

IPM-4-Citrus, Final Meeting (19<sup>th</sup> & 20<sup>th</sup> December 2022, Nabeul & Hammamet, TUN)

**IPM-4-CITRUS**

From Research  
From Lab

...to Market  
...to Field

CELEBRATES ITS 80<sup>th</sup> ANNIVERSARY  
AND CHANGES

# IDB2022

THE 20<sup>th</sup> INTERNATIONAL DAYS OF  
BIOTECHNOLOGY

December 19<sup>th</sup> - 20<sup>th</sup> 2022  
Hotel Occidental Platanos, Nabeul \*\*\*\*\*  
YASMINE HAMMAMET, TUNISIA

## Toxicity assays on Rabbit, Rat and Guinea pig: Biosafety assessment of new BLB1 and LIP *Btk*- based formulated products

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## Challenges in Plant Protection

- **Invertebrates** cause significant cultivated plant losses;
- **Plant Protection** is essential for securing the quality of crops;
- In 2006, a new legislative approved the use of pesticides, establishing a framework for Community action to achieve the sustainable use of pesticides by reducing the risks and impacts of pesticide use on human health and the environment and by promoting the use of Integrated Pest Management (IPM);
- Up-to-date with all aspects of the directive **2009/128/EC** adopted on 21 October) on **sustainable Use of Pesticides (SUD)**, an IPM program has been set up;


**There are specific criteria**

- for chemical substances and
- for micro-organisms

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## Challenge in Low-Risk Criteria Assessment

- The aim of this study is to support the process development of Btk-based new biopesticides by the evaluation of risks on Human and Environment;
- Possible (microbial Plant) products biosafety could be assessed only based on results studying the impact on health, food quality and environment;
- Toxicity assays on target and non-targets organisms must be carried out, according to ISO and OECD guidelines → EU regulations;

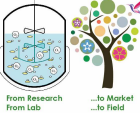
**An active substance can be approved as a low-risk substance if it meets the regular approval criteria and the low-risk criteria as specified in Annex II, point 5 of Regulation (EC) 1107/2009**



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## Criteria for approval of active substances

- **General rules (since 2011)**
- The **substitution principle** to be applied to the substances identified as candidates for substitution, as incentives for **low-risk active substances**;
  - The **zonal system** for the assessment and authorizations of plant protection products (**PPP**);
  - The mutual recognition of such authorizations with **strict deadlines**;


**The risk assessment to define at higher tiers the safe use are fundamental to define the conditions for use of pesticides in each crop**



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## Plant Protection Product Risk Evaluation

- **Eco-toxicology risk assessment**
  - **Risk Assessment for terrestrial and aquatic environment**
    - → PPP evaluation (cf. Dr Rim El Jeni presentation)

- **Toxicological assessment**
  - **Risk assessment for Human Health**
    - → PPP evaluation


**Effects on animal health and the environment must be evaluated**



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## Bacillus Thuringiensis as microbial biopesticide for sustainable agriculture

- Bt-based products have been recognized as valuable environment-friendly biopesticides because of the insecticidal proteins;
- Current and previous studies indicate their high potential for better Integrated Pest Management;
- Secreted  $\delta$ -endotoxins have insecticidal property and exert their toxic effects towards specific insect species;




**Targeted pests**  
Phyllocnistis citrella & Prays Citri  
Insect larvae



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## Scientific Background

- Two new *Bacillus thuringiensis*-based biopests, called *Bt kurstaki strains* efficiently targeting lepidopteron pests;
- Namely, BLB1 (from CBS) and LIP strains (from USJ) were recently formulated as dried products (JKI Lab);

### General Objective

- To assess their biosafety, toxicity assays were carried out on lab animals (i.e. rabbits, rats and guinea pigs), according to the European ISO and OECD guidelines;


**with an ultimate dual goal of risk and impact characterization**



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## Overview: Btk Risk characterization

