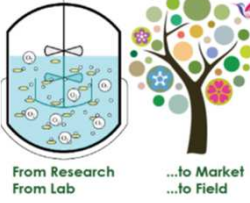



IPM-4-CITRUS



From Research
From Lab

...to Market
...to Field




Risk Assessment of New *Btk* Isolates BLB1 and LIP Formulations via Toxicity Assays on Lab Animals

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1



Overview



Introduction

- Project (IPM-4-Citrus)
- Btk* new isolates
- Objective of the study

Material & Method

- Test materials
- Action Plan
- Dermal irritation, Eye irritation and Skin sensitization tests

Results

- Dermal irritation, Eye irritation and Skin sensitization tests` results

Summary & Conclusion

- The new formulations are safe?

2

Introduction

IPM-4-CITRUS

From Research From Lab **...to Market ...to Field**

This project has received funding from the European Union's Horizon 2020 Research and Innovation programme under Grant Agreement No 734921 (2017-2023).

GOALS:

- Strengthening Academia & Industrial collaborations
- Optimising bioproduction processes for the new *Btk* isolates BLB1 (Tunisian) and LIP (Lebanon)
- Developing the new *Btk* isolates formulations in the Mediterranean region for Citrus pests

11 partners from 6 countries

3

Introduction

Germany

Lab-scale fermentation & formulation + Efficacy

Tunisia

Risk assesment



Türkiye

Risk assesment + Efficacy


4

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Introduction

Commonly employed, human and environmental toxicity tests provide a safe and highly effective method of biopesticide control.



 Toxicity assays on target and non-target organisms must be carried out by following **ISO** and **OECD** guidelines with **EU regulations**.

ISO: the International Organization for Standardization,
OECD: Organisation for Economic Co-operation and Development

5

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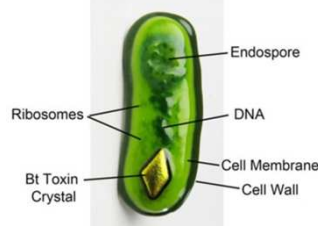
Introduction


Bt as biopesticide

- *Bacillus thuringiensis* (Bt) is the best known and widely used pesticidal bacteria with a high potential of better IPM.
- Described by [Berliner in 1911](#);
 - is a gram-positive and
 - spore-forming soil bacterium.
- Its **cryproteins** with **δ -endotoxins** are highly toxic to a wide variety of agricultural insect pests as well as other invertebrates ([Sauka and Benintende, 2008](#)).
- Registered for use in pesticides by the U.S. EPA since 1961.

Bacillus thuringiensis (Bt)



<https://u.osu.edu/cmifsud7588/2019/05/18/bacillus-thuringiensis/>



<https://doi.org/10.3390/toxins6123296>

6

IPM-4-CITRUS


Introduction

The new Btk isolates


- *Bacillus thuringiensis* ssp. *kurstaki* (Btk) is the most widely used to protect agricultural crops and fruit trees from defoliating lepidopteran larvae.
- In this project, two new Btk-based formulations efficiently targeting lepidopteran pests:
 - **BLB1** (Tunisia strain) and
 - **LIP** (Lebanon strain)

were produced at lab-scale as dried formulations at JKI Lab/ Germany.

Phyllocnistis citrella (Citrus leafminer)



Prays citri (Citrus flower moth; damaged ovarium of citrus flower)




7

IPM-4-CITRUS

Introduction

Objective

To support the development process of new Btk formulations (LIP and BLB1) by evaluating the risks to **Human Health** and the **Environment**.

