“Acute Systemic Toxicity via Oral gavage in CD-1 Mice, Repeat Dose”

Hydroethanolic extract of Vernonanthura nudiflora

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SPONSOR 15980

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5750 SW 6th Place
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United States

23E0241G-X01G

STUDY NUMBER

23E0241G-X01G

FINAL REPORT

STUDY TITLE

Acute Systemic Toxicity via Oral gavage in CD-1 Mice, Repeat Dose

TEST ARTICLE

Hydroethanolic extract of *Vernonanthura nudiflora*
Lot Number: 008

STUDY DIRECTOR

Simret Beraki, Ph.D.
Director, *In Vivo* Services

PERFORMING LABORATORY

Pacific BioLabs
551 Linus Pauling Drive
Hercules, CA 94547
United States

TABLE OF CONTENTS

SIGNATURE PAGE

This report is being submitted by the following personnel:

Study Director: Simret Beraki, Ph.D., Director, *In Vivo* Services

20 Jul 2023

X Simret Beraki

I approve the content of this document.
Signed by: Simret Beraki

RESPONSIBLE PERSONNEL

1. F. Michael Yakes, Ph.D., Chief Operating Officer
2. Michael Spalding, Chief Executive Officer

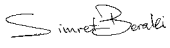
:lkv

STATEMENT OF COMPLIANCE

All aspects of the study contained in this report were conducted according to Pacific BioLabs Standard Operating Procedures (SOPs) and in compliance with the United States Food and Drug Administration (FDA) Good Laboratory Practice (GLP) for Nonclinical Laboratory Studies, Title 21 of the U.S. Code of Federal Regulations, Part 58.

Study Director Signature

20 Jul 2023

X 

I approve the content of this document.
Signed by: Simret Beraki

QUALITY STATEMENT

QUALITY ASSURANCE UNIT GLP MONITORING AND INSPECTION SUMMARY

In accordance with 21 CFR 58, this study, 23E0241G-X01G, was inspected by Quality Assurance at intervals adequate to assure the integrity of the study. The phase(s) of the study inspected, the date(s) of the inspection, QA auditor, and the date(s) that the QAU inspection report for this study were reported to the Study Director and Management are provided below.

Phase of Study	Date of Inspection	QA Auditor	Date QA Report Provided to Study Director and Management
144 Hour Observation	29 Jun 2023	RA	29 Jun 2023

The QAU inspection summary is routinely reviewed by the study director and management of Pacific BioLabs. Management is notified immediately if there are any deviations which might affect the integrity of the study data.

DATA/REPORT REVIEW

Quality Assurance has conducted a thorough review of the test data generated during this study. Report Number 23E0241G-X01G represents an accurate description of the conduct and final results of the study. To the best of my knowledge and ability, this study has been conducted in compliance with applicable Good Laboratory Practice regulations.

20 Jul 2023

X

QA Review
Signed by: Raffie Agojo

STUDY SUMMARY

Purpose: The purpose of this test was to evaluate systemic responses to test article following injection into mice. This test was conducted according to Pacific BioLabs SOP 16G-63, which followed procedures outlined in ISO 10993-11.

Procedures: The test article "Hydroethanolic extract of *Vernonanthura nudiflora*" was prepared per Sponsor's instructions. The test article is a diluted plant extraction a liquid form and was tested as received. Physiological saline was used as a control.

Twenty (20) CD-1 albino mice (10 male and 10 female; 5 males and five females for test article and five test article and 5 males and five females for corresponding control) were used. Animals were randomly assigned to test and control group.

Each animal was administrated with 0.1 mL (approximately 5 mL/kg as per average mice weight) of the test article or control according to Table 1. The doses were adjusted based on last obtained body weight. The doses were rounded to the nearest 0.1 mL. The test article or control was administered via oral gavage.

Interpretation: According to ISO 10993-11, the test article meets the requirements for this test if none of the animals treated with the test article exhibits a greater biological reactivity when compared to those treated with the control.

The test article does not meet the ISO 10993-11 requirements if two or more animals die, if abnormal behavior such as convulsions or prostration occurs in two or more animals, or if a body weight loss greater than 10% occurs in three or more animals.

If any animals treated with the test article show only slight signs of biological reactivity, and not more than one animal shows gross signs of biological reactivity or dies, a repeat test is required using 10 mice per group. The test article meets the ISO 10993-11 requirements if during the repeat test all 10 of the animals treated with the test sample show no scientifically meaningful biological reactivity when compared to the vehicle control animals during the observation period.

Results: All test article and control group animals (male and female) survived the test period. None of the test group animals exhibited any biological reactivity at any of the specified time points during the fourteen-day observation period. All animals from the test group and control group (male and female) gained weight at the end of the test period. No abnormalities were noted at gross necropsy.

Conclusion: This test was conducted according to ISO 10993-11. All animals appeared healthy during the course of the study and gained weight at the end of the test. No biologically significant differences were noted between the test and control animals. No acute systemic toxicity was observed in tested animals. The test article met the requirements for the Acute Systemic Toxicity test using conditions listed in this report.