	Tests	Faisability	Risk characterization/toxicity value	
Human Risk assessment	Acute and subacute eye assay (OECD 405)	✓		
	Acute dermal assay (OECD 404)	✓		
	Skin sensitization (ISO 10993-10 (2010))	✓		
	Oral toxicity test (OECD 425)	✓		
	Inhalation (EU regulation 528/2012 (8.7.2) or OECD 433 (2018)).	Needs inhalation Chamber	Clinical observation and grading of eventual lesions observed	
Environmental risk assessment	Aquatic organisms	Freshwater Daphnids ( <i>Daphnia magna</i> )	✓	50% effect concentration (EC50)
		Aliivibrio Fischeri luminescent bacteria	✓	
		Fish. (Zebrafish)	✓	
	Aquatic plants	Freshwater Algae ( <i>Pseudokirchneriella subcapitata</i> )	✓	
Terrestrial invertebrates	Warm	No applicable*		



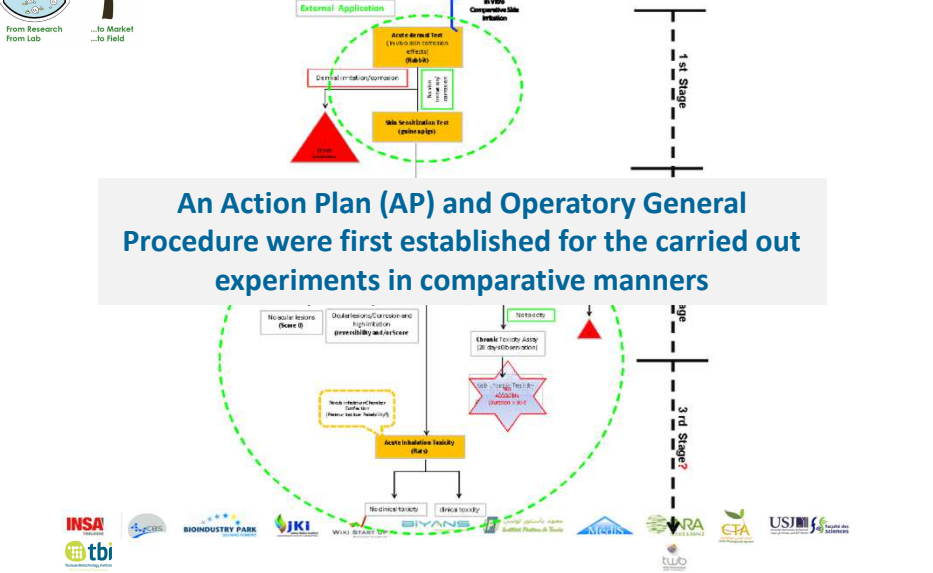
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## Action Plan : Stop and Go




An Action Plan (AP) and Operatory General Procedure were first established for the carried out experiments in comparative manners



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## Technical informations

- **Unformulated products**
  - **Liquid Formulations**

Concentrated fermented broth  
 Crude mixture  
 endotoxin  
 Containing  
 endotoxin  
**2mg/ml** ( )  
 For Bt-LIP pH value of 8.28  
 (compatible with dermal contact test)


- **Formulated products**
  - **Dried Formulation**

- Concentrated fermented of Btk mixtures

**BLB1 and LIP Btk-based products:**

2019-2020 batches: liquid forms  
 2021 batches: Dried forms  
 2022 batches: Optimized forms

- Additives  
 (non disclosures' agreement)



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## Toxicity assays on Lab animals


### Unformulated products (1)



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### Acute eye irritation test LIP Active ingredients ( $\delta$ -endotoxins and spores) (Unformulated liquid products, 2019-2020)


**STEP 1:** 100  $\mu$ l of Btk-LIP applied into lower conjunctival sac

**STEP 2:** Animals were examined for injury at 24, 48, and 72 hours

**After exposure:**  
No redness in conjunctiva  
No corneal opacity  
No conjunctival irritation  
No discharge  
No sign of iridial or corneal change  
No effect was observed 7 days after;

Classified as a non-irritant for eye according to U.S. EPA






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## Acute dermal irritation test

### LIP Active ingredients ( $\delta$ -endotoxins and spores)

(Unformulated liquid product)


**STEP 1:** shaving the rabbit's trunk (dorsal area, 6 cm<sup>2</sup>)

**STEP 2:** Up to 3 test patches are applied on different shaved sites at different time


**STEP 3:** The 3 patches are removed after 3 min, 1 and 3 hours if no serious skin reaction observed.

**STEP 3 - Clinical observation**


State of skin irritation/corrosion after 3 minutes of exposure.