→  Focused on toxicity assays via lab animals in the frame of human risk assessment

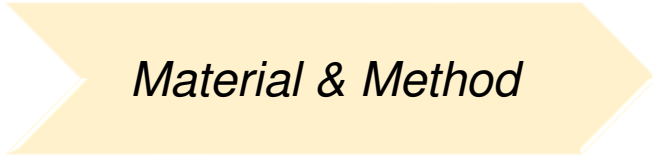
8

Introduction

First risk assessment tests conducted within the scope of IPM-4-CITRUS

Risk Assessment type	Tests		Risk characterization/ toxicity value
Human Risk Assessment	Acute and Subacute eye irritation/corrosion test (OECD 405, ISO10993-10:2010)		Clinical observation and grading eventually lesions
	Acute dermal irritation/corrosion test (OECD 404, ISO10993-10:2010)		
	Skin sensitization test (ISO 10993-10:2010)		
	Oral toxicity test (OECD 407)		
Environmental Risk Assessment	Aquatic microorganisms	Freshwater Daphnids (<i>Daphnia magna</i>)	50% effect concentration (EC50)
		Luminescent Bacteria (<i>Aliivibrio fischeri</i>)	
	Terrestrial invertebrates	Freshwater Algae (<i>Pseudokirchneriella subcapitata</i>)	
		Soil Worm	




9



Material & Method

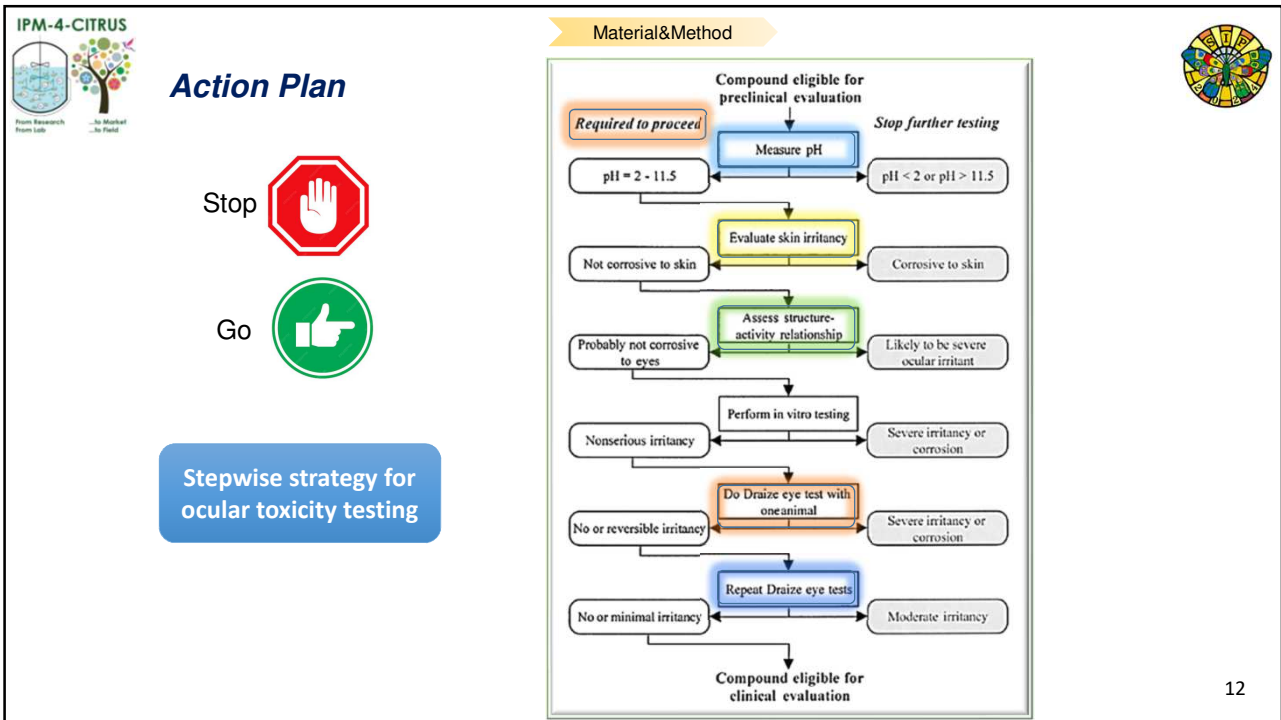
10

IPM-4-CITRUS Material&Method

TEST NAME (In vivo)	STANDARDS (guidance & regulations)	Risk characterization	Number of animals per product	Strain, age and weight of animals
 Ocular (eye) Irritation/corrosion	OECD 405 (2020) ISO10993-10:2010 (ISO10993-23:2021)*	Clinical observation and grading reactions	3 Rabbits/ acute (single exposure) 3 Rabbits/ subacute (repeated exposure)	New Zealand, 4-4.5 months, 2-3 kg
 Acute Dermal Irritation/corrosion	ISO10993-10:2010 (ISO10993-23:2021)*	Clinical observation and grading reactions	3 Rabbits (single exposure)	New Zealand, 4-4.5 months, 2-3 kg
 Skin Sensitization	ISO10993-10:2010 (ISO10993-10:2021)*	Clinical observation and grading reactions	10 Guinea pigs (single exposure), 5 Guinea pigs as a control	Dunkin-Hartley, 300-500 g

