. At 1600 ppm (achieved dose 123 and 135 mg NeemAzal/kg bw/d for males and females, respectively) increased incidence and severity of periportal fat deposition was noted in females only, while slightly increased total protein levels were noted for both sexes and prolonged APTT values for males only. At 400 ppm (achieved dose 32 and 36 mg NeemAzal/kg bw/d for males and females, respectively) and 100 ppm (achieved dose 8 and 9 mg NeemAzal/kg bw/d for males and females, respectively) no signs of toxicity were observed.

The NOAEL was established at a dose level of 400 ppm (32 or 36 mg/kg bw/d for males or females, respectively). The LOAEL was 1600 ppm (123 or 135 mg/kg bw/d for males or females, respectively).

Studies performed with Fortune Aza

Reference:	SIP IIA 5.3.1 / 01
Report:	Waterson, L. A., Dawe, I. S. (1997) Fortune Aza technical toxicity study in rats by dietary administration for 4 weeks Huntingdon Life Sciences Limited, England Report-no. FBT 3/961630; TOX2005-2385
Guidelines:	OECD Guideline 407 (1987)
Deviations:	None (report number on the title page (FBT 3/961630) is different from the number inside the report (FBT 3/961640))
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be acceptable.

Material and Methods:

Fortune Aza technical (batch no.: 110301195, purity: 13.3% azadirachtin A+B) was offered for 4 weeks to groups of 10 Crl: CD (SD) BR rats (animals provided by Charles River Breeding Laboratories, England; 5 of each sex) in the diet at concentrations of 0, 4000, 8000 and 16000 ppm of Fortune Aza technical (mean actual achieved intakes of Fortune Aza technical were calculated and averaged 400, 780 and 1420 mg/kg bw/d for males and 400, 880 and 1420 mg/kg bw/d for females, respectively). Observations were carried out on mortality, clinical signs, bodyweights, and food consumption. Following the 4-week treatment period all animals were sacrificed, weights were recorded for specific organs (adrenals, brain, epididymides, heart, kidneys, liver, ovaries, pituitary, spleen, testes, thyroid, uterus), detailed macroscopic examinations were performed. Organs were fixed in appropriate solutions and preserved for potential future microscopic analysis.

Statistics: Statistical analysis were carried out separately for either sex. Data relating to food and water consumption were analysed on a cage basis and thus could not be analysed, all other parameters were analysed using individual animals as the basic experimental unit. Bodyweight gain, clinical pathology and organ weight data were analysed for heterogeneity of variance between treatment with Bartlett's test. Where significant heterogeneity (at the 1% level) was found a logarithmic transformation was tried to test for more stable variance. If no significant variance was detected, a one-way analysis of variance was carried out. If significant heterogeneity of variance was present, a Kruskal-Wallis-analysis of ranks was used.

Findings:

Compound concentration in feed was within 2% of nominal concentration. Under the conditions of this 4-week feeding study, no mortalities occurred. During the first four days of treatment both sexes receiving the 8000 ppm or 16000 ppm dose showed weight loss, and in the low dose group bodyweight gain was significantly reduced in both sexes (Table 67). Thereafter, weight gain improved in the two higher dose groups but remained significantly lower as compared to control. In the low dose group, weight gain was comparable to control animals from day 4 onwards. Clinical signs included piloerection in three males and one female of the high dose group.

Table 67: Bodyweight gain (g)

Dosage level	Male						Female					
	Day 1-4		Day 4-29		Day 1-29		Day 1-4		Day 4-29		Day 1-29	
0	25	(100%)	183	(100%)	208	(100%)	15	(100%)	67	(100%)	82	(100%)
4000	13**	(52%)	184	(101%)	196	(94%)	10**	(67%)	66	(99%)	76	(93%)
8000	-18**	(-72%)	106**	(58%)	88**	(42%)	-7**	(-47%)	41*	(61%)	34**	(41%)
16000	-34**	(-136%)	25**	(14%)	-9**	(-4%)	-19**	(-127%)	23**	(34%)	4**	(5%)

* p <0.05, ** p <0.01

Both sexes receiving the 16000 ppm and 8000 ppm diets and females receiving 4000 ppm diet showed lower mean food intakes as compared to the controls. During the first week food intake was reduced in the 4000 ppm group in males also; thereafter, food consumption improved and was in this group comparable to controls. There were no further observations that were considered treatment related. All treated female groups showed higher mean absolute liver weights (Table 68), lower mean absolute adrenal and ovary weights in comparison with controls, statistical significance being attained by females receiving 16000 ppm for the liver finding and all treated groups for the adrenal and ovary finding. These findings were (relative to bodyweight) dose-related (Table 69). At

the 16000 ppm level nearly all relative and several absolute mean organ weight values were affected.

Table 68: Absolute organ weights –group means

Males										
Dose group (ppm)	**Body-weight (g)**	**Liver (g)**	**Brain (g)**	**Thyroids (mg)**	**Pituitary (mg)**	**Spleen (g)**	**Heart (g)**	**Adrenals (mg)**	**Testes (g)**	**Epididymi-des (g)**
0	415	19.8	1.96	19.9	10.7	0.85	1.40	57.6	3.306	0.892
4000	405	23.5	1.97	19.4	11.0	0.87	1.45	57.0	3.229	0.831
8000	303	19.0	1.87	13.8*	8.9*	0.54**	1.03**	39.9**	2.763	0.715
16000	210	16.5	1.76**	13.7*	6.2**	0.41**	0.84**	41.3**	2.920	0.690
Females										
Dose group (ppm)	**Body-weight (g)**	**Liver (g)**	**Brain (g)**	**Thyroids (mg)**	**Pituitary (mg)**	**Spleen (g)**	**Heart (g)**	**Adrenals (mg)**	**Ovaries (mg)**	**Uterus (g)**
0	235	10.9	1.82	14.8	11.8	0.58	0.93	75.7	91.7	0.45
4000	230	13.5	1.80	16.0	11.5	0.50	0.88	60.3*	72.5*	0.38
8000	194	13.2	1.72*	12.2	8.4**	0.43**	0.73**	49.4**	58.1**	0.36
16000	172	13.9*	1.68**	13.0	6.5**	0.39**	0.66*	40.1**	37.2**	0.15**

*, p <0.05; **, p <0.01

Table 69: Relative organ weights –group means (in percent x 100)

Males										
Dose group (ppm)	**Body-weight (g)**	**Liver**	**Brain**	**Thyroids**	**Pituitary**	**Spleen**	**Heart**	**Adrenals**	**Testes**	**Epididymi-des**
0	415	477	47	0.48	0.26	20	34	1.4	40	10.8
4000	405	578*	49	0.47	0.27	19	36	1.4	40	10.3
8000	303	626**	62**	0.46	0.30*	18	34	1.3	45	11.7
16000	210	783**	84**	0.65*	0.30*	20	40**	2.0**	70**	16.3**
Females										
Dose group (ppm)	**Body-weight (g)**	**Liver**	**Brain**	**Thyroids**	**Pituitary**	**Spleen**	**Heart**	**Adrenals**	**Ovaries**	**Uterus**
0	235	464	78	0.63	0.51	25	39	3.2	3.9	19
4000	230	585**	79	0.69	0.50	22	38	2.6	3.2	17
8000	194	683**	89*	0.65	0.43	22	38	2.6	3.0*	18
16000	172	800**	99**	0.74	0.38**	22	38	2.3**	2.3**	8**

*, p <0.05; **, p <0.01

Various macroscopic findings in high and mid dose groups were considered to be a result of the effect on bodyweight: A reduction in adipose tissue was noted in 2/5 and 3/5 females receiving 8000 and 16000 ppm, respectively, compared with zero incidences in controls.Small seminal vesicles were observed in 4/5 males receiving 16000 ppm, compared with zero incidences in controls.Small prostate glands were observed in all males of the high dose group, compared with zero incidences in controls. Small ovaries were observed in 3/5 females of the high dose group, compared with zero incidences in controls. Small uteri were observed in 3/5 and 4/5 females receiving 8000 and 16000 ppm, respectively, compared with zero incidences in controls.

Conclusions:

Clear evidence of toxicity was observed at the 16000 and 8000 ppm dose levels, where reduced bodyweight gain was noted for both sexes, reduced feed intakes were also observed at these levels. Various macroscopic findings in these two dose groups were considered to be a result of the effect on bodyweight. Clinical signs included piloerection in three males and one female of the high dose group. At 4000 ppm bodyweight was affected only during the first four days of the study. However, dose-related changes were noted in liver weights of both sexes, adrenal and ovary weights in females. In the absence of histological examination, these findings account as adverse effects.

A NOAEL could not be determined. The LOAEL was the lowest dose level, 4000 ppm (males: 400 mg/kg bw/d; females: 401 mg/kg bw/d).

Reference:	SIP IIA 5.3.2 / 01
Report:	Waterson, L. A. and Dawe, I. S. (1997)
	Fortune Aza technical – Toxicity Study in Rats by Dietary Administration for 13 Weeks
	Huntingdon Life sciences Ltd., Huntingdon, England
	unpublished report No. FBT 4/962744; TOX2005-2386
Guidelines:	EPA FIFRA
	OECD Guideline 408 (1987),
	EEC Directive 92/69/EEC B.26
Deviations:	none
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be acceptable.

Material and Methods:

Fortune Aza technical (batch no.: 110301195, purity: 13.3% azadirachtin A+B) was offered for 13 weeks to groups of 20 Crl: CD (SD) BR rats (animals provided by Charles River Breeding Laboratories, England; 10 of each sex) in the diet at concentrations of 0, 100, 400, 1600 and 6400 ppm. Mean achieved doses of Fortune Aza technical were 0, 8.5, 33.5, 140 and 520 mg/kg bw/day in males and 0, 11, 40, 180 and 550 mg/kg bw/day in females. Animals were observed with respect to mortality, clinical signs, bodyweight and food consumption, water consumption was recorded, blood samples were taken for haematology and biochemistry, samples of urine were obtained for the determination of specific parameters. Each animal was examined ophthalmoscopically at the beginning of the study and during week 13 all animals of the control group and the high dose group were examined. Following the 13-week treatment period all animals were sacrificed, weights were recorded for specific organs, detailed macroscopic and microscopic

(lungs, livers, kidneys, thyroids, sciatic nerve, uterus, ovaries, testes and epididymides) examinations were performed.

Statistics: Statistical analyses were carried out separately for either sex. Data relating to food and water consumption were analysed on a cage basis, all other parameters were analysed using individual animals as the basic experimental unit. Bodyweight gain, food and water consumption, clinical pathology and organ weight data were analysed for heterogeneity of variance between treatment using Bartlett's test. Where significant heterogeneity (at the 1% level) was found a logarithmic transformation was tried to test for more stable variance. If no significant variance was detected a one-way analysis of variance was carried out. If significant heterogeneity of variance was present, a Kruskal-Wallis-analysis of ranks was used. Analysis of variance were followed by Student's t test and William's test. Kruskal-Wallis analyses were followed by the non-parametric equivalent of these test (Shirley).

Findings:

Concentration of azadirachtin in feed was determined chromatographically. Mean analytical results were within 3% of nominal concentrations. Under the conditions of this 13-week rat-feeding study, no mortalities occured.

In the high dose group (6400 ppm) generalised hair loss was noted in 8 of 10 female animals, apparent from week 7 onwards. While in male rats of all treatment groups and also in control animals localised hair loss was observed from week 1, males of the high dose group tended to show generalised hair loss. During week 1 both sexes receiving 6400 ppm showed significantly lower weight gain as compared to the controls. Thereafter, weight gain improved in this high dose group but remained statistically lower as compared to control (Table 70).

Table 70: Bodyweight gain over the study period

	Male		Female	
Dosage level (ppm)	**Weight gain (g)**	**% of control**	**Weight gain (g)**	**% of control**
0	325	-	154	-
100	363	112	154	100
400	326	100	147	95
1600	337	104	152	99
6400	213**	66	92**	60

**, $p < 0.01$

During week 1 both sexes receiving 6400 ppm showed significantly lower mean food intakes as compared to the controls. Thereafter, weekly food consumption improved in this high dose group but remained statistically lower as compared to control (Table 71). The overall mean food intake during the treatment period for both sexes receiving 100, 400 and 1600 ppm were similar to controls. Water consumption was notably lower for males receiving 6400 ppm. Statistically significance was not attained. No effects were observed for females or any other treatment group.

Table 71: Average food consumption and Fortune Aza technical intake

Dosage level	Male		Female	
	Mean food intake (g/animal/day)	Mean compound intake (mg/kg bw/day)	Mean food intake (g/animal/day)	Mean compound intake (mg/kg bw/day)
0	29.1	0	23.8	0
100	31.6	8.5	28.7	11.1
400	29.4	33.5	24.0	39.2
1600	30.9	137	27.4	176
6400	23.6*	516	18.4**	553

* p <0.05; ** p <0.01

There were no findings noted at the ophthalmoscopic examinations in week 13. Statistically significant elevated red blood cell counts and concomitant lower mean corpuscular values (MCV) were noted for the 6400 ppm dose group for both sexes. These effects were considered treatment-related. MCVs were also reduced for males receiving 400 or 1600 ppm as compared to controls but a clear dose-response was not evident. Similarly, lower packed cell volume counts observed for females (dose groups 1600 and 6400 ppm) were not considered treatment related. Effects regarding blood coagulation were minimal, specifically for females of the high dose group thrombotest (TT) values were elevated (but within the range of controls) and activated partial thromboplastin times (APTT) were marginally reduced. Lower mean neutrophil, eosinophil, monocyte and large unstained cells (LUC) counts were observed for females in the 6400 ppm group while males showed a lower mean eosinophil count. However, total white cell counts were generally similar to control animals.

Elevated globulin and total protein concentrations in the blood were noted for males of the high dose group (Table 72). Creatinine levels for both sexes in the 6400 ppm-group and for males in the 1600 ppm group were significantly higher. Significantly increased values were observed for alkaline phosphatase (AP) in females of the 6400 ppm group, while lower values were observed for males in all but the 100 ppm group. Similarly, reduced glutamic-pyruvate transaminase (GPT, alanine aminotransferase) for both sexes and glutamic-oxaloacetic transaminase (GOT, aspartate aminotransferase) for males only were observed in the 6400 ppm group. Since lowering of enzyme values is generally not a sign of (hepato-)toxic response these differences were not considered of toxicological importance. The statistically significant higher values in the 6400 ppm group for calcium in males and for potassium and chloride in females were not considered dose related because individual values were generally within the concurrent range. Differences in females were mainly attributable to a single outlier.

No further differences were noted in biochemical parameters.

Table 72: Biochemical parameters week 13 (group mean values)

Males

ppm	Globulin g/dL	Protein g/dL	Creatinine mg/dL	AP mU/mL	GPT mU/mL	GOT mU/mL	Na mEq/L	K mEq/L	Ca mEq/L	Cl mEq/L
0	3.7	6.6	0.5	191	27	60	145	3.4	5.5	102
100	3.8	6.5	0.5	187	29	63	145	3.6	5.5	101
400	3.7	6.5	0.5	159**	29	54	144	3.6	5.5	102
1600	3.8	6.5	0.6**	150**	30	60	144	3.7	5.4	102
6400	4.1**	7.0**	0.7**	162**	23*	50**	145	3.4	5.7**	101

Females

ppm	Globulin g/dL	Protein g/dL	Creatinine mg/dL	AP mU/mL	GPT mU/mL	GOT mU/mL	Na mEq/L	K mEq/L	Ca mEq/L	Cl mEq/L
0	3.7	6.8	0.5	99	25	54	144	3.3	5.6	102
100	3.7	6.7	0.6	103	28	58	145	3.3	5.4	103
400	3.7	6.8	0.6	102	29	61	144	3.3	5.5	102
1600	4.0	7.2	0.6	82	26	51	144	3.2	5.6	101
6400	3.8	6.8	0.8**	159**	19*	46	146**	3.6*	5.5	103*

* $p < 0.05$; ** $p < 0.01$

Significantly higher urine output volumes and associated lower specific gravity and protein values and also higher pH-values were observed for females of the 6400 ppm group. Minimal hair loss was noted at macroscopic examination in 8/10 female rats of the 6400 ppm group (none were observed in the control group). Small uteri were noted in six of ten females in the high dose group (6400 ppm) compared to none in the control group. For females of all doses except the 100 ppm increased absolute and bodyweight adjusted mean liver weights were noted with a dose response relationship (Table 73). For males elevated liver weights were only observed in the highest dose level. Significant reduced organ weights were noted for uteri and ovaries in the 6400 ppm group, a slightly reduced bodyweight-adjusted mean ovary weight was noted at 1600 ppm. Bodyweight adjusted mean heart weights were noted in all treated females but there was no dose response. Testes and epididymides weights were reduced, albeit not significantly, at 6400 ppm. The apparent effects on these organs in the 1600 ppm group wereattributable to a single animal. Lower bodyweight adjusted mean adrenal and absolute pituitary weights were noted for females in the 6400 ppm dosage group. No further abnormalities were found at macroscopic post mortem examination of the animals.

Table 73: Organ weights – bodyweight adjusted means

Males							
ppm	**Liver (g)**	**Heart (g)**	**Adrenals (mg)**	**Pituitary (mg)**	**Seminal vesicle (mg)**	**Testes§ (g)**	**Epididymides§ (g)**
0	19.8	1.45	53.7	13.3	1.32	3.51	1.21
100	18.8	1.51	55.5	12.5	1.29	3.68	1.21
400	18.1	1.45	55.2	12.2	1.44	3.56	1.25
1600	20.3	1.50	57.9	12.0	1.29	3.37	1.18
6400	22.5*	1.45	53.0	13.0	1.48	3.30	1.12
Females							
ppm	**Liver (g)**	**Heart (g)**	**Adrenals (mg)**	**Pituitary§ (mg)**	**Uterus§ (g)**	**Ovaries (mg)**	
0	10.6	0.94	71.2	15.0	0.78	82.4	
100	11.0	1.03**	71.1	17.4	0.65	84.6	
400	11.8*	1.08**	74.8	17.1	0.71	81.2	
1600	12.8**	1.04**	70.4	18.2	0.63	71.5	
6400	16.5**	1.00**	55.7**	11.8**	0.28**	65.4*	

§: unadjusted means Fisher's exact test: * p <0.05; ** p <0.01

At microscopic examination the following findings were noted:

Liver: In all animals receiving 6400 ppm and most males (9/10) receiving 1600 ppm, periportal hepatocyte eosinophilia with clumping and bile duct hyperplasia was observed (Table 74). In males the incidence and degree of these changes increased in a dose dependent manner. In two males receiving 6400 ppm hypertrophy was also noted in periportal hepatocytes. These findings are in accordance with observed changes in liver weights.

Thyroid: In the 6400 ppm dosage group trace follicular epithelial hypertrophy was seen in 3 males and 4 females (Table 74).

Ovaries: In all females receiving 6400 ppm and in one animal each of the 1600 ppm and 400 ppm groups as well as in one animal of the control group apparently decreased numbers of corpora lutea was observed (Table 74). Corpora lutea were absent in a single female of the 1600 ppm dose level. The number of corpora lutea was counted in each animal and decreased numbers were observed at the 1600 ppm and 6400 ppm dose levels. This correlated with the decreased mean ovary weights observed in these groups.

Uterus: In six females of the 6400 ppm dosage group endometrial atrophy was observed, correlating with decreased uterus weight at this dose level (Table 74). No effects were observed at the other dose levels.

Testes and epididymides: In two males receiving 6400 ppm marked seminiferous tubular atrophy was seen concomitant with absence or decreased spermatozoa in the epididymides (Table 74). In addition one male in the 1600 ppm group, where the testes had been reported as small macroscopically, had moderate seminiferous tubular atrophy and abnormal spermatids in the ducts of the epididymides. Trace seminiferous tubular atrophy was seen in one male of the 400 ppm group. As this effect is sometimes seen in control animals this finding in a single male was considered unrelated to treatment.

Sciatic nerve: As compared to controls an increased incidence and degree of nerve fibre degeneration was observed in rats receiving 6400 ppm of both sexes (Table 74). In a single female rat receiving 400 ppm moderate nerve fibre degeneration was noted. This was mainly in one area

and was considered to be a result of trauma and, thus, unrelated to treatment. No microscopic findings could account for lower adrenal and pituary weights observed for females receiving 6400 ppm. Similarly no microscopic findings were observed accounting for the higher heart weights.

Table 74: Microscopical findings

			Male					Female				
Dose level (ppm)			**0**	**100**	**400**	**1600**	**6400**	**0**	**100**	**400**	**1600**	**6400**
Liver	Number of organs examined		10	10	10	10	10	10	10	10	10	10
	Hepatocyte hypertrophy – periportal	Minimal	0	0	0	0	2	0	0	0	0	0
	Bile duct hyperplasia	Total	0	0	0	8**	10**	0	0	0	0	10**
		Trace	0	0	0	8**	0	0	0	0	0	10**
		Minimal	0	0	0	0	10**	0	0	0	0	0
	Hepatocyte cytoplasmic eosinophilia with clumping – periportal	Total	0	0	0	9**	10**	0	0	0	0	10**
		Trace	0	0	0	9**	0	0	0	0	0	6**
		Minimal	0	0	0	0	10**	0	0	0	0	4*
Thyroid	Number of organs examined		10	10	10	10	10	10	10	10	10	10
	Follicular epithelial hypertrophy	Trace	0	0	0	0	3	0	0	0	0	4*
Ovaries	Number of animals examined							10	10	10	10	10
	Absent corpora lutea							0	0	0	1	0
	Apparent decreased numbers of corpora lutea							1	0	1	1	10**
	Group mean number of corpora lutea[§]							36	39	38	28	21
Uterus	Number of organs examined							10	10	10	10	10
	Endometrial atrophy							0	0	0	0	6**
Testes	Number of organs examined		10	10	10	10	10					
	Seminiferous tubular atrophy	Total	0	0	1	1	2					
		Trace	0	0	1	0	0					
		Moderate	0	0	0	1	0					
		Marked	0	0	0	0	2					
Epidi-dymides	Number of organs examined		10	10	10	10	10					
	Absence of spermatozoa		0	0	0	0	1					
	Decreased spermatozoa	Marked	0	0	0	0	1					
	Abnormal spermatids in ducts	Moderate	0	0	0	1	0					
	Ductal epithelial vacuolisation	Trace	0	0	0	0	1					
Sciatic nerve	Number of organs examined		10	10	10	10	10	10	10	10	10	10
	Nerve fiber degeneration	Total	4	5	5	4	8	1	2	4	3	7**
		Trace	4	4	5	3	5	1	2	3	3	2
		Minimal	0	1	0	1	3	0	0	0	0	5*
		Moderate	0	0	0	0	0	0	0	1	0	0

Fisher's Exact Test: *p <0.05; ** p <0.01 §: Statistical analysis not performed

Conclusions:

A wide range of signs of toxicity were observed in the high dose group (6400 ppm, corresponding to 520 and 550 mg/kg bw/d for males and females, respectively) including reduced bodyweight, hepatotoxicity, altered haematologic parameters, hair loss, effects on the female and male reproductive organs and sciatic nerve degeneration. At 1600 ppm (corresponding to 140 and 180 mg/kg bw/d for males and females, respectively) hepatotoxicity and toxic effects on the ovaries (slightly reduced weight, reduced number of corpora lutea) were noted. At 400 ppm (corresponding to 33 and 40 mg/kg bw/d for males and females, respectively) increased bodyweight adjusted liver weights in females was noted. As the effect on liver weight is not supported by histological findings, this dose level is considered the NOAEL for treatment with Fortune Aza over a period of 90 d.

Studies performed with ATI 720

Reference:	MAS IIA 5.3.2 / 02
Report:	Johnson, W. D. (1994) 90-day oral (diet) toxicity study of ATI-720 in rats. IIT Research Institute, Life Science Research, 10 West 35th Street, Chicago, Illinois, USA Project No L 08424 Study No 4; TOX2005-2388
Guidelines:	OECD Guideline 408 (1987), EEC Directive 92/69/EEC B.26
Deviations:	Page 204 is reproduced incompletely in the report. No information on validation of analytical method given. Detection limit of azadirachtin not stated.
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be acceptable.

Material and Methods:

Azadirachtin ATI-720 (batch no.: 21380, purity: 7.74% azadirachtin) was offered for 13 weeks to groups of 20 Sprague Dawley rats (animals provided by Charles River Laboratories, USA; 10 of each sex) in the diet at concentrations of 0, 500, 2500 and 10000 ppm (mean achieved doses were 0, 30, 145, and 585 mg/kg bw/day in males and 0, 35, 180 and 680 mg/kg bw/day in females). Dose selection was based on a 14-d rangefinding study (there are no further information available on this study). Animals were observed with respect to mortality, clinical signs; bodyweight and food consumption were recorded, blood samples were taken for haematology and biochemistry. Each animal was examined ophthalmoscopically at the beginning of the study and after 90 days of feeding. Following the 13-week treatment period all animal were sacrificed. Weights were recorded for specific organs (kidneys, liver, testes, ovaries), detailed macroscopic and microscopic (complete set of collected tissues from the control and high dose animals, any macroscopically abnormal tissue, as well as lungs, livers, kidney from animals of the low and medium dose) examinations were performed.

All data were analysed using analysis of variance (ANOVA) followed by the post hoc Dunnett's test for comparing multiple treatment groups to a single control. This was done automatically for bodyweights, weekly bodyweight gains, weekly food consumption and haematology data. Absolute and relative organ weights, food conversion ratios and clinical chemistry data were analysed by ANOVA and Dunnett's test using SYSTAT software.

Findings:

Concentration of azadirachtin in feed was determined chromatographically relatively to a standard of azadirachtin (98%). Mean compound concentrations in feed were within 7.2% of nominal concentrations. Feed was found to be homogenous. Preparations were stable for up to 14 d when stored at room temperature or in freezer. Under the conditions of this 13-week rat feeding study, no mortalities occurred. Hair loss (alopecia) was noted especially in female animals of the high dose groups (5/10 animals) and the mid dose group (2/10). For males, hair loss was reported only for 1/10 of each of these two treatment groups. These observations were not considered treatment related. No other treatment related sign were observed. From week 3 (males) or week 4 (females) through the duration of the feeding period significantly lower bodyweights were observed in the high dose group (10000 ppm) as compared to the controls. Weight gain improved in high dose group from week 6 on, but remained over the study period statistically lower as compared to control (Table 75). For females in the 500 ppm group significantly elevated cumulative bodyweight gains were recorded. For the other treatment groups no differences were observed.

Table 75: Bodyweight gain over study period

	Male		Female	
Dosage level	**Weight gain (g)**	**% of control**	**Weight gain (g)**	**% of control**
0	315	-	109	-
500	310	98.4	129*	118.3
2500	315	100	110	101
10000	230**	73.0	78**	71.6

* $p<0.05$; ** $p<0.01$

In the high dose group, mean weekly food consumption was decreased for both sexes from the first week (Table 76). This decrease only failed to reach significance in weeks 1, 2, 7 and 12 for males and in weeks 1 and 12 for females. The mean food intake during the treatment period for both sexes receiving 500 and 2500 ppm were similar to controls.

Table 76: Average food consumption and compound intake

Dosage level (ppm)	**Male**		**Female**	
	Mean food intake (g/animal/day)	**Mean compound intake (mg/kg bw/day)**	**Mean food intake (g/animal/day)**	**Mean compound intake (mg/kg bw/day)**
0	26.6	0	17.3	0
500	26.3	29.6	17.6	34.5
2500	26.0	145.2	17.4	178
10000	23.2	585	15.2	680

There were no findings noted at ophthalmoscopic examination in week 13. Statistically significant lower mean corpuscular volumes (MCV) were noted for the 10000 ppm dose group for both sexes (Table 77). Similarly, mean corpuscular haemoglobin (MCH) was reduced, and red blood cell count was elevated in males receiving 10000 ppm. Decreases in haemoglobin and haematocrit were

observed for females (dose group 10000 ppm). These effects were considered treatment-related. MCVs and MCH were reduced for males receiving 500 ppm as compared to controls but a dose-response was not evident since no effects were seen in the 2500 ppm dose group and, thus, these differences were not considered of toxicological significance.