State of skin irritation/corrosion after 1 hour of exposure.




State of skin irritation/corrosion after 3 hours of exposure.



**STEP 4: Observation after patch removal**

No sign of erythema and oedema  
 No defatting of the skin  
 No systemic adverse effects  
 → scored at 1, 24, 48 and 72 hours;

→Based on the results, Btk unformulated product is classified as a **NON-IRRITANT** for skin according to U.S. EPA



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# Toxicity assays on Lab animals

## Formulated products (2)

### External application-based assays

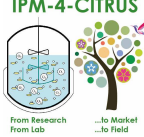




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## External applications

**In comparative manners:**

- Tested formulations (LIP & BLB1)
- Negative control
- Blank formulation (Additives without active ingredients);
- **DELFIN®** and **Bactospein®** reference products;




- 1. Eye irritation tests on New Zealand rabbits were carried out :**
  - **2.1-** Acute assays (2-3 kg/rabbit, (1 instillation);
  - **2.2-** Sub-acute assays (2-3 kg/rabbit, daily instillation, 5 days);
  - *Maximum mean total scores (MMTS) calculated;*
- 2. Dermal (skin) irritation assays on New Zealand rabbits :**
  - Three rabbits/product (2-3 kg/rabbit, single exposure);
  - *Primary Irritation Index (PII) determined;*



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## Regulations and Guidelines

*Ocular and dermal applications*

Effects of Btk-based new formulations (LIP and BLB1) on eye and skin

TEST TECHNIQUE	STANDARDS (guidance & regulations)	Risk characterization	Number of animals per product	Strain, age and weight of animals
<i>Ocular (eye) Irritation</i>	ISO 10993-10 OECD 405	Clinical observation and grading reactions	3 Rabbits/acute 3 Rabbits/subacute	New Zealand 4-4,5 months 2-3 kg
<i>Dermal (Skin) Irritation</i>	ISO 10993-10:2010; in vivo skin irritation test	Clinical observation and grading reactions	3 Rabbits single exposure	New Zealand 4-4,5 months 2-3 kg




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## Products' Features

Product features	DELFIN-WG Positive control-1	BACTOSPEIN-DF Positive control-2	BLB1 (2021)	LIP (2021)	BLANK (2021) Negative control
Active ingredients (Btk solids, spores, endotoxins)	85%	54%			
Other ingredients	15%	46%			
Potency	32000 IU/mg Btk	32000 IU/mg Btk			
Form	Wettable granules	Dry Flowable	Wettable granules	Wettable granules	Wettable granules
Company	CERTIS/ USA	VALENT /USA	TUNISIA	LEBANON	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

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## Acute and Subacute Eye Irritation Assays

The protocol was followed according to **OECD/OCDE 405** (14 June 2021- In vivo acute eye irritation/corrosion test) **for Acute eye irritation test (single exposure)** and **ISO 10993-10-2010 for Subacute eye irritation test (repeated exposure)**













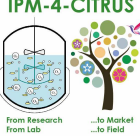





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## Eye irritation tests on New Zealand rabbits


### Acute Eye Irritation Assay



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## Acute eye irritation assay

Initial concentration (g/L)	<b>1g/L (based on Turkish DELFIN)</b>
Final concentration (mg/mL)	<b>X200 fold concentration (200mg/mL)</b>
Dose volume	100µL
Dose location	into the lower conjunctival sac
Eyelide closure	one second
Instillation	1 instillation on the 1 <sup>st</sup> day ( <i>single-exposure</i> )
Observation	1h and during 14 days post-instillation
Scale for scoring ocular reactions	OECD-405 (Based on Draize Grading scale)



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**Table 2: Grading Scale and Multiplication Factors**  
(Draize et al., 1944)