ISO10993-10:2010:Biological evaluation of medical devices —Part 10: Tests for irritation and skin sensitization
OECD 405: Test Guideline No. 405 Acute Eye Irritation/Corrosion

11



IPM-4-CITRUS

Material&Method

Formulations used for the Risk Assessment

Formulation features	DELFIN-WG Positive control-1 (Golden standard)	BACTOSPEIN-DF Positive control-2	BLB1 (2021) Tunisian strain	LIP (2021) Lebanon strain	BLANK (2021) co-formulants
Active ingredients (Btk solids, spores, endotoxins)	85%	54%	14%	14%	No active ingredients
Other ingredients	15%	46%	86%	86%	100%
Potency /CFU	32000 IU/mg Btk	32000 IU/mg Btk	-	-	-
Form	Wettable granules	Dry Flowable	Wettable granules	Wettable granules	Wettable granules
Producer/Country	Certis/ USA	Valent /USA	JKI/ Germany	JKI/ Germany	JKI/ Germany
Date of production	30.09.2020	11.2020	29.06.2021	16.04.2021	30.06.2021
Date of expiry	30.09.2022	11.2023	-	-	-
Field Usage dose for citrus	100g/ 100L	75g/ 100L	-	-	-
pH	4.92	4.29	5.58	6.26	5.85

eligible for the toxicity tests ✓

13

IPM-4-CITRUS

Material&Method

Morpho-granulometric analysis of the formulations

Wet way

20 µL



Formulation
~ 1mg/1ml
distilled water

SOP

Measurement control	Fixed number of slides/plates A new result for each slide/plate
Sample carrier	4-slide plate (75x25mm) / Compensate for plate tilt / Coverslip (WW)
Illumination	Diascopic light source (bright mode) Light intensity: 80% ± 0.2
Optic selection	x 10 (WW)
Threshold	~ 180 (WW)
Scan area (square)	10 x 10 mm ²
Analysis setting	Trash size: 2 pixels No fill holes / No watershed
Filters	None


(x10, BF, 10x10 mm²)


14

Material&Method



Acute Dermal Irritation/ Corrosion Test (single exposure)






TEST NAME (In vivo)	STANDARDS (guidance & regulations)	Risk characterization	Number of animals per product	Strain, age and weight of animals
Acute Dermal Irritation/corrosion	ISO10993-10:2010 (ISO10993-23:2021)*	Clinical observation and grading reactions	3 Rabbits (single exposure)	New Zealand, 4-4.5 months, 2-3 kg

15

Material&Method



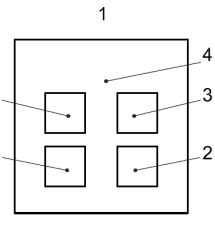
Animal husbandry → ISO 10993-2

Preparation of test materials and animals → ISO 10993-10:2010


Acute Dermal Irritation/ Corrosion Test (single exposure)	
Base concentration (g/L)	1g/L (DELFIN golden standard dose)
Final concentration (mg/mL)	X200 fold suspension (200mg/mL)
Dose volume	500µL/patch
Dose location	Dorsal region on 4 patches /rabbit
Patch size	2,5 cm x 2,5 cm per patch
Instillation	Single exposure
Exposure time	4h
Observation	1h , 24h, 48h, 72h following removal of the patches
Scale for scoring reactions & grading system	ISO 10993-10:2010; in vivo skin irritation test

Key

- 1 cranial end
- 2 test site
- 3 control site
- 4 clipped dorsal region
- 5 caudal end



Location of skin application sites



16

IPM-4-CITRUS

Material&Method

Acute dermal irritation/corrosion test (single exposure)
ISO 10993-10:2010; skin reaction scoring system

Reaction	Irritation score
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate erythema	3
Severe erythema (beet-redness) to eschar formation preventing grading of erythema	4
Oedema formation	
No oedema	0
Very slight oedema (barely perceptible)	1
Well-defined oedema (edges of area well-defined by definite raising)	2
Moderate oedema (raised approximately 1 mm)	3
Severe oedema (raised more than 1 mm and extending beyond exposure area)	4
Maximal possible score for irritation	8
Other adverse changes at the skin sites shall be recorded and reported.	