1. GENERAL INFORMATION

1.1. Study Dates

Study Authorization:	Signed Protocol
Date Test Article Received:	12 Apr 2023
Study Initiation Date:	09 Jun 2023
Date On Test:	23 Jun 2023
Date Off Test:	06 Jul 2023

1.2. Protocol

This test was conducted according to Protocol Number: 23E0241G-X01G, which incorporates, by reference Standard Operating Procedure 16G-63, and is on file at Pacific BioLabs. There were two amendments to the Protocol (Appendix III).

1.3. Deviations from Protocol

There were no deviations from the Protocol.

1.4. Key Personnel and Laboratories

Study Director:	Simret Beraki, Ph.D. Director, *In Vivo* Services Pacific BioLabs 551 Linus Pauling Drive Hercules, CA 94547 United States Phone: (510) 964-9000
Study Sponsor:	William Denizard Immune & Genetics Protocols, LLC 5750 SW 6th Place Ocala, FL 34474 United States Phone: 407-616-2970
Veterinarian:	Sophie Russell, DVM, MPVM Pacific BioLabs 551 Linus Pauling Drive Hercules, CA 94547 United States Phone: (510) 964-9000

2. INTRODUCTION

Purpose: The purpose of this test was to evaluate systemic responses to test article following injection into mice. This test was conducted according to Pacific BioLabs SOP 16G-63, which followed procedures outlined in ISO 10993-11.

Justification for Test System: Justification for the use of animals in this study is based on the premise that animal testing is an appropriate and ethical prerequisite to testing new medical devices in humans and that data obtained from nonclinical animal models will have relevance to the behavior of the test material in humans. Because of the complex interactions that occur *in vivo*, an *in vitro* system does not provide sufficient information for evaluation of a compound's *in vivo* activities. The use of the mouse in this study is specified in current ISO 10993-11 guidelines.

Justification for Number of Animals: The current ISO 10993-11 guidelines require a minimum of five animals in the test group and a minimum of five animals in the control group be evaluated. The number of animals used in this study was the minimum number that could be used for evaluating the test article.

Justification for Route of Administration: The oral route of administration was selected based on clinical route.

Justification for Dose: Doses were specified by Sponsor.

3. MATERIALS AND METHODS

3.1. Test Materials

3.1.1. Test Article Identification

Test Article Name:	Hydroethanolic extract of *Vernonanthura nudiflora*
Physical Description:	Liquid
Total Quantity Received for Testing:	1 bottle containing ~450 mL
Total Quantity Used for This Study:	14.0 mL
Lot Number:	008
Part Number:	Not provided by Sponsor
Other Identifier:	Not provided by Sponsor
Expiration Date:	Feb 2026
Special Handling and/or Precautions:	None
Sterilization Data:	Non-sterile
Storage Conditions:	Room Temperature
Final Intended Use:	Dietary supplement

3.1.2. Negative Control Article Identification

Name:	Sterile Saline (0.9% Sodium Chloride Injection, USP)
Manufacturer:	Nova-Tech, Inc.
Physical Description:	Clear liquid
Total Quantity Used for This Study:	14.0 mL
Lot Number:	B2303032
Expiration Date:	Mar 2026
Sterility Status:	Sterile
Storage Conditions:	Room Temperature

3.1.3. Reserve Sample and Sample Disposition

All remaining test articles will be disposed per Pacific BioLabs SOPs. No reserve samples of the test or control articles will be retained by Pacific BioLabs.

FDA and US Environmental Protection Agency (EPA) regulations require that, for studies of more than four weeks duration, reserve sample from each batch of material be retained for the period of time provided in FDA GLP Regulations 21 CFR Parts 58.105 and 58.195; EPA FIFRA GLP Regulations 40 CFR Parts 160.105 and 160.195; and EPA TSCA GLP Regulations 40 CFR Parts 792.105 and 792.195. The various agencies have, in the past, recommended that the amount of reserve sample be enough to repeat the study two or three times. Sponsor is responsible for retention of test and control article reserves.

3.1.4. Test and Control Article Characterization

Test Article: The Sponsor is responsible for all test article characterization specified in the Good Laboratory Practices (GLP) regulations (21 CFR 58.105). Because this is a solid material(s) containing no drug(s), characterization of the test article strength and purity are not considered applicable requirements. The Sponsor has supplied sufficient information to Pacific BioLabs to assure characterization of the test article meets applicable requirements, including the unique identification and stability of the test article (Appendix I). The Sponsor is responsible for maintaining records of manufacture that would provide information on the composition of the test article and would be able to supply those records if requested by regulatory authorities.

Control Article: The control articles were supplied by Pacific BioLabs and information related to the characterization of the control articles can be found in Appendix II. The control articles were adequately characterized as specified in the Good Laboratory Practices (GLP) regulations (21 CFR 58.105).

3.1.5. Test and Control Article Dose Solution Characterization

Test Article Dose: The test article was tested as received from the Sponsor. No further characterization of the test article, beyond that provided by the Sponsor, was conducted.

Control Article Dose: The control articles were used without modification. No further characterization of the control articles, beyond that provided by the supplier, was conducted.

3.1.6. Test and Control Article Description and Preparation

Test Article Description: The test article was "Hydroethanolic extract of *Vernonanthura nudiflora*". The test article was prepared per Sponsor instructions. The test article is a diluted plant extraction in liquid form and was tested as received. Physiological saline was used as a control.