Table 77: Haematological parameters, week 13

	Male				Female			
Dose level (ppm)	**0**	**500**	**2500**	**10000**	**0**	**500**	**2500**	**10000**
Mean corpuscular volume (fL)	50.8	48.9**	49.6	47.9**	52.4	52.6	53.6	50.0*
Mean corpuscular haemoglobin (pg)	19.1	18.2*	18.5	17.5**	19.7	19.5	20.2	18.7
Red blood cells (10^6/mm^3)	8.08	8.45	8.30	8.82*	7.78	7.91	7.39	7.72
Haemoglobin (g/dL)	15.4	15.3	15.4	15.4	15.2	15.4	14.9	14.4**
Haematocrit (%)	41.0	41.3	41.2	42.3	40.6	41.5	39.6	38.5*

Dunnett's test: *p <0.05

Mean biochemical data are summarised in Table 78. Significant increases were observed in the high dose group for GGT in both sexes and for urea nitrogen and creatinine in females only. Decreased values were observed for chloride, and ALT in the high dose group for females. Decreased values for AST and alkaline phosphatase in the mid dose group (females) only, were considered not treatment related because of the lack of dose response. Furthermore, reduced enzyme activities are generally not considered of toxicological relevance. Chloride values were within the range of historical controls (105-111 meq/L, n=20). The increased levels of GGT (high and mid dose) and creatinine (high dose) and urea nitrogen (high dose) in females were considered treatment induced effects, although no concomitant histopathological changes were observed.

Table 78: Biochemical parameters, week 13 (group mean values)

	Male				Female			
Dose level (ppm)	**0**	**500**	**2500**	**10000**	**0**	**500**	**2500**	**10000**
Globulin (g/dL)	2.4	2.4	2.5	2.5	2.2	2.3	2.3	2.3
Protein (g/dL)	6.2	6.2	6.4	6.5	6.2	6.5	6.4	6.6
Creatinine (mg/dL)	0.47	0.52	0.52	0.54	0.5	0.51	0.51	0.59*
AP (mU/mL)	65	71	67	72	54	51	37*	61
ALT (mU/mL)	27	28	24	22	28	27	23	22*
AST (mU/mL)	85	91	85	82	96	89	70*	77
Na (mEq/L)	144	143	144	143	142	141	143	142
K (mEq/L)	4.4	4.4	4.5	4.6	4.1	4.3	4	4.4
Ca (mEq/L)	10	10.1	10.4	10.3	10.1	10.3	10.4	10.4
Cl (mEq/L)	105	106	107	106	111	109	110	108*
GGT (IU/L)	1	2	2	7*	2	2	4*	15*
BUN (mg/dL)	13.9	15.5	15.2	16.2	17.1	16.3	16.2	20.6*

Dunnett's test: *p <0.05

The most common gross lesion was red mandibular lymph nodes. One control and one low dose male had urinary bladder calculus and one low dose female exhibited unilateral dilation of the kidney pelvis. These lesions were not considered dose related. Mean absolute kidney weights were significantly decreased in high dose males (Table 79 and Table 80). Relative liver and testes weights were elevated for males in the high dose group only. Increased relative kidney weight and absolute and relative liver weight was noted for females in the high dose group and increased liver weight was also observed for females in the mid dose group. Fasted bodyweights were significantly decreased for animals of both sexes treated with 10000 ppm. It is likely that this reduction accounted for all the increased relative organ weights except for increased liver weights in females in the mid- and high dose groups.

Table 79: Organ weights – absolute and relative means (males)

Dose (ppm)	Fasted bodyweight (g)	Liver		Kidney		Testes	
		absolute (g)	relative (%)	absolute (g)	relative (%)	absolute (mg)	Relative (%)
0	528	14.9	2.81	3.37	0.64	3.49	0.66
500	520	15.2	2.91	3.40	0.66	3.45	0.67
2500	530	15.6	2.94	3.31	0.63	3.49	0.66
10000	442*	15.1	3.41*	3.03*	0.69	3.51	0.81*

*, p <0.05

Table 80: Organ weights – absolute and relative means (females)

Dose (ppm)	Fasted bodyweight (g)	Liver		Kidney		Ovaries	
		absolute (g)	relative (%)	absolute (g)	relative (%)	absolute (mg)	Relative (%)
0	262	6.55	2.50	1.81	0.69	90	0.035
500	282	7.19	2.56	1.87	0.67	92	0.033
2500	263	7.66*	2.91*	1.83	0.70	84	0.032
10000	229*	9.52*	4.16*	1.73	0.76*	74	0.032

*, p <0.05

No substance related microscopic abnormalities were seen in any organ or tissue from any animal examined at the end of the treatment period.

Conclusions:

Administration of ATI-720 at a dietary level of 10000 ppm (corresponding to 585 mg and 680 mg/kg bw/d for males and females, respectively) resulted in several toxicological effects related to the test compound including hepatotoxicity, altered biochemical parameters, and hair loss. Decreased palatability of the test diet resulted in decreased feed intake, and, consequently, decreased bodyweight gain and bodyweight were observed in both sexes. Statistically significant changes were observed in haematological and biochemical parameters.

Both, absolute and relative liver weights in females were significantly increased also in the mid dose group at a dietary level of 2500 ppm (corresponding to 145 mg and 180 mg/kg bw/d for males and females, respectively). Additionally, GGT levels were increased in this dose level group (females). No treatment related histopathological changes were observed in any of the treatment groups. Based on these observations the NOAEL was 500 ppm for females (corresponding to 35 mg/kg bw/d) and 2500 ppm (145 mg/kg bw/d) for males.

9.7.2 Studies in other mammalian species

No guideline compliant studies in other species than in rats have been submitted.

For purpose of better information, the whole justification submitted by Trifolio is printed. A discussion is given below.

Statement by Trifolio:

Introduction:

Practically all parts of the Neem- tree have been used since thousands of years for different medical (human and veterinary) and nutritional purposes (1 - 6). During the last 40 years the traditional knowledge has been reviewed critically (1-7) in order to optimise usage and application.

Due to the very large number of applications, observed effects in animals and humans as well as the large number of active compounds, which varies considerably in nature and composition in the different parts of the tree (leaves, stem, bark, twigs, seed, fruit pulp, seed kernels and roots) it is very difficult to draw totally precise conclusions from these reports for extrapolation of properties of purified extracts, like NeemAzal.

However, it is clear that the constituents of NeemAzal are present in Neem Seed Kernels (NSK) (or powdered NSK), Neem Oil (NO) as well as Neem Seed Cake (NSC). In addition to the constituents (predominantly azadirachtins) of NeemAzal, other active substances of varying amounts are present in NSK, NO and NSC, which may have relevant properties for an estimation of toxicological properties.

For a safe judgement of possible risks after application of NeemAzal and its formulations toxicological information on various mammals is desirable in addition to studies with mice and/or rats. Therefore we have summarised reports especially on the internal uptake of NSK, NSC and NO in order to analyse critically whether any non-desirable side-effects can be expected.

Discussion:

Neem Oil (NO):

NO has been used in cases as an additive to cattle or poultry feed on behalf of its nutritional value. Additionally it was used as a remedy against different diseases in humans. As Niemann (9) and Niemann and Hilbig (8) point out intoxications which have occasionally occurred with NO may probably be caused by the presence of aflatoxins, which are usually not controlled in traditional use. Thus experience with NO is not a valid model for the above purpose.

Neem Seed Kernels or powder thereof (NSK):

Some reports describe the use of NSK or Neem fruits for medical purposes or as a cattle feed for example. However, under practical conditions NSK is not used frequently since it is economically preferable to farmers to sell the NSK to oil mills and obtain payment and NSC in return.

Neem Seed Cake (NSC):

Due to the favourable composition of NSC with respect to protein (amino acids) and other constituents (7) and its abundance (low cost) NSC had been used as an additive to animal feed frequently (7). Due to the very bitter taste of NSC, which is due to limonoids, the animals have to be adapted to taking it in; alternatively the NSC can be debitterised by washing with water (Water washed Neem Seed Kernel Cake WWNSKC) (7). Usually NSC contains between 0.5 to 5 mg azadirachtin A/g. According to our own experience even after repeated treatment with water it will be a good estimate that NSC contains still 1/10 of the original amount of azadirachtins.

For the following discussion it seems reasonable and safe to assume that NSC contains 1 g azadirachtin A/kg and debitterised NSC contains 0.1 g azadirachtin A/kg. The aflatoxin content of the material is unknown and depends on the care taken for selection of the appropriate material. In several cases of feeding animals with NSC the observations may be influenced by its aflatoxin content or by the impalatability of the bitter NSC to the animals. It may be assumed that in controlled tests material which is strongly infested by fungi was not used.

The recorded studies show the following results:

WWNSKC (crude protein approx. 40%) “has been tried on growing cow calves (10), growing buffalo calves (11), growing pigs (12) and cows (7, 14). The results were:

WWNSKC “could easily replace groundnut cake without affecting the quality and quantity of milk. Studies included determination of milk yield, milk quality (both chemical and organoleptic), digestibility of nutrients, blood parameters and reproductive ability of cows (13)" (7, 14). Semen characteristics of 4 cross bred bulls did not show any adverse effects on volume, colour, density, initial motility, live and dead sperm count, total count, deformities and fructose content even after 12 months of feeding (7). Semen quality was tested after 1, 2, 6, 9, and 12 months. No adverse effects on libido were observed (7). Piglets fed with a 10% ration of WWNSKC in replacement of groundnut cake for 5 months gave significant higher growth rate (7). After addition of NSC or NSKC to feed, effects on the growth of cow calves are unclear (11, 14). However, 45% WWNSKC addition to the ration resulted in normal development of the animals for a period of 6 months (10, 14). Substitution of groundnut cake for WWNSKC in pigs diet as a protein source did not show significant effects on live weight, carcass characteristics, chemical composition, cooking yield and sensory quality of pork (7). Later studies indicate a faster growth of the pigs after receiving WWNSKC (14, 17). Kumar et al (14, 19) observed no adverse effects after addition of 30% WWNSKC to the ration of dairy cattle as judged by red and white blood cells, SGPT and SGOT levels and haemoglobin content.

Gupta and Bhaid reported results of feeding studies with growing sheep: Feeding of 100% Neem Fruit Cake NFC resulted in weight loss of the sheep, “however, the animals did not exhibit any symptoms of toxicity by continous feeding (in increasing portions) of deoiled NFC for a period of about 4 months” (14, 15). 75% of deoiled NFC with corn could be used as a maintenance mixture (14, 15). Tests with lambs using (obviously?) Neem cake - with or without purification with alcohol - resulted in poor acceptance of the feed as well

as changes in kidney and liver values (aflatoxins?) (see 14, 15). Addition of deoiled NC had no detrimental effect on development, body weight or milk yield of cows (14, 18).

Applications in Unani Medicine:

In traditional Unani medicine all parts of the Neem tree are used one way or the other in humans (2). Neem seeds are used internally and externally against different diseases (2). One method of application which is regarded highly beneficial against piles is to take Neem seeds beginning from one seed on the first day and then increasing it daily by one seed up to 40 days and then decreasing it similarly till day 80 (2).

According to this prescription an average intake of 20 seeds (approx. seed weight: 80 mg/seed) per day with an average azadirachtin A content of 3 mg/g seeds leads to an average daily intake of 4.8 mg azadirachtin A - obviously without adverse effects over a period of 80 days.

Conclusions by Trifolio:

From the above cited experiences and studies with various Neem preparations it seems justifiable to conclude that no effects after the intake of azadirachtin-containing preparations can be expected which would not have been anticipated on the basis of the available thorough toxicological studies with mice and rats. Thus it can not be expected that long term toxicological tests with a dog for example with NeemAzal and/or the formulation NeemAzal-T/S will bring up principally new insights. Thus the lives of the animals should be saved.

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Conclusions:
Similar justifications were provided by Trifolio and SIPCAM/MITSUI (Anonymous, 2002, TOX2005-2335; Pfau, 2005, TOX2005-2389). The applicant presented published reports on the use of neem seed products in feeding studies with farm animals. In particular, feeding studies with sheep, growing pigs, buffalo calves and milk cows over periods of up to 12 months were summarised.Feeding with water-washed Neem seed kernel cake as protein source resulted in no signs of toxicity regarding a diverse spectrum of parameters tested including milk production in cows, sperm quality in bulls, growth rate in piglets and cattle, meat characteristics. Also red and white blood cell counts as well as haemoglobin and liver enzymes were unaffected by Neem feeding of cattle.

Furthermore, the neem tree component nimbin was tested for subacute toxicity in adult rats and mongrel dogs. Rats were administered daily oral doses of 25, 50 or 100 mg/kg bw for a 6-week exposure period whereas dogs were treated over 28 days at dose levels of 10 or 20 mg/kg bw/d. In both species, no evidence of toxicity was obtained (Pillai & Santhakumari 1984, TOX2006-3045 as cited in Niemann et al., 2002, TOX2006-3044).

Unfortunately, the available data allow only a very rough estimate of the amount of azadirachtin to which the farm animals were exposed. According to the applicant, the highest concentration of neem extract in the diet of goats receiving 25% WWNSKC as protein concentrate mixture was 375 ppm. Growing calves were fed a concentrate mixture containing 45% WWNSKC and received a daily dietary dose of *approx.* 675 ppm NeemAzal. Using standard conversion factors for goats and cattle to adjust dietary concentrations to a mean daily intake per kg bodyweight, assuming a fraction of one third of the protein concentrate mixture in the total diet and taking into account the variability in azadirachtin A content in the extracts and other neem products, a mean daily dose of azadirachtin A in the range of 3-9 mg/kg bw (equivalent to 9-27 mg NeemAzal/kg bw) may be calculated. This would be in the same order of magnitude as the NOAEL in the subchronic study in rats and is much lower than doses that produced adverse effects in those experiments.

9.7.2.1 Repeated dose toxicity: inhalation

No studies submitted by the applicants

9.7.2.2 Repeated dose toxicity: dermal

No studies submitted by the applicants

9.7.2.3 Repeated dose toxicity: other routes

No studies submitted by the applicants

9.7.2.4 Human information

No studies submitted by the applicants

9.7.2.5 Other relevant information

No studies submitted by the applicants

9.8 Germ cell mutagenicity (Mutagenicity)

9.8.1 Non-human information

9.8.1.1 In vitro data

Studies performed with NeemAzal

Reference: TRF IIA 5.4.1 / 01

Report: Jones, E., Gant, R. A. (1997)
NeemAzal technical – Bacterial mutation assay
Huntingdon Life Sciences Limited, England
Report-no. EIP 11/950642
published: no; TOX9700511

Guidelines: EPA FIFRA Guideline 152-16 (1984)
Corresponding to OECD 471
EC Directive 92/69/EEC B.14

Deviations: No strain used to detect cross-linking mutagens (TA102 or *E. coli*).

GLP: Yes (certified laboratory)

Acceptability: The study is considered to be acceptable.

Material and Methods:

In a reverse gene mutation assay in bacteria, strains TA98, TA100, TA1535, TA1537 and TA1538 of *S. typhimurium* (provided by B. Ames, University of California, Berkley, CA, USA) were exposed to NeemAzal technical (batch IV, purity: 36.6% azadirachtin A), using ethanol as a vehicle (0.1 mL/plate) at concentrations of up to 5000 µg/plate, with and without S9 activation (Aroclor 1254 induced Sprague Dawley rat liver). *Preliminary toxicity study:* Dose levels of the test article up to 5000 µg/plate induced no toxicity, both in the presence and absence of liver enzyme

preparation. *Mutagenicity Assay:* The test article was tested at six dose levels (50, 150, 500, 1500 and 5000 µg/plate) along with vehicle and positive controls (without activation: 2-nitrofluorene (TA98, TA1538), N-ethyl-N'-nitro-N-nitrosoguanidine (TA100, TA1535), 9-aminoacridine (TA1537); with metabolic activation: 2-aminoanthracene (all strains)) in the presence and absence of S9-mix. All dose levels, vehicle and positive controls were plated in triplicate. *Statistics:* For all replicate platings, the mean revertants per plate and the standard deviation were calculated. The test was considered positive, when the average number of revertants was dose responsive in two separate experiments and at least one dose was ≥ 2x the solvent control spontaneous revertant value for at least one tester strain.

Findings:

The results of the dose range-finding study indicate that no appreciable toxicity was observed up to 5000 µg per plate. Plates treated with 5000 µg were contaminated, therefore this solution of test compound was filter sterilised (0.2 µm). No positive responses were observed with any of the strains used, in the presence as well as in the absence of microsomal enzymes. These results were confirmed in an independent assay. Plates treated with positive controls, showed an increase in the number of revertants, indicating the sensitivity of the assay and the metabolising activity of the S9-mix.

Conclusions:

NeemAzal technical was not mutagenic when tested on *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538, with or without S9-mix activation.

Reference:	TRF IIA 5.4.2 / 01
Report:	Stien, J. (2006) *In vitro* assessment of the clastogenic activity of NeemAzal in cultured human peripheral lymphocytes LPT, Laboratory of Pharmacology and Toxicology, Germany Unpublished Report No. 19026/1/05; TOX2006-739
Guidelines:	OECD Guideline 473 EC guideline B.10
Deviations:	None (LPT employs two different concentrations of each of its positive controls mitomycin C and cyclophosphamid, it is unclear, which concentrations are summarised in the table on historical control data.)
GLP:	Yes (certified laboratory)

Acceptability: The study is considered to be acceptable.

Material and Methods:

Cultures of human lymphocytes (blood obtained from healthy donors) were exposed to NeemAzal technical (batch: 05, purity: 37.4±1.5% azadirachtin A, 10 µg/kg Aflatoxin B_1 + B_2 + G_1 + G_2, dissolved in DMSO) with and without metabolic activation (S9 liver fraction was obtained from Aroclor 1254 induced rats, Analabs, North Haven, CT, USA). A preliminary cytotoxicity test was performed in order to determine the concentrations used for the main study: for tests with and without metabolic activation, concentrations of 10 – 5000 µg/mL were used. Cytotoxicity was characterised by the percentages of mitotic suppression in comparison to the control. Based on this experiment, dose levels of up to 5000 mg/mL (4 h exposure, with and without metabolic activation) and 2500 µg/mL (24 h exposure) were chosen. Concentrations higher than 2500 µg/mL precipitated, concentrations of 5000 µg/mL (4 h exposure) and 2500 µg/mL (24 h exposure) were cytotoxic. For the main study, duplicate cultures per concentration were incubated for 4 h or for 24 h with the test compound without metabolic activation; sampling was performed 24 h after incubation start. For tests with metabolic activation, cells were incubated for 4 h, only, and harvested 24 h after incubation start (this experiment was performed twice). 2 hours before harvesting of cells, colcemid was added. Additional cultures were treated with solvent control (DMSO, 1% v/v) as well as positive control (mitomycin C and cyclophosphamide for tests without and with metabolic activation, respectively). Evaluation criteria: Breaks, fragments, deletions, exchanges and chromosomal disintegration were recorded (100 metaphases per culture were investigated); gaps were recorded, but were not included in the calculation of aberration rates. Number of aberrations in control and treated cells were compared statistically (Fisher's exact test).

Findings:

No relevant increase in the structural chromosomal aberration rate could be found when compared with the range of aberrations in the corresponding controls at dose levels up to approximately 1250 µg/mL at any time interval investigated, with and without metabolic activation (Table 81). The aberration rates (exclusive gaps) of the cells after treatment with NeemAzal technical (0.0 – 4.0) were considered in the range of control values (0.0 – 2.0, historical control: 0.0 – 4.0). Incubation with higher concentrations (approximately 2500 µg/mL) led to increases of chromosomal aberrations, these concentrations induced cytotoxicity. The positive controls showed distinct increases of structural chromosomal aberrations.

Table 81: Results of chromosomal aberration assay

Treatment (µg/mL)	4 h exposure						24 h exposure	
	Without metabolic activation		With metabolic activation				Without metabolic activation	
	MI	CA	MI	CA	MI	CA	MI	CA
Solvent	1.00	1.5	1.00	0.0	1.00	2.0	1.00	2.0
312.5			0.93	1.5			1.35	2.5
625	1.33	1.5	0.94	0.0	1.50	2.5	1.23	2.5
1250	1.46	2.0	1.12	1.0	0.95	2.0	1.29	4.0
2500§	1.38	2.5	1.34	0.0#	0.66	0.5	0.08	0#
5000§	0.68	6.1#			0.64	3.8		
MMC (0.1)							0.86	11.0*
MMC (0.2)	1.09	11.5*						
CP (10)			0.65	8.5*				
CP (20)					0.76	11.0*		

MI: mitotic index (solvent = 1); CA: mean chromosome aberrations in 100 metaphases excl. gaps; MMC: mitomycin C; CP: cyclophosphamide; *: p ≤0.05; #: due to cytotoxicity not enough metaphases found; §: test compound precipitated

Conclusions:

The results of this study indicate that under the test conditions used NeemAzal technical was clastogenic in cytotoxic concentrations in chromosomal aberration assay in cultured human lymphocytes.

Reference: TRF IIA 5.4.3/01

Report: Adams, K., Kirkpatrick, D. (1997)

NeemAzal technical Mammalian cell mutation assay

Huntingdon Life Sciences Limited, England

Report-no. EIP 12/950657

published: no; TOX9700512

Guidelines: OECD Guideline 476

Deviations: None

GLP: Yes

Acceptability: The study is considered to be acceptable.

The test substance NeemAzal technical (Batch: IV, purity: 36.6% azadirachtin A, dissolved in ethanol) was examined for its potential to induce gene mutations at the HPRT-locus of CHO-K1-BH4 cells (provided by British Industrial Biological Research Association, UK) in both the absence and presence of an S9-activation system (Aroclor 1254 induced Sprague Dawley rat liver fraction). As negative control solvent alone (ethanol, 1% v/v) was used, as positive control without and with activating system ethyl methanesulfonate (250 µg/mL, solvent: ethanol) and 20-methylcholanthrene (5 µg/mL, solvent: DMSO) were used, respectively. Cells were exposed to the test substance,

solvent and positive control for 4 h at 37 °C after attachment (with or without S9-mix). Preliminary cytotoxicity was assessed by plating efficiency (3 plates, 200 cells, each) using concentrations of up to 1250 µg/mL. Cell survival was in the range of 140 – 60%. Following treatment (up to 1250 µg/mL, duplicate incubations), cells were incubated for seven days, sub-cultivating once. Mutant cells were selected with 6-thioguanine (10 µg/mL) in 5 plates containing 2 x 10^5 cells, each. After further 7 days of incubation, colonies were fixed, stained and counted. Two independent tests were carried out. The data were evaluated for statistical significance following the methods described by Arlett, C. F. et al. (1989) [Mammalian cell gene mutation assays based upon colony formation. In: Kirkland, D. J. (Ed.) UKEMS Sub-committee on Guidelines for Mutagenicity testing, Report, Part III. Statistical evaluation of Mutagenicity data.CambridgeUniversity Press, Cambridge, UK].

Findings

Slight cytotoxicity was observed at higher concentrations.

NeemAzal technical did not induce an increase in mutant frequency, neither in the S9-activated nor in the non-activated system (Table 82).

Both positive control compounds led to an increase of mutant frequency.

Table 82: Cytotoxicity and mean mutant frequency

Treatment (µg/mL)	Test 1				Test 2			
	Without metabolic activation		With metabolic activation		Without metabolic activation		With metabolic activation	
	CS	MF	CS	MF	CS	MF	CS	MF
Solvent	100	9	100	4	100	5	100	6
25	61§	-	113§	-	123§	-	115§	-
50	69§	-	85§	-	122§	-	126§	-
100	69§	-	81§	-	109§	-	110§	-
200	93	6	70	7	106	5	132	7
400	101	11	59	9	125	6	113	3
800	90	8	80	11	92	10	90	3
1000	64	4	79	9	98	5	95	5
1250	60	4	74	7	92	8	104	7
EMS	83	268***			128	189***		
MC			80	212***			100	156***

CS: cell survival determined after treatment (% of solvent control); MF: mutant frequency; EMS: ethyl methansulfonate; MC: 20-methylcholanthren; §: cultures discarded due to excess toxicity or because they were not needed in the test; ***: $p < 0.001$; grey fields: not done.

Conclusions:

Based on the results of this study it is concluded that the test substance NeemAzal technical was not mutagenic at the HPRT-locus of CHO cells.

Studies performed with Fortune Aza

Reference: SIP IIA 5.4.1 / 02

Report: Jones, E., Gant, R. A. (1997)
Fortune Aza technical – Bacterial mutation assay
Huntingdon Life Sciences Limited, England
Report No. EIP 11/952556; TOX2005-2393

Guidelines: EPA FIFRA Guideline 152-16 (1984)
Corresponding to OECD 471
EC Directive 92/69/EEC B.14

Deviations: No strain used to detect cross-linking mutagens (TA102 or *E. coli*).

GLP: Yes (certified laboratory)

Acceptability: The study is considered to be acceptable.

Material and Methods:

In a reverse gene mutation assay in bacteria, strains TA98, TA100, TA1535, TA1537 and TA1538 of *S. typhimurium* (provided by B. Ames, University of California, Berkley, CA, USA) were exposed to Fortune Aza technical (batch: 0010195-0050195; 8.5% azadirachtin A+B), using ethanol as a vehicle (0.1 mL/plate) at concentrations up to 5000 µg /plate, with and without S9 activation (Aroclor 1254 induced Sprague Dawley rat liver). *Preliminary toxicity study:* Dose levels of the test article up to 5000 µg/plate induced no toxicity, both in the presence and absence of microsomal enzymes.

Mutagenicity Assay: The test article was tested at six dose levels (50, 150, 500, 1500 and 5000 µg/plate) along with vehicle and positive controls (without activation: 2-nitrofluorene (TA98, TA1538), N-ethyl-N'-nitro-N-nitrosoguanidine (TA100, TA1535), 9-aminoacridine (TA1537); with metabolic activation: 2-aminoanthracene (all strains)) in the presence and absence of S9-mix. All dose levels, vehicle and positive controls were plated in triplicate.

Statistics: For all replicate platings, the mean revertants per plate and the standard deviation were calculated. The test was considered positive, when the average number of revertants was dose responsive in two separate experiments and at least one dose was ≥ 2x the solvent control spontaneous revertant value for at least one tester strain.

Findings:

The results of the dose range-finding study indicate that no appreciable toxicity was observed up to 5000 µg per plate. No mutagenic responses were observed with any of the strains used, in the presence as well as in the absence of microsomal enzymes. These results were confirmed in an independent assay. Plates treated with positive controls, showed an increase in the number of revertants, indicating the sensitivity of the assay and the metabolising activity of the S9-mix.

Conclusions:

Fortune Aza technical was not mutagenic when tested on *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538, with or without S9-mix activation.

Reference: SIP IIA 5.4.2 / 01

Report: Stien, J. (2006)

In vitro assessment of the clastogenic activity of Azadirachtin (A+B) in cultured human peripheral lymphocytes

LPT, Laboratory of Pharmacology and Toxicology, Germany

Unpublished Report No. 19026/3/05; TOX2006-464

Guidelines: OECD Guideline 473

EC guideline B.10

Deviations:	None (LPT employs two different concentrations of each of its positive controls mitomycin C and cyclophosphamid, it is unclear, which concentrations are summarised in the table on historical control data.)
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be acceptable.

The technical product "Azadirachtin (A+B) technical" was provided by SIPCAM, the producer of the extract Fortune Aza technical.

Material and Methods:

Cultures of human lymphocytes (blood obtained from healthy donors) were exposed to azadirachtin (A+B) technical (batch: E240, purity: 15% azadirachtin A the notifier stated, that the composition of this batch was within the typical range], dissolved in DMSO) with and without metabolic activation (S9 liver fraction was obtained from Aroclor 1254 induced rats, Analabs, North Haven, CT, USA). A preliminary cytotoxicity test was performed in order to determine the concentrations used for the main study: for tests with and without metabolic activation, concentrations of 10 – 5000 µg/mL were used. Cytotoxicity was characterised by the percentages of mitotic suppression in comparison to the control. Based on this experiment, dose levels of up to 1000 mg/mL (4 h exposure, with and without metabolic activation) and 250 µg/mL (24 h exposure) were chosen. Concentrations higher than 2500 µg/mL precipitated, concentrations of above 1000 µg/mL (4 h exposure) and 250 µg/mL (24 h exposure) were cytotoxic. For the main study, duplicate cultures per concentration were incubated for 4 h or for 24 h with the test compound without metabolic activation; sampling was performed 24 h after incubation start. For tests with metabolic activation, cells were incubated for 4 h, only, and harvested 24 h after incubation start (this experiment was performed twice). 2 hours before harvesting of cells, colcemid was added. Additional cultures were treated with solvent control (DMSO, 1% v/v) as well as positive control (mitomycin C and cyclophosphamide for tests without and with metabolic activation, respectively). Evaluation criteria: Breaks, fragments, deletions, exchanges and chromosomal disintegration were recorded (100 metaphases per culture were investigated); gaps were recorded, but were not included in the calculation of aberration rates. Number of aberrations in control and treated cells were compared statistically (Fisher's exact test).