I. Cornea	Grade
<b>A. Opacity - degree of density (area most dense taken for reading):</b>	
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>B. Area of cornea involved:</b>	
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
<b>Score (A x B) x 5</b>	<b>Total Maximum = 80</b>
<b>II. Iris</b>	
<b>C. Values:</b>	
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
<b>Score C x 5</b>	<b>Total Maximum = 10</b>
<b>III. Conjunctivae</b>	
<b>D. Redness (refers to palpebral conjunctivae only):</b>	
Vessels normal	0
Vessels definitely injected above normal	1
More diffuse, deeper crimson red, individual vessels not easily discernible	2
Diffuse beefy red	3
<b>E. Chemosis (Oedema):</b>	
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
<b>F. Discharge:</b>	
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
<b>Score (D + E + F) x 2</b>	<b>Total Maximum = 20</b>
<b>Maximum possible score = 80 + 10 + 20 = 110</b>	

**Grading Scale and Multiplication Factors**  
(Draize et al., 1944)

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**EYE IRRITATION CLASSIFICATION SYSTEM**  
(Based on Draize eye irritation test by Kay&Calandra, 1962)  
(Max. Mean Total Score)

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).

**Formula for MMTS:**

$\Sigma$  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

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**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	
	Area involved	B	0	0	0	0	0	
	(AxB)x5		0	0	0	0	0	
Iris		C	0	0	0	0	0	
	Cx5		0	0	0	0	0	
	Conjunctiva	D	1	1	1	1	0	
	Redness	E	0	0	0	0	0	
	Chemosis	F	0	1	0	0	0	
	Discharge		2	4	2	2	0	
	(D+E+F)x2		2	4	2	2	0	
<b>TOTAL</b>			2	4	2	2	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	
	Area involved	B	0	0	0	0	0	
	(AxB)x5		0	0	0	0	0	
Iris		C	0	0	0	0	0	
	Cx5		0	0	0	0	0	
	Conjunctiva	D	1	1	1	1	0	
	Redness	E	0	0	0	0	0	
	Chemosis	F	0	1	1	0	0	
	Discharge		2	4	4	2	0	
	(D+E+F)x2		2	4	4	2	0	
<b>TOTAL</b>			2	4	4	2	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	
	Area involved	B	0	0	0	0	0	
	(AxB)x5		0	0	0	0	0	
Iris		C	0	0	0	0	0	
	Cx5		0	0	0	0	0	
	Conjunctiva	D	1	1	1	1	1	
	Redness	E	0	0	0	0	0	
	Chemosis	F	1	1	1	1	0	
	Discharge		4	4	4	4	2	
	(D+E+F)x2		4	4	4	4	2	
<b>TOTAL</b>			4	4	4	4	2	

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

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**Calculation of Maximum Mean Total Score for DELFIN**

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

Classification of eye irritation scores from the Draize eye irritation test


MMTS*	Irritation classification
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25.1-50.0	Moderately irritative
50.1-80.0	Severely irritative
80.1-100.0	Extremely irritative
100.1-110	Maximally irritative

**4 ; Minimally irritating**

\*MMTS = Maximum Mean Total Score

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### Calculation of Maximum Mean Total Score for BLANK, BLB1, LIP and BACTOSPEIN


“Maximum Mean Total Score” (MMTS) for **BLANK** = (0+0) / 2 rabbits = **0**  
 “Maximum Mean Total Score” (MMTS) for **BLB1** = (0+0+0) / 3 rabbits = **0**  
 “Maximum Mean Total Score” (MMTS) for **LIP** = (0+0+0) / 3 rabbits = **0**  
 “Maximum Mean Total Score” (MMTS) for **BACTOSPEIN** = (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
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25.1–50.0	Moderately irritative
50.1–80.0	Severely irritative
80.1–100.0	Extremely irritative
100.1–110	Maximally irritative

→ **0 ; NON-irritating**


\*MMTS = Maximum Mean Total Score



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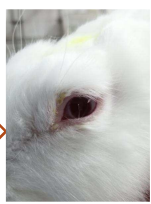