3.2. Test System

Species:	Mouse
Strain:	CD-1
Source:	Charles River, Hollister, CA
Number Used:	20 mice: 10 Males and 10 Females (five per test group and five per control group)
Sex:	Male and Female (Naïve)
Age:	Young adult
Initial Weight:	Female Test Article Group: 20 to 21 grams Female Control Group: 21 grams Male Test Article Group: 21 to 23 grams Male Control Group: 20 to 23 grams
Identification:	Cage labeling and tail marking

Environment: Mice were housed in groups in polycarbonate cages. Animals were maintained in a controlled environment at a nominal temperature range of 20 to 26°C, a humidity range of 50 ± 20%, and a light/dark cycle of 12 hours. Animals were maintained in rooms with at least ten room air changes per hour. Room logs documenting temperature and humidity are kept on file at Pacific BioLabs.

Diet and Feed: Mice received Certified Laboratory Rodent Diet *ad libitum*. The feed was analyzed by the supplier for nutritional components and environmental contaminants. There were no known contaminants in the feed that are reasonably expected to interfere with the conduct of this study.

Water: Fresh, potable drinking water was provided *ad libitum* to all animals via a sipper tube. Water testing is conducted two times a year for total dissolved solids and specified microbiological content and selected elements, heavy metals, organophosphates, and chlorinated hydrocarbons. Results of water analyses are archived at Pacific BioLabs. There were no known contaminants in the water that are reasonably expected to interfere with the conduct of this study.

Acclimation: Mice were acclimated for at least five days prior to test. Health observations were performed prior to the study to ensure that the animals were acceptable for study use.

Veterinary Care: Veterinary care was available during the course of the study. No veterinary treatment was necessary during the course of the study.

Disposition: Disposition of study animals is documented in the Pacific BioLabs study records. Alternate animals not selected for the study were returned to the Pacific BioLabs animal colony for use in subsequent studies or procedures.

3.3. Experimental Design

The study design is presented in Text Table 1. A total of twenty albino mice (ten male and ten female; Five males and five females for test article and five males and five females for corresponding control) were used. Animals were randomly assigned to test article or control group.

Each animal will be administrated with 0.1 ml (approximately 5 ml/kg as per average mice weight) of the test article or control according to Table 1. The doses will be adjusted based on last obtained body weight. The doses will be rounded to the nearest 0.1 ml.

Table 1. Study Design

Group	Sex	Number of Animals (n)		Route of Administration	Dose volume	Observations	Body Weights	Gross Necropsy
		Test	Control					
1	M	5	5	Oral Gavage	0.1 ml	Immediately; 4 hours ± 15 minutes after dosing; daily	Prior to Dose 1, Dose 7, and Dose 14	Yes
2	F	5	5	Oral Gavage	0.1 ml	Immediately; 4 hours ± 15 minutes after dosing; daily	Prior to Dose 1, Dose 7, and Dose 14	Yes

3.4. In Life Observations and Measurements

3.4.1. Mortality/Moribundity Checks

General morbidity and moribundity checks (cage side observations) were performed once daily.

3.4.2. Clinical Observations

The animals were observed prior to and immediately after dosing. In addition, animals were observed at 4 hours ± 15 minutes following dose administration of each day. Cage-side observations were included, but not be limited to, changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous system, somatomotor activity, and behavior patterns.

3.4.3. Body Weight Measurement

Animals were weighed prior to dosing on Day 1, Day 7, and Day 14. Additional observations or body weight measurements were performed if deemed necessary by the Study Director.

3.4.4. Gross Necropsy

At the end of the study, animals were euthanized as per Pacific BioLabs SOPs and the method of euthanasia was consistent with the recommendations of the American Veterinary Medical Association guidelines on euthanasia.

Gross necropsy was performed and no abnormality was detected.

3.5. Interpretation and Analysis

According to ISO 10993-11, the test article meets the requirements for this test if none of the animals treated with the test article shows a significantly greater biological reactivity compared to those animals treated with the negative control.

The test article does not meet the ISO 10993-11 requirements if two or more animals die, if abnormal behavior such as convulsions or prostration occurs in two or more animals, or if a body weight loss greater than 10% occurs in three or more animals.

If any animals treated with the test article show only slight signs of biological reactivity, and not more than one animal shows gross signs of biological reactivity or dies, a repeat test is required using ten mice per group. The test article meets the ISO 10993-11 requirements if during the repeat test all ten of the animals treated with the test sample show no scientifically meaningful biological reactivity when compared to the vehicle control animals during the observation period.

3.6. Statistical Analysis

No statistical analyses were performed.

3.7. Data Acquisition and Analysis

Major computer software systems used on this study included Microsoft Word® and the Vaisala Environmental Monitoring System® for environmental control of chambers used in this study.

3.8. Maintenance of Raw Data, Records, and Specimens

Following issuance of the Final Report, records (including, but not limited to, protocol, protocol amendment(s), in-life records, pathology records, dose preparation records, correspondence related to the study, Final Report, and histopathology records) and materials (including, but not limited to, slides, specimens, wet tissues, and blocks) will be archived at Pacific BioLabs (Hercules, CA) for a period of one year after issuance of the Final Report. After one year, the Sponsor will be contacted concerning continued storage or return of materials.