Findings:

No relevant increase in the structural chromosomal aberration rate could be found when compared with the range of aberrations in the corresponding controls at dose levels up to 62.5 µg/mL (24 h exposure) or up to approximately 250 µg/mL (4 h exposure), with and without metabolic activation (Table 83). The aberration rates (exclusive gaps) of the cells after treatment with azadirachtin (A+B) technical (0.0 – 3.9) were considered in the range of control values (0.0 – 1.5, historical control: 0.0 – 4.0). Incubation with concentrations of 500 µg/mL (4 h exposure) or 125 µg/mL (24 h exposure) led to (significant) increases of chromosomal aberrations, these concentrations induced cytotoxicity. The positive controls showed distinct increases of structural chromosomal aberrations.

Table 83: Results of chromosomal aberration assays

Treatment (µg/mL)	4 h exposure						24 h exposure	
	Without metabolic activation		With metabolic activation				Without metabolic activation	
	MI	CA	MI	CA	MI	CA	MI	CA
Solvent	1.00	1.0	1.00	1.5	1.00	0.5	1.00	0.0
15.6							1.27	1.0
31.3							1.11	1.0
62.5	1.41	0.5			1.39	0.0	1.01	3.0
125	1.04	1.5	1.04	3.0	0.78	1.5	0.40	5.4*#
250	0.88	2.5	0.92	1.5	0.73	1.5		
500	0.59	3.4#	0.67	3.5	0.32	3.9#		
1000			0.00	0.0#				
MMC (0.1)	1.25	10.0*						
MMC (0.2)							0.67	19.0*
CP (10)					1.10	6.0*		
CP (20)			0.68	10.0*				

MI: mitotic index (solvent = 1); CA: mean chromosome aberrations in 100 metaphases excl. gaps; MMC: mitomycin C; CP: cyclophosphamide; *: p ≤0.05; #: due to cytotoxicity not enough metaphases found

Conclusions:

The results of this study indicate that under the test conditions used azadirachtin (A+B) technical was clastogenic in cytotoxic concentrations in chromosomal aberration assay in cultured human lymphocytes.

Reference: SIP IIA 5.4.3 / 01

Report: Adams, K., Ransome, S. (1997)

Fortune Aza technical Mammalian cell mutation assay

Huntingdon Life Sciences Limited, England

Report No. FBT 12/952792; TOX2005-2395

Guidelines: OECD Guideline 476

Deviations: None

GLP: Yes

Acceptability: The study is considered to be acceptable.

Material and Methods:

The test substance Fortune Aza technical (Batch: 0010195-0050195, purity: 8.5% azadirachtin A+B, dissolved in ethanol) was examined for its potential to induce gene mutations at the HPRT-locus of CHO-K1-BH4 cells (provided by British Industrial Biological Research Association, UK) in both the absence and presence of an S9-activation system (Aroclor 1254 induced Sprague Dawley rat liver fraction). As negative control solvent alone (ethanol, 1% v/v) was used, as positive control without and with activating system methyl methanesulfonate (10 µg/mL) and 20-

methylcholanthrene (5 µg/mL) were used, respectively. Cells were exposed to the test substance, solvent and positive control for 4 h at 37 °C after attachment (with or without S9-mix). Preliminary cytotoxicity was assessed by plating efficiency (3 plates, 200 cells, each) using concentrations of up to 2000 µg/mL. Cell survival was dose-dependently inhibited (between 110% and 0%). Following treatment (up to 750 µg/mL, duplicate incubations), cells were incubated for seven days, sub-cultivating once. Mutant cells were selected with 6-thioguanine (10 µg/mL) in 5 plates containing 2 x 10^5 cells, each. After further 7 days of incubation, colonies were fixed, stained and counted. Two independent tests were carried out. The data were evaluated for statistical significance following the methods described by Arlett, C. F. et al. (1989) [Mammalian cell gene mutation assays based upon colony formation. In: Kirkland, D. J. (Ed.) UKEMS Sub-committee on Guidelines for Mutagenicity testing, Report, Part III. Statistical evaluation of Mutagenicity data.CambridgeUniversity Press, Cambridge, UK].

Findings

Cytotoxicity was observed at lower concentrations (100 µg/mL and above). Cytotoxicity was slightly reduced when S9-mix was added. Fortune Aza technical did not induce an increase in mutant frequency, neither in the S9-activated nor in the non-activated system (Table 84).

Both positive control compounds led to an increase of mutant frequency.

Table 84: Cytotoxicity and mean mutant frequency

Treatment (µg/mL)	Test 1				Test 2			
	Without metabolic activation		With metabolic activation		Without metabolic activation		With metabolic activation	
	CS	MF	CS	MF	CS	MF	CS	MF
Solvent	100	3	100	7	100	1	100	4
5	86	4	119§	-				
10	99	3	130	5				
25	99	4	129	12	96§	-		
50	99	0	96	4	99	4	104§	-
75					93	2		
100	31	1	142	4	73	6	100	3
150					60	5	92	0
200					58	0	88	9
250	2§	-	27	1	9§	-	98	10
300					0§	-	89	1
400							4§	-
500	0§	-	8§	-			1§	-
750	0§	-	6§	-				
MMS	62	37***			153	51***		
MC			113	421***			102	399***

CS: cell survival determined after treatment; MF: mutant frequency; MMS: methyl methansulfonate; MC: 20-methylcholanthren; §: cultures discarded due to excess toxicity or because they were not needed in the test; ***: $p < 0.001$; grey fields: not done

Conclusions:

Based on the overall results of this study it is concluded that the test substance Fortune Aza technical was not mutagenic at the HPRT-locus of CHO cells.

Studies performed with ATI 720

Reference:	MIT IIA 5.4.1 / 01
Report:	Barbera, P. W. (1990) Ames Salmonella mammalian microsomal test of test article no. NPI-720 IIT Research Institute, Life Science Research, 10 West 35th Street, Chicago, Illinois, USA Project No L 08270 Study No 7; TOX2005-2392
Guidelines:	EPA FIFRA Guideline 152-17 (1984) Corresponding to OECD 471 EC Directive 92/69/EEC B.13/14
Deviations:	The results were not confirmed in an independent assay. No strain used to detect cross-linking mutagens.
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be acceptable.

Material and Methods:

In a reverse gene mutation assay in bacteria, strains TA98, TA100, TA1535, TA1537 and TA1538 of *S. typhimurium* (provided by B. Ames, University of California, Berkley, CA, USA) were exposed to NPI 720 (batch 13, purity: 8.6% azadirachtin, 20 – 100 ppb aflatoxin), using DMSO as a vehicle (0.1 mL/plate). The test article was tested at five dose levels (5, 50, 500, 1000 and 5000 µg/plate) along with vehicle and positive controls on the tester strains mentioned above in the presence and absence of S9-mix (Aroclor 1254 induced Sprague Dawley rat liver). All dose levels, vehicle and positive controls were plated in triplicate. As positive control in the absence of metabolic activation served 2-nitrofluoren (TA98, TA1538), sodium azide (TA1535, TA100) and 9-aminoacidine (TA1537), furthermore in the presence of metabolic activation 2-anthramine (TA98, TA100, TA1535, TA1537, TA1538). For all replicate platings, the mean revertants per plate and the standard deviation were calculated. The test was considered positive, when the average number of revertants was dose responsive and at least one dose was ≥ 2x the solvent control spontaneous revertant value for at least one tester strain.

Findings:

The results of the study indicate that no appreciable toxicity was observed up to 5000 µg per plate. No positive responses were observed with any of the strains used, in the presence as well as in the

absence of microsomal enzymes. Plates treated with positive controls, showed an increase in the number of revertants, which were within the historical range of the laboratory.

Conclusions:

NPI 720 was not mutagenic when tested on *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538, with or without S9-mix activation.

Reference:	MIT IIA 5.4.2 / 02
Report:	Stien, J. (2006) *In vitro* assessment of the clastogenic activity of Neem Seed Extract in cultured human peripheral lymphocytes LPT, Laboratory of Pharmacology and Toxicology, Germany Unpublished Report No. 19026/2/05; TOX2006-463
Guidelines:	OECD Guideline 473 EC guideline B.10
Deviations:	None
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be acceptable.

The technical extract "Neem Seed Extract" was provided by PJ Margo, the producer of the extract ATI 720.

Material and Methods:

Cultures of human lymphocytes (blood obtained from healthy donors) were exposed to Neem Seed Extract (batch: AZ/01/04-05, purity: 22.0% azadirachtin A [the notifier stated, that the composition of this batch was within the typical range], dissolved in DMSO) with and without metabolic activation (S9 liver fraction was obtained from Aroclor 1254 induced rats, Analabs, North Haven, CT, USA). A preliminary cytotoxicity test was performed in order to determine the concentrations used for the main study: for tests with and without metabolic activation, concentrations of 10 – 5000 µg/mL were used. Cytotoxicity was characterised by the percentages of mitotic suppression in comparison to the control. Based on this experiment, dose levels of up to 1000 mg/mL (4 h exposure, with and without metabolic activation) and 250 µg/mL (24 h exposure) were chosen. Concentrations higher than 2500 µg/mL precipitated, concentrations of above 1000 µg/mL (4 h exposure) and 250 µg/mL (24 h exposure) were cytotoxic. For the main study, duplicate cultures per concentration were incubated for 4 h or for 24 h with the test compound without metabolic activation; sampling was performed 24 h after incubation start. For tests with metabolic activation, cells were incubated for 4 h, only, and harvested 24 h after incubation start (this experiment was

performed twice). 2 hours before harvesting of cells, colcemid was added. Additional cultures were treated with solvent control (DMSO, 1% v/v) as well as positive control (mitomycin C and cyclophosphamide for tests without and with metabolic activation, respectively). Evaluation criteria: Breaks, fragments, deletions, exchanges and chromosomal disintegration were recorded (100 metaphases per culture were investigated); gaps were recorded, but were not included in the calculation of aberration rates. Number of aberrations in control and treated cells were compared statistically (Fisher's exact test).

Findings:

No relevant increase in the structural chromosomal aberration rate could be found when compared with the range of aberrations in the corresponding controls at dose levels up to 125 µg/mL (24 h exposure) or 250 µg/mL (4 h exposure), with and without metabolic activation (Table 85). The aberration rates (exclusive gaps) of the cells after treatment with Neem Seed Extract (0.0 – 4.0) were in the range of control values (0.5 – 2.5, historical control: 0.0 – 4.0). Incubation with concentrations of 500 µg/mL led to significant increases of chromosomal aberrations, this concentration induced cytotoxicity. The positive controls showed distinct increases of structural chromosomal aberrations.

Table 85: Results of chromosomal aberration assay.

Treatment (µg/mL)	4 h exposure						24 h exposure	
	Without metabolic activation		With metabolic activation				Without metabolic activation	
	MI	CA	MI	CA	MI	CA	MI	CA
Solvent	1.00	0.5	1.00	2.5	1.00	0.0	1.00	0.5
15.6							0.96	1.0
31.3							0.87	1.0
62.5	0.74	1.0	1.33	0.5	1.00	0.0	0.73	0.5
125	1.14	0.5	0.77	1.5	0.70	0.5	0.18	4.0
250	0.71	2.5	0.62	4.0	0.60	2.5		
500	0.23	19.0*#	0.47	14.8*#	0.69	6.5*#		
MMC	1.20	10.5*					0.64	19.5*
CP			0.74	15.5*	0.91	15.0*		

MI: mitotic index (solvent = 1); CA: mean chromosome aberrations in 100 metaphases excl. gaps; MMC: mitomycin C (0,2 µg/mL); CP: cyclophosphamide (20 µg/mL); *: p ≤0.05; #: due to cytotoxicity not enough metaphases found

Conclusions:

The results of this study indicate that under the test conditions used Neem Seed Extract was clastogenic in cytotoxic concentrations in chromosomal aberration assays in cultured human lymphocytes.

Reference: IIA 5.4.3/03

Report: Cifone, M.A. (1993), The L5178Y TK+/- mouse lymphoma forward mutation assay with Neem concentrate TGAI, Hazleton Washington, Virginia, USA, Unpublished report No. 15032-1-431R

Guidelines: US EPA 152-17, OECD 476

Deviations: Individual data are missing in the report

GLP: Yes

Acceptability: The study is considered to be supplementary.

Material and Methods:

1. Test Material:	Neem concentrate TGAI
Description:	Brown liquid
Lot/Batch #:	17285-74B
Purity:	3.15%
2. Control Materials:	
Negative:	Vehicle (DMSO)
Positive controls:	
Without activation	Methyl methanesulfonate 10 and 15 nL/mL
With activation	2-Methylcholanthrene 2.0 and 4.0 µg/mL
3. Activation:	S9 derived from male Sprague Dawley rats (Aroclor 1254 induced rat liver).
4. Test organisms:	Mouse lymphoma cell line clone 3.7.2C (BorroughsWellcome Company, Research Triangle Park, USA) RPMI 1640 medium supplemented with L-glutamine, antibiotics and 5-10% horse serum
5. Locus examined	TK locus, selection agent used: 5-trifluorothymidine

TEST PERFORMANCE

In life dates: 10.06. - 02.08.1993

In a preliminary dose finding test concentrations of 1.95 – 1000 µg Neem Concentrate TGAI technical per mL medium were evaluated.

Neem Concentrate TGAI was non-toxic from 1.95 - 62.5 µg/mL without and with metabolic activation; higher doses induced cytotoxicity. High levels of toxicity were observed at 250 µg/mL and above in the absence and presence of rat liver S9-mix.

Four mutation assays were performed without activation, one of which was terminated because of insufficient toxicity. Dose levels included in these assays ranged from 50 - 600 µg/mL, 75 – 225 µg/mL and 50 – 350 µg/mL.

The mutation assay was repeated independently.

Cell treatments

Cells were exposed to the test substance, solvent and positive control for 4 h at 37 °C in suspension (with or without S9-mix).

Following treatment, cells were incubated for two days. For each treatment group three plates were seeded with 200 cells each in basal medium and three plates with 1 x 10^6 cells (each) in selective medium.

Following 10-14 days of incubation colonies were counted.

Evaluation criteria

a) Assessment of cytotoxicity:

The cytotoxicity of the test substance was determined by exposure for four hours and subsequent determination of the cell count.

b) Assessment of mutagenicity

A response is considered to be positive, if the induced mutant frequency (MF) was as more than twice than the concurrent background level.

The test substance is considered to be mutagenic if a concentration-related increase in MF was observed or if a reproducible positive response for at least one of the test substance concentrations was observed.

If the test substance produced neither a dose-related increase in the MF nor a reproducible positive response at any of the test points, it was considered as non-mutagenic.

Findings:

A. ANALYTICAL DETERMINATION

Selected test solutions from all trials were analysed for the azadirachtin A and B by HPLC. Compond concentrations were within 20% of the planned concentrations.

B. CYTOTOXICITY

Neem Concentrate TGAI was non-toxic from 1.95 - 62.5 µg/mL without metabolic activation. Moderate reduction of cell counts were seen at 125 µg/mL. High levels of toxicity were observed at 250 µg/mL and above in the absence and presence of S9-mix.

C. MUTATION ASSAYS

The mutation frequency of the solvent controls ranged from 32 to 70 per 10^6 clonable cells in the experiments with and without metabolic activation and, hence, was well within the historical data-range.

The mutation frequencies of the cultures treated with Neem Concentrate TGAI ranged from 44 to 68 per 10^6 in the experiments with and without metabolic activation. These results were within the range of the solvent controls and, hence, no mutagenicity was observed according to the criteria for assay evaluation. At 250 µg/mL (without S9) the mutant frequency was apparently elevated, however cytotoxicity at this concentration was severe with a relative growth of 1.3% of control values.

The positive controls methylmethanesulfonate (MMS) and 3-methylcholanthrene (MCA) caused pronounced increases in the mutation frequency. Remark by RMS: cytotoxicity was quite high in incubations with MMS.

Table 86: Effects of Neem Concentrate TGAI on gene mutations at the TK-locus of mouse lymphoma cells in the absence of S9-mix

TGAI concentration (µg/mL)	Trial 1		Trial 2		Trial 4	
	Relative growth (%)	Mutant frequency ($x10^{-6}$)	Relative growth (%)	Mutant frequency ($x10^{-6}$)	Relative growth (%)	Mutant frequency ($x10^{-6}$)
0	100	49-55.6	100	63-70	100	32-52
50	76.0	50.2			47.1	56.3
75	63.5	53.2	47.3	71		
100	46.3	70.7	33.0	100		
150			33.5 5.7	91.2 154.5		
175			39.6 25.4	81.1 79.1	33.5	47.6
200					32.1	68.3
250					1.3	195
MMS (15 µg/mL)	1.7	980	2.5	771	0.1	857
MMS (10 µg/mL)	11.7	855	8.8	815	0.9	1106

The concentrations used in three incubations of trial 4 could not be identified from the report. The results of these incubations are not given above.

Table 87: Effects of Neem Concentrate TGAI on gene mutations at the TK-locus of mouse lymphoma cells in the presence of S9-mix

TGAI concentration (µg/mL)	Trial 5		Trial 6	
	Relative growth (%)	Mutant frequency ($x10^{-6}$)	Relative growth (%)	Mutant frequency ($x10^{-6}$)
0	100	36-51	100	63-67
12.5			51.3	123.2
25.0			44.7	110.6
50			65.2	73.1
75.5	56.6	49.2		
101	68.7	46		
151 / 150 #	29.5	54	21.9	66.4
176 / 175 #	29.6	54.3	29.8	45.1
201 / 200 #	21.2	33.6	18.4	59.7
226	10.1	55.6		
251	4.9	53.4		
MCA (2 µg/mL)	52.4	241	13.6	374.8
MCA (4µg/mL)	64.9	266		

The concentrations used in two incubations of trial 6 could not be identified from the report. The results of these incubations are not given above.
#, concentrations in trials 5 or 6, respectively.

Conclusions:

Based on the overall results of this study it is concluded, that the test substance Neem Concentrate TGAI was not mutagenic at the TK-locus of mouse lymphoma cells under the conditions of this study. High concentrations induced equivocal increases in mutant frequency at cytotoxic concentrations.

Comment by RMS:

The notifier provided an *in vitro* gene mutation assay in mammalian cells. The study was negative to equivocal [at high cytotoxic concentrations] (individual data are missing in the report).

However the test material in the study is unclear: purity was stated to be 3.15% or 4.5%, respectively, which is lower than the purity of the test material used in the other studies conducted with ATI 720 (*i.e.*, acute toxicity studies, 90-d study in rats, Ames test, chromosomal aberration study). Further on, it is unclear on which parameter the purity was based on. A statement concerning the test material of the *in vivo* study (Murli, 1992, see below) was provided. However, comparing this statement with the test material, there seem to be some discrepancies ("wet cake" vs. "cloudy brown fluid"). Based on these considerations, the submitted study give only little further information on the genotoxic potential of ATI 720 (i.e, the technical extract).

The applicant submitted a study and provided the following summary of the study (extract from Pfau, 2012 ASB2012-6696):

Report: **IIA 5.4.3/04** Flügge, C. (2011a) MUTAGENICITY STUDY OF AZATIN TECHNICAL IN MAMMALIAN CELLS (V79) IN THE IN VITRO GENE MUTATION ASSAY (HPRT TEST)
LPT, Laboratory of Pharmacology and Toxicology GmbH&Co KG, Hamburg, Germany
Report No. 27740

Guideline

OECD Guideline 476

GLP

Yes

Executive Summary

The test substance Azatin technical was examined for its potential to induce gene mutations at the HPRT-locus of V79 cells in both the absence and presence of an S9-activation system. Two independent trials in both the absence and the presence of S9-mix activation system were conducted preceded by a dose finding cytotoxicity study. For the cytotoxicity and gene mutation tests substance doses in the different assays ranged from 9.77 - 5000 µg/mL.

Cytotoxicity was observed at the higher doses in the absence of rat liver S9-mix only. Azatin technical did not induce an increase in mutant frequency, neither in the S9-activated nor in the non-activated system.

On the basis of this study it is concluded that the test substance Azatin technical is not mutagenic at the HPRT-locus of CHO cells.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material:		Azatin technical	
Description:		Yellow powder	
Lot/Batch #:		AZ/11-12/B-006a	
Purity:		Azadirachtin (A+B): 16.174% (12.718% + 3.456%	
Stability:		Expiry date 14.08.2013	
2. Control Materials:			
Negative:		Vehicle (DMSO)	
Solvent:		DMSO at 1% v/v	
Positive controls:			
Without activation		Ethyl methanesulfonate 600 and 700 µg/mL	
With activation		9,10-Dimethyl-1,2-benzanthracene 20 and 30 µg/mL	
3. Activation:		S9 (Aroclor 1254 induced rat liver).	
		Protein: 34.2 mg/mL	
		Cytochrome P-450 0.36 nmol/mg protein	
	S-9 mix composition	**Component**	**Concentration**
		Dulbecco's phosphate buffered saline-HEPES buffer	100 mM
		Glucose-6-phosphate	7.1 mM
		NADP	1 mM
		S-9	10 mL
4. Test organisms:		V79 cells	
		DSMZ, Braunschweig, Germany	
		Dulbecco's modified Eagle-medium supplemented with 10% fetal calf serum, penicillin (100U/mL) and streptomycin (100µg/mL)	

5. Locus examined HPRT locus,
selection agent used: 6-thioguanine

B. TEST PERFORMANCE

1. In life dates: 18.10. – 28.11.2011

The total study consists of three assays.
In a preliminary dose finding test concentrations of 9.77 –5000 µg Azatin technical per mL medium were evaluated.
In the actual mutation assays the concentrations of 9.77 – 156.3 and 78.13 – 1250 mg/mL were tested in the absence and presence of rat liver homogenate (S9-mix), respectively.
The mutation assay was repeated independently.

2. Cell treatments
Cells were exposed to the test substance, solvent and positive control for 4 h at 37°C (with or without S9-mix).
A second independent experiment cells were exposed for 4h (With S9) or 24h (without S9).
Afterwards the cells were trypsinised and a relative plating efficiency (PE1) was determined for each dose to obtain an accurate measure of the toxic effect of the chemical. Three replicate plates (60 mm diameter) were used with a known number of cells. The remaining cells were replated and the culture incubation continued until day 8 with 30 mL normal DMEM-FCS with one subcultivation on day 5.
Following trypsinisation and replating at a density of 1000000 cells per 150 mm diameter dish in DMEM-FCS containing 6-thioguanine1 (10 µg/mL) for selection of mutants (5 replicate plates), or at approx. 100 to 150 cells (exact number known) per 60 mm diameter dish in medium without 6-thioguanine for the estimation of plating efficiencies (PE2), (3 replicate plates). The plates were fixed and stained after about 8 days (plating efficiency plates) or 12 days (6-thioguanine plates).

3. Evaluation criteria
a) Assessment of cytotoxicity:
The cytotoxicity of the test substance was determined by exposure for 4 h (+S9) or 24 h (-S9) and subsequent determination of the plating efficiency in the preliminary test and as indicated above as PE_1 and PE_2 in the mutagenicity experiment.

b) Assessment of mutagenicity
The mutagenicity of the test substance was determined by the mutant frequency (MF), the ratio of MCE (mutant cloning efficiency) and CE (cloning efficiency).
A response was considered to be positive, if the induced mutant frequency was at least more than 40 mutants per 1,000,000 clonable cells and at least twofold the mutant frequency of the solvent control.
The background mutation frequency at LPT ranges from 1.30 to 38.36 x 10^{-6} clonable cells for the negative controls. The mutation frequency of the positive controls at LPT ranges from 112.1 to 1708.4 x 10^{-6} clonable cells for EMS and 130.0 to 2693.3 x 10^{6} clonable cells for DMBA (see table below).

II. RESULTS AND DISCUSSION

A. CYTOTOXICITY ASSAY

In a preliminary cytotoxicity assay reduced plating efficiency was observed at concentrations of 156.3 µg/mL and above in the absence of rat liver S-9 mix. Cytotoxicity was reduced when S9-mix was added.

In the main test decreased plating efficiency was observed at the highest dose levels tested.

[1] SIGMA-ALDRICH Chemie GmbH, 82024 Taufkirchen, Germany

Table 0-1 Cytotoxicity of Azatin technical in V79 cells in absence or presence of rat liver S9 mix

	Cell survival absolute plating efficiency										
	0 µg/mL	9.77 µg/mL	19.53 µg/mL	39.06 µg/mL	78.13 µg/mL	156.3 µg/mL	312.5 µg/mL	625 µg/mL	1250 µg/mL	2500 µg/mL	5000 µg/mL
- S9	0.90	1.09	0.91	0.88	0.98	0.28	0	0	0	0	0
+ S9	1.01	0.96	1.11	1.10	0.97	1.02	1.00	1.05	0.55	0	0

B. MUTATION ASSAYS

In the absence of S9 mix, the mutation frequency observed for the negative control DMSO was 5.78 and 6.54 × 10^{-6} clonable cells, and the mutation frequency of the cultures treated with concentrations of 9.77 - 156.3 µg Azatin technical/mL culture medium ranged from 5.32 to 8.20 × 10^{-6}clonable cells. These results are within the normal range of the negative controls.

Table 5.4.3-2: Effects of Azadirachtin on gene mutations at the HPRT-locus of V79 cells in the absence of S9-mix

Concentration (µg/mL)	S9 mix	Trial 1 (4-h exposure)			Trial 2 (24-h exposure)		
		Plating efficiency		Mutant frequency (× 10^{-6})	Plating efficiency		Mutant frequency (× 10^{-6})
0	-	0.54	1.04	5.78	0.98	0.92	6.54
9.77	-	0.75	0.86	5.32	0.93	1.02	7.24
19.53	-	0.91	0.86	6.06	0.93	0.98	8.20
39.03	-	0.96	0.90	6.65	0.93	1.01	6.51
78.13	-	1.13	0.90	5.81	1.02	0.96	5.86
156.3	-	0.25	0.30	6.57	0.23	0.20	7.00
EMS (600 µg/mL)	-	0.30	0.48	135	0.30	0.24	469
EMS (700 µg/mL)	-	0.35	0.28	272	0.22	0.23	468

Table 5.4.3-3: Effects of Azadirachtin on gene mutations at the HPRT-locus of V79 cells in the presence of S9-mix

Concentration (µg/mL)	S9 mix	Trial 1 (4-h exposure)			Trial 2 (4-h exposure)		
		Plating efficiency		Mutant frequency (× 10^{-6})	Plating efficiency		Mutant frequency (× 10^{-6})
0	+	0.68	0.99	5.68	1.04	1.06	7.58
78.13	+	1.07	1.03	4.06	1.02	1.01	9.54
156.3	+	1.14	0.86	6.05	1.01	0.96	5.86
312.5	+	1.03	0.91	4.63	0.87	1.03	10.30
625	+	1.00	0.91	5.74	0.96	1.02	5.47
1250*	+	0.50	0.49	9.04	0.52	0.44	7.81
DMBA (20 µg/mL)	+	0.24	0.31	225	0.26	0.26	439
DMBA (30 µg/mL)	+	0.26	0.24	230	0.26	0.23	428

*Test item precipitation

In the presence of S9 mix, the mutation frequency observed for the negative control DMSO was 5.68 and 7.58 × 10^{-6} clonable cells and the mutation frequency of the cultures treated with concentrations of 9.77 - 156.3 µg Azatin technical/mL culture medium ranged from 4.06 to 10.3 × 10^{-6}clonable cells. These results are within the normal range of the negative controls
Azatin technical did not induce an increase in mutant frequency. Both positive control compounds fulfilled the requirements for a valid test.