17

IPM-4-CITRUS

Material&Method

PRIMARY IRRITATION INDEX (PII) Formula for Dermal Irritation Test

European Centre for Ecotoxicology and Toxicology of Chemicals
(ECETOC Technical Report No.66)


The skin irritation potential of chemicals is often summarised as the "Primary Irritation Index" (PII) calculated from erythema and oedema grades.

PII's are not always calculated the same way. For the purpose of this data bank, PII was calculated according to the following formula :

$$PII = \frac{\sum \text{ERYTHEMA at 24/48/72 hrs} + \sum \text{OEDEMA at 24/48/72 hrs}}{3 \times \text{no. of animals}}$$

The maximum possible PII is 8.

18




From Research
From Lab


...to Market
...to Field

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Material&Method


Acute and Subacute Eye Irritation/ Corrosion Tests





TEST NAME (In vivo)	STANDARDS (guidance & regulations)	Risk characterization	Number of animals per product	Strain, age and weight of animals
Ocular (eye) Irritation/corrosion	OECD 405 (2020) ISO10993-10:2010 (ISO10993-23:2021)*	Clinical observation and grading reactions	3 Rabbits/ <i>acute</i> (single exposure) 3 Rabbits/ <i>subacute</i> (repeated exposure)	New Zealand, 4-4.5 months, 2-3 kg

19



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Material&Method

Acute eye irritation/corrosion test (single exposure)

Base concentration (g/L)	1g/L (DELFIN golden standard dose)
Final concentration (mg/mL)	X200 fold suspension (200mg/mL)
Dose volume	100µL
Dose location	into the lower conjunctival sac
Eyelide closure	one second
Instillation	1 instillation on the 1st day (single exposure)
Observation	1h and during 14 days post-instillation
Scale for scoring reactions & grading system	OECD-405 (Based on Draize Grading scale)







Animal husbandry


→ ISO 10993-2

Preparation of test materials and animals

→ ISO 10993-10:2010







20



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Material&Method




Subacute eye irritation/ corrosion test (repeated exposure)


Base concentration (g/L)	1g/L (DELFIN golden standart dose)
Final concentration (mg/mL)	X30 fold suspension (30mg/mL)
Dose volume	100µL
Dose location	into the lower conjunctival sac
Eyelide closure	one second
Instillation	Every single day during 5 day-period
Observation	Every single day during 14 day-period
Scale for scoring reactions & grading system	ISO 10993-10-2010* (Based on Draize Grading scale)

Animal husbandry
→ ISO 10993-2

Preparation of test materials and animals
→ ISO 10993-10:2010




21



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Material&Method



Grading Scale And Multiplication Factor For Eye Irritation Test
(Draize et al, 1944)