Records and materials associated with activities external to Pacific BioLabs (including, but not limited to, clinical pathology, and histopathology) and activities conducted by the Sponsor will be archived by the individual performing laboratories or the Sponsor in a manner consistent with their individual operating SOPs and regulatory requirements.

4. RESULTS AND DISCUSSION

4.1. In Life Observations and Measurements

4.1.1. Survival

No mortality occurred during the study; all animals survived until scheduled termination. At the end of the study, all animals were euthanized with CO_2 as per Pacific BioLabs SOPs.

4.1.2. Clinical Observations

Clinical observations are presented in Summary Tables 1 and 2.

Female: All animals from the female test and control group appeared healthy and no abnormalities were observed at any of the specified time points during the fourteen-day observation period. No biologically significant differences were noted between the test and control animals.

Male: All animals from the male test and control group appeared healthy and no abnormalities were observed at any of the specified time points during the fourteen-day observation period. No biologically significant differences were noted between the test and control animals.

4.1.3. Body Weights

Animal body weights and dose volumes are presented in Summary Tables 3 and 4.

Female: All of the animals in the test article group gained weight at the end of the test. The weight gain range was from 1 to 3 grams in the animals dosed with the test article. The weight gain range was from 2 to 5 grams in the animals dosed with the control.

Male: All of the animals in the test article group gained weight at the end of the test. The weight gain range was from 5 to 8 grams in the animals dosed with the test article. The weight gain range was from 4 to 9 grams in the animals dosed with the control.

4.1.4. Gross Necropsy

Gross necropsy findings are presented in Summary Tables 5 and 6.

Females: No abnormalities were observed in any of the test or control animals.

Males: No abnormalities were observed in any of the test or control animals.

5. CONCLUSION

This test was conducted according to ISO 10993-11. All animals appeared healthy during the course of the study and gained weight at the end of the test. No biologically significant differences were noted between the test and control animals. No acute systemic toxicity was observed in tested animals. The test article met the requirements for the Acute Systemic Toxicity test using conditions listed in this report.

6. REFERENCES

ISO 10993-11:2017 (E), *Biological Evaluation of Medical Devices – Part 11: Tests for Systemic Toxicity*

ISO 10993-12:2021, *Biological Evaluation of Medical Devices – Part 12: Sample Preparation and Reference Materials*

Pacific BioLabs SOP 16G-63, rev. 3G.00, *ISO Acute Systemic Toxicity Test (Mice/Rats)*

7. SUMMARY OF RESULTS

Summary Table 1. Clinical Observations (Females)

Group	Animal Number	Immediately after dosing	4 hours	24 hours	48 hours	72 hours	96 hours	120 hours	144 hours	168 hours	192 hours	216 hours	240 hours	264 hours	288 hours	312 hours	336 hours
Test Article	1	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	2	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	3	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	4	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	5	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Control	6	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	7	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	8	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	9	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	10	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

*NBR – No Biological Reactivity

Summary Table 2. Clinical Observations (Males)

Group	Animal Number	Immediately after dosing	4 hours	24 hours	48 hours	72 hours	96 hours	120 hours	144 hours	168 hours	192 hours	216 hours	240 hours	264 hours	288 hours	312 hours	336 hours
Test Article	11	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	12	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	13	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	14	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	15	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Control	16	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	17	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	18	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	19	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	20	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

*NBR – No Biological Reactivity

Summary Table 3. Animal Body Weights and Dose Volumes (Females)

Group	Animal Number	Dose (mL)	Pre-Test Weight (g)	144-Hour Weight (g)	312-Hour Weight (g)	Weight Change (g)*
Test Article	1	0.1	21	23	24	+ 3
	2	0.1	21	21	22	+ 1
	3	0.1	21	23	24	+ 3
	4	0.1	21	23	24	+ 3
	5	0.1	20	22	23	+ 3
Control	6	0.1	21	24	24	+ 3
	7	0.1	21	23	24	+ 3
	8	0.1	21	24	24	+ 3
	9	0.1	21	24	26	+ 5
	10	0.1	21	23	23	+ 2

*Body Weight change was calculated by subtracting the Pre-Test Weight from the 72-Hour Weight.

Summary Table 4. Animal Body Weights and Dose Volumes (Males)

Group	Animal Number	Dose (mL)	Pre-Test Weight (g)	144-Hour Weight (g)	312-Hour Weight (g)	Weight Change (g)*
Test Article	11	0.1	21	27	28	+ 7
	12	0.1	23	29	29	+ 6
	13	0.1	22	26	27	+ 5
	14	0.1	22	25	27	+ 5
	15	0.1	23	29	31	+ 8
Control	16	0.1	22	28	30	+ 8
	17	0.1	22	24	26	+ 4
	18	0.1	23	28	32	+ 9
	19	0.1	23	28	30	+ 7
	20	0.1	20	27	30	+ 8

*Body Weight change was calculated by subtracting the Pre-Test Weight from the 72-Hour Weight.

Summary Table 5. Gross Necropsy Findings (Females)

Group	Animal Number	Observations
Test Article	1	No abnormalities observed.
	2	No abnormalities observed.
	3	No abnormalities observed.
	4	No abnormalities observed.
	5	No abnormalities observed.
Control	6	No abnormalities observed.
	7	No abnormalities observed.
	8	No abnormalities observed.
	9	No abnormalities observed.
	10	No abnormalities observed.