III. CONCLUSIONS

Based on the overall results of this study it is concluded, that the test substance Azatin technical is not mutagenic at the HPRT-locus V79 cells.

(Flügge, 2011)

Conclusion by RMS:

The summary prepared by the applicant adequately reflects the study conduct and study results as described in the study report. The study is considered acceptable.

Under the conditions of this study, the test material was not mutagenic in cultured mammalian V79 cells.

9.8.1.2 In vivo data

Studies performed with NeemAzal

Reference:	TRF IIA 5.4.4 / 01
Report:	Proudlock, R. J., Statham, J., Howard, W. R., Dawe, I. S. (1997) NeemAzal technical Mouse micronucleus test Huntingdon Life Sciences Limited, England Report-no. EIP 13/952782 published: no; TOX9700513
Guidelines:	OECD 474 (1983)
Deviations:	None
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be acceptable.

Material and Methods:

Following a dose finding study, CD-1 outbred mice of Swiss origin (animals provided by Charles River UK Ltd., England; 5 animals/sex / dose group / treatment time) were treated by gavage of NeemAzal technical (Batch: VII, purity: 27.2% azadirachtin A). Following overnight fast, animals received dose levels of 1250, 2500 and 5000 mg/kg bw. A negative control group was treated with the vehicle, (aqueous 1% methyl cellulose) a positive control group received mitomycin C

(12 mg/kg bw, solvent: saline). 24, 48 and 72 hours after dosing, animals were killed by cervical dislocation and femur bone marrow smears prepared. After staining with Giemsa, cells were analysed microscopically by counting micronuclei in 1000 polychromatic erythrocytes per animal. The ratio of polychromatic to normochromatic erythrocytes for each animal was assessed by examination of at least 1000 erythrocytes. Results for both sexes were combined. For comparison of an individual treatment group with the control group Wilcoxon's sum of rank test is used and inter group comparisons are performed with an adaptation of this method. Jonckheere's test is used for analysis of dose related trends. A positive response is indicated by a substantial, statistically significant increase in the incidence of micronucleated polychromatic erythrocytes compared to the vehicle control group for at least one sampling time.

Findings

Concentrations of solutions used for dosing were controlled analytically. Analysed concentrations were within 98 and 107% of nominal concentration. No animal died in the range finding study or main study, nor were there any clinical signs of toxicity. NeemAzal technical did not induce micronucleated polychromatic or normochromatic erythrocytes up to the highest dose of 5000 mg/kg bw at any of the three sampling times (Table 88). Mitomycin C caused large significant increases in the frequency of micronucleated polychromatic erythrocytes. The ratio of normochromatic to polychromatic erythrocytes was significantly decreased at the highest dose and there was a significant trend for dose related reduction of this value at the 24 h sampling time, indicating that the test item had indeed reached the target organ bone marrow.

Table 88: Summary of micronucleus results in male and female mice combined

Treatment (mg/kg bw)		**Sampling time 24 h**			**Sampling time 48 h**			**Sampling time 72 h**		
		pe/ne	**mnp**	**mne**	**pe/ne**	**mnp**	**mne**	**pe/ne**	**mnp**	**mne**
Vehicle	0	0.843§	0.5	0.3	0.844	0.5	0.2	0.850	0.3	0.3
NeemAzal technical	1250	0.797§	0.8	0.3	0.866	1.3	0.5	0.847	1.0	0.7
	2500	0.823§	0.8	0.9	0.873	0.8	0.0	0.876	0.6	0.5
	5000	0.666*§	0.8	0.7	0.825	1.3	0.7	0.795	0.7	0.7
Mitomycin C	12	0.536**	20.9**	1.7						

pe/ne: Ratio polychromatic to normochromatic erythrocytes; mnp: micronuclei per 1000 polychromatic erythrocytes; mne: micronuclei per 1000 normochromatic erythrocytes; *P <0.01, **P < 0.001; § significant trend

Conclusions:

NeemAzal technical did not induce micronucleated polychromatic erythrocytes up to a dose of 5000 mg/kg bw. NeemAzal did not show clastogenic potential *in vivo*.

Studies performed with Fortune Aza

Reference:	SIP IIA 5.4.4 / 01
Report:	Proudlock, R. J., Statham, J., Howard, W. R., Dawe, I. S. (1997) Fortune Aza technical Mouse micronucleus test Huntingdon Life Sciences Limited, England Report No. EIP 13/952782; TOX2005-2399
Guidelines:	OECD 474 (1983) EC Directive 92/69/EEC B.12 EPA TSCA Guideline 798 5385
Deviations:	None
GLP:	Yes (certified laboratory)
Acceptability:	The study is considered to be acceptable.

Material and Methods:

Following a dose finding study, CD-1 outbred mice of Swiss origin (animals provided by Charles River UK Ltd., England; 5 animals / sex / dose group / treatment time) were treated by gavage of Fortune Aza technical (Batch: 0010195-0050195, purity: 8.5% azadirachtin A+B). Following overnight fasting, animals received dose levels of 1250, 2500 and 5000 mg/kg bw. A negative control group was treated with the vehicle, (aqueous 1% methyl cellulose) a positive control group received mitomycin C (12 mg/kg bw, solvent: saline). 24, 48 and 72 hours after dosing, animals were killed by cervical dislocation and femur bone marrow smears prepared. After staining with Giemsa, cells were analysed microscopically by counting micronuclei in 1000 polychromatic erythrocytes per animal. The ratio of polychromatic to normochromatic erythrocytes for each animal was assessed by examination of at least 1000 erythrocytes. Results for both sexes were combined. For comparison of an individual treatment group with the control group Wilcoxon's sum of rank test is used and inter group comparisons are performed with an adaptation of this method. Jonckheere's test is used for analysis of dose related trends. A positive response is indicated by a substantial, statistically significant increase in the incidence of micronucleated polychromatic erythrocytes compared to the vehicle control group for at least one sampling time.

Findings

Concentrations of solutions used for dosing were controlled analytically. Analysed concentrations were within 3.2% deviation of nominal concentration. No animal died in the range finding study. During the main study, three females of the high dose group died within 18 h after dosing, another female of this dose group died approximately 42-48 h after dosing. Fortune Aza technical did not induce micronucleated polychromatic or normochromatic erythrocytes up to the highest dose of 5000 mg/kg bw at any of the three sampling times (Table 89). Mitomycin C caused large significant increases in the frequency of micronucleated polychromatic erythrocytes. The ratio of normochromatic to polychromatic erythrocytes was significantly decreased at the highest dose and

there was a significant trend for dose related reduction of this value at the 24 h sampling time, indicating that the test item had indeed reached the target organ bone marrow.

Table 89: Summary of micronucleus results in male and female mice combined

Treatment (mg/kg bw)		Sampling time 24 h			Sampling time 48 h			Sampling time 72 h		
		pe/ne	mnp	mne	pe/ne	mnp	mne	pe/ne	mnp	mne
Vehicle	0	0.843 §	0.5	0.3	0.844§	0.5	0.2	0.850	0.3	0.3
Fortune Aza technical	1250	0.720*§	1.2	0.7	0.765§	1.0	0.2	0.907	0.7	0.2
	2500	0.711*§	1.1	0.6	0.717§	0.7	0.3	0.947	1.0	0.4
	5000	0.629*§	1.8	0.8	0.487**§	0.4	0.6	0.846	0.9	0.4
Mitomycin C	12	0.536**	20.9**	1.7						

pe/ne: Ratio polychromatic to normochromatic erythrocytes; mnp: micronuclei per 1000 polychromatic erythrocytes; mne: micronuclei per 1000 normochromatic erythrocytes; *P <0.01, **P < 0.001; § significant trend

Conclusions:

Fortune Aza technical did not induce micronucleated polychromatic erythrocytes up to a dose of 5000 mg/kg bw. Fortune Aza did not show clastogenic potential *in vivo*.

Studies performed with ATI 720

Reference: IIA 5.4.4/03

Report: Murli, H. (1992): Dose rangefinding and mutagenicity test on Neem concentrate TGAI in an in vivo mammalian mutagenicity assay

Hazleton Washington Inc., USA, Unpublished report No. 15032-0-455

Guidelines: US EPA 152-17

Deviations: None

GLP: Yes (certified laboratory)

Acceptability: The study is considered to be supplementary due to the test material (*c.f.*, comment by RMS below the study evaluation; the study itself is acceptable)

Material and Methods:

A. MATERIALS

1. Test Material:	Neem Concentrate TGAI
Description:	Cloudy brown liquid
Lot/Batch #:	3/3/92
Purity:	4.5%

2. Vehicle and/or positive control:	None (test compound was administered undiluted) Saline (for the vehicle control group) 80 mg/kg bw cyclophosphamide in distilled water
3. Test animals	
Species:	Mice
Strain:	ICR strain
Age:	8-10 weeks
Weight at dosing:	Males: ~30-40 g; females: ~20-30 g
Source:	Harlan Sprague-Dawley Inc., Frederick, MD, USA
Acclimation period:	7-8 days
Diet:	Purina Certified Laboratory Chow #5002, ad libitum
Water:	Tap water, ad libitum
Housing:	5 animals of the same sex per polycarbonate cage
4. Environmental conditions	
Temperature:	72 ± 6 °F (22 ± 3.5 °C)
Humidity:	Relative humidity 50 ± 20%
Air changes:	No data
Photoperiod:	Alternating 12-hour light and dark cycles,

B. STUDY DESIGN AND METHODS:

In life dates: November 3 – November 18, 1992 (main study)

Preliminary toxicity tests

Following an overnight fast three male and three female mice per test group were administered by gavage suspensions of Neem Concentrate TGAI in corn oil (Trial I-III) or the neat test compound (Trial IV-V). Animals were observed for 72 h and any mortalities and signs of toxicity were recorded. Results are summarised in Table 92.

When diluted in corn oil, the test compound had a tendency to aggregate and to adhering to the dilution vial, despite of constant mixing.

Table 90: Mortality incidences in dose range finding studies.

Dose level	Trial I[1)]		Trial II[1)]		Trial III[1)]		Trial IV[2)]		Trial V[2)]	
(mg/kg bw)	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
1500	0/3	0/3	-	-	-	-	0/3	0/3	-	-
2125	0/3	0/3	-	-	-	-	-	-	-	-
2250	-	-	-	-	-	-	0/3	0/3	-	-
2750	0/3	0/3	-	-	-	-	-	-	-	-
3000			-	-	-	-	0/3	0/3	-	-
3375	0/3	0/3	-	-	-	-	-	-	-	-
3500	-	-	-	-	3/3	3/3	-	-	-	-
3750	-	-	-	-	-	-	0/3	0/3	-	-
4000	0/3	0/3	-	-	3/3	3/3	-	-	-	-
4500	-	-	3/3	3/3	-	-	0/3	0/3	-	-
5000	-	-	3/3	3/3	-	-	-	-	0/3	0/3

1) Test item administered as suspension in corn oil
2) neat test item administered

Micronucleus test

Fifteen male and fifteen female mice were dosed with 1250, 2500 and 5000 mg/kg bw Neem Concentrate TGAI. Negative controls received saline (4.8 mL/kg bw), while positive controls (five of each sex) received 80 mg/kg bw cyclophosphamide. Following administration the animals were allowed food and water ad libitum.

Five mice of either sex per dose group were killed after 24, 48 and 72 h, positive and negative controls were killed after 24 h with carbon dioxide.

Both tibiae were dissected and bone marrow smears were prepared. Smears were fixed in methanol, stained in May-Grunwald solution followed by Giemsa. The stained smears were examined by light microscopy to determine the incidence of micronucleated cells per 1000 polychromatic erythrocytes per animal and polychromatic to normochromatic cell ratio.

Statistics

Analyis of variance of the square root arcsine transformed data. For significance of difference from the vehicle control group was tested using Tukey’s Studentized range test with adjustment for multiple comparisons.

Findings:

A. MORTALITY

Since treatment with the test item suspended in corn oil gave non-reproducible results in the dose-range finding trials I-III, the test item was subsequently administered undiluted. With the neat test item administered by gavage no mortalities occurred and no signs of toxicity were noted at dose levels up to 5000 mg/kg bw.

No mortality and no clinical signs of toxicity during the observation period were reported for treated animals.

B. MICRONUCLEUS TEST

1. Micronucleated polychromatic erythrocytes

The mean micronucleated cell count for all dose groups of Neem Concentrate TGAI were essentially comparable with the concurrent vehicle control group, at any of the three sampling times. Cyclophosphamide caused significant increases in the frequency of micronucleated polychromatic erythrocytes. Results are summarised in Table 93.

2. Ratio of normochromatic to polychromatic erythrocytes

The ratio of polychromatic to normochromatic erythrocytes was significantly decreased in females treated with cyclophosphamide. No effects were noted in any other group.

Table 91: Summary of micronucleus results

TREATMENT	DOSE	HARVEST TIME (HR)	% MICRONUCLEATED PCEs MEAN OF 1000 PER ANIMAL ± S.E.			RATIO PCE:NCE MEAN ± S.E.	
			MALES	FEMALES	TOTAL	MALES	FEMALES
VEHICLE CONTROL SALINE	4.8 ml/kg	24	0.04 ± 0.02	0.04 ± 0.02	0.04 ± 0.02	0.72 ± 0.13	0.93 ± 0.04
POSITIVE CONTROL CP	80 mg/kg	24	3.26 ± 0.86*	4.00 ± 0.32*	3.63 ± 0.45*	0.40 ± 0.06	0.46 ± 0.07*
TEST ARTICLE		24	0.04 ± 0.02	0.04 ± 0.02	0.04 ± 0.02	0.50 ± 0.08	0.88 ± 0.22
	1250 mg/kg	48	0.00 ± 0.00	0.06 ± 0.04	0.03 ± 0.02	0.66 ± 0.16	0.56 ± 0.13
		72	0.04 ± 0.02	0.00 ± 0.00	0.02 ± 0.01	0.62 ± 0.16	0.58 ± 0.13
		24	0.04 ± 0.02	0.04 ± 0.02	0.04 ± 0.02	0.43 ± 0.15	0.81 ± 0.16
	2500 mg/kg	48	0.04 ± 0.04	0.06 ± 0.04	0.05 ± 0.03	0.70 ± 0.14	0.53 ± 0.11
		72	0.02 ± 0.02	0.02 ± 0.02	0.02 ± 0.01	0.63 ± 0.08	0.73 ± 0.16
		24	0.00 ± 0.00	0.02 ± 0.02	0.01 ± 0.01	0.54 ± 0.17	0.81 ± 0.15
	5000 mg/kg	48	0.00 ± 0.00	0.08 ± 0.02	0.04 ± 0.02	0.71 ± 0.19	0.77 ± 0.17
		72	0.06 ± 0.02	0.02 ± 0.02	0.04 ± 0.02	0.62 ± 0.16	0.99 ± 0.12

* Significantly different from the corresponding vehicle control, $p<0.05$.

Conclusions:

Neem Concentrate TGAI technical did not induce micronucleated polychromatic erythrocytes up to a dose of 5000 mg/kg bw under the conditions of this study.

Comment by RMS:

The notifier provided an *in vivo* MN assay in mice. The study was negative up to the top dose level of 5000 mg/kg bw [PCE/NCE-ratio not altered].

However the test material in the study is unclear: purity was stated to be 3.15% or 4.5%, respectively, which is lower than the purity of the test material used in the other studies conducted with ATI 720 (*i.e.*, acute toxicity studies, 90-d study in rats, Ames test, chromosomal aberration study). Further on, it is unclear on which parameter the purity was based on. A statement concerning the test material of the *in vivo* study was provided. However, comparing this statement with the test material, there seem to be some discrepancies ("wet cake" vs. "cloudy brown fluid"). Based on these considerations, the submitted study give only little further information on the genotoxic potential of ATI 720 (i.e, the technical extract).

The applicant submitted a study and provided the following summary of the study (extract from Pfau, 2012 ASB2012-6696):

Report: IIA 5.4.4/04 Flügge, C. (2011b) MICRONUCLEUS TEST OF AZATIN TECHNICAL IN BONE MARROW CELLS OF THE NMRI MOUSE BY ORAL ADMINISTRATION
LPT Laboratory of Pharmacology and Toxicology GmbH & Co. KG, Hamburg, Germany
Report No.: 27510

Guidelines

OECD 474 (1997), EC Dir. 2000/32/EC B.12 (2000)

GLP

Yes (certified laboratory)

Executive Summary

Following a dose finding study, NMRI mice (5 animals per sex and dose group) were treated by gavage at dose levels of 250, 500 and 1000 mg per kg bodyweight. A negative control group was treated with the vehicle, (0.8% hydroxypropylmethyl cellulose) a positive control group received cyclophosphamide (27 mg/kg bw).

Twentyfour and 48 hours after dosing, animals were killed by cervical dislocation and bone marrow smears were analysed microscopically by counting micronuclei in 2000 erythrocytes per animal.

There was no increase in the number of micronuclei in treated animals as compared to controls.

Azatin technical did not induce micronucleated polychromatic erythrocytes up to a dose of 1000 mg per kg bw. Validity of the test performed was shown with a vehicle treated control group with no effects, a cyclophosphamide treated positive control group with a marked response and signs of systemic toxicity in the group treated with 1000 mg/kg bw, indicating that the test item was systemically available.

I. MATERIALS AND METHODS

A. MATERIALS

1 Test Material:	Azatin technical
Description:	Mustard yellow powder
Lot/Batch #:	AZ/11-12/B-006a
Purity:	16.175% Azadirachtin (A+B)
2 Vehicle and/or positive control:	0.8% hydroxypropyl-methylcellulose 27 mg/kg bw cyclophosphamide (CPA) in saline administered i.p.

3 Test animals

Species:	Mouse
Strain:	NMRI / Crl: NMRI
Age:	30 - 33 days
Weight at dosing:	Male and Female 18 - 29 g
Source:	Charles River Laboratories, Sulzfeld, Germany
Acclimation period:	At least 5 days
Diet:	ssniff® R/M-H V1534 (ssniff Spezialdiäten GmbH, 59494 Soest, Germany), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	2-3 animals of the same sex per cage (MAKROLON)

4 Environmental conditions

Temperature:	22 ± 3 °C
Relative humidity:	55 ± 15%
Air changes:	12-18 per hour
Photoperiod:	Alternating 12-hour light and dark cycles, artificial fluorescent light

B. STUDY DESIGN AND METHODS:

1 In life dates: 07.09. - 29.09.2011

2 Animal assignment and treatment

Preliminary toxicity test

Following an overnight fast one male and one female mouse per test group were administered Azatin technical in 0.8% aqueous hydroxypropylmethylcellulose by gavage at 500, 1000 and 2000 mg/kg bw. No signs of systemic toxicity were noted at the dose level of 500 mg/kg bw. At 1000 mg/kg bw reduced motility was seen in both animals; and at 2000 mg/kg bw reduced motility in both animals and, in addition, tremor, ataxia, dyspnoea and death in the female animal were observed.

Micronucleus test

Five male and five female mice per group were dosed with 250, 500 and 1000 mg/kg bw Azatin technical in 0.8% aqueous hydroxypropylmethylcellulose by gavage. Negative controls received vehicle only, while positive controls (five of each sex) received 27 mg/kg bw cyclophosphamide. Additional groups of five animals per sex received the highest dose or the vehicle control for the 48 hour sampling time. Following administration the animals were allowed food and water *ad libitum*.

Five mice of either sex per dose group were killed 24 h after dosing. The additional high dose group and control group were killed after 48 hours.

Both femurs were dissected out and bone marrow smears were prepared. Two smears per femur were fixed in methanol, defatted in xylene and May-Grünwald/Giemsa-stained. The stained smears were examined by light microscopy to determine the incidence of micronucleated cells per 2000 polychromatic erythrocytes per animal.

3 Statistics

After completion of scoring and decoding of slides, the ratio of PCE/NCE for each animal and the mean for each group was calculated. The individual and group mean frequencies of micronucleated PCE/1000 were also determined.

PCE/NCE ratios were determined in order to evaluate possible bone marrow toxicity.

The assessment was carried out by a comparison of the samples with the positive and the vehicle control, using a chi-square test corrected for continuity as recommended by the UKEMS guidelines (The United Kingdom Branch of the European Environmental Mutagen Society: Report of the UKEMS subcommittee on guidelines for mutagenicity testing, part III, 1989: Statistical evaluation of mutagenicity data).

II. RESULTS AND DISCUSSION

A. RANGE-FINDING TEST

No signs of systemic toxicity were noted at the dose level of 500 mg/kg bw. At 1000 mg/kg bw reduced motility was seen in both animals; and at 2000 mg/kg bw reduced motility in both animals and, in addition, tremor, ataxia, dyspnoea and death in the female animal were observed.

Hence, for the main study three doses of 250, 500 and 1000 mg Azatin technical/kg bw were administered.

B. MICRONUCLEUS TEST

1 Mortality and clinical signs of toxicity

There was no mortality. At 1000 mg/kg bw slightly reduced motility was noted.

2 Micronucleated polychromatic erythrocytes

The mean micronucleated cell count for all dose groups of Azatin technical were essentially comparable with the concurrent vehicle control group, at any of the three dose level and at both sampling times.

The positive control agent cyclophosphamide caused large significant increases in the frequency of micronucleated polychromatic erythrocytes.

3 Micronucleated normochromatic erythrocytes

Azatin technical did not cause any substantial increases in the incidence of micronucleated normochromatic erythrocytes at any of the three sampling times.

4 Ratio of normochromatic to polychromatic erythrocytes

The ratio of normochromatic to polychromatic erythrocytes was decreased at the highest dose indicating a slight, transient bone marrow suppression.

Table 5.4.4-1 Summary of micronucleus results in male and female mice combined

Sampling time	Treatment	Dose mg/kg bw	Ratio polychromatic to normochromatic erythrocytes	Micronuclei per 1000 polychromatic erythrocytes
24 h	Vehicle	0	0.58	1.1
	Azatin technical	250	0.51	1.0
		500	0.56	1.7
		1000	0.51	1.0
	Cyclophosphamide	27	0.42	**18.8***
48 h	Vehicle	0	0.64	1.9
	Azatin technical	1000	0.57	2.0

*p< 0.05

III. CONCLUSIONS

Azatin technical did not induce micronucleated polychromatic erythrocytes up to a dose of 1000 mg per kg bw. Validity of the test performed was shown with a vehicle treated control group with no effects, a cyclophosphamide treated positive control group with a marked response, and signs of systemic toxicity and bone marrow depression in the group treated with 1000 mg/kg bw, indicating that the test item had indeed reached the target organ bone marrow.

(Flügge, 2011)

Conclusion by RMS:

The summary prepared by the applicant adequately reflects the study conduct and study results as described in the study report. The study is considered acceptable.

Under the conditions of this study, the test material did not induce micronuclei in mouse bone marrow. The top dose was limited by toxicity observed in the range-finding study.

9.8.2 Human information

No studies submitted by the notifiers.

9.8.3 Other relevant information

No studies submitted by the notifiers.

9.9 Carcinogenicity

9.9.1 Non-human information

9.9.1.1 Carcinogenicity: oral

Studies performed with NeemAzal

Reference: TRF IIA 5.5.2 / 01

Report: Kumar, T. (2000)

Long term carcinogenicity study of NeemAzal in Wistar Rats, Fredrick Institute of Plant Protection and Toxicology, Padappai, Tamil Nadu, India.

Report No. 7291 This study is presented in six parts, Part I- Part VI.; TOX2001-170

Guidelines: Gaitonde Committee Guideline (No. 6.3.0.c.4) corresponds to OECD Guideline 451

Deviations: In addition to OECD guideline 451 clinical chemistry data are presented.

No historical control data provided. Data on test item analysis in feed (level, stability, homogeneity) are missing, even though, according to the report, these analyses were done. Mean daily compound intake is only summarised in a graphical presentation, there are no actual numbers reported. The data on compound intake were calculated by the notifier, based on the data on feed intake, bodyweight and compound concentration in feed. Urine analysis not performed.

The specification of the test compound is unclear, the report states a concentration of 37.3% azadirachtin (page RUN-MAIN-5). TRIFOLIO submitted an undated analytical report prepared by EID Parry, which gives a concentration of 27.34% azadirachtin A (TRIFOLIO stated, that the analysis was performed on 18th July 1997).

GLP: No

Acceptability: Concerning oncogenicity, the study is considered to be acceptable.

Concerning long-term toxicity the study is considered to be supplementary.

The study was performed according to the Indian Gaitonde Guidelines and, thus, contain additional data (clinical chemistry) that can cover the endpoints required in a chronic oral exposure study. Urinalysis, as recommended by OECD guidelines 452 and 453, was not performed in this study. All animals were treated for 105 weeks.

Trifolio submitted (IIA 5.5.1 / 01 [TOX2005-2336]) a letter by Dr. Murthy (Director of the Fredrick Institute of Plant Protection and Toxicology, Padappai, Tamil Nadu, India) which describes the differences between OECD guideline 451 and Gaitonde Guideline 6.3.0.c.4:

	OECD	**Gaitonde**
GLP required	yes	no
age at start of the experiment	less than 6 weeks old	adult
number of animals	50 / sex / group	25 / sex / group
number of groups	min. 3 groups and control group	depending on substance 1 treated + 1 control group allowed
Dose levels	control: vehicle high dose: sufficiently high to elicit signs of minimal toxicity without altering the normal life span intermediate dose: mid range	control: pure vehicle or solvent high dose: should be within toxic range but majority of animal should survive intermediate dose: All animals

	between high and low low dose: should be lower than 10% of the high dose	should survive but can produce symptoms low dose: should permit animals to survive in good health for their natural life span
Observations	Toxic signs & mortality Tumor grows Bodyweight feed consumption blood collection	as per prolonged toxicity studies
Clinical chemistry	not needed	needed
Pathology	All organs, tissues and tumours should be preserved for microscopic examination	All organs and carcass should be fixed, processed and examined microscopically

Material and Methods:

NeemAzal technical (batch: CC86, purity: 27.34% or 37.3% azadirachtin) was offered in the diet at dosage levels corresponding to 0, 400, 1600 and 6400 ppm to Wistar rats for 105 weeks. Fifty Wistar rats (animals provided by Fredrick Institute of Plant Protection and Toxicology, India) per sex were treated at each dosage level and a control group. Diet was prepared weekly by diluting a premix (20000 ppm) with plain diet. Animals were observed daily for clinical signs, mortality, morbidity and overt toxicity. Weekly detailed observations were conducted on bodyweights and food and compound consumption. Blood was analysed initially, on month 6 and 12 (10 animals per dose and sex) and from all animals at the end of the treatment period. A differential cell count was determined on smears from animals in both control groups and the high dose group. RBC and WBC were estimated, haemoglobin, PCV and thrombocyte count were performed. Plasma was analysed for total proteins, albumin, GPT, ALP, BUN, and cholesterol. Sodium, potassium and calcium were estimated by flame photometry.

Macroscopic and microscopic post-mortem examinations were performed on all animals. Moribund animals and those died during treatment were autopsied. Organ weights were recorded for liver lungs, spleen, heart, kidney, gonads, brain, thyroid, pituary, and uterus. For histopathological examination 41 different organs and tissues were excised and preserved in formalin.

Statistics: Data on bodyweight, feed consumption, haematology and biochemistry were compared between treated and control group using student's t-test. Prior to application of the t-test data were tested for homogeneity of variance between treatments by applying Bartlett's test. If heterogeneity was found, modified t-test was applied for comparison of means.

Findings:

Survival during the study was similar between control groups and the treated dosage levels (male and female). Most mortalities occurred when animals were 52 weeks and older (Table 92). No clinical signs were observed during the treatment period.

Table 92: Number of rats found dead or found to be moribund after treatment with NeemAzal

Treatment ppm	males	females
Control	4 / 50	2 / 50
400	6 / 50	4 / 50
1600	2 / 50	5[§] / 50
6400	10 / 50	5 / 50

§, including two females that died during blood collection

No significant differences between mean values for bodyweight in the control groups and the corresponding mean values in the treated groups were noted. Male control group showed slightly lower bodyweights in comparison to the other groups throughout the study. Females receiving 400 ppm showed slightly lower bodyweight gain in comparison to the other groups.