I. Cornea
 Opacity
 Area of cornea involved

II. Iris (reacting to light?)

III. Conjunctivae
 Redness
 Chemosis (Oedema)
 Discharge


I. Cornea		Grade
A. Opacity - degree of density (area most dense taken for reading):		
No opacity		0
Scattered or diffuse area, details of iris slightly obscured		1
Easily discernible translucent areas, details of iris slightly obscured		2
Opalescent areas, no details of iris visible, size of pupil barely discernible		3
Opaque, iris invisible		4
B. Area of cornea involved:		
One quarter (or less) but not zero		1
Greater than one quarter but less than half		2
Greater than half but less than three quarters		3
Greater than three quarters, up to whole area		4
Score (A x B) x 5		Total Maximum = 80
II. Iris		
C. Values:		
Normal		0
Folds above normal, congestion, swelling, circumcorneal injection (any or all of these or combinations of any thereof), iris still reacting to light (sluggish reaction is positive)		1
No reaction to light, haemorrhage, gross destruction (any or all of these)		2
Score C x 5		Total Maximum = 10
III. Conjunctivae		
D. Redness (refers to palpebral conjunctivae only):		
Vessels normal		0
Vessels definitely injected above normal		1
More diffuse, deeper crimson red, individual vessels not easily discernible		2
Diffuse beet red		3
E. Chemosis (Oedema):		
No swelling		0
Any swelling above normal (includes nictating membrane)		1
Obvious swelling with partial eversion of lids		2
Swelling with lids about half closed		3
Swelling with lids about lids about half closed to completely closed		4
F. Discharge:		
No discharge		0
Any amount of difference from normal (does not include small amounts observed in inner canthus of normal animals)		1
Discharge with moistening of the lids and hairs just adjacent to lids		2
Discharge with moistening of the lids and hairs, and considerable area around the eye		3
Score (D + E + F) x 2		Total Maximum = 20
Maximum possible score = 80 + 10 + 20 = 110		

22




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Material&Method



EYE IRRITATION CLASSIFICATION SYSTEM

(Based on Draize eye irritation evaluation by Kay&Calandra, 1962)

Ocular irritation (status of the cornea, iris and conjunctiva) on the treated eye was evaluated at 1 hour and at 1, 2, 3, 4 and 7 days post instillation (Draize et al., 1944). Individual scores were recorded for each animal. The time interval with the highest mean score (Maximum Mean Total Score – MMTS) for all rabbits was used to classify the test substance by the system of Kay and Calandra (1962) (Table 2).

Max.Mean Total Score

Formula for MMTS:

Σ Max. Mean total Score for each rabbit /
number of rabbits


Table 2

Classification of eye irritation scores from the Draize eye irritation test

MMTS*	Irritation classification
0.0–0.5	Non-irritative
0.6–2.5	Practically non-irritative
2.6–15.0	Minimally irritative
15.1–25.0	Mildly irritative
25.1–50.0	Moderately irritative
50.1–80.0	Severely irritative
80.1–100.0	Extremely irritative
100.1–110	Maximally irritative


*MMTS = Maximum Mean Total Score

23




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Material&Method




Skin Sensitization Test on Guinea pigs; GPMT (single exposure)



TEST NAME (In vivo)	STANDARDS (guidance & regulations)	Risk characterization	Number of animals per product	Strain, age and weight of animals
Skin Sensitization	ISO10993-10:2010 (ISO10993-10:2021)*	Clinical observation and grading reactions	10 Guinea pigs (single exposure), 5 Guinea pigs as a control	Dunkin-Hartley, 300-500 g

24

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منهجية باسستور تونس
Industrial Production of Tonic


BIYANS

KCBAY

Animal husbandry
→ ISO 10993-2





Preparation of test materials and animals
→ ISO 10993-10:2010

Material&Method



Skin Sensitization assay on Guinea pigs (single exposure)

Base concentration (g/L)	
Final concentration (mg/mL)	X250 fold suspension (250mg/mL isotonic 0.9% NaCl)
Dose volume	100µL/intradermal injection; 100µL/subcutaneous
Dose location	Dorsal region: 1 Intradermal and 2 Subcutaneous
Instillation	Single exposure
Exposure time	
Observation	After 24h and 48h
Scale for scoring reactions & grading system	ISO 10993-10:2010; in vivo skin sensitization test (GPMT, Magnusson and Kligman rating scale)







Intradermal injections (ID) are administered **into the dermis**, just below the epidermis.

Subcutaneous (SC) injections are administered **into the adipose tissue** layer just below the epidermis and dermis.