Summary Table 6. Gross Necropsy Findings (Males)

Group	Animal Number	Observations
Test Article	11	No abnormalities observed.
	12	No abnormalities observed.
	13	No abnormalities observed.
	14	No abnormalities observed.
	15	No abnormalities observed.
Control	16	No abnormalities observed.
	17	No abnormalities observed.
	18	No abnormalities observed.
	19	No abnormalities observed.

APPENDIX I

Test Article Information

IMMUNE & GENETICS PROTOCOLS
URUGUAY

Analytical Specifications

Product: Vernonanthura nudiflora extract

Analysis N°: 20230047 Lot N° 008
Elaboration date: February 2023
Expiration date: August 2024

General Specifications	
Herb Compund:	Vernonanthura nudiflora
Part of the plant utilized:	Aerial part
Carrier:	Distilled water/Ethanol 1:9
Preservative:	None

Analysis	Specification	Result
Aspect	Solution	Acceptable
Rate Herb/Extract	1:80	Acceptable
Identification	TLC Positive	Acceptable
Dry Residue	0.22% - 0.40%	0.34%

Microbiological parameters

Analysis	Specification	Result
Aerobial Bacteria	Max. 10^3 UFC/g	Acceptable
Fungi and Yeast	Max. 10^2 UFC/g	Acceptable
Salmonella	Absence	Acceptable
E. Coli	Absence	Acceptable

February 27th 2023
Storage: Store in original well closed container, in a cool area, protected from direct solar light and humidity

Supervised by

Chem. Daniel Kerekes

Inmune & Genetic Protocols Uruguay
Diego Lamas 1471
Tel: 0059827085446 / 0059899288758
CP 11600
Montevideo-Uruguay

APPENDIX II

Certificate of Analysis for Saline

NOVA-TECH
ANIMAL HEALTH

CERTIFICATE OF ANALYSIS

PRODUCT:	Sterile Saline
CAT. #:	510224
LOT #:	B2303032
MANUFACTURE DATE:	March 8, 2023
EXPIRATION DATE:	MAR 2026

TEST	SPECIFICATION	RESULT
Sterility	Sterile	Sterile
Volume	≥ 250 mL	> 250 mL
Appearance	Clear, colorless	Pass
pH	4.5 - 7.0	6.2
Sodium Chloride Assay	855 - 945 mg/dL	903 mg/dL
Chloride Identification	Pass	Pass
Sodium Identification (Method I)	Pass	Pass
Sodium Identification (Method II)	Pass	Pass
Heavy Metals	≤ 0.001% w/v	Pass
Iron Test	≤ 2 ppm	Pass
Bacterial Endotoxin	≤ 0.5 EU/mL	< 0.0250 EU/mL
Particulates ≥ 10 µm	≤ 25 counts/mL	Pass
Particulates ≥ 25 µm	≤ 3 counts/mL	Pass
Visible Particulates	Essentially free from Visible Particulates	Pass

This product meets specifications and is eligible for release.

23MAR23
Quality

APPENDIX III

Protocol and Protocol Amendment 1 and 2

PACIFIC BIOLABS PBL
The Service Leader in Life Science Testing

PROTOCOL TITLE

Acute Systemic Toxicity via Oral gavage in CD-1 Mice, Repeat Dose

GLP PROTOCOL

23E0241G-X01G

STUDY SPONSOR

Immune & Genetics Protocols, LLC
5750 SW 6th Place
Ocala, FL 34474
United States

PERFORMING LABORATORY

Pacific BioLabs
551 Linus Pauling Drive
Hercules, CA 94547
United States

APPROVALS:

Designation	Reviewed By	Signature	Date
Study Sponsor	William Denizard Immune & Genetics Protocols, LLC		5/jun/2023
Study Director	Simret Beraki *In Vivo* Services	Simret Beraki	09jun2023

551 Linus Pauling Drive | Hercules CA 94547 | 510.964.9000 | PacificBioLabs.com

1. GENERAL INFORMATION

This GLP Protocol (Protocol) describes testing for test and control articles (TACA) submitted by the Sponsor in compliance with the Food and Drug Administration's Good Laboratory Practice (GLP) Regulations (21CFR Part 58). Pacific BioLabs will require a *Laboratory Service Request* (LSR) form with each TACA that details the characteristics of the TACA submitted for testing.

1.1. Study Number

23E0241G-X01G

1.2. Study Title

Acute Systemic Toxicity via Oral gavage in CD-1 Mice, Repeat Dose

1.3. Test Facility

Pacific BioLabs
551 Linus Pauling Drive
Hercules, CA 94547
United States

1.4. Responsible Personnel

Sponsor Representative:
William Denizard
Immune & Genetics Protocols, LLC
5750 SW 6th Place
Ocala, FL 34474
United States
Phone: 407-616-2970
Email: billydenizard@gmail.com

Study Director:
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United States
Phone: (510) 964-9000
Email: simretberaki@pacificbiolabs.com

1.5. Proposed Study Dates

The study dates may change due to unexpected events and major delays in the study conduct will be communicated with the Sponsor. The actual study dates will be specified in the Study Report and will not be added by amendment to the Protocol.