Table 93: Mean bodyweights (g) of rat treated with NeemAzal in selected weeks

Treatment (ppm)	males					females				
	week									
	initial	26	52	80	105	initial	26	52	80	105
Control	51	397	419	410	433	50	256	278	294	298
400	61	398	421	434	447	53	236	258	264	271
1600	45	442	441	427	427	45	238	256	289	294
6400	62	432	455	433	448	58	249	271	288	290

Mean food consumption values were comparable between control and the treated dosage level groups. Mean compound intake during study was not calculated. There are graphics in the report with the mean weekly compound intake. The notifier calculated the mean compound intake, based on feed intake, compound concentration in feed and bodyweight: 29, 114, or 448 mg/kg bw/d for males or 38, 167, 635 mg/kg bw/d for females for 400, 1600, or 6400 ppm dose levels, respectively (Table 93). No effects on haematologic (Table 95) or blood biochemical (Table 96) parameters were noted.

Table 94: Mean achieved intake in rats treated with NeemAzal (mg/kg bw/d; calculated by notifier)

Treatment (ppm)	males					females				
	week									
	26	52	80	103	mean	26	52	80	103	mean
Control	-	-	-	-	-	-	-	-	-	-
400	27.2	25.8	32.4	30.9	29.1	40.3	39.9	37.1	34.7	38.0
1600	110.2	104.1	122.4	119.6	114.1	176.3	175.6	163.6	153.5	167.2
6400	396.7	403.4	497.6	494.6	448.1	693.8	680.0	600.0	566.0	634.9

Table 95: Coagulation time for males and females (s)

Dose level (ppm)	male				female			
	Day 0	Day 190	Day 360	Day 730	Day 0	Day 190	Day 360	Day 730
0	134.6	134.5	134.5	156.3	139.6	131.3	124.9	157.7
400	142.0	140.1	133.9	153.1	134.9	135.0	139.6	154.9
1600	136.7	136.4	135.8	153.2	145.4	149.1	142.5	165.2
6400	130.3	140.5	137.9	152.0	130.9	137.6	131.2	155.6

Table 96: Serum protein values for male and female rats (g/dL)

Dose level (ppm)	male				female			
	Day 0	Day 190	Day 360	Day 730	Day 0	Day 190	Day 360	Day 730
0	5.40	6.73	7.47	5.86	6.35	6.60	6.79	5.72
400	6.47	6.62	7.07	5.82	6.87	6.54	6.92	5.82
1600	5.75	6.60	6.81	5.71	6.2	6.89	7.44	5.95
6400	6.56	6.76	7.01	5.82	6.46	6.65	7.15	5.92

There were no relevant effects on organ weights (Table 97). Some mean values were statistically significantly different from control animals but differed by only 10% or affected only one side of paired organs or there was no dose related trend.

Table 97: Mean bodyweight (g) and organ weight (g) of rats treated with NeemAzal

Males

Dose level (ppm)	Body-weight	Liver	Heart	Brain	Kidney (left)	Spleen	Thyroid (left)	Gonads (left)
Control	433	13.689	1.151	2.019	1.318	1.246	0.016	1.596
400	447	13.036	1.142	2.026	1.276	1.249	0.015	1.579
1600	427	13.306	1.152	2.023	1.301	1.224	0.016	1.566
6400	448	14.074	1.223	2.041	1.280	1.266	0.014	1.572

Females

Dose level (ppm)	Body-weight	Liver	Heart	Brain	Kidney (left)	Spleen	Thyroid (left)	Gonads (left)
Control	298	10.579	0.938	1.885	1.046	0.877	0.010	0.068
400	271	9.945	0.902	1.832§	1.008	0.855	0.010	0.067
1600	294	10.409	0.943	1.858	1.025	0.891	0.010	0.069
6400	290	10.415	0.909	1.830§	0.999	0.859	0.010	0.066

§: significantly lower than control p <0.05

Rounded or irregular growths were noted in the teat region of female rats at all doses (incidence were 2, 1, 3 and 3 in the control, low dose, intermediate and high dose group). In male rats, rounded or irregular growths were observed in the lower abdomen of one animal of the low and two animals of the high dose group and one male in the prostate of the high dose group. Further recurring significant lesions included custodial enteritis, hepatitis due to *taenia talniformis*. Dose dependant infestation of liver with taeniae might indicate an influence of compound at very high doses on the immune system (Table 98). Due to the low incidence, all these effects were considered incidental.

Tumours observed included mammary tumours (mixed types), lymphosarcoma, and prostatic tumours. These occurred at very low incidences both in control and treatment groups (Table 98).

Table 98: Histopathological lesions

	males				females			
Dose level (ppm)	0	400	1600	6400	0	400	1600	6400
Liver cysts (taenial)	0	3	4	6	2	3	3	8
Mammary tumours	0	0	0	0	0 or 2#	1	3	3
Prostatic carcinoma	0	0	0	1				
Subcutis lymphosarcoma/fibrosarcoma	0	1	1	2	0	0	0	0

#: in the report, there are two different information on the number of mammary tumours in control group females

In summary, it is concluded that there was no test substance related carcinogenic effect in this study. All other gross and histopathologic findings were considered incidental and typical of the rat strain employed.

Conclusions:

No clinical signs were observed during the treatment period. No treatment related mortalities occurred. No effects on bodyweights or feed intake were noted. No effects on haematological or blood biochemical parameters were noted. Tumours observed included mammary tumours, lymphosarcoma, and prostatic tumours. These occurred at very low incidences both in control and treatment groups. In summary, it is concluded that there was no test substance related carcinogenic effect in this study. All other gross and histopathological findings were considered incidental and typical of the rat strain employed. No effects were found, thus a NOAEL of 6400 ppm (corresponding to about 448 mg/kg bw for males and 635 mg/kg bw/d for females) may be derived from this study.

Remarks concerning chronic toxicity:

The rat long-term dietary study was conducted according to the Gaitonde Committee Guideline 6.3.0.C.iv. The applicant argued that, while designed as carcinogenicity study, the observations reported exceeded the requirements of OECD guideline 451 on carcinogenicity studies. According to the applicant, these studies may therefore be considered as covering the chronic toxicity. The RMS's evaluation of this justification is the following:

The applicant's justification is accepted for the rat long-term study. Some deviations from the OECD test guideline 452/453 can be reported in this study but they are considered to be acceptable:

- The haematological and clinical chemistry analyses are not complete and were performed only at study initiation, after 6 and 12 months of treatment and after the final sacrifice. A full micro- and macroscopic pathological investigation was however performed and showed no adverse findings (histopathologic findings were considered incidental and typical of the rat strain employed). A full haematological and clinical chemistry analysis was furthermore carried out in the rat subchronic toxicity study, in which only few parameters (MCV, MCHC, globulin) not investigated in the rat long-term study were modified.
- A urinalysis was not performed, since there is no such requirement in the Gaitonde Committee Guideline 6.3.0.C.iv. The histopathological investigation of the kidneys and measurements of BUN concentration are however provided and do not show signs of nephrotoxicity. Furthermore, the urinalysis in the rat subchronic toxicity study did not reveal any findings.

In conclusion, the list of parameters examined in this study was incomplete as compared to requirements of OECD guidelines 452 and 453. However, it appears unlikely that toxicologically relevant adverse changes with respect to these parameters have been overlooked by these omissions.

Based on these considerations as well as for reasons of animal welfare, it is considered acceptablethat no additional chronic toxicity study was submitted.

Reference:	TRF IIA 5.5.3 / 01
Report:	Moorthy, M. V. (1996) Carcinogenicity study of NeemAzal-F 5% in mice, Department of Toxicology, JAI Foundation, Valdvada- 396108 Gujarat, India– Report No. 1544; TOX9700523
Guidelines:	OECD Guideline 451
Deviations:	Pages 307 and 308 (and 3 more, yet unidentified pages) are missing. No analysis of diet. No analysis of test compound. Clinical signs and physical/veterinary examinations were not reported. Normal background incidence of pathological findings not reported. Appendix 44 – 47 and 52 – 55 report the testes weights of female animals.
GLP:	Yes
Acceptability:	The study is considered to be supplementary.

This study was performed with the formulation NeemAzal-F 5%.

Material and Methods:

NeemAzal-F 5% (batch: 1; NeemAzal technical dissolved in polyethylene oxide; purity: 5% azadirachtin) was offered in the diet at dosage levels corresponding to 0, 100, 300 and 1000 ppm to Swiss albino mice (animals provided by the animal house of Jai Research Foundation, India) for 18 months (mean achieved doses were 0, 6.6, 18.4 and 63 mg/kg bw/day in males and 0, 7.0, 21 and 72 mg/kg bw/day in females). Feed mixture was prepared once per week. Fifty mice per sex were initiated at each of the dosage levels and a control group. Animals were observed daily for mortality, morbidity and overt toxicity. Weekly detailed observations were conducted on clinical signs, bodyweights and food and compound intake. On initiation and at monthly intervals thereafter physical/veterinary examinations were carried out including palpation on all animals. Haematological studies were conducted on all surviving animals at months 12 and 18. Macroscopic post-mortem examinations were performed on all animals, weights of selected organs were determined. Tissues were preserved in 10% formaline and those from control and high dose group animals, along with all gross lesions from low and intermediate dose group, were subjected to histopathological evaluation.

Statistics: Raw data were processed to give group means with standard deviations with significance between treated and control groups, using suitable software.

Findings:

Survival during the study was similar between control groups and the treated dosage levels (male and female) (Table 99).

Table 99: Mean achieved intake, study design and survival data

Dose level (ppm)	Mean achieved intake (mg/kg bw/d)		Number of animals		Survival (%)		
	male	female	male	female	male	female	combined
Control	0.0	0.0	50	50	64	50	57
100	6.6	7.0	50	50	70	64	67
300	18.4	21.1	50	50	68	72	70
1000	63.2	72.4	50	50	80	70	75

Statistically significant differences between mean values for bodyweight in the control groups and the corresponding mean values in the treated groups were noted (Table 100). However, these differences were already apparent at initiation of the study. Overall bodyweight gain was significantly higher in the high and mid dose (males) or high and low dose group (females) as compared to controls.

Table 100: Mean bodyweights (g) and overall weight gain

Dose level (ppm)	Male					Female				
	week				weight gain	week				weight gain
	initial	26	52	80	(80-0,%)	initial	26	52	80	(80-0,%)
Control	24.4	41.5	40.9	41.4	72.0	21.8	35.0	36.0	35.5	63.5
100	22.1*	39.3*	39.9	41.1	80.0	19.9*	31.5*	33.9*	35.3	80.0§
300	23.9	41.0	40.4	43.4	84.6§	20.2*	32.4*	33.0*	35.1	71.2
1000	21.0*	37.6*	38.9*	39.4*	88.7§	18.2*	31.0*	34.0	33.9	85.1§

*, significantly lower than control p <0.05; §, significantly higher than control p <0.05

Mean food consumption values were comparable between control and the treated dosage level groups with only sporadic instances of statistically significant differences from control groups. Achieved intake of NeemAzal-F 5% (Table 99) was calculated from group mean individual bodyweight and feed consumption data. Both absolute and relative testes weights were significantly reduced in the high dose (1000 ppm) group. Both absolute and relative kidney weights were elevated in females in the low dose group and in males in the mid dose group. Absolute kidney weights and heart weights were also elevated in male mice maintained on the low dose.

The statistically significant effects on testes, kidney and heart weights in the animals did not show a clear dose relation and were only marginal and thus were considered not treatment related. All other organ weights were not affected.

Table 101: Mean bodyweights and mean organ weights

Males

Dose level (ppm)	Number of mice	Bodyweight (g)	Liver (g)	Brain (g)	Heart (g)	Kidneys (g)	Spleen (g)	Testes (g)
Control	32	41.3	2.53	0.47	0.25	0.85	0.19	0.24
100	35	41.3	2.59	0.48	0.28*	0.92*	0.17	0.23
300	34	44.1*	2.54	0.48	0.27	0.94*	0.18	0.23
1000	40	40.5	2.39	0.46	0.25	0.79	0.15	0.21§

Females

Dose level (ppm)	Number of mice	Bodyweight (g)	Liver (g)	Brain (g)	Heart (g)	Kidneys (g)	Spleen (g)	Ovaries (g)
Control	25	35.5	2.00	0.48	0.21	0.58	0.17	0.26
100	32	35.5	2.14	0.47	0.20	0.67*	0.30	0.16
300	36	35.7	2.00	0.48	1.03[a]	0.64	0.20	0.28
1000	35	36.5	2.03	0.48	0.19	0.58	0.16	0.24

*, significantly higher than control $p < 0.05$; §, significantly lower than control $p < 0.05$

a, sic! The number could not be verified, as pages 307 and 308 (of the report) with the individual organ weights are missing.

Differential blood count revealed no effects.

The lesions noted upon external and internal examination were found at low level of incidence in all treatment groups and control animals. No treatment related findings were noted. In males, lesions in adrenals, bladder, kidneys, liver, lung and ileum were noted. In female mice affected organs were ileum, kidney, liver, lung, ovary, spleen and uterus. However, microscopic examination revealed similar lesions in the control and high dose groups, they were of low incidence or showed no dose response. Therefore these findings were considered incidental.

Conclusions:

No signs of overt toxicity were observed and survival of animals was similar in treated and control groups. Pathologic evaluation revealed that NeemAzal-F 5% is not carcinogenic and also no treatment related findings were noted. At 1000 ppm the NOAEL was established in this study. This corresponds to a dose of 63 and 72 mg NeemAzal-F 5%/kg bw/d for male and female mice, respectively.

Studies performed with Fortune Aza

No studies submitted by the notifiers.

Studies performed with ATI 720

No studies submitted by the notifiers.

9.9.1.2 Carcinogenicity: inhalation

No studies submitted by the notifiers.

9.9.1.3 Carcinogenicity: dermal

No studies submitted by the notifiers.

9.9.2 Human information

No studies submitted by the notifiers.

9.9.3 Other relevant information

No studies submitted by the notifiers.

9.10 Toxicity for reproduction

9.10.1 Effects on fertility

9.10.1.1 Non-human information

Studies performed with NeemAzal

Reference: TRF IIA 5.6.1 / 01 and IIA 5.6.1 / 01 Addendum

Report: Ramamoorthy, S. (2000)

Evaluation of toxicity of NeemAzal technical to reproductive process in Wistar rats – Segment IV – Toxicity to two generation reproductive process, Fredrick Institute of Plant Protection and toxicology, Padappai, Tamil Nadu, India– published: no, report No. 4826; TOX2001-173

Guidelines: Gaitonde Committee Guideline (No. 6.3.0.c.4)

Corresponds to OECD Guideline 416

Deviations: Three matings in the second generation instead of normally one. Data on test item analysis in feed (level, stability, homogeneity) are missing. Data on feed intake, bodyweight, compound intake limited to 15 weeks (up to the first mating). Time to fertilisation not reported.

Data reported on “weekly mean feed consumption” (e.g., table 4) are unclear: it is vague whether these data are the mean amount of feed

consumed per cage or per animal and whether it is consumed within one day or one week (TRIFOLIO stated, they were measured once per week, and cover intake during one day for all animals in one cage (i.e., 5)). The historical data reported as "bodyweight gain" seem to be "bodyweights" (confirmed by TRIFOLIO). For the historical data, the number of studies and the time-range within they were conducted is not given.

GLP: No

Acceptability: The study is considered to be acceptable.

Material and Methods

In a two-generation study, groups of 10 male and 20 female Wistar rats (animals provided by Fredrick Institute of Plant Protection and Toxicology, India) per dose group received diets containing NeemAzal technical (batch no.: CC86, purity: 27.3% or 37.3% azadirachtin) at concentrations of 0, 250, 500, or 750 ppm (prepared weekly). Samples of formulated diet were taken during the course of the experiment and analysed. The concentration of the test compound was within the acceptable limits. The P0 parental generation were treated for 105 days before the first mating (1 male: 2 females). The resulting F1a generation was weaned at 21 days, grossly observed and sacrificed. After a resting period of 10 days, P0 animals were mated again and from the resulting F1b generation 40 males and 80 females were allowed to grow as P1 parents. After weaning at 21 days these were maintained on test diets from 15 weeks before being mated thrice to produce the F2a, F2b and F2c litters. Treatment continued through pre-mating, mating, gestation, lactation, or weaning of the animals.

All animals were observed daily for mortality, behavioural changes and clinical signs of toxicity during premating dosing period, mating, pregnancy and during the resting period before second mating. Individual bodyweights were recorded weekly. Feed consumption was recorded twice or thrice a week and recalculated into weekly data. Information on fertility, reproductive performance, still births and live births were collected. On sacrifice, parental animals (10 animals/sex/group) were subjected to gross and histopathological examinations.

For all litters, information on the sex ratio, litter size, viablility, and bodyweights on day 0, 4, 7 and 21 of weaning were collected. Upon sacrifice of litters F1a, F2a, F2b, and F2c on day 22, necropsy was performed. Histopathological examinations were carried out on F2b litters.

Data on weekly bodyweights, feed consumption, fertility index of parents, litter size, sex-ratio and viability index of offspring of controls and treated groups were analysed statistically by Students t-test or Chi-square test.

Findings:

Achieved doses were to 0, 17, 34 and 50 mg/kg bw/d for male and 0, 20, 40 and 60 mg/kg bw/d for females (Table 102).

Table 102: Mean daily test compound consumption (mg/kg bw/day) of P0 animals as calculated by the submitter

	Dose level (ppm)			
	0	**250**	**500**	**750**
males	0.0	16.8	34.0	50.7
females	0.0	19.9	38.9	59.6

No treatment-related effects were noted with respect to clinical signs, bodyweights or food consumption in the parental rats of the P0 and P1 generations. In male rats of the P0 generation elevated absolute and relative mean brain weights were noted at the highest dose (Table 103). Also reduced relative heart weights in the high dose group and reduced relative testes weight were observed in the 500 and 750 ppm treatment group. No significant changes in relative or absolute means of organ weights were observed in females of the P0 generation. The effects seen in males were considered of doubtful toxicological relevance.

Table 103: Bodyweights and organ weights of males P0 animals (absolute and relative values)

Absolute values

Dose level (ppm)	**Fasted body-weight (g)**	**Liver (g)**	**Brain (g)**	**Kidney§ (g)**		**Heart (g)**	**Adrenal§ (mg)**		**Gonads§ (g)**	
0	273.8	10.59	1.79	0.99	0.99	0.93	31	33	1.48	1.47
250	300.0	11.20	1.82	1.02	1.02	0.91	32	33	1.46	1.47
500	287.3	10.77	1.79	1.04	1.04	0.93	33	34	1.46	1.45
750	310.4	11.61	1.84*	1.05	1.02	0.92	34*	33	1.48	1.49

Relative values

Dose level (ppm)	**Liver (%)**	**Brain (%)**	**Kidney§ (%)**		**Heart (%)**	**Adrenal§ (%)**		**Gonads§ (%)**	
0	3.86	0.66	0.36	0.36	0.34	0.011	0.012	0.54	0.54
250	3.74	0.62	0.35	0.35	0.31	0.011	0.011	0.49	0.50
500	3.75	0.62	0.36	0.36	0.32	0.012	0.012	0.51*	0.51*
750	3.73	0.59**	0.34	0.34	0.30**	0.011	0.011*	0.48**	0.48**

*, p <0.05; **, p <0.01; §, left and right organs

In male rats of the P1 generation a reduced relative mean brain weight noted at the lowest dose was considered incidental. Also reduced relative testes weight wasobserved in the 250 and 500 ppm treatment group. However, these effects were marginal and only confined to one side and thus considered no signs of toxicity. No significant changes in relative or absolute means of organ weights were observed in females of the P1 generation.

Table 104: Bodyweights, absolute and relative organ weights in male P1 animals – means

Dose level (ppm)	Fasted bodyweight (g)	Brain (g)	Brain (%)	Heart (g)	Heart (%)	Gonads§ (mg)		Gonads§ (%)	
0	344.1	1.81	0.52	0.93	0.27	1.42	1.46	0.41	0.42
250	348.5	1.79	0.51*	0.90	0.26	1.42	1.41	0.41	0.40*
500	349.5	1.81	0.52	0.93	0.27	1.44	1.41	0.42	0.41*
750	347.9	1.81	0.53	0.93	0.27	1.44	1.44	0.42	0.42

*, p <0.05; §, left and right organs

Administration of NeemAzal did not influence pup bodyweights for the male and female offspring for all matings of both generations (Table 105). Total number of live pups was reduced in the litter from the first mating of the P1 generation.Both number of male and female pups wasreduced in the 500 and 750 ppm dose groups. However, in the subsequent matings, number of pups (F2b and F2c) was not different from control animals and thus this effect is considered not treatment related. The proportion of male pups was reduced in the F1a litter in the highest dose group. However, since sex ratio was normal (48.1% male) in the litters of the subsequent mating (F1b), this observation was not considered treatment related. Reproductive performance and the other litter parameters assessed, e.g. bodyweight and sex ratio were not affected by ingestion of test diets at any level tested.

Table 105: Effect of treatment on mean bodyweights (g) for the offspring from all matings of both generations

Litter	Dose level (ppm)	Total number of live pups		Sex ratio (% male)	Mean bodyweight at lactation day					
					0		4		21	
		M	f		m	f	m	f	m	f
F1a	0	69	81	46.0	5.10	5.06	9.26	9.12	25.25	25.76
	250	74	77	49.0	5.14	5.06	9.31	9.16	25.78	25.93
	500	73	97	42.9	5.14	5.16	9.26	9.23	24.71	24.77
	750	62	97	39.0	5.08	4.93	9.00	9.12	24.34	24.43
F1b	0	78	78	50.0	5.24	5.32	8.38	8.35	33.92	33.86
	250	70	67	51.1	5.33	5.40	8.08	8.00	33.76	34.00
	500	73	71	50.7	5.44	5.44	8.16	7.96	34.96	35.14
	750	74	80	48.1	5.47	5.40	8.11	8.01	35.23	34.70
F2a	0	72	75	49.0	4.22	4.25	8.73	8.83	30.03	29.05
	250	68	66	50.7	4.44	4.42	8.54	8.40	30.53	30.43
	500	63	58	52.1	4.54	4.55	8.19	8.59	29.54	30.24
	750	61	51	54.5	4.75	4.76	8.77	8.76	31.44	30.98
F2b	0	79	66	54.5	4.71	4.41	8.72	8.41	29.80	29.64
	250	74	57	56.5	4.59	4.32	8.47	8.16	29.12	29.32
	500	64	64	50.4	4.89	4.84	8.45	8.39	31.45	30.81
	750	78	64	54.9	4.50	4.25	8.29	8.15	29.37	28.72
F2c	0	67	62	51.9	4.49	4.34	8.48	8.42	28.03	29.42
	250	71	79	47.3	4.49	4.46	8.18	8.20	27.73	29.15
	500	75	63	54.4	4.64	4.70	8.44	8.35	29.23	29.76
	750	69	70	49.6	4.48	4.38	8.29	8.37	28.98	29.98

P0 generation: In the testes of two animals of the high dose group tubular hypoplasia was noted. This was not observed in any other dose group and only in one male of the control group. In three cases of the high dose group hyperaemia of substance was reported in the testes of the high dose group. This was not observed in any other dose or control group.

P1 generation: Tubular atrophy and focal interstitial oedema were noted in two males each of the high dose and the intermediate dose level, while this observation was reported in one male of the low dose and control group of the P1 parental generation. Hyperaemia of uterus was noted in three and two females of the high and mid dose respectively, while this was noted only in one case of the control group. Several other sporadic effects were noted but there was no substance related effects since similar observations were made in control animals. No lesions were noted in F2b that were subjected to necropsy neither with regard to gross pathology nor histopathological examinations.

Conclusions:

There were no treatment related reproductive and developmental effects reported regarding litter size, fertility, pup weight or any other signs in the offspring. The NOEL/NOAEL was 750 ppm with regard to reproductive and developmental parameters, corresponding to 51 mg and 60 mg NeemAzal/kg bw/day for males and female respectively. No dose related effects were noted in parental animals, the NOAEL is, thus, equivalent to the maximal dose tested, 750 ppm corresponding to 51 or 60 mg NeemAzal/kg bw/d for males or females respectively.

Reference:	TRF IIA 5.6.1 / 02
Report:	Mani, B. (1996) Reproduction toxicity study (Segment IV) of NeemAzal-F 5% in Charles Foster rat, JAI Foundation, Department of Toxicology, Gujarat, India– Report No. 1542/JRF/Tox/96; TOX9700522
Guidelines:	Gaitonde Committee Guideline (No. 6.3.0.c.4) corresponds to OECD Guideline 416
Deviations:	Bodyweights of 4/10 males (Group 3, F1 generation) was in week 1 10 times higher than that of the other animals (page 494), in later weeks it was as low as measured in the other animals. Time to fertilisation and duration of gestation not reported. Data on test item analysis in feed (level, stability, homogeneity) are missing. The chemical polyethylene oxide was not further characterised. On the first page of tables 3 and 4 (weekly bodyweight data of males or females) N=10, on the second page N=30, whereas there were a total of 10 males and 20 females in each group. In tables 36 to 39 organ weights of males and females are reported; in the header it is stated, that the data are mean and standard deviation of 10 animals, whereas in the table itself the number of animals surviving until sacrifice is reported (i.e. up to 10 for males and 20 for females).

GLP: No (in life study period: December 7, 1994 till June 06, 1996; laboratory's conformity with OECD principles of GLP was assessed on January 9-12, 1996 by The Netherlands GLP authority)

Acceptability: The study is considered to be not acceptable.

This study was conducted with the formulation NeemAzal-F 5% containing 20% NeemAzal technical.

Material and Methods:

In a two-generation study, groups of 10 male and 20 female Charles Foster (animals provided by the animal house unit of Jai Research Foundation, India) rats per dose group received diets containing NeemAzal-F 5% (batch no.: 11; NeemAzal technical dissolved in polyethylene oxide; purity: 5% azadirachtin) at concentrations of 0, 200, 1000 or 5000 ppm throughout the whole study, including mating, gestation, and lactation. The P0 parental generation were treated with the compound for approximately 10 weeks before the first mating. For mating, two females were caged with one male. The resulting F1a generation was weaned at 21 days, grossly observed and sacrificed. After a resting period of 90 d (control and low dose groups) or 44 d (mid and high dose groups) P0 animals were mated again and from the resulting F1b generation 10 males and 20 females of each dose group were allowed to grow as P1 parents. After weaning at 21 days these were maintained on test diets for 70 d and being mated twice to produce the F2a and F2b litters. After selection of siring animals for the second generation, P0 animals were sacrificed and were subjected to gross pathological observations. Tissues from the control and high dose group were examined microscopically.

All animals were examined for overt signs of toxicity, illness and behavioural changes once daily. Bodyweights were recorded at the start of treatment and weekly after that and finally at necropsy. Food consumption was recorded daily for each cage. Sex ratio, litter size, bodyweights, live-birth index, survival index were taken on days 1, 4, 7, 14 and 21 after parturition for all litters. Upon sacrifice on day 22, necropsy was performed and histopathological examinations were carried out on all litters, excluding the new parental generation animals.

Data on bodyweights, feed consumption, fertility index of parents, litter size, sex ratio and viability index of offspring of controls and treated groups were analysed statistically by suitable statistical methods (viz. Students t-test etc.). All statistical analyses compared the treatment groups with the control group with the level of significance.

Findings:

No treatment related effects were noted with respect to clinical signs for the parental rats in the P0 generation. Mortalities occurred on treatment days 253 – 306 and were considered incidental (Table 106).

Table 106: Mortalities in the parental generations P0 and P1

Dosage level (ppm)	Number of animals		Mortalities P0		Mortalities P1	
	female	male	female	male	female	male
0	20	10	2	1	2	0
200	20	10	1	1	0	0
1000	20	10	2	0	0	0
5000	20	10	0	0	1	0

Mean weekly bodyweight values for the males from the high and mid dose groups were generally lower as compared to the control group reaching significance in more than half of the weeks for males (Table 107). For females mean bodyweights in the high dose group were generally lower as compared to the control group but significance was reached only in one third of the weeks. During the first gestation there were no consistent differences in bodyweights but in the second gestation maternal rats of both the high and mid dose groups had significantly reduced bodyweights as compared to the control group (Table 108). Similarly, bodyweights during lactation were significantly reduced in the mid and high dose groups during both lactation periods.