25

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منهجية باسستور تونس
Industrial Production of Tonic

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Material&Method



Skin Sensitization Test Rating Scale (single exposure)

Magnusson and Kligman rating scale

Patch test reaction	Rating scale
No visible change	0
Discrete or patchy erythema	1
Moderate or contiguous erythema	2
Evident	3
Severe erythema or swelling	4

26

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Results

27

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Results

Morpho-granulometric analysis of the formulations

MG-analysis → Composite images (x10, BF, 10x10 mm²)

Coarse population (number / concentration)-Wet Way of granulometry

Wet-Way	Raw	Filter 1 (<20 pixels)	Coarse (Dce>30µm)	Coarse [%]	D(4,3)
BLB1 2021	12229	5080	86	1,7%	58,72
LIP 2021	45869	14641	41	0,28%	41,01
Delfin 2022	52852	19646	1342	6,83%	61,8
Bactospeine	363412	102268	441	0,43%	81,01


Raw : raw data extracted from morphogranulometer (no filter)

Filter 1 (<20 pixels): application of a filter to remove noise background. In Wet way (WW), magnification used is x10 (with 0.28µm/pixel) it means that particles with size <1.4µm will be removed.


Dce > 30µm : the critical diameter is used to quantify the number of particles with a size superior to 30µm. This data could be interpreted from the cumulative number and volume distribution.

D[4,3]: volume diameter.

28




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Results


Dermal Irritation/Corrosion Test (Single Exposure) Results



$$PII = \frac{\sum \text{ERYTHEMA at 24/48/72 hrs} + \sum \text{OEDEMA at 24/48/72 hrs}}{3 \times \text{no. of animals}}$$


Primary Irritation Index categories in a rabbit

Mean score	Response category
0 to 0,4	Negligible
0,5 to 1,9	Slight
2 to 4,9	Moderate
5 to 8	Severe




Formulation	(PII) Score	Response category
BLANK	0	Negligible (non-irritating)
DELFIN	0	Negligible (non-irritating)
BACTOSPEIN	0	Negligible (non-irritating)
BLB1	0	Negligible (non-irritating)
LIP	0	Negligible (non-irritating)

29




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Results

Acute Eye Irritation/ Corrosion Tests Results (Based on Draize Grading scale)





→

Calculation of Maximum Mean Total Score for DELFIN


Animal No	P2R1	Observation period (hour / day)						
		1 hour	24h	48h	72h	4th day	7th day	
Cornea	Opacity A	0	0	0	0	0	0	
	Area involved B	0	0	0	0	0	0	
	(AxB)x5	0	0	0	0	0	0	
Iris	Cx5 C	0	0	0	0	0	0	
	Conjunctiva Redness D	1	1	1	1	0	0	
	Chemosis E	0	0	0	0	0	0	
TOTAL	Discharge F	0	1	0	0	0	0	
	(D+E+F)x2	2	4	2	2	0	0	
	TOTAL	2	4	2	2	0	0	

Animal No	P2R2	Observation period (hour / day)						
		1 hour	24h	48h	72h	4th day	7th day	
Cornea	Opacity A	0	0	0	0	0	0	
	Area involved B	0	0	0	0	0	0	
	(AxB)x5	0	0	0	0	0	0	
Iris	Cx5 C	0	0	0	0	0	0	
	Conjunctiva Redness D	1	1	1	1	0	0	
	Chemosis E	0	0	0	0	0	0	
TOTAL	Discharge F	0	1	1	0	0	0	
	(D+E+F)x2	2	4	4	2	0	0	
	TOTAL	2	4	4	2	0	0	


Animal No	P2R3	Observation period (hour / day)						
		1 hour	24h	48h	72h	4th day	7th day	
Cornea	Opacity A	0	0	0	0	0	0	
	Area involved B	0	0	0	0	0	0	
	(AxB)x5	0	0	0	0	0	0	
Iris	Cx5 C	0	0	0	0	0	0	
	Conjunctiva Redness D	1	1	1	1	1	0	
	Chemosis E	0	0	0	0	0	0	
TOTAL	Discharge F	1	1	1	1	0	0	
	(D+E+F)x2	4	4	4	4	2	0	
	TOTAL	4	4	4	4	2	0	