Proposed Start Date:	To Be Determined
Proposed Termination Date:	To Be Determined
Proposed Report Date:	To Be Determined

1.6. Alterations to the Protocol

Alterations to the general scope of the Protocol may be made over the period that the Protocol is in effect. Alterations to the Protocol that apply to all subsequent testing will be documented by an amendment to the Protocol and signed and dated by Pacific BioLabs and the Sponsor. Administrative protocol changes may not require Sponsor signature. All Protocol amendments will be issued to the Sponsor and will be included in the Study Report.

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All deviations to the Protocol during the course of a study will be justified by the Study Director as to impact on the study. All deviations will be documented in the Study Report.

1.7. Statement of Compliance

This nonclinical laboratory study will be conducted in accordance with the appropriate Standard Operating Procedures of Pacific BioLabs (Hercules, CA) and the Food and Drug Administration Good Laboratory Practice (GLP) Regulations For Nonclinical Laboratory Studies (21 CFR Part 58). This nonclinical study will be inspected by the Quality Assurance Unit (QAU) at Pacific BioLabs at intervals adequate to assure the integrity of the studies. QAU inspection findings will be reviewed by the management of Pacific BioLabs; and the Study Director and management will be notified immediately if there are any deviations which might affect the integrity of the study data.

Supporting Studies Conducted by Pacific BioLabs Designated Laboratories. There are no supporting studies conducted by outside laboratories, designated by Pacific BioLabs that contribute to this Protocol.

Supporting Studies Conducted by Sponsor. This Protocol does not incorporate supporting studies conducted by the Sponsor. All studies conducted by the Sponsor in conjunction with this Protocol will be reported separately by the Sponsor and will be the sole responsibility of the Sponsor.

1.8. Animal Welfare

This study will comply with all applicable sections of the Final Rules of the Animal Welfare Act regulations (9 CFR 1-3), the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and the Guide for the Care and Use of Laboratory Animals. Test procedures were reviewed and approved by Pacific BioLabs' Institutional Animal Care and Use Committee (IACUC) in compliance with Animal Welfare Act.

Requirement for this study by regulatory agencies is based on the premise that animal testing is a prerequisite for testing new drugs and medical devices in humans, and that animal testing results will predict effects in humans. Because of the complex and multiple interactions that occur *in vivo*, an *in vitro* system would not necessarily provide sufficient information for evaluation of test article toxicity (NIH, 1993). By signature of this protocol, the Sponsor provides assurance that the study is not an unnecessary duplication of previous work, and that documentation for the necessity of this study may be obtained from the Sponsor.

1.9. Safety to the Laboratory

The Sponsor will provide safety information to Pacific BioLabs in the form of a Material Safety Data Sheet (SDS) for each test article, if available. In the absence of specific safety requirements, standard laboratory safety procedures will be employed for handling the test and control articles, including the use of appropriate personal protective equipment. Eye protection/goggles should be worn when handling the test article.

1.10. Declaration of Intent

The design and scope of this study are consistent with the overall development strategy of the Sponsor, and this study may be submitted to regulatory agencies, including the United States Food and Drug Administration (FDA).

2. PURPOSE

The purpose of the study is to evaluate systemic responses to test article following oral gavage into mice. This test will be conducted according to this Protocol.

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2.1. Justification of Test System

Justification for the use of animals in this study is based on the premise that animal testing is an appropriate and ethical prerequisite to testing new medical devices in humans, and that data obtained from nonclinical animal models will have relevance to the behavior of the test material in humans. Because of the complex interactions that occur *in vivo*, an *in vitro* system does not provide sufficient information for evaluation of a compound's *in vivo* activities. The mouse is the species required by ISO 10993-11 guidelines.

2.2. Justification of Number of Animals

The current guidelines (e.g. ISO 10993-11) require a minimum of five animals in the test and five animals in the control group. The minimum number of animals required for this test will be used.

2.3. Justification of Route of Administration

The oral route of administration is based on clinical route.

2.4. Dose Rationale

Doses are specified Sponsor.

3. PROCEDURES

3.1. Test Materials

3.1.1. Test and Control Articles

Identification and characterization of test articles will be specified in the Study Report of test results, and will not be added by amendment to the Protocol. The following information, supplied by the Sponsor, may be included in the Study Reports:

Test Article Name:	Hydroethanolic extract of *Vernonanthura nudiflora*
Physical Description:	Liquid
Lot Number:	008
Part Number:	Not provided by Sponsor
Other Identifier:	Not provided by Sponsor
Expiration Date:	Feb 2026
Special Handling and/or Precautions:	None
Sterilization Data:	Non-sterile
Storage Conditions:	Room Temperature
Final Intended Use:	Dietary supplement

The Control Article will be provided by Pacific BioLabs and will be specified in the Final Report.