Table 107: Mean bodyweights (g) of P0 males and P0 females (selected weeks)

Week of treatment	Dose level (ppm)							
	0	200	1000	5000	0	200	1000	5000
	male				female			
1	251	259	250	230*	191	188	197	183*
10	376	369	366	359	246	250	244	235*
20	372	376	327*	344	270	251*	236*	251*
30	462	420*	406*	394*	293	304	290	276
37	460	414	423*	408*	302	317	304	270*

*Significantly different from control

Table 108: Mean bodyweights (g) of P0 animals during gestation and lactation

day	Dose level (ppm)							
	0	200	1000	5000	0	200	1000	5000
	F1a				F2a			
Gestation								
0	255	267*	278*	264	317	314	287*	270*
6	280	283	289	277	321	320	295*	276*
14	297	313*	289	280*	327	332	299*	281*
20	303	314	296	291	322	332	292*	281*
Lactation								
1	296	297	261*	262*	316	327	290*	287*
4	301	283	263*	253*	319	321	289*	287*
7	293	277*	267*	259*	319	322	288*	287*
14	271	264	246*	253	320	319	285*	278*
21	233	233	230	231	322	312	285*	262*
Pregnant animals	20	18	20	19	18	17	18	19

*Significantly different from control

During the premating period there were no consistent differences in feed consumption in either sex. During both cohabitation/mating periods no difference were recorded between dose groups and control group. There were no clear trends for differences in feed consumption during post-mating/resting periods.

No substance related effects were observed regarding the number of pregnancies resulting from the first (F1a) or second cohabitation (F1b), fertility indices were between 85 and 100% for all treatment groups including control group.

The time to fertilisation was not reported.

No substance related effects were noted on the live index in the first (F1a) or second litters (F1b), live birth indices were between 96 and 100% for all treatment groups including control group. Similarly, survival during lactation was unaffected by treatment, survival indices ranging from 90 to 100% on days 4, 14 and 21 with no significant effect of treatment. Litter weight was generally lower in treated groups reaching significance on days 4, 7 and 14. However, this effect was not observed in the F1b litters resulting from the second mating.

Table 109: Litter weight in the F1a and F1b generation

day	Dose level (ppm)							
	0	200	1000	5000	0	200	1000	5000
	F1a				F1b			
1	72.8	72.7	66.4	67.1	65.2	66.2	54.6	53.2
4	130.5	114.8	94.5*	89.3*	99.3	99.4	86.6	93.0
7	168.5	152.5	119.9*	107.2*	127.3	135.5	124.6	121.5
14	223.8	195.1*	173.7*	158.1*	206.9	186.5	182.1	190.4
21	255.3	281.7	283.4	262.5	260.5	289.3	242.7	294.4

*p <0.05

Total number of pups, proportion of live and pup weights were not affected (Table 110, Table 111). Mortality among male pups was increased in the high and mid dose groups of the second mating but this was not observed on the first mating or among female pups and thus was considered incidental.

Table 110: Litter parameters in the F1a generation

		Control	200	1000	5000
Total no of male pups	male	95	93	95	112
Mortalities		5 (5.2%)	11 (11.8%)	10 (10.5%)	7 (6%)
Total no female pups	female	117	107	120	87
Mortalities		17 (14.5%)	7 (6.5%)	5 (4.2%)	13 (15%)
Sex ratio (% male)		45	47	44.2	56
Total mortalities		10.4%	9%	7%	10%

Table 111: Litter parameters in the F1b generation

		Control	200	1000	5000
Total no of male pups	male	79	75	59	80
Mortalities		10 (12.6%)	13 (17.3%)	17 (28.8%)	23 (28.7%)
Total no female pups	female	79	90	74	80
Mortalities		10 (12.5%)	9 (10%)	12 (16.2%)	11 (13.7%)
Sex ratio (% male)		50	45	44	50
Total mortalities		12.6%	13.3%	21.8%	15%

No macroscopic or microscopic abnormalities were recorded in F1a and F1b generation. Terminal organ weights in F1a and F1b generation were effected as summarised below (Table 112). Terminal bodyweights were not affected by treatment with NeemAzal technical.

Table 112: Significantly altered organ weights in F1a and F1b animals

Dose (ppm)	**Male**		**female**	
	Absolute	relative	Absolute	relative
200	Liver↓	Adrenal↑, brain↑, gonads↑		
1000	Brain↑, liver↓	Liver↓	Brain↑, kidney↑, Adrenals↓	Spleen↓
5000	Brain↑, kidney↑	Liver↓, spleen↑		

No treatment related macroscopic findings were noted in the parental generation P0. For one female of the high dose group a tumour was noted near the lower mandible. This finding was considered incidental. Several significant changes in terminal organ weights were noted for the P0 generation (Table 113). Relative weights of ovaries and spleen in maternal rats were consistently increased in all treatment groups and, although not following a dose response, may be related to treatment (Table 114 and Table 115).

Table 113: Significantly elevated organs weights (P0 generation)

Dose (ppm)	**male**		**female**	
	Absolute	**relative**	**absolute**	**relative**
200	Adrenal, brain	Adrenal	Spleen	Ovary, liver, spleen
1000	Testes, spleen	Heart, kidney, testes	Ovary, spleen, liver,	Ovary, spleen
5000	Adrenal, brain, heart	Adrenal, kidney	Ovary, spleen	Ovary, spleen

Table 114: Bodyweights and relative organ weights of P0 males – means

Dose ppm	**Bodyweight**	**Liver (%)**	**Brain (%)**	**Kidney (%)**	**Heart (%)**	**Adrenal (%)**	**Spleen (%)**	**Testes (%)**
0	490	3.76	0.41	0.65	0.29	0.012	0.19	0.51
200	429	3.73	0.48*	0.71	0.36*	0.019*	0.26*	0.58
1000	407	3.78	0.50	0.72	0.32	0.014	0.28	0.76*
5000	421	3.61	0.48*	0.64	0.34	0.019*	0.25	0.65

*p <0.05; ** p <0.01

Table 115: Bodyweights and relative organ weights of P0 females – means

Dose ppm	**Bodyweight**	**Liver (%)**	**Brain (%)**	**Kidney (%)**	**Heart (%)**	**Adrenal (%)**	**Spleen (%)**	**Ovaries (%)**
0	280	3.94	0.66	0.72	0.36	0.026	0.18	0.032
200	296	4.22	0.65	0.75	0.37	0.028	0.26*	0.070*
1000	288	3.83	0.63	0.69	0.36	0.024	0.22*	0.040*
5000	278	4.14	0.65	0.71	0.38	0.044	0.25*	0.045*

*p <0.05; ** p <0.01

All females and males from the high dose and control groups were examined histopathologically. There were numerous microscopic findings in several organs of the high dose groups. However,

since similar findings were observed in controls at comparable levels of incidence, these findings were considered not dose related.

In animals of the P1 generation, males showed no compound induced clinical signs of toxicity, whereas females, showed hyperactivity, discharge from vagina, lacrimation and mortality (one incidence, each in the high dose group); animals in the control group showed discharge from vagina (2 animals), lacrimation (1 animal), and mortality (2 animals).

Bodyweight was significantly increased or decreased in certain weeks, showing no clear trend (Table 116).

Table 116: Mean bodyweights (g) of P1 males and P1 females (selected weeks)

Week of treatment	Dose level (ppm)							
	0	200	1000	5000	0	200	1000	5000
	male				female			
1	52.5	43.1*	215.8*[a]	44.5*	39.9	44.5	42.4	42.3
10	175.1	150.9*	195.3	201.1	145.3	154.0	159.4	147.7
20	350.8	317.7	306.4	335.6	319.3	307.6	239.7*	217.9*
30	389.1	379.3	425.2	432.2*	342.0	332.8	274.9*	261.2*

*, Significantly different from control; a, sic!

Mean bodyweight of dams in the intermediate dose group was significantly higher on gestation day 0 after both matings (Table 117). Other significant changes were seen only in one mating and thus considered incidental. Bodyweight of females in intermediate dose group was significantly higher compared to control group animals. Feed consumption showed only minor significant variations during pre-mating, mating, resting, and post-mating periods.

Table 117: Mean bodyweights (g) of P1 animals during gestation and lactation

day	Dose level (ppm)							
	0	200	1000	5000	0	200	1000	5000
	F2a				F2b			
Gestation								
0	239	241	262*	243	271	271	301*	266
6	253	253	272	258	295	291	315	277
14	285	283	289	274	316	318	334	293*
20	320	326	322	311	364	364	364	322*
Lactation								
1	252	246	276*	257	290	292	322*	292*
4	357	252	279	260	299	300	326*	293*
7	261	258	291*	266	308	302	327	294
14	267	263	303*	273	295	291	326*	292*
21	247	241	292*	262	274	270	302*	272*
Pregnant animals	18	15	20	19	18	18	19	16

*, Significantly different from control ($p < 0.05$)

Litter weight of the F2b generation was generally lower in high and mid dose groups reaching significance on days 1 and 7 in the high dose group (Table 118). This effect was not observed in the F2a litters resulting from the first mating.

Table 118: Litter weight of F2a and F2b generation

day	Dose level (ppm)							
	0	200	1000	5000	0	200	1000	5000
	F2a				F2b			
1	64.6	75.7*	55.2	62.0	70.4	78.3	62.8	57.5*
4	87.5	100.6	79.6	90.5	98.3	103.9	85.7	84.3
7	114.6	127.3	104.1	113.6	133.0	134.3	125.1	107.2*
14	207.5	211.9	182.5	197.8	209.7	211.6	183.7	187.6
21	280.8	282.2	268.8	276.2	289.3	305.1	303.9	269.4

*p <0.05

Total number of pups, proportion of live pups, sex ratio and pup weights were not affected (Table 119). Total number of pups was reduced in the high and mid dose groups of the second mating but this was not observed on the first mating and was, thus, considered incidental.

Table 119: Litter parameters F2a and F2b generation

		Dose level (ppm)							
		0	200	1000	5000	0	200	1000	5000
		F2a				F2b			
Total no of pups	Male	87	98	79	99	89	99	71	76
Mortalities		6	20	14	13	15	27	16	19
Total no pups	Female	92	83	91	85	99	106	93	65
Mortalities		11	15	20	12	24	23	23	19
Sex ratio (% male)		49	54	46	54	47	48	43	54
Total mortalities (%)		9.6	19.5	21.4	14.1	20.7	19.5	23.8	24.8

Macroscopic abnormalities, recorded in F2a and F2b generations, were of low incidence and occurred in comparable frequency in all groups.

All findings noted during necropsy of P1 animals were found to be incidental. Microscopic lesions observed, had low levels of incidence, which were comparable in high dose group and control group.

Compound intake was not calculated/reported in the study report, therefore, it is estimated as 13, 67, or 333 mg/kg bw/d for 200, 1000 or 5000 ppm dose level.

Conclusion:

There were no treatment related developmental effects reported regarding litter size, fertility, pup weight or any other signs in the offspring. There were no treatment related reproductive effects reported. Several significant changes in bodyweight and terminal organ weights were noted for the P0 generation. Relative weights of ovaries and spleen in maternal rats were consistently increased in all treatment groups and, although not following a dose response, this may be related to dosing. Based on the reduction of bodyweight, and the increase in organ weights in all treatment groups in the P0 parental generation, a NOAEL with regard to parental toxicity could not be established in this study.

Comment:

The maternal weight difference between gestation day 20 and lactation day 1 should be at least as high as the litter weight (Table 120). For litters F2a and F2b the difference and litter weight are considered equal, whereas with litters F1a and F1b the difference and litter weight have unacceptable large differences.

Table 120: Comparison of maternal weight loss due to birth and litter weight

	Dose level (ppm)							
	0	**200**	**1000**	**5000**	**0**	**200**	**1000**	**5000**
	F1a				**F1b**			
Gestation day 20	303	314	296	291	322	332	292	281
Lactation day 1	296	297	261	262	316	327	290	287
Difference	7	17	35	29	6	5	2	-6
Litter weight, day 1	72.8	72.7	66.4	67.1	65.2	66.2	54.6	53.2
	F2a				**F2b**			
Gestation day 20	320.3	326.4	321.6	311.1	364.4	363.7	364.0	322.1
Lactation day 1	252.4	245.7	275.7	256.9	289.5	292.3	322.4	291.6
Difference	67.9	80.7	45.9	54.2	74.9	71.4	41.6	30.5
Litter weight, day 1	64.6	75.7	55.2	62.0	70.4	78.3	62.8	57.5

Reference: IIA 5.6.3/01

Report: Ramamoorthy (2000) Evaluation of toxicity of Neemazal technical to general reproductive process and fertility in Wistar rats - Segment I

Fredrick Institute of Plant Protection and Toxicology, Padappai, Tamil Nadu, India

Report No.: 4823, Project No: 05-512-97

TOX2001-171, 1863425

Guidelines: Gaitonde Committee Guideline (No. 6.3.0.Ciii-1)

Sim. OECD 415 (1983)

Deviations: Page 116 is missing

Due to the watermark on each page, some information is not/hardly readable.

Deviations compared to OECD TG 415:

- Only 2 dose levels and a control group (OECD: 3 dose levels, a limit test is possible)
- The sum of dead & live foetuses is not in agreement with number of corpora lutea
- Data on test item analysis in vehicle (level, stability,

homogeneity) are missing
- Feed intake not measured
- Pre-mating phase in males only 60 d (OECD: 70 d)
- Duration of mating period and time to successful mating not reported
- No indication of mating pairs (assignment of males to females)
- One male was mated with 3 females (OECD: 1:1 or 1:2)
- No sex determination of offspring
- Dead or moribund foetuses were not examined for defects
- Only testes of parental generation males were examined by microscopy
- Interim sacrifice of dams to evaluate number of CL and implantations (additionally to OECD TG)

GLP: No (but Gaitonde quality assurance scheme)

Acceptability: The study is considered to be not acceptable.

Material and Methods:

1. Test and Control Materials:	NeemAzal technical
Purity:	37.3% azadirachtin A
Batch/Lot No.:	CC 86
Description:	Light brown powder with mild odour
Stability:	Stored at 5 – 8 °C
2. Test animals:	
Species:	Rattus norvegicus
Strain:	Wistar
Age:	8-10 weeks
Weight	154-170 g
Sex:	males and females
Source:	Fredrick Institute of Plant Protection and Toxicology, Padappai, 601301, India
Acclimation:	Yes (duration not stated)
Housing:	Standard polypropylene rat cages (with stainless steel top grill), animals were housed individually except during mating

Food: Water:	Standard pellet feed (Lipton India Ltd, Bangalore) ad libitum Aquagard filtered water ad libitum
3. Environmental conditions: Temperature: Humidity: Photoperiod:	 22 ± 3 °C 55 ± 5% relative humidity 12 hour light/12 hour dark

In life dates: 9 May – 27 June 1998

Groups of 10 males and 30 female Wistar rats received NeemAzal technical at 100 and 1000 mg/kg bw/d in distilled water by gavage and a control group received distilled water only. Males were treated for 60 days, females for 14 days before mating. Dosing was continued through mating and females were further dosed during gestation and lactation. After mating, males were sacrificed and testes subjected to histopathological investigation.

All animals were examined for mortality, overt signs of toxicity throughout the observation period. Bodyweights were recorded at the start of treatment and weekly after that and finally at necropsy.

On day 13 of gestation one half of female rats was sacrificed and subjected to a full external and internal macroscopic examination, uterine horns were exposed and observed for implantations and corpora lutea, live and dead implantations and ovaries were screened for corpora lutea and other uterine abnormalities.

For the offspring of the remaining dams, litter size, and litter bodyweights of pups were taken on days 0, 4, 7, 14 and 21. During this period viability, growth and weaning indices of litters were also recorded.

Effects of the test item on general reproduction parameters (fertility index, total implantation, and dead implantation rates) were determined.

Data were analysed statistically by suitable statistical methods (viz. Students t-test or chi-square test).

Findings:

No treatment related effects were noted with respect to mortality or clinical signs for the parental rats. Bodyweights were not affected during the pre-mating period, during gestation and lactation periods.

Testes weights were not affected. Gross pathology revealed no gross recurrent abnormalities. No recurrent lesions were noted upon histopathologic examination of testes, solitary lesions were noted in the control and treated groups.

Uterine contents (number of live and dead embryos, number of corpora lutea) and mean uterus weights were not affected by treatment.

Fertility was not affected by treatment with NeemAzal technical: 2/30, 3/30 and 3/30 females were found non-pregnant in the control group, 100 and 1000 mg/kg bw/d groups, respectively.

Table 121: Mean organ weights (g)

	Control	**100 mg/kg bw/d**	**1000 mg/kg bw/d**
Testes (left)	1.472 ± 0.014	1.478 ± 0.015	1.451 ± 0.023
Testes (right)	1.484 ± 0.013	1.447 ± 0.016	1.444 ± 0.020
Uterus	2.79 ± 0.14	2.86 ± 0.14	2.84 ± 0.18

Table 122: Mean litter size (determined after spontaneous birth), ovarian and intrauterine content (determined on GD 13)

	Control	**100 mg/kg bw/d**	**1000 mg/kg bw/d**
Litter size	9.57 ± 0.29	9.92 ± 0.21	9.90 ± 0.20
Live embryo	7.79 ± 1.12	8.00 ± 1.11	8.29 ± 1.27
Dead embryo	1.75 ± 0.89	1.67 ± 0.82	1.43 ± 0.53
Corpora lutea	8.79 ± 1.37	8.71 ± 1.27	9.00 ± 1.47

The number of pups was not affected by treatment. There was no treatment-related effect on pup bodyweight and pup bodyweight gain.

Table 123: Pup bodyweight (g)

Lactation day	**Control**	**100 mg/kg bw/d**	**1000 mg/kg bw/d**
0	3.98	4.00	4.01
4	8.17	8.22	8.26
7	11.48	11.44	11.52
14	22.82	22.67	22.78
21	33.47	33.13	33.03

Conclusions:

Groups of Wistar rats received NeemAzal technical at 100 and 1000 mg/kg bw/d by gavage for 60 days (males) and 14 days (females) before mating. Dosing was continued through mating and females were further dosed during gestation and lactation. After mating, males were sacrificed and testes subjected to histopathological investigation. No adverse effects were noted on testes.

No adverse effects on parental animals, fertility or reproductive parameters were described.

According to the report, there were no treatment related developmental effects regarding litter size, fertility, pup weight or any other signs in the offspring.

Under the conditions of this study, the NOEL/NOAEL was equivalent to the highest dose tested, 1000 mg/kg bw/d with regard to maternal, reproductive and developmental/offspring parameters. This corresponds to a dose level of azadirachtin A of 373 mg/kg bw/d.

Reference: KIIA 5.6/1

Report: Pfau W (2009): Evaluation of the reproductive toxicity of azadirachtin

Report No. 379234-A2-050601-01

1863427

Summary (taken from the report)

Summary

Azadirachtin as notified for use as insecticidal pesticide in the EU is a refined medium polarity extract from the kernels of the Neem tree. Various parts of the Neem tree (Azadirachta indica) are being used in India in traditional folk medicine. Concern for reproductive toxicity stems from the traditional use of aqueous Neem leave extracts to reduce male fertility or reports on local contraceptive effects of Neem seed kernel oil upon intra-uterine application supported by spermicidal effects in vitro.

However, adverse effects of medium polarity Neem kernel extracts, Azadirachtin, on fertility were not observed in dedicated 2-generation-feeding studies or a segment I study or in published studies in rats. Also, circumstantial evidence confirms the lack of adverse effects of Azadirachtin on male or female fertility.

Despite the common use of Neem products in Indian folk medicine there are no epidemiological studies and no casuistic reports on teratogenic or other adverse developmental effects of Azadirachtin in humans. No developmental toxicity was noted in 2-generation studies or a segment I study.

Six teratogenicity studies according to relevant guidelines and GLP employing Azadirachtin demonstrated either the lack of adverse developmental effects, or developmental toxicity was noted only at maternally toxic dose levels.

Teratogenicity in rats was reported only in one of these studies but incidences were within the range of historical control values. Based on initially reduced bodyweight and food consumption in the high and mid-dose groups the no observable adverse effect level for maternal toxicity was at the low dose level. Increased incidences of visceral malformations noted in the fetuses of the high dose treatment group were within the range of historical control values for the rat strain employed. Thus, developmental effects (not significantly increased incidence of supernumerary ribs at high dose) were only observed at maternally toxic levels.

In a rabbit teratogenicity study maternal toxicity was also noted both at mid dose and high dose level whereas adverse effects on the foetuses were confined to the high dose level. No significant increase of adverse developmental effects was noted at mid dose and low dose. The high dose effects are considered secondary to maternally toxic effects which were observed at high and also at mid dose level and included body weight loss, reduced feed intake and clinical signs. Developmental effects at high dose level may cause concern. However, this level apparently exceeded the maximum tolerated dose (=mid dose) by a factor of 5. As there were no adverse effects on development observable at mid dose it is safe to conclude that there are no developmental effects at maternally non-toxic dose levels.

Male or female reproductive organs were not affected in most repeated dose studies conducted with Azadirachtin. Apparent effects on testes weights in two 2-generation studies with NeemAzal (reduced or increased organ weight) and a 90 day feeding study with ATI-720 in rats or an 18-months study in mice were not toxicologically relevant. Spurious effects (increased ovary weight) were noted in a two generation study with the formulation NeemAzal F 5%, attributable to a component in this formulationnot related to Azadirachtin. Only in one 90-day feeding study adverse effects were noted including reduced organ weights for uterus and ovaries concomitant with endometrial hypertrophy and reduced number of *corpora lutea*. Effects on testes weight in this study were not significant and histological observations affecting this organ were of low incidence and observed also in historical controls. Effects on female reproductive organs were confined to the high dose level where also strong signs of systemic toxicity were noted affecting liver, body weight, sciatic nerve, thyroids, biochemical and haematological parameters.

Even at high dose levels the reproductive organs of males or females are not a main target organ for Azadirachtin induced toxicity.

While for other Neem products such as Neem oil or aqueous leaf extracts contraceptive or anti-fertility effects are reported, no adverse effects on male or female fertility were observed in a number of dedicated studies with Azadirachtin. Despite the common use of Neem products in Indian folk medicine there are no human data on adverse developmental effects of Azadirachtin. Teratogenicity studies employing Azadirachtin demonstrated either the lack of adverse developmental effects or developmental toxicity was noted only at maternally toxic dose levels. Male or female reproductive organs were not affected in nine out of ten available repeated dose studies conducted with Azadirachtin. Only in one study, adverse effects were noted affecting uterus and ovaries in female rats but only at the highest dose level where also strong signs of systemic toxicity were noted.

Azadirachtin induced no reproductive toxicity; neither affecting fertility nor development and the reproductive organs of male or female experimental animals are no targets for Azadirachtin induced toxicity.

Based on the available data classification and labelling regarding reproductive toxicity is not warranted.

Comment by RMS:

For the evaluation of effects on fertility or reproduction, findings in single-dose (e.g., histopathology of testes [however not done for the azadirachtin technical extracts]), short-term, long-term, multi-generation and one-generation studies can be used. All azadirachtin technical extracts (evaluated in this AR) were evaluated in short-term studies in rats. Additionally, NeemAzal was evaluated in a long-term as well as a 2-generation and a 1-generation study.

In the 28-d, 90-d and long-term studies in rats with NeemAzal no findings on sex organs were reported in the study reports. No effects on fertility or reproduction were observed in the submitted 1-generation (considered not acceptable) or 2-generation (considered acceptable) toxicity studies with NeemAzal. Dose levels in the 2-generation study were calculated as mean of the compound intake in weeks 0, 5, 10 and 15 (Pfau, 2009, 1863427). Therefore, compound intake was based only on the intake during the pre-mating period.

EFSA proposed to discuss the acceptability of the 2-generation study: It should be noted that DE does not reject studies out of formal reasons (e.g., GLP status or guideline compliance). The studies are assessed for their scientific results.

In the 28-d study in rats with Fortune Aza findings on sex organs were reported in the study report (ovary weight ↓). In the 90-d study, reduced number of corpora lutea and slightly reduced ovary weights were observed at 1600 ppm. At 6400 ppm, uteri (small, lower weight and endometrial atrophy), ovaries (lower weights, reduced number of corpora lutea) and testes (seminiferous tubular atrophy) exhibited findings. Compared to the control groups, animals treated with 6400 ppm had a bodyweight gain of 60-66% and a feed intake of 77-81%. No long-term or multi-generation studies performed with Fortune Aza were submitted.

In the 90-d study in rats with ATI 720 findings on sex organs (relative testes weight ↑) were reported. However, absolute testes weight was unchanged, therefore, this finding was considered to be not adverse. No long-term or multi-generation studies performed with ATI 720 were submitted.

In reports from open literature, various findings with respect to fertility or reproduction are described. However, in the literature reports different test compounds (other extraction methods, other starting materials, etc.) were used when compared to the technical extracts used for PPP. There seem to be some differences in properties, when comparing different preparations of different parts of neem tree (e.g., flower, leaves, seed kernel). In the available reproductive toxicity study, no effects on fertility were observed. Therefore, the proposal to classify for toxicity to fertility/reproduction is not supported by the RMS.

The reproductive NOAEL (expressed as Aza A-dose level) in the 2-generation study (with NeemAzal) was as high as the LOAEL in the 90-d study with Fortune Aza. Therefore, it can be concluded (under the condition that the bridging concept presented in the DAR is accepted) that these effects at 1600 ppm had no impact on reproductive performance of the animals. Effects at 6400 ppm might be associated with the marked decrease of bodyweight gain.

Studies performed with Fortune Aza

No studies submitted by the notifiers.

Studies performed with ATI 720

No studies submitted by the notifiers.

9.10.1.2 Human information

9.10.2 Developmental toxicity

9.10.2.1 Non-human information

Studies performed with NeemAzal

Reference:	TRF IIA 5.6.10 / 01
Report:	Myers, D. P., Dawe, I. S. (1997) NeemAzal technical – A Preliminary Study of the Developmental Toxicity in Rats Huntingdon Life Sciences Ltd., Huntingdon, England unpublished report No. EIP 2/951879; TOX9700510
Guidelines:	OECD guideline 414 (1981)
Deviations:	This is a pre-study, thus only macroscopic examination of external foetal morphology was performed. Only 10 females per dose group.
GLP:	Yes
Acceptability:	The study is considered to be supplementary.

Material and Methods:

Time-mated Crl:CD BR VAF/plus female rats (animals provided by Charles River, England), assigned to one control and three treatment groups of 10 animals each, were used to determine the teratogenic potential of NeemAzal technical (batch no.: IV, purity: 36.6% azadirachtin A). Dosage levels of 0, 100, 300 and 1000 mg/kg bw/d were administered orally by gavage on days 6 through 19 of gestation in a volume of 10 mL/kg in 1% aqueous methylcellulose in this study. Dosage solution was prepared daily. Solution of day 1 was analysed and found to be homogenous, and stable for up to 24 h. Achieved concentrations were within 10% of nominal concentrations. Observations on mortality, clinical signs of toxicity and bodyweights were recorded. Feed intake and water consumption were measured. On gestation day 20, all females were sacrificed and the number and location of viable and nonviable foetuses, early and late resorptions and corpora lutea were recorded. Foetus and uterus weights were determined. Gross lesions were recorded.

Findings:

Survival was 100% for all groups during the course of the study. No gross lesions were seen at necropsy of the study animals. Post-dosing salivation was seen intermittently in 9/10 animals in the high and mid dose groups, first observed after the third dosing. Generally, this salivation was clear or brown. Wet coat was noted for 9 animals from day 11 post coitum. Post-dose salivation was observed in one animal at days 14 and 15 of presumed pregnancy, but no other treatment-related clinical signs of toxicity were seen at low dose. Bodyweight gain was reduced in the high and mid dose group on the first two days of treatment, but improved thereafter. Final bodyweights were equivalent to controls. The bodyweight changes in the low dose group were comparable with those of the control throughout the study. Concomitant to the initial reduced bodyweight gain statistically significant reduced food intake was noted on days 6-7 at the high and mid dose levels compared to control animals. Increased water consumption was noted throughout the treatment in the 1000 mg/kg bw/d group and a slight increase in water consumption was observed at the mid dose. No effects were noted in the low dose group and control. There was one non-pregnant female in each group. The mean foetal weight in one litter of each of the treated groups was noticeably heavier than in the other litters on the study, suggesting that the stage of development of these litters was later than day 20 of pregnancy; this finding is presumed to reflect an error in the mating of these females by the animal supplier, thus these animals and litters were excluded from data analysis. Mean foetal weight and the number of *in utero* deaths were comparable in all treatment groups and in controls. The incidences of early resorptions were slightly higher in the high dose group.