### After 72h obsv. of post-instillation (x200)

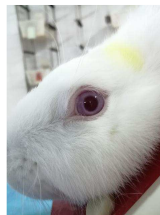
**BACTO Acute**



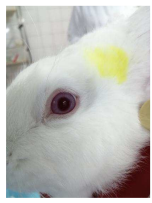
**DELFIN Acute**



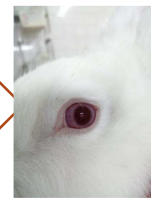
**BLANK Acute**




**LIP Acute**



**BLB1 Acute**

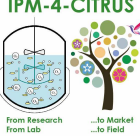




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## Eye irritation tests on New Zealand rabbits

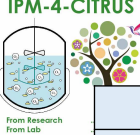
### Subacute Eye Irritation Assays



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<b>Subacute eye irritation assay (repeated exposure)</b>	
Initial concentration (g/L)	1g/L (based on Turkish DELFIN)
Final concentration (mg/mL)	<b>X30 fold concentration (90mg/3mL)</b>
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 ocular reactions	ISO 10993-10-2010*(Based on Draize Grading scale)

\* The detailed (full) grading table is found on ISO 10993-10-2010 regulation









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### Calculation of Maximum Mean Total Scores

“Maximum Mean Total Score” (MMTS) for **BLANK**= (0+0) / 2 rabbits= **0**

“Maximum Mean Total Score” (MMTS) for **BLB1**= (0+0+0) / 3 rabbits= **0**

“Maximum Mean Total Score” (MMTS) for **LIP**= (0+0+0) / 3 rabbits= **0**

“Maximum Mean Total Score” (MMTS) for **BACTOSPEIN**= (0+0+0) / 3 rabbits= **0**


“Maximum Mean Total Score” (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

→ **0 ; NON-irritating**

\*MMTS = Maximum Mean Total Score

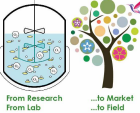




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## Dermal irritation tests on New Zealand rabbits

### Skin Irritation Assays (single exposure)











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<b>Dermal (Skin) irritation assay (single exposure)</b>	
Initial concentration (g/L)	1g/L (based on Turkish DELFIN)
Final concentration (mg/mL)	<b>X200 fold concentration (200mg/mL)</b>
Dose volume	500µL/patch
Dose location	Dorsal region on 4 patches /rabbit
Patch size	2,5 cm x 2,5 cm per patch
Instillation	<i>single-exposure</i>
Exposure time	<b>4h</b>
Observation	1h , 24h, 48h, 72h following removal of the patches
Scale for scoring ocular reactions	ISO 10993-10:2010; in vivo skin irritation test








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## Scoring for Skin Reaction

**Table 2. Scoring System for Skin Reaction.**

Skin Reaction	Skin Reaction
<b>Erythema and eschar formation</b>	
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
<b>Oedema formation</b>	
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 edema (raised more than 1 mm and extending beyond exposure area)	4
<b>Total possible score for irritation</b>	<b>8</b>

doi:10.1371/journal.pone.0158513.t002
















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## Dermal (Skin) irritation assay


**PRIMARY IRRITATION INDEX (PII) Formula**

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

(single exposure)

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

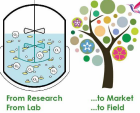
Formulation	(PII) Score	Response category
BLANK	0	Negligible (non-irritating)
DELFIN	0	Negligible (non-irritating)
BACTOSPEIN	0	Negligible (non-irritating)
BLB1	0	Negligible (non-irritating)
LIP	0	Negligible (non-irritating)



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## Toxicity assays on Lab animals

Formulated products (2)

Oral and Intradermal administration assays





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## Oral toxicity assays on Wistar Rats

### Acute and Subacute toxicity assays

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## Oral administration assays

1. **Oral toxicity assays on rats (200-250g/rat) (Kobay lab animal facilities)**
  - **4.1- Acute** (one gavage and 14 days observations);
  - **4.2- Subacute** (daily gavage during 28 days).