Negative Control Article Name:	0.9% Sodium Chloride Injection, USP
Physical Description:	Clear liquid
Manufacturer:	Will be provided in the Final Report
Lot Number:	Will be provided in the Final Report
Sterility Status:	Sterile (Passed Parametric Release)
Expiration Date:	Will be provided in the Final Report
Special Handling and/or Precautions:	None
Storage Conditions:	Room Temperature

Test and Control Article Characterization. The Sponsor will supply Certificates of Analyses and stability certifications for GLP required characterization of the purity, composition, stability and other pertinent information for the test and control article(s). Similar information for materials (e.g., excipients) used in preparation of dose solutions, if applicable, will be obtained by Pacific BioLabs. Documentation of the characterization of test articles, control articles and excipients (as applicable) will be included in the Study Report. The absence of documentation of the identity, composition, strength and stability of the test articles or control articles (e.g., a CofA) will be considered noncompliance with GLP expectations and will be documented in the Final Report.

The Sponsor's signature and approval of this Protocol indicates that appropriate documentation of the method of synthesis, fabrication or derivation of the test and control article(s) is available to the appropriate regulatory agencies if requested.

Dose Formulation Analysis. Dose formulation analysis will not be conducted for prepared test articles.

Reserve Sample and Sample Disposition. Unless requested otherwise, unused test articles or control articles will be discarded or destroyed at the end of the study according to Pacific BioLabs SOP.

FDA and US Environmental Protection Agency (EPA) regulations require that, for studies of more than four weeks duration, reserve sample from each batch of material be retained for the period of time provided in FDA GLP Regulations 21 CFR Parts 58.105 and 58.195; EPA FIFRA GLP Regulations 40 CFR Parts 160.105 and 160.195; and EPA TSCA GLP Regulations 40 CFR Parts 792.105 and 792.195. The various agencies have, in the past, recommended that the amount of reserve sample be enough to repeat the study two or three times. Sponsor is responsible for retention of test and control article reserves.

3.2. Test System

Species:	Mouse
Strain:	Albino (from the same source)
Source:	Approved vendor
Number*:	20 mice; 10 males/10 females; 5 test/5 control
Gender:	Male and Female
Age:	Young adult
Initial Weight:	The weight variation of animals used within a gender will not exceed ± 20% of the mean weight
Identification:	Cage labeling and tail marking

*Alternate animals will be obtained for this study and will be used as a replacement if necessary. Unused animals will be released into Pacific BioLabs animal colony.

Environment. Mice will be housed in groups in polycarbonate cages. Animals will be maintained in a controlled environment at a nominal temperature range of 20 to 26°C, a humidity range of 50 ± 20%, and a light/dark cycle of 12 hours. The 12-hour lighting cycle may be briefly interrupted to accommodate study procedures. These brief interruptions will be considered as unlikely to have an effect on the outcome of a study and will not be considered study deviations. Animals will be maintained in rooms with at least ten room air changes per hour. Room logs documenting temperature and humidity are kept on file at Pacific BioLabs.

Diet and Feed. Mice will receive Certified Laboratory Rodent Diet *ad libitum*. The feed is analyzed by the supplier for nutritional components and environmental contaminants. There are no known contaminants in the feed that are reasonably expected to interfere with the conduct of this study. It may be necessary during the course of the study to offer supplemental food as part of standard veterinary care. This may not be a certified diet, but will be commercially available food that contains no known contaminants that would interfere with the conduct of this study. Food will be withheld during dosing.

Water. Fresh, potable drinking water will be provided *ad libitum* to all animals via a sipper tube. Water testing is conducted two times a year for total dissolved solids and specified microbiological content and

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selected elements, heavy metals, organophosphates, and chlorinated hydrocarbons. There are no known contaminants in the water that are reasonably expected to interfere with the conduct of this study. Water will be withheld during dosing.

Acclimation. Mice will be acclimated for at least 5 days prior to initiation of the study. Health observations will be performed prior to the study to ensure that the animals are acceptable for study use.

Veterinary Care. Veterinary care will be available during the course of the study. Due to the nature of this test, animals will not be treated for mild to moderated signs of toxicity (lethargy, unkempt appearance) as assessment of toxicity is the primary objective. No adverse effects are expected but humane endpoints will be in effect. Animals in physical distress (labored breathing, convulsion) or moribund, including animals that have lost greater than 15% of their body weight, will be immediately euthanized at the discretion of the veterinarian.

Assignment to Study and Disposition. Animals will be examined prior to study initiation, and determined (based on clinical observations) if suitable as test subjects. Eligibility for inclusion on test will be established by the Study Director (or alternate). Disposition of study animals is documented in the Pacific BioLabs study records. Alternate animals not selected for the study will be returned to the Pacific BioLabs animal colony for use in subsequent studies or procedures.

3.3. Experimental Design

Test Article Preparation. The test article will be prepared per Sponsor instructions. The test article is a diluted plant extraction a liquid form and will be tested as received. Physiological saline will be used as a control.

Procedures. A total of twenty albino mice (male/female; ten five for test article and five for corresponding control) will be used. Animals will be randomly assigned to test or control group.

Each animal will be administered 6.4 ml/kg of the test article or control according to Table 1. The doses will be adjusted based on the last obtained body weight (Day 1, Day 7, and Day 14). The doses will be rounded to the nearest 0.1 mL.

The animals will be observed prior to and immediately after dosing. In addition, animals will be observed at 4 hours ± 15 minutes following dose administration of each day. Cage-side observations will include, but not be limited to, changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous system, somatomotor activity, and behavior patterns.

Animals will be weighed prior to dosing on Day 1, Day 7, and Day 14. Additional observations or body weight measurements may be performed if deemed necessary by the Study Director.