Table 124: Cesarean section observations

Observations	**Dose level (mg/kg bw/d)**			
	0	100	300	1000
Total number of females	10	10	10	10
Females excluded from analysis:				
# non pregnant	1	1	1	1
# litter to heavy	0	1	1	1
Females analysed	9	8	8	8
corpora lutea/dam	14.0	14.0	13.3	16.0
Total implantation/dam	13.1	12.8	12.9	14.6
Live foetuses/dam	12.4	12.3	12.3	12.9
Resorptions				
Early	0.7	0.5	0.5	1.5
Late	0.0	0.0	0.1	0.3
Fetal weight (g)	3.82	3.77	3.90	3.73

Conclusion:

Based on the initial reduced bodyweight (high and mid dose groups) and food consumption in the high dose group the NOAEL was 100 mg/kg bw/day for maternal toxicity. The post dose salivation observed for dams at 1000 and 300 mg/kg bw/day is a common observation in studies employing the oral gavage route and is possibly a reaction to the bitter taste of the test substance. No effects on foetal number and development or incidences of malformations were observed at any treatment levels. Thus, the NOAEL for developmental toxicity was 1000 mg/kg bw/day.

Reference:	TRF IIA 5.6.10 / 02
Report:	Myers, D. P., Dawe, I. S. (1997) NeemAzal technical – A Study of the Developmental Toxicity in Rats (Gavage administration) Huntingdon Life Sciences Ltd., Huntingdon, England unpublished report No. EIP 2/952493; TOX9700514
Guidelines:	OECD guideline 414 (1981) EC 83/571/ES Annex 1(1983) US EPA Pesticide Assessment Guidelines, Subdivision F, 83-3, (1982)
Deviations:	None
GLP:	Yes
Acceptability:	The study is considered to be acceptable.

Material and Methods:

Time-mated Charles River (England) Crl: CD BR VAF/plus female rats, assigned to one control and three treatment groups of 25 animals each, were used to determine the teratogenic potential of NeemAzal technical (batch no: IV, purity: 36.6% azadirachtin A). Dosage levels of 50, 225 and 1000 mg/kg bw/d were administered orally by gavage on days 6 through 19 of gestation at a volume of 10 mL/kg in 1% aqueous methylcellulose. Suspensions were prepared daily. Compound suspension prepared for the first dosage, was analysed and found to be within 6% of nominal concentration. Observations on mortality, clinical signs of toxicity and bodyweights were recorded. Food consumption and water consumption was measured per cage from weighday to weighday from day 3 of pregnancy. Immediately following sacrifice on day 20 of pregnancy, animals were dissected and examined for congenital abnormalities and macroscopic pathological changes in maternal organs. Uterus and ovaries were exposed by an abdominal incision and the number and location of viable and nonviable foetuses, early and late resorptions and corpora lutea were recorded. The gravid uterus was then excised, weighed and the foetuses removed. Foetuses were individually weighed, sexed, and examined for external malformations and variations. Approximately one-half of the foetuses were prepared for subsequent soft tissue examination,the remaining one-half of the foetuses stained for skeletal examination. Foetal findings were classified as malformations or developmental variations. Bodyweight change, food and water consumption of adult animals were analysed by significance tests employing analysis of variance followed by inter-group comparison with the control using parametric or non-parametric tests, as appropriate. For litter data and foetal changes the basic sample unit was the litter and non-parametric analyses were routinely used: Linear-Linear Association test, Kruskal-Wallis test and pairwise permutation test. Analysis of mean values for corpora lutea, implantations, litter size, sex ratio, litter weight, foetal weight, and gravid uterine weight were performed using Kruskal-Wallis test followed by Shirley's test.

Findings:

Post-dosing salivation was seen intermittently in all animals treated with 1000 mg/kg bw/d. A total of 2/25 animals showed brown coloured salivation on one or more days. Post-dosing wet coat was noted for five animals on day 19 post coitum. Turquoise or red staining on the traypaper under the cage was noted on three or one days for two different cages of animals in the high dose group. Occasionally, a total of 4 animals (16%) showed post dose salivation in the mid dose group between day 17 and 19. No treatment-related clinical signs of toxicity were seen at 50 mg/kg/day. Survival was 100% for all groups during the course of the study.

Bodyweight gain was significantly reduced in the high dose group on the first two days of treatment, but improved thereafter (Table 125). Final bodyweights were equivalent to controls. The bodyweight changes in the mid dose group were initially slightly reduced, while bodyweight changes of low dose animals were comparable with those of the control throughout the study period. Concomitant to the initial reduced bodyweight gain statistically significant reduced food intake was noted on days 6-7 at the high and mid dose groups compared to control animals. As the bodyweight and food intake were altered in the mid dose group only on single instances, these effects were considered to be not adverse.

Table 125: Maternal bodyweights and bodyweight changes

	Dose level (mg/kg bw/d)			
	0	50	225	1000
Number of animals §	23	23	23	23
Weight gain Day 2-Day 6	40.1	39.9	36.9	34.3
Weight gain Day 6-Day 8	10.4	10.5	8.5	6.1**
Weight gain Day 8-Day 20	133.1	143.8	138.7	143.0
Final bodyweight	408.7	420.3	409.7	408.1

**, $p<0.01$; §, excluding non-pregnant animals

Significantly increased water consumption was noted throughout the treatment in the 1000 mg/kg bw/d group only. No effects on water consumption were noted in the low and mid dose groups.

Macroscopic post mortem examination of females did not indicate any adverse effects of treatment. There were two non-pregnant females in each of the treatment groups and the control group (Table 126). There were no instances of total litter loss *in utero*. Mean foetal weight and the number of *in utero* deaths were comparable in all treatment groups and in controls.

Table 126: Caesarean section observations

Observations	Dose level (mg/kg bw/d)			
	0	**50**	**225**	**1000**
No. of animals assigned	25	25	25	25
Females gravid	23	23	23	23
Females excluded of analysis: # non pregnant	2	2	2	2
Corpora lutea/dam	15.2	15.8	16.0	15.3
Total Implantation/dam	13.7	14.7	14.7	14.3
Live foetuses/dam	13.3	14.0	13.3	13.4
Resorptions				
Early	0.4	0.7	1.2	0.7
Late	0.1	0.0	0.2	0.2
Mean uterus weight (g)	78.2	83.9	80.8	70.7
Sex ratio (% male)	51.5	50.9	55.0	46.9
Fetal weight (g)	3.88	3.94	3.94	3.85

While only 1/305 malformed foetus was observed in the control group there were 8/308 foetuses classified as malformed (5/23 litters affected) in the high dose treatment group (Table 127). Four of these from one litter showed mottled foetus syndrome, a syndrome occurring spontaneously in this rat strain and thus considered incidental. The remaining 4 malformed foetuses at this dose level showed visceral changes associated with the heart, or thoracic circulatory system (interventricular septal defect, duplicated inferior vena cava). These incidences were just outside the historical control values and may be related to treatment. Furthermore, there was a clear increase in the percentage of foetuses showing supernumerary ribs in the high dose group as compared to the controls (Table 129). In the mid dose group 5/306 foetuses were affected (3/23 litters). Three of these foetuses from one litter showed squat foetus syndrome, a syndrome occuring spontaneously in this rat strain and thus considered incidental. One of the remaining two malformed foetuses showed interventricular septal defect and a further three (from different litters) showed small interventricular septal defect(Table 129). Because of the similarity to the high dose group it was considered that these observations may be related to treatment. At the lowest dose 5/323 foetuses were classified as malformed, while four of these showed diaphragmatic hernia. This was considered incidental because similar effects were not observed at higher dose levels.

Table 127: Foetal abnormalities – prevalence and distribution in litters

Dose level (mg/kg bw/d)		0	50	225	1000
Number of litters examined		23	23	23	23
Observation	**Number of affected foetuses per litter (n)**	**No. of litters with n foetus affected**			
Malformations	0	22	20	20	18
	1	1	2	2	4
	2				
	3		1	1	
	4				1
Visceral anomaly	0	15	11	15	14
	1	5	8	6	5
	2	3	2	2	4
	3		1		
	4		1		
Skeletal anomaly	0	13	16	13	14
	1	6	6	6	6
	2	2	1	3	2
	3	1		1	1
	4				
	5	1			

Table 128: Foetal (litter) incidences of selected findings

Observation		**Dose level (mg/kg bw/d)**			
		0	**50**	**225**	**1000**
Number of foetus (litters) examined:		305 (23)	323 (23)	306 (23)	308 (23)
Visceral findings					
Thoracic (malformations)	Malformed systemic/pulmonary arteries	0 (0)	0 (0)	0 (0)	1 (1)
	Atrial septal defect with narrow pulmonary vein	0 (0)	0 (0)	0 (0)	1 (1)
	Interventricular septal defect	0 (0)	0 (0)	1 (1)	2 (2)
	Malrotated heart	0 (0)	0 (0)	1 (1)	1 (1)
	Duplicated inferior vena cava	0 (0)	0 (0)	0 (0)	2 (2)
Thoracic (anomalies)	Anomalous cervicothoracic arteries	1 (1)	0 (0)	0 (0)	0 (0)
	Interventricular septal defect (small)	0 (0)	1 (1)	3 (3)	2 (2)

Table 129: Skeletal variants in foetuses after treatment with NeemAzal

Dose level (mg/kg bw/d)	**Foetuses examined**	**Foetuses with**							
		13 ribs		14 ribs		Normal sternebrae		Variant sternebrae	
	N	n	%	n	%	n	%	n	%
0	152	137	90.6	15	9.4	75	47.7	77	52.3
50	159	145	91.4	14	8.6	92	59.0	67	41.0
225	149	138	93.3	11	6.7	86	57.9	63	42.1
1000	149	114	75.7	35	24.3	77	51.0	72	49.0

No statistically significant differences were observed.

Conclusions:

Based on the initial reduced bodyweight gain, food consumption and the increased water consumption in high dose animals, the no observable adverse effect level was 225 mg/kg bw/day for maternal effects. The post dose salivation observed for dams at 225 and 1000 mg/kg bw/day is a common observation in studies employing the oral gavage route and is possibly a reaction to the bitter taste of the test substance. Increased incidences of malformations were noted in the foetuses of the high and mid dose treatment groups affecting the heart (ventricular septal defect, malrotation of heart) and an increased incidence of supernumerary ribs occurred in the high dose group. Even though maternal toxicity was not observed in this study, liver toxicity in dams can be expected, which had a LOAEL of 123 mg NeemAzal/kg bw/d (1600 ppm) in the 90-d study in rats (NOAEL: 32mg/kg bw/d (400 ppm)).Additionally, incidences were increased only slightly. Therefore, a classification with R63 (possible risk of harm to unborn child; toxic to reproduction category 3) according to the criteria laid down in Directive 67/548/EEC (as amended in Directives 96/56/EC and 2004/73/EC) was considered warranted.

No effects on foetal number and development were observed at the lowest dose. Thus, a NOAEL for developmental toxicity was 50 mg/kg bw/day.

A further study (Pugazhenthi, 1998, TOX1999-225) with NeemAzal was submitted, which could not be evaluated due to great deficiencies in the report.

Reference: KIIA 5.6.10/06

Report: Anonymous (1996): Historical Control Data (1992-1994) for Developmental and Reproductive Toxicity Studies using the Crl:CD®(SD)BR Rat; MARTA (Middle Atlantic Reproduction and Teratogenicity Association)

1863426

Summary:

Collection of findings observed in control groups (Sprague-Dawley rats provided by Charles River Laboratories) as reported by 15 American laboratories.

Information on visceral alterations:

Total studies: 229

Total litters: 4935

Total foetuses: 24340

	Foetal incidence				Litter incidence			
Finding	No.	Avg (%)	S.D.	Max	No.	Avg (%)	S.D.	Max
Atrial septa (defect)	0	0.000	0.00	0.00	0	0.000	0.00	0.00
Ventricular septal defect, membran.	44	0.260	1.44	10.30	30	1.018	5.61	40.90
Ventricular septal defect, muscular	4	0.018	0.13	1.34	4	0.134	0.98	10.00
Vena cava, any alteration	0	0.000	0.00	0.00	0	0.000	0.00	0.00

Avg.: calculated from all studies

Comment by RMS:

The historical control data summarised by MARTA are considered less relevant as compared to the historical control data of the performing laboratory (Huntingdon Life Sciences). In the study report the following incidences were given:

Control incidence of interventricular septal defects

Study	1	2	3	4	5	6	7	8	9	10	11
Animal source	CR/UK	CR/UK	CR/UK	CR/UK	CR/UK	CR/UK	CR/UK	CR/UK	CR/UK	CR/UK	CR/UK
Date of sacrifice	07.94	07.94	09.94	09.94	10.94	11.94	11.94	01.95	01.95	02.95	02.95
No. foetuses examined	144	146	144	161	158	164	171	139	147	160	156
No. litters examined	22	22	22	24	24	24	25	23	24	23	24
Description	Incidence (Foetuses (litters))										
Interventricular septal defect A	-	-	-	-	1(1)	-	1(1)	-	-	-	-
Interventricular septal defect (small) B	3(3)	-	-	1(1)	-	-	2(2)	2(2)	1(1)	2(2)	1(1)
Total (anomalous and malformed)	3(3)	-	-	1(1)	1(1)	-	3(3)	2(2)	1(1)	2(2)	1(1)

A Classified as malformation
B Classified as visceral anomaly
CR/UK Charles River UK rats

Studies performed with Fortune Aza

Reference: SIP IIA 5.6.10 / 01

Report: Waterson, L. A. (1997)

Fortune Aza technical – A Preliminary Study of the Developmental Toxicity in Rats

Huntingdon Life Sciences Ltd., Huntingdon, England

unpublished report No. FBT 1/952837; TOX2005-2400

Guidelines: OECD Guideline 414 (1981)

Deviations: Only 10 animals per dose group. Only gross external examination of foetuses.

GLP: Yes

Acceptability: The study is considered to be supplementary.

Material and Methods:

Mated Charles River (England) Crl: CD BR VAF/Plus female rats, assigned to one control and three treatment groups of 10 animals each, were used to determine the teratogenic potential of Fortune Aza technical (Batch no.: 0010195-0050195, purity: 8.5% azadirachtin A+B). Dosage levels of 0, 100, 300 and 1000 mg/kg bw/d were administered orally by gavage on days 6 through 19 of gestation at a volume of 10 mL/kg bw in 1% aqueous methylcellulose. Dosage suspension was prepared daily. Stability, homogeneity and stability of suspension prepared for the first dosing was assessed analytically. The suspension was stable for up to 24 h and within 2.3% of nominal concentration. Observations on mortality, clinical signs of toxicity, bodyweights, feed and water consumption were recorded. On gestation day 20, all females were sacrificed and the number and location of viable and nonviable foetuses, early and late resorptions and corpora lutea were recorded. Uterus weights were determined. Gross lesions were recorded.

Findings:

Post-dosing salivation was seen intermittently in all animals treated with 1000 mg/kg bw/d. Generally this salivation was clear and lasted for one hour after dose administration. A total of 3/10 animals showed brown coloured salivation on one or more days. A total of 4 animals showed occasional post-dose salivation in the mid dose group, first observed after the third dosing. Brown post-dosing salivation was observed in one animal on day 16 of pregnancy. No treatment-related clinical signs of toxicity were seen at 100 mg/kg bw/day. One animal in this dose group showed poor clinical condition (reduced body tone, piloerection, inability to stand on its right hindfoot) and was found at autopsy to show total resorption. This was considered not dose related.

Bodyweight gain and feed intake was reduced in the high dose group on the first two days of treatment, but improved thereafter. Final bodyweights were equivalent to controls. The pattern of bodyweight gain and food intake at dosages 300 and 100 mg/kg bw/d was similar to controls. Increased water consumption was noted throughout the treatment in the 1000 mg/kg bw/d group especially during the first two days. No effects were noted in the low and mid dose groups.

Survival was 100% for all groups during the course of the study. No gross lesions were seen at necropsy of the study animals. One instance of total litter loss *in utero* was observed in the female with poor clinical condition (low dose). This was considered unrelated to the treatment. One female of the high dose group was not pregnant. Mean foetal weight and the number of *in utero* deaths were comparable in all treatment groups and in controls.

Table 130: Cesarean section observations

Observations	Dose level (mg/kg bw/d)			
	0	**100**	**300**	**1000**
Dams with live young at day 20	10	9	10	9
Corpora lutea/dam	15.2	14.1	14.5	15.7
Total implantation/dam	14.3	13.4	14.1	14.8
Live foetuses/dam	13.4	12.6	13.1	14.1
Resorptions				
early	0.9	0.6	0.9	0.7
late	0.0	0.3	0.1	0.0
Fetal weight (g)	3.79	3.79	3.71	3.88

Conclusions:

Based on the initial reduced bodyweight and food consumption and increased water consumption in the high dose group the no observable adverse effect level was 300 mg/kg bw/day for maternal toxicity. The post dose salivation observed for dams at 300 and 1000 mg/kg bw/day is a common observation in studies employing the oral gavage route and is possibly a reaction to the bitter taste of the test substance. No effects on foetal number and development or incidences of malformations were observed at any treatment levels. Thus, the NOAEL for developmental toxicity was 1000 mg/kg bw/day.

Reference: SCM IIA 5.6.10 / 02

Report: Waterson, L.A. (1997)

Fortune Aza technical – A Study of the Developmental Toxicity in Rats

Huntingdon Life Sciences Ltd., Huntingdon, England

unpublished report No. FBT 2/960340;

TOX2005-2401, 1893597

Guidelines: OECD guideline 414 (1981)

EC 83/571/ES Annex 1(1983)

US EPA Pesticide Assessment Guidelines, Subdivision F, 83-3, (1982)

Deviations: None

GLP: Yes

Acceptability: The study is considered to be acceptable.

Materials and Methods:

Time-mated Charles River (England) Crl: CD BR VAF/Plus female rats, assigned to one control and three treatment groups of 25 animals each (treated in two batches of 15 and 10 animals), were

used to determine the teratogenic potential of Fortune Aza technical (batch no.: 0010195-0050195, purity: 8.5% azadirachtin A+B). Dosage levels of 100, 300 and 1000 mg/kg bw/d were administered orally by gavage on days 6 through 19 of gestation at a volume of 10 mL/kg bw in 1% methylcellulose. Suspensions used for dosing, were prepared daily. Compound concentration in the suspension prepared for the first dosing was assessed analytically, it was found to be within 3.3% of nominal concentration. Observations on mortality, clinical signs of toxicity, bodyweights, food and water consumption were recorded.

On gestation day 20, all females were sacrificed and the number and location of viable and nonviable foetuses, early and late resorptions and corpora lutea were recorded. Uterus weights were determined. Gross lesions were recorded. Sex ratio and foetal abnormalities were recorded. Bodyweight change, food and water consumption of adult animals were analysed by significance tests employing analysis of variance followed by inter-group comparison with the control using parametric or non-parametric tests, as appropriate. For litter data and foetal changes the basic sample unit was the litter and non-parametric analyses were routinely used: Linear-Linear Association test, Kruskal-Wallis test and pairwise permutation test. Analysis of mean values for corpora lutea, implantations, litter size, sex ratio, litter weight, foetal weight, and gravid uterine weight were performed using Kruskal-Wallis test followed by Shirley's test.

Findings:

Post-dosing salivation was seen intermittently in all but one animals of the high dose group treated with 1000 mg Fortune Aza technical/kg bw/day. Generally this salivation was clear and lasted for one hour after dose administration. A total of 14/25 animals showed brown coloured salivation on one or more days. Post-dosing wet coat (ceasing one hour after salivation) was noted for four animals. A total of 11 animals (44%) showed post dose salivation in the mid dose group lasting for one hour post administration. Salivation was clear in most animals but in five animals, brown salivation was observed. No treatment-related clinical signs of toxicity were seen in animals of low dose group. Bodyweight gain was reduced in the high dose group in the first week of treatment, but improved thereafter (Table 131). Final bodyweights were equivalent to controls. The bodyweight changes in the low and mid dose group were comparable to those of the control group throughout the treatment (gestation days 6 through 15) and overall gestation (gestation days 0 to 20) periods. No statistically significant differences in food intake were noted between treated and control animals. Water consumption of high dose group was markedly higher during the first 2 days of treatment in comparison to control and pre-treatment values, thereafter, the magnitude of the finding was marginally less than that noted during the first 2 days of treatment.

Table 131: Maternal bodyweights (g) and bodyweight changes (g)

	Dose level (mg/kg bw/d)			
	0	100	300	1000
Number of animals §	25	25	22	24
Weight gain Day 2-Day 6	31.2	32.7	30.2	30.3
Weight gain Day 6-Day 8	10.6	8.6	10.1	6.9**
Weight gain Day 8-Day 20	121.5	118.8	120.1	121.2
Final bodyweight	365.7	361.7	363.6	360.6

§, Excluding non-pregnant animals; **, p <0.01

Survival was 100% for all groups during the course of the study. No gross lesions were seen at necropsy of the study animals.

There were three non-pregnant females in the high dose group and one non-pregnant female in the mid dose group (Table 132). The mean number of implantations was slightly lower in these two treatment groups. However, since treatment started only after implantation, this was not considered related to treatment. There were no instances of total litter loss *in utero*. Mean foetal weight and the number of *in utero* deaths were comparable in all treatment groups and in controls.

Table 132: Cesarean section observations

Observations	**Dose level (mg/kg bw/d)**			
	0	100	300	1000
No. of females assigned	25	25	25	25
Females gravide	25	25	22	24
Females excluded form analysis: # non pregnant	0	0	3	1
Corpora lutea/dam	13.5	13.5	12.9	13.0
Total implantation/dam	12.8	12.7	12.4	12.0
Live foetuses/dam	12.4	12.0	11.7	11.4
Resorptions early late	 0.3 0.1	 0.6 0.1	 0.6 0.0	 0.5 0.1
Mean gravid uterus weight (g)	72.2	69.1	68.3	66.7
Sex ratio (% male)	50.5	51.4	41.7	56.4
Foetal weight (g)	3.86	3.79	3.81	3.78

Malformations observed among the treated groups were not considered an adverse effect of treatment with the compound (Table 133, Table 134, Table 135, Table 136). Considering the lack of a dose-response and the low and similar incidences of findings in all dose groups, no effects on foetuses were recognised.

Table 133: Foetal abnormalities – prevalence and distribution in litters

Dose level (mg/kg bw/d)		**0**	**100**	**300**	**1000**
Number of litters examined		25	25	22	24
Observation	**Number of affected foetuses per litter (n)**	**No. of litters with n foetus affected**			
Malformations	0	23	24	21	23
	1	2	1	1	0
	2	0	0	0	1
Visceral anomaly	0	10	17	10	10
	1	7	3	10	9
	2	6	5	1	5
	3	1	0	1	0
	4	1	0	0	0
Skeletal anomaly	0	12	12	12	11
	1	7	8	4	8
	2	4	2	6	3
	3	2	3	0	2

Table 134: Incidence of skeletal variants and mean proportions

Dose level (mg/kg bw/d)	Foetuses examined	Foetuses with							
		13 ribs		14 ribs		Normal sternebrae		Variant sternebrae	
	n	n	%	n	%	n	%	n	%
0	152	139	91.4	13	8.6	66	43.4	86	56.6
100	149	134	87.7	15	10.1	58	38.9	91	61.1
300	127	116	91.5	11	8.7	78	61.4	49	38.6
1000	135	123	91.1	12	8.9	64	47.4	71	52.6

No statistically significant differences were observed.

Table 135: Skeletal and visceral malforamtions – incidence summary

Skeletal and visceral malformations - incidence summary

	Group/dosage (mg/kg/day)							
	Foetuses				Litters			
	1 Control	2 100	3 300	4 1000	1 Control	2 100	3 300	4 1000
No. examined	310	300	257	274	25	25	22	24
No. affected	2	1	1	2	2	1	1	1
Region/Description	Incidence*							
CRANIAL								
Hydrocephaly	1	-	-	-	1	-	-	-
Microphthalmia	1	-	-	-	1	-	-	-
Exophthalmia with ablepharia	1	-	-	-	1	-	-	-
Orbital socket reduced in size	1	-	-	-	1	-	-	-
Misshapen centres	1	-	-	-	1	-	-	-
Flushed centres	1	-	-	-	1	-	-	-
Absent buccal cavity	1	-	-	-	1	-	-	-
Cleft palate	1	-	-	-	1	-	-	-
Mandible reduced in size	1	-	-	-	1	-	-	-
CERVICAL								
Termination vertebral column	-	-	1	-	-	-	1	-
THORACIC								
Sternebral irregularities	-	-	1	-	-	-	1	-
Absent rib cage	-	-	1	-	-	-	1	-
Kinked/irregular ossification ribs	-	-	-	2	-	-	-	1
LUMBAR/ABDOMINAL								
Displaced and fused kidneys	-	-	1	-	-	-	1	-
Displaced uterine horns/ovaries	-	-	1	-	-	-	1	-
APPENDICULAR								
Curved scapulae, radii, ulnae	-	-	-	1	-	-	-	1
Forelimb flexure	-	-	1	-	-	-	1	-
Fore and hindlimb brachymelia	-	1	-	-	-	1	-	-

* Individual foetuses may occur in more than one category

Table 136: Visceral anomalies– incidence summary

Visceral anomalies - incidence summary

	Group/dosage (mg/kg/day)							
	Foetuses				Litters			
	1 Control	2 100	3 300	4 1000	1 Control	2 100	3 300	4 1000
No. examined#	156	150	129	137	25	25	22	24
No. affected#	26	13	15	19	15	8	12	14
Region/Description	Incidence*							
Subcutaneous haemorrhage:								
cranium	-	-	-	1	-	-	-	1
trunk	-	-	1	-	-	-	1	-
CRANIAL								
Haemorrhages affecting:								
brain	2	2	-	3	2	2	-	3
eyes and surrounding tissue	2	-	2	-	2	-	2	-
CERVICAL								
Thyroid reduced in size	1	-	-	-	1	-	-	-
THORACIC								
Anomalous cervicothoracic arteries	1	1	-	-	1	1	-	-
Interventricular septal defect (small)	3	-	1	-	3	-	1	-
LUMBAR/ABDOMINAL								
Thin diaphragm with protrusion liver	3	6	4	2	3	4	4	2
Liver: abnormal lobation	6	1	1	2	4	1	1	2
haemorrhage within lobe	2	-	3	2	2	-	2	2
Intraabdominal haemorrhage	2	1	2	2	1	1	2	2
Dilated renal pelvis/ureter	4	3	1	2	3	2	1	2
Displaced testis(es)	5	-	3	5	4	-	3	5

* Individual foetuses may occur in more than one category
\# Excludes malformed foetuses

Conclusions:

Based on the initial reduced bodyweight and food consumption in the high dose group the no observable adverse effect level was 300 mg/kg bw/day for maternal effects. The post dose salivation observed for dams at 300 mg/kg bw/day is a common observation in studies employing the oral gavage route and is possibly a reaction to the bitter taste of the test substance. No effects on foetal number and development were observed. Thus, the NOAEL for developmental toxicity was 1000 mg/kg bw/day for Fortune Aza technical.

Studies performed with ATI 720

Reference: MIT IIA 5.6.11 / 01

Report:	Ryan, B. (1994) A developmental toxicity study of orally administered ATI-720 in rabbits IIT Research Institute, Life Science Research, 10 West 35th Street, Chicago, Illinois, USA unpublished report Project No L 08424 Study No2b; TOX2005-2402
Guidelines:	US EPA Pesticide Assessment Guidelines, Subdivision F, 40 CFR Part 158; 83-3, (1982) Corresponding to OECD guideline 414 (1981) EC 83/571/ES Annex 1(1983)
Deviations:	None
GLP:	Yes
Acceptability:	The study is considered to be acceptable.