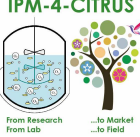
Type of tests	High toxicity (danger)	Moderate toxicity (warning)	Low toxicity (caution)	Very low toxicity (caution)
Oral LD <sub>50</sub>	Less than 50 mg kg <sup>-1</sup>	50 – 500 mg kg <sup>-1</sup>	500 – 5000 mg kg <sup>-1</sup>	Greater than 5000 mg kg <sup>-1</sup>
Dermal LD <sub>50</sub>	Less than 200 mg kg <sup>-1</sup>	200 – 2000 mg kg <sup>-1</sup>	2000 – 5000 mg kg <sup>-1</sup>	Greater than 5000 mg kg <sup>-1</sup>
Inhalation LC <sub>50</sub>	Less than 0.05 mg litre <sup>-1</sup>	0.05 – 0.5 mg litre <sup>-1</sup>	0.5 – 2 mg litre <sup>-1</sup>	Greater than 2 mg litre <sup>-1</sup>
Eye effects	Corrosive	Irritation persisting for 7 days	Irritation reversible within 7 days	Minimal effects, gone within 24 hr
Skin effects	Corrosive	Severe irritation at 72 hr	Moderate irritation at 72 hr	Mild or slight irritation

Source: NPIC (2000).


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## Product estimated quantity and volume


	Animal Assays	Number of Animals	Volume of each product	Estimated quantity of each product	Acute, 250mg/ml Sub-acute, 100mg/ml
1.	ACUTE TOXICITY TEST ORAL	25 Wistar Rats (200 g) 5 rats per product	2 ml / rat (0.5 g product)	0.5g x 5 +1 dose suppl. = 3 g  (2.5 g without 1 additional dose)	-Single gavage
2.	SUBACUTE TOXICITY TEST ORAL	50 Wistar Rats (200 g) (25 female; 25 male)  5 female+ 5 male rats per product	2 ml / rat (0.2 g product)	0.2g x2x5+1 dose suppl) x 28 days= 61.6 g (56 g without 1 additional dose)	-Daily gavage (28 days )
3.	SKIN SENSITIZATION TEST (GPMT)	10 Guinea pigs / product (and 5 animals as a control group )	100µl/intradermal inj. (1 Intra-Dermal and 2 dermal applications) (250 mg/ml final concentration)	For 0.1 ml (100µl= per injection solution  0,025gx3x10+1=0.775g	



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## Experimental conditions

- **Acute oral toxicity assay**
  - Test substances: BLB1 & LIP
  - Ref. substances: DELFIN & BACTOSPEIN & BLANK (additives)
  - **Single administration**
  - Observation: 14 days
    - Recorded Data : body weight, behaviour & symptomatology






- **Subacute oral toxicity assay**
  - Test substances: BLB1 & LIP
  - Ref. substances: DELFIN & BACTOSPEIN & BLANK (additives)
  - **Daily Oral gavage (28 days)**
  - Recorded Data :
    - body weight, behaviour & symptomatology
    - Histo-, Hemato- and Biochem- parameters



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## Acute Oral Toxicity Assay: Results


### OBSERVATIONS AND CONCLUSIONS

**5.2.7.1. Mortality**  
NO mortality were observed in any animal during the test period.

**5.2.7.2. Evaluation of Clinical Findings**  
No abnormal clinical findings were detected in animals during the testing period.

**5.2.7.4. Body Weight Change**  
No significant weight loss was observed in the animals during the test period.

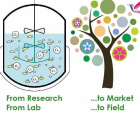
**5.2.7.5. Feed/Water Consumption**  
No reduction in feed and water consumption was observed in animals during the test period.



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## Subacute Oral Toxicity Assay: Results


### 7.2.7. OBSERVATIONS AND CONCLUSIONS

**7.2.7.1. mortality**  
No mortality observed in any animal during the test period.

**7.2.7.2. Evaluation of Clinical Findings**  
No abnormal clinical findings were detected in animals during the testing period.

**7.2.7.4. Body Weight Change**  
No significant weight loss was observed in the animals during the test period.

**7.2.7.5. Feed/Water Consumption**  
No reduction in feed and water consumption was observed in animals during the test period.