"Maximum Mean Total Score" (MMTS) for DELFIN= (4+4+4) / 3 rabbits= 4

30




From Research
From Life



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Institut Pasteur de Tunisie

Results

Acute Eye Irritation/ Corrosion Tests Results



MMTS for **BLB1** = (0+0+0) / 3 rabbits = **0**

MMTS for **BLANK** = (0+0) / 2 rabbits = **0**

MMTS for **LIP** = (0+0+0) / 3 rabbits = **0**

MMTS for **BACTOSPEIN** = (0+0+0) / 3 rabbits = **0**


MMTS for **DELFIN** = (4+4+4) / 3 rabbits = **4**

Classification of eye irritation scores from the Draize eye irritation test


MMTS*	Irritation classification	
0.0-0.5	Non-irritative	→ 0 ; Non-irritative for BLB1, BLANK, LIP and BACTOSPEIN
0.6-2.5	Practically non-irritative	
2.6-15.0	Minimally irritative	→ 4 ; Minimally irritative for DELFIN
15.1-25.0	Mildly irritative	
25.1-50.0	Moderately irritative	
50.1-80.0	Severely irritative	
80.1-100.0	Extremely irritative	
100.1-110	Maximally irritative	

*MMTS = Maximum Mean Total Score

31




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Results

After 72h obsv. of post-instillation (x200)




BLANK
Acute




Non-irritative

LIP
Acute




Non-irritative

BLB1
Acute




Non-irritative

BACTO
Acute



Non-irritative



DELFIN
Acute



Minimally irritative

32

IPM-4-CITRUS

Results

Subacute Eye Irritation/Corrosion Test Results

MMTS for **BLB1** = (0+0+0) / 3 rabbits = **0**

MMTS for **BLANK** = (0+0) / 2 rabbits = **0**

MMTS for **LIP** = (0+0+0) / 3 rabbits = **0**


MMTS for **BACTOSPEIN** = (0+0+0) / 3 rabbits = **0**

MMTS for **DELFIN** = (0+0+0) / 3 rabbits = **0**

Classification of eye irritation scores from the Draize eye irritation test



MMTS*	Irritation classification
0.0-0.5	Non-irritative
0.6-2.5	Practically non-irritative
2.6-15.0	Minimally irritative
15.1-25.0	Mildly irritative
25.1-50.0	Moderately irritative
50.1-80.0	Severely irritative
80.1-100.0	Extremely irritative
100.1-110	Maximally irritative

*MMTS = Maximum Mean Total Score



0 ; **Non-irritative** for all formulations

IPM-4-CITRUS

BIYANS


KCBAY

Results

Magnusson and Kligman rating scale

Patch test reaction	Rating scale
No visible change	0
Discrete or patchy erythema	1
Moderate or contiguous erythema	2
Evident	3
Severe erythema or swelling	4

Formulations	Score	Reaction
BACTOSPEIN	0.49	No visible change
BLANK	0.30	No visible change
BLB1	0.51	No visible change
DELFIN	1.65	Discrete or patchy erythema
LIP	0.57	No visible change



Skin Sensitization Test Result


⚡

A couple of mortality was observed in → LIP and DELFIN



However, no mortality is expected for this test

???

Technical difficulties???



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Summary&Conclusion

36

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Summary&Conclusion

Summary

- **Dce > 30 μ m** : the number of particles with a size superior to 30 μ m
DELFIN (1342) > BACTOSPEIN (441) > BLB1 (86) > LIP (41)
- **Acute Dermal Irritation: 0 ; Non-irritating** for all formulations
- **Acute Eye Irritation: 0 ; Non-irritative** for BLB1, BLANK, LIP and BACTOSPEIN
4 ; Minimally irritative for DELFIN
- **Subacute Eye Irritation: 0 ; Non-irritative** for all formulations
- **Skin sensitization: 0 ; No visible change** for BLB1, BLANK, LIP and BACTOSPEIN
1.65; Discrete or patchy erythema for DELFIN
- No weight loss and reduction in feed and water consumption were observed in any of these tests

37




IPM-4-CITRUS Summary&Conclusion


Conclusion

- All the protocols received an early approval from the Ethical Committee
 - Ethical Turkish Authorities Approval
 - CEBM Ethical Committee Approval (IPT)
- The first evaluation of risk on human of the new *Btk* isolates BLB1 and LIP formulations is promising
- Further Risk characterization tests necessary as new *Btk* isolates BLB1 and LIP formulation processes are still in progress
- Considering The Skin Sensitization test needs to be carried out again

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Thanks for your kind attention


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22