Material and Methods:

Four groups of pregnant New Zealand White rabbits (animals provided by Myrtle's Rabbitry; Thompson Station, TN, USA) were treated daily on gestation days 6 to 18 by gavage with suspensions of ATI-720 (batch no: 21380, 1111-10, purity: 8.3-9.5% Aza A) in 0.5% aqueous carboxymethyl cellulose at 20, 100 and 500 mg/kg bw/d and a control group was treated with vehicle alone (5 mL/kg bw). Suspensions were prepared two days before first usage and used approximately 4 days. Compound concentrations of two preparations were confirmed analytically, and proved to be within 7% of nominal concentration. The suspension was homogenous and stable for 7 days. Throughout the study, the females were observed at least daily for mortality and overt changes in appearance and behaviour. The presence and duration of clinical signs of toxicity were recorded once daily. Individual maternal bodyweights were recorded on gestation days 0, 5, 6, 12, 18, 24 and 29. Food consumption was measured by weighing the feeder every other day. Immediately following sacrifice on gestation day 29, animals were dissected and examined for congenital abnormalities and macroscopic pathological changes in maternal organs. Uterine horns, foetuses and ovaries were exposed by an abdominal incision and the number and location of viable and nonviable foetuses, early and late resorptions and corpora lutea were recorded. The gravid uterus was then excised, weighed and the foetuses removed. Foetuses were individually weighed, sexed, tagged and examined for external malformations and variations. For approximately one third of the foetuses, decapitated heads were fixed in Bouin's solution and examined using a modified Wilson's sectioning technique. All received a wet visceral examination, and all fetal carcasses were processed for skeletal evaluations. Bodyweight, uterus weight, bodyweight change and food consumption of adult animals were analysed by significance tests employing analysis of variance (ANOVA) for repeated measures or a multivariate ANOVA. For viability data, a one-factor ANOVA was used for corporea lutea, total implants, the percent live implants, the percent resorptions, and percent pre-implantation loss. In the presence of significant main effects, all post

hoc comparisons between the treated and control group were conducted using Dunnett's test. Skeletal, visceral and gross external malformation data were statistically analysed by Chi-square when the incidence in the treatment groups was higher than controls.

Findings:

Clinical signs related to treatment included scant faeces in 2/16 mid dose and 16/17 high dose animals concomitant with reduced food intake (Table 137). However, scant faeces were also observed in 3/17 control animals. Two cases of diarrhoea were recorded in the high dose group. The bloody urine recorded for one mid dose and 12 high dose animals was considered to be vaginal discharge associated with abortion of foetuses. Other observations were considered incidental and unrelated to treatment.

One animal in the high dose and control group respectively died during the study. Ruptured esophagi indicated that these deaths resulted from gavage trauma and were not substance related. No gross lesions were seen at necropsy of the study animals that survived until the end of the study.

Table 137: Clinical observations in maternal rabbits

Clinical observation	**Dose level (mg/kg bw/d)**			
	0	20	100	500
Number of sperm positive does	17	16	16	17
Death	1	-	-	1
Scant feces	3	-	2	16
Redness around nose fur	-	1	-	-
Hypoactivity	-	1	-	-
Bloody urine	-	-	1	12
Hair loss (Abdominal)	-	-	-	1
Diarrhea	-	-	-	2
Malocclusion	1	-	-	-
Ocular Opacity	-	-	-	1

Bodyweight gain was reduced in the high- and mid dose groups throughout the experiment also after termination of dosing (Table 138). During dosing, bodyweight loss was observed in these groups. In the low dose group, the bodyweight changes were comparable with those of the control throughout the treatment (gestation days 6 through 18) and overall gestation (gestation days 0 to 29) periods. Corresponding to the bodyweight data, food consumption was reduced in the high and mid dose groups during treatment period and improved later on. No difference from control was noted in the low dose group.

Table 138: Maternal cumulative bodyweight gain (g)

	Dose level (mg/kg bw/day)			
	0	20	100	500
Number of animals[§]	14	14	14	15
Weight gain Day 0-Day 6	0.14	0.10	0.13	0.17
Weight gain Day 0-Day 12	0.22	0.14	0.09*	-0.30*
Weight gain Day 0-Day 18	0.36	0.29	0.18*	-0.27*
Weight gain Day 0-Day 24	0.47	0.43	0.30*	-0.25*
Weight gain Day 0-Day 29	0.55	0.56	0.42*	-0.11*

*, $p < 0.05$ significantly different from control group; §, gravid animals

Significantly decreased uterine weights were noted in the high dose (500 mg/kg bw/d) group only (Table 139). No signs of maternal toxicity were observed at necropsy in the mid and low dose groups. Mean foetal weight, number of corpora lutea, live foetuses and viable litters were significantly reduced and the number of *in utero* deaths was significantly elevated in the high dose group but in the other treatment groups these were comparable to controls.

Table 139: Cesarean section observations

Observations	**Dose level (mg/kg bw/day)**			
	0	20	100	500
No. assigned (sperm-positive)	17	16	16	17
Females gravid	14	14	14	15
Viable litters	13	14	12	5
Corpora lutea/dam	10.2	10.8	10.0	8.5 [a]
Total implantation/dam	9.8	9.6	9.0	10.2
Live foetuses/dam	8.4	8.6	8.0	0.9*
Post implantation loss	1.34	1.07	1.0	9.26*
Mean uterus weight (kg)	0.55	0.59	0.52	0.09*
Sex ratio (% male)	49.2	44.2	52.7	57.1
Foetal weight (g)	44.3	45.6	42.1	28.6*

*, p <0.05 significantly different from control group; a, sic! Animals 270, 271, and 277 were reported to have 0 corpora lutea and 14, 12, or 7 implants, respectively.

Foetal abnormalities were significantly more frequent in foetuses of high dose animals as compared to controls, low and mid dose treatment groups. Consistent with the low foetal weight in the high dose group, foetuses had domed shaped heads. Additional gross external foetal malformations, consisting of intestines and liver outside body, umbilical hernia with exposed intestines, clubbed feet/forelimbs, absence of forelimbs (abrachia) or forelimbs digits, and absence of eyelids, were seen only in the high dose group. Hypoplasia or absence of cerebellum was seen in all dose groups including the control group, in the latter, the highest incidence of this finding was seen. The skeletal malformations in the pups of the control group had fused ribs or fused thoracic centrae. In the mid dose group fused ribs (2 pups) and fused vertebrae (one of the aforementioned) were seen. Anomaly findings in the high dose pups were incompletely ossified skull bones and enlarged fontanels. The animal with abrachia missed, of course, the respective bones. No historical control data were included in the study report.

Table 140: Summary of gross external, visceral and cephalic anomalies

	Study Group			
	Vehicle Control	ATI-720 (mg/kg)		
	F/L[a]	20 F/L	100 F/L	500 F/L
Number Examined:	118/13	120/14	112/12	14/5
Gross External Variations				
Dome-Shaped Head	-/-[b]	-/-	-/-	6/2
Malformations				
Spinal bifida	-/-	-/-	1/1	-/-
Gastroschisis/Umbilical Hernia	-/-	-/-	-/-	4/3
Ectodactyly	-/-	-/-	-/-	1/1
Ablepharia	-/-	-/-	-/-	1/1
Clubbed feet	-/-	-/-	-/-	2/1
Abrachia	-/-	-/-	-/-	1/1
Visceral Malformations				
Heart, Malformed	1/1	-/-	-/-	-/-
Subclavions absent	-/-	-/-	-/-	1/1
Kidney, Absent	-/-	1/1	-/-	-/-
Total Gross and Visceral Malformations	1	1	1	5
% Malformations	0.85	0.83	0.89	36.0
Number Examined:	40/13	40/14	37/12	5/5
Cephalic Malformations				
Cerebellum, Hypoplastic/Absent	10/7	7/4	5/3	-/-
Hydrocephaly	4/4	2/2	5/4	2/2
Anencephaly	-/-	-/-	-/-	1/1
Cerebrum, Hypoplastic	1/1	1/1	-/-	1/1
Total Cephalic Malformations	14	9	8	3
% Malformations	35	23	22	60

[a] F/L = Number of Fetuses (F)/Number of Litters (L)
[b] -/- = zero incidence

Table 141: Summary of skeletal anomalies

	Study Group			
	Vehicle Control	ATI-720 (mg/kg)		
	F/L[a]	20 F/L	100 F/L	500 F/L
Number Examined:	80/12	80/14	77/12	9/5
Skull				
Any Variation	-/-[b]	-/-	2/2	7/4
% Affected	-/-	-/-	3/17	78/80
Body of	80/12	80/14	77/12	9/5
Any Variation	-/-	-/-	1/1	-/-
% Affected	-/-	-/-	1/8	-/-
Number Examined:	118/13	120/14	112/12	14/5
Sternabrae				
Any Variation	49/10	55/14	31/10	5/2
% Affected	42/77	46/100	28/83	36/40
Ribs				
12 pairs, normal	43/10	53/12	31/9	1/1
% 12 pairs	36/77	44/86	28/75	7/20
13 pairs, normal	38/7	37/11	39/10	10/5
% 13 pairs	32/54	31/79	35/83	71/100
13th pairs, variations	19/10	15/10	21/8	1/1
% Affected	16/77	13/71	19/65	7/20
13th unilateral variations	17/7	16/8	18/9	2/2
% Affected	14/54	13/57	16/75	14/40
Any Other variations	1/1	-/-	-/-	-/-
% Affected	1/8	-/-	-/-	-/-
Malformation	1/1	-/-	2/2[c]	-/-
% Affected	1/8	-/-	2/17	-/-
Thoracic Centrae				
Any Variation	-/-	-/-	1/1	-/-
% Affected	-/-	-/-	1/8	-/-
Any Malformation	1/1	-/-	-/-	-/-
% Affected	1/8	-/-	-/-	-/-

[a] F/L = Number of Fetuses (F)/Number of Litters (L)
[b] -/- = zero incidence
[c] One of the two fetuses had spinal bifida

	Study Group			
	Vehicle Control	ATI-720 (mg/kg)		
	F/L[a]	20 F/L	100 F/L	500 F/L
Number Examined:	118/13	120/14	112/12	14/5
Thoracic Vertebrae				
Any Variation	-/-[b]	-/-	1/1	-/-
% Affected	-/-	-/-	1/8	-/-
Any Malformation	-/-	-/-	1/1[c]	-/-
% Affected	-/-	-/-	1/8	-/-
Pectoral Girdle				
Any Variation	-/-	-/-	-/-	1/1
% Affected	-/-	-/-	-/-	7/20
Any Malformation	-/-	-/-	-/-	1/12[d]
% Affected	-/-	-/-	-/-	7/20
Pelvic Girdle				
Any Variation	-/-	-/-	-/-	-/-
% Affected	-/-	-/-	-/-	-/-
Any Malformation	-/-	-/-	-/-	1/1[d]
% Affected	-/-	-/-	-/-	7/20
Total Malformed	2/2	-/-	2/2	1/1
% Affected	2/15	-/-	2/17	7/20

[a] F/L = Number of Fetuses (F)/Number of Litters (L)
[b] -/- = zero incidence
[c] fetus with spinal bifida
[d] same fetus, consistent with gross external appearance ectodactyly, clubbed feet, and abrachia

Conclusions:

Based on the reduced bodyweight and food consumption in the high dose and mid dose group the no observable adverse effect level was 20 mg ATI-720/kg bw/d for maternal effects.

Significant signs of developmental toxicity were observed in the high dose group only and may be related to maternal toxicity. No effects on foetal number and development were observed in the mid dose and low dose group. Thus, the NOAEL for developmental toxicity was 100 mg/kg bw/day.

9.10.2.2 Human information

No studies submitted by the notifiers.

9.10.3 Other relevant information

No studies submitted by the notifiers.

9.11 Other effects

9.11.1 Non-human information

9.11.1.1 Neurotoxicity

Studies performed with NeemAzal

Reference:	TRF IIA 5.7.3 / 01
Report:	Chandrasekaran, R. (1998)
	Neurotoxicity study with NEEMAZAL technical (27.3% azadirachtin) in chicken
	Fredrick Institute of Plant Protection and Toxicology, Padappai, 601301 Tamil Nadu, India
	unpublished report No. 4813; TOX1999-226
Guidelines:	Gaitonde Committee Guideline 6.3.0.C.i
	Similar to OECD Guideline 419 (Delayed neurotoxicity of organophosphorus substances: 29-day repeated dose study)

Deviations:	Only 21 days of dosing (instead of 28 days), 21 days of recovery (instead of 14 days). Neuropathy target esterase activity not measured. Only three hens per group and treatment duration instead of 6 animals. Acetylcholinesterase measured in serum and red blood cells. No *in situ* fixation of neuronal tissue by perfusion. Clinical observations reported in appendix I are in unreadable small print.
GLP:	No
Acceptability:	The study is considered to be not acceptable.

Material and Methods

In a dose finding pilot study, two groups of each three White leghorn layers (*Gallus domesticus*, animals provided by Poultry Research Station, Tamil Nadu Veterinary and Animal Sciences University, India) were treated with single doses of 5000 or 10000 mg/kg bw of an aqueous suspensions of NeemAzal technical (batch no.: CC86; purity: 27.3% azadirachtin A+B). Birds were observed for signs of toxicity and mortality for seven days. In the main study, three groups of six white leghorn chicken each were dosed daily by gavage with aqueous suspensions of NeemAzal technical at dose levels of 0, 500, 750 and 1000 mg/kg bw/d for 21 days. The control group received distilled water (10 mL/kg bw). On day 22, 50% of the birds were sacrificed and the remaining birds were observed for another 21 days. On day 43 all birds were sacrificed. The following parameters of neurotoxicological relevance were investigated: a daily behavioural test for locomotive ataxia, activity of acetylcholine esterase in blood and serum on day 0, 23 and 43. Histopathological examination of the brain (cerebrum, cerebellum, medulla oblongata), spinal cord (thoracic, cervical, lumbo-sacral) and sciatic nerve (proximal to distal length on either side) following sacrifice. In addition, animals were observed for clinical signs of toxicity, bodyweights and food consumption as well as number and weight of eggs laid were noted. Haematological and biochemical parameters were investigated. Bodyweight, food consumption, egg weight, egg yield, haematological and biochemical parameters were analysed by significance tests (student's t test) comparing treated and control groups.

Findings

In the dose finding study, birds were observed for signs of toxicity and mortality for seven days but no effects were noted in both groups during the observation period. In the main study, no treatment induced effects were observed regarding mortality, clinical signs, bodyweight, feed consumption, and egg yield/weight. No ataxia was seen in treated groups of birds throughout the observation period. There were no remarkable changes in haematological and biochemical parameters including acetylcholinesterase (serum and red blood cells) of the treated birds compared with control animals. Gross pathology and histopathology revealed no treatment induced lesions.

Conclusions

After treatment of chicken with NeemAzal technical a NOAEL of 1000 mg/kg bw/d was established in this study.

Studies performed with Fortune Aza

No studies submitted by the notifiers.

Studies performed with ATI 720

No studies submitted by the notifiers.

9.11.1.2 Immunotoxicity

No studies submitted by the notifiers.

9.11.1.3 Specific investigations: other studies

No other/special studies were submitted.

Neem extracts were found to be contaminated with aflatoxins. Aflatoxins B_1, B_2, G_1, and G_2 are mycotoxins that may be produced by three moulds of the *Aspergillus* species: *A. flavus, A. parasiticus* and *A. nomius,* which contaminate plants and plant products. Of the aflatoxins, aflatoxin B_1 is the most frequent one present in contaminated samples and aflatoxins B_2, G_1 and G_2 are generally not reported in the absence of aflatoxin B_1.

Toxicological properties of aflatoxins were assessed and described extensively by international scientific bodies (e.g., Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1998 [WHO FOOD ADDITIVES SERIES 40], IARC in 1993 and 2002 [IARC Monographs Vol. 56, p. 245 and Vol. 82, p. 171]). Aflatoxins are genotoxic carcinogens. Aflatoxins B_1 and G_1 can be activated by cytochrome P_{450} enzymes, leading to epoxides which can bind covalently to DNA. The International Agency for Research on Cancer (IARC) has concluded that naturally occurring aflatoxins are carcinogenic to humans (group 1), with a role in aetiology of liver cancer, notably among subjects who are carriers of hepatitis B virus (HBV) surface antigens. In experimental animals there was sufficient evidence for carcinogenicity of naturally occurring mixtures of aflatoxins and of aflatoxins B_1, G_1 and M_1, limited evidence for aflatoxin B_2 and inadequate evidence for aflatoxin G_2. The principal tumours were in the liver, although tumours were also found at other sites including the kidney and colon. AFB_1 is consistently genotoxic *in vitro* and *in vivo* (IARC, 1993 and 2002).

Hence, exposure to aflatoxins should stay as low as reasonable achievable. In the EU there are regulations on the acceptable maximum level of aflatoxins in food (Regulation (EC) No. 1881/2006):

- for groundnuts to be subjected to sorting, or other physical treatment, before human consumption or use as an substance in foodstuffs there is a maximum limit of 15 µg/kg (sum of B_1, B_2, G_1 and G_2)
- for food (nuts, dried fruit, maize) to be subjected to sorting, or other physical treatment, before human consumption or use as an substance in foodstuffsthere is a maximum limit of 10 µg/kg (sum of B_1, B_2, G_1 and G_2)
- for food (dried fruit, all cereals, groundnuts and nuts and processed products thereof) intended for direct human consumption or use as an substance in foodstuffs there is a maximum limit of 4 µg/kg (sum of B_1, B_2, G_1 and G_2)

It is proposed to set the maximum level relative to the Aza A level, i.e, to set a maximum level of 300 µg aflatoxin (sum of B_1, B_2, G_1 and G_2) per kg Aza A in the specification of the technical extract. The plant protection products have a content of 1% or 3% Aza A for NeemAzal-T/S or Fortune Aza 3% EC / ORIS-Aza. This would lead to an aflatoxin content of 3 µg/kg NeemAzal-T/S or 9 µg/kg Fortune Aza 3% EC / ORIS-Aza.

The plant protection products are not intended for intake as food. They are used up to 3 times with intervals of 5-10 days. Therefore, it is considered acceptable to have concentrations of aflatoxins in the products as stated above.

9.11.1.4 Human information

No studies submitted by the notifiers.

9.11.2 Report on medical surveillance on manufacturing plant personnel

9.11.2.1 NeemAzal

Reference: TRF IIA 5.9.1 / 01

Report: Venkataram, T. V. (2002)

Employees Health Record 2001

EID Parry India Ltd., Cuddalore, India

TOX2005-2337

Acceptability: The report is considered to be acceptable.

Monthly observations on 15 employees working in the NeemAzal production at the company EID Parry in India are presented as a summary. With 64 parameters routinely tested, no adverse occupational health effects were reported.

Reference: TRF IIA 5.9.1 / 02

Report: Venkataram, T. V. (2003)

Employees Health Record 2002

EID Parry India Ltd., Cuddalore, India

TOX2005-2338

Acceptability: The report is considered to be acceptable.

Monthly observations on 17 employees working in the NeemAzal production at the company EID Parry in India are presented as a summary. With 64 parameters routinely tested, no adverse occupational health effects were reported.

Reference: TRF IIA 5.9.1 / 03

Report: Venkataram, T. V. (2004)

Employees Health Record 2003

EID Parry India Ltd., Cuddalore, India

TOX2005-2339

Acceptability: The report is considered to be acceptable.

Monthly observations on 17 employees working in the NeemAzal production at the company EID Parry in India are presented as a summary. With 64 parameters routinely tested, no adverse occupational health effects were reported.

9.11.2.2 Fortune Aza

Reference: SIP IIA 5.9.1 / 01

Report: Kumar, A. D. (2005)

Statement

Fortune Bio-tech Ltd., Secunderabad, India

Unpublished

TOX2005-2403

Acceptability: The report is considered to be acceptable.

It is stated that in seven years of manufacturing of neem extract with currently 42 employees exposed to the product, no adverse health effects were noted and no worker has fallen sick due to the process environment.

Reference:	SIP IIA 5.9.1 / 02
Report:	Mahesh, A. (2005)
	To whomsoever it may concern
	Sri Satya Sai Clinic, Secunderabad, India
	TOX2005-2404
Acceptability:	The report is considered to be acceptable.

It is stated that in five years of manufacturing of neem extract in a plant of the Fortune Biotech Ltd., located in RaigiriVillage, Nalgonda District of Andhra Pradesh State, India, no health effects including allergy or hypersensitivity of eyes, skin or respiratory tract or other symptoms of toxicity were noted. The workers have been exposed seasonally for 4-5 months per year.

9.11.2.3 ATI 720

No studies/information submitted by the notifiers.

9.11.3 Report on clinical cases and poisoning incidents

There are reports of intoxications from India and Malaysia including death or irreversible brain damage after treatment of children with neem seed oil. Signs of toxicity were seen within minutes or few hours after intake of an estimated volume of 5 to 50 mL neem oil as drug against a range of different diseases. Initial clinical signs included vomiting, convulsion, and at later stages metabolic acidosis with coma. Post-mortem examination revealed histological liver damage, such as lipid infiltration in hepatocytes, damage of mitochondria, and sometimes encephalopathy (Sundaravalli *et al.,* 1982, TOX2006-3064; Sinniah *et al.,* 1981, TOX2006-3062; Sinniah *et al.,* 1982, TOX2006-3061). In some reports, relatively high case numbers are given, e.g. more than 60 (supposed or verified) intoxications of children with neem oil within 5 years in one hospital in Madras/India (Sinniah *et al.,* 1981, TOX2006-3062). Neem oil is a common treatment in southern Asia, therefore, the incidence of cases with such severe adverse effects can not be judged. Clinical signs, occurrence in children often following an infection, and pathology results are similar to Reye-syndrome. It occurs rarely but most times after virus infections (influenza, chicken pox) and subsequent treatment with certain drugs (e.g., acetyl salicylic acid) (Sinniah & Baskaran, 1981, TOX2006-3060; Beers & Berkow, 1999, TOX2006-3056; Gerok, 1996, TOX2006-3058). A Reye-like syndrome was induced by treatment of rats and mice with neem oil. In contrast to humans, however, microsomal liver enzymes were not decreased, and brain oedema did not occur (Sinniah *et al.,* 1985, TOX2006-3063). The toxic substance and the mode of action are unknown. It was hypothesised that the neem substances picrin and nimbidin where the cause, but it could not be verified in experimental animals (Sundaravalli *et al.,* 1982, TOX2006-3064; Pillai & Santhakumari,

1984, TOX2006-3045). Aflatoxins B and G could be detected (250 – 1000 µg/kg) in crude neem oil (Sinniah *et al.*, 1981, TOX2006-3062; Jacobson, 1995, TOX2006-3059). Contamination with aflatoxins might explain the intoxications, as it is effective in relatively low concentrations and liver is one of its target organs, where it can induce acute liver toxicity (Westendorf, 1994, TOX2006-3065).

During the PPP peer-review, RMS was asked to provide more information on the medicinal use/clinical cases/poisoning incidences. Following further information was provided:

It is difficult to gain reliable information on the medical use of neem-derived products in India (and other countries). In open literature[2], similar lists of traditional uses according to Ayurveda are given in the various articles. The following list was taken from Ketkar & Ketkar (2002):

> Green leaves: Constipate, and thus causing production and accumulation of gas, but used as a cure or treatment for epistaxis, eye trouble and leprosy.
> Leaves: Advantageous for all types of eye troubles, intestinal worms, biliousness, toxic manifestations, lack of appetite, and leprosy, but also create gas.
> Old leaves: Generally relieve and heal boils and skin ulcers.
> Flowers: Suppress bile and eliminate intestinal worms and phlegm.
> Young twigs: Relieve coughs, asthma, piles, excess stomach gas. Also effective against worms and spermatorrhea.
> Unripe fruits: Bitter and pungent in taste, a mild irritant while undergoing metabolism; viscid, light and producing warmth in the system; effective against flatus accumulation, piles, intestinal worms and urinary troubles in general.
> Ripe neem fruit: Sweetish-bitter taste, relieve epistaxis, phlegm, eye troubles, wounds and when taken orally, have a soothing effect on the system. The seed kernels relieve leprosy and intestinal worms.
> Bark: An analgesic, alterative and curative of fever (liquid extract). All the five parts of the neem tree (leaves, fruits, bark, resin and root) are taken together.
> Neem toddy (gum): In some old trees, when they are fully mature, a kind of juice or toddy (gum) begins to flow out and in some cases continues to flow for one year or more. This thick juice is sweet in taste but has an unpleasant, pungent odor. It is a valuable medicine, used as a specific for skin diseases like scabies, wounds, ringworms, ulcers, etc.

Ketkar & Ketkar stated:

> "The neem tree has been used as a traditional remedy in Ayurvedic medicine in India since antiquity and medicinal properties have been ascribed especially to the leaves, fruits and bark […]. Neem oil and extracts of various parts of the neem tree, especially the bark and leaves, have been used in Indian folk medicine as a therapy for leprosy, intestinal helminthiasis and respiratory disorders in children […]. Occasionally it is administered for constipation and also as a general health promoter. It is also used for treatment of rheumatitis, chronic syphilitic scores and indolent ulcer […]. Furthermore, neem oil is used

[2] E.g, Ketkar & Ketkar, 2002, Medicinal uses including pharmacology in asia, in: Schmutterer: The neem tree, 2nd ed., Neem foundation, Mumbai
Biswas et al., 2002, Current science, 82, 1336-1345
Brahmachari, 2004, ChemBioChem, 5, 408-421
Singh & Singh, 2002, Journal of Herbal Pharmacotherapy, 2, 13-28

as an antiseptic and acaricide (parasiticide), and in various skin infections like ringworm and scabies, respectively […].

In the view of the curative properties attributed in folklore and traditional medicine to neem, it has been subjected to chemical and therapeutic studies from about the beginning of the present century.

Neem preparations have been used to treat blood disorders, hepatitis, eye diseases, cancer, ulcers, constipation, diabetes, indigestion, sleeplessness, stomach ache, boils, burns, cholera, gingivitis, malaria, measles, nausea, snakebites, rheumatism and syphilis […]. Numerous formulations are used as antiseptics, astringents, emollients, febrifuges, anodynes, diuretics, parasiticides, pediculicides, purgatives, sedatives, stomachics, and tonics […]. Neem products with these reported activities are available commercially" [c.f., Table 142].

Table 142: Selected neem-based commercial medicinal products in India (taken from Ketkar & Ketkar)

Product	Plant parts	Use	Manufacturer
'Nimbola'	Oil	Antihyperglycemic	Kee Pharma, New Delhi
'JK 22'	Leaf decoction	Diabetes mellitus, nonketonic diabetes	Charak Pharmaceuticals, Mumbai
'Clean'N Cure'	Leaf extract	Pimple cure	Dabur (India) Ltd., Ahmedabad
'Greneem Capsules'	Extract of neem leaves	Blood purifier, acne, skin disorders, bacterial and viral infections	Asoj Soft Caps Pvt. Ltd. Asoj Dist., Baroda
'Curoline'	Oil	Protective and soothing emollient	Chemicure Laboratories Pvt. Ltd., Udaipur
'Neem Cure'	Oil, leaf extract	Antiseptic	Excelsior Enterprises, Kanpur
'Kailas Jeevan'	-	Diseases caused by heat and acidity	Ayurvedic Sumsodhanalaya, Pune
'Pasutone'	Leaf powder	Intestinal worms (for veterinary use)	Domesto Pvt. Ltd., Vijaywada-4, Andhra Pradesh
'Marguentum Forte' ointment	-	Dermatological infections	Calcutta Chemical Co. Ltd., Calcutta
'Nemlent'	Oil	Wound dressing	Domesto Pvt. Ltd., Vijaywada-4, Andhra Pradesh
'Loquin' tablets	Leaf based extract	Chronic malaria	J. and J. Dechance Laboratories Pvt. Ltd., Hyderabad
'Olosyn'	-	Local sedative	J. and J. Dechance Laboratories Pvt. Ltd., Hyderabad

The RMS has no knowledge about the extent of the usage of neem-based medicinal products, nor on the constituents of the products or the safety and efficacy of their uses.