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## Biochem- & Hematological assessments

- **Blood sera were separated to evaluate hepatic cytolysis and renal failure (500 µl serum):**
  - Alanine amino transférse (ALT)
  - Aspartate Aminotransférse (AST)
  - alkaline phosphatase (ALP)
  - Gama Glutamyl Transferase (GGT)
  - Creatinine
  - Uric acid
  - Cholestase
  - Albumin
- **Hematological parameters were recorded (20 µl serum):**
  - White Blood Cell (WBC)
  - Red Blood Cell (RBC)
  - Hemoglobin (Hb)
  - Hematocrit (Hct)
  - Mean Cell Volume (MCV)
  - Mean Cell Hemoglobin (MCH)
  - Mean Cell Hemoglobin Concentration (MCHC),
  - Red Cell Distribution Width (RDW)
  - Platelet count (Plt)
  - Mean Platelet Volume (MPV)

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## Hemograms and Blood Biochemistry analysis

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## Histological observations

- **In progress to be analyzed**

Organ by organ :

- Uterus
- Testis
- Ovary
- Colon
- Secum
- Karaciger
- Bobrek
- **Hepar**
- Etc.

*The intestinal tracts were stored at -80C, using DNA/RNA preservation solution*






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## Sensitization assay on Guinea pigs

### Intradermal and Subcutaneous assays





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## Sensitization assay on Guinea pigs



### SENSITIZATION (GUINEA PIG MAXIMIZATION) TEST

- Quarantine
- Animal's Shaving
- 
- **Initiation of the intradermal induction phase**
- Intradermal induction phase ends
- **Superficial ( topical ) induction phase**
- Superficial ( topical ) induction phase ends
- **Stimulation phase**
- Stimulation phase end

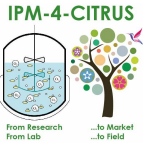
**Result report**





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## Sensitization assay : Results

- ✓ Few mortality was observed and animals were euthanized by decision of the veterinarian, in a study product (LIP) and a reference product (Delfin);
- ✓ However, no mortality is expected for this test  
→ technical difficulties


**The Skin Sensitization test will be carried out once again**





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## Conclusion


- All the protocols received an early approval from the Ethical Committee of Institut Pasteur Tunis and Turkey Authorities
  - Ethical Turkish Authorities Approval
  - CEBM Ethical Committee Approval (IPT)
- The effects of BLB1 and LIP Bt-based formulations were compared to the equivalent DELFIN reference product;
- **Based on collected data :**
- → No significant weight loss and reduction in feed and water consumption were observed;
- → No mortality and abnormal clinical findings were observed following external and gavage toxicity tests;



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## First Human Risk characterization

- The first evaluation of the new *Btk*-based biopests risk on human is promising;
- The active ingredients as well as the formulated products are non-irritating eye/skin and non-toxic on rat by gavage;

### Further needed Risk characterization

→ Toxicogenic

→ Mutagenic

→ Carcinogenic

→ Toxic for reproduction

→ Endocrine disrupting

→


❖ EC Regulations

❖ Guidelines

❖ Technical requirements

❖ Data analysis

❖ Risk assessment




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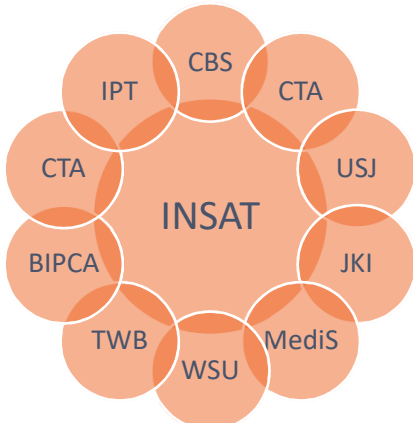

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## DEEP ACKNOWLEDGMENTS THE CONSORTIUM OF PARTNERS IPM-4-CITRUS EU PROJECT

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## IPM-4 CITRUS - BIYANS Members TURKEY



**Dr Zeynep Yurtkuran**  
Ceterez  
BIYANS CEO

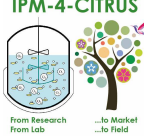


**Dr Gül Ayyıldız**  
BIYANS team  
member



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