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Table 1. Study Design

Group	Sex	Number of Animals (n)		Route of Administration	Dose volume	Observations	Body Weights	Gross Necropsy
		Test	Control					
1	M	5	5	Oral Gavage	6.4 ml/kg	Immediately; 4 hours ± 15 minutes after dosing; daily	Prior to Dose 1, Dose 7, and Dose 14	Yes
2	F	5	5	Oral Gavage	6.4 ml/kg	Immediately; 4 hours ± 15 minutes after dosing; daily	Prior to Dose 1, Dose 7, and Dose 14	Yes

3.4. In-Life Observations

Mortality/Moribundity Checks. General health of animals will be observed prior and during the test. All of the animals will be observed for adverse reactions immediately after dosing, approximately 4 hours after dosing and daily until the end of the study. Abnormal behavior or evidence of poor health will be noted in the study file and the Study Report. Moribund animals, those in respiratory distress or animals exhibiting convulsions will be humanely euthanized.

Body Weight. Animals will be weighed prior to Dose 1, Dose 7 and Dose 14.

3.5. Terminal Procedures and Measurements

Moribund Animals. Animals in physical distress (e.g. labored breathing, seizures) or moribund will be euthanized at the discretion of the attending veterinarian. Animals removed from the study may be replaced at the discretion of the Study Director, if replacement does not adversely affect study conduct.

Post mortem Examinations. Attempts will be made to conduct a gross necropsy on animals that die during the study. Gross necropsy may have limited value due to rapid autolysis that occurs in small animals.

At the end of the study, animals will be euthanized as per Pacific BioLabs SOPs and the method of euthanasia will be consistent with the recommendations of the American Veterinary Medical Association guidelines on euthanasia.

Gross necropsy will be performed and observed lesions will be collected and preserved in 10% formalin. Histopathology may be performed if requested by Sponsor. The disposition of study animals will be documented in the Pacific BioLabs study records.

4. DATA ACQUISITION AND ANALYSIS

4.1. Descriptive Statistics

No descriptive statistics will be generated by Pacific BioLabs for these studies.

4.2. Statistical Analysis

No statistical analyses will be performed by Pacific BioLabs for these studies.

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5. REPORTS

5.1. General Description of Study Report

The Study Report will include all information necessary to provide a complete and accurate description of the experimental procedures and results. The Study Report will include a compliance statement signed by the Study Director that the report accurately reflects the raw data obtained during the performance of the study and that all applicable GLP regulations were followed in the conduct of the study.

5.2. Study Report

The individual Study Report will include, but not be limited to, the following:

Name and address of the test facility
Study dates
Study summary
The objective of the study
Test and control article identification
A full description of the test system
A full description of the experimental design and methods
Study results in prose and tabular form as appropriate
Any deviations from the Protocol
Signed statement of compliance from the Study Director

The Study Report will not include results of analyses performed by the Sponsor. Communication of the results of these Sponsor-conducted analyses to the appropriate regulatory agencies will be the responsibility of the Sponsor. Upon finalization, copies of the Final Reports will be provided to the Sponsor as hardcopies or PDF files.

6. MAINTENANCE OF RAW DATA, RECORDS AND SPECIMENS

Original data, specimens and reports from this study are the property of the Sponsor. These materials will be available to the Sponsor to facilitate reviewing the study during its progress and before issuance of the Final Report. Records (including, but not limited to, protocol, protocol amendments(s), in-life records, pathology records, dose preparation records, correspondence related to the study, Final Report, and histopathology records) and materials (including, but not limited to, slides, specimens, wet tissues and blocks) will be archived at Pacific BioLabs (Hercules, CA) for a period of one year after issuance of the Final Report. After one year, the Sponsor will be contacted concerning continued storage or return of materials.

Records and materials associated with activities external to Pacific BioLabs (including, but not limited to, clinical pathology, histopathology, and bioanalysis) and activities conducted by the Sponsor (including, but not limited to, dose solution analysis), will be archived by the individual performing laboratories or the Sponsor in a manner consistent with their individual operating SOPs and regulatory requirements.

7. REFERENCES

PBL ACUP 17C-09, rev. IACUC 9.0, *Toxicity Study in Mice*
Good Laboratory Practice Regulations; Food and Drug Administration: 21 CFR Part 58.
Good Laboratory Practice Regulations; Environmental Protection Agency: 40 CFR Part 160
National Institutes of Health. Position statement on the Use of Animals in Research, NIH Guide 22(8), Feb 26, 1993.

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PROTOCOL

AMENDMENT 1

Sponsor:	Immune & Genetics Protocols, LLC 5750 SW 6th Place Ocala, FL 34474 United States
Sponsor Representative:	William Denizard Phone: 407-616-2970 Email: billydenizard@gmail.com
Test Facility:	Pacific BioLabs 551 Linus Pauling Drive Hercules, CA 94547 United States Phone: (510) 964-9000
Study Director:	Simret Beraki, Ph.D. Phone: (510) 964-9000 E-mail: simretberaki@pacificbiolabs.com
Pacific BioLabs Study Number:	23E0241G-X01G

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Change (1) From:

Table 1. Study Design

Group	Sex	Number of Animals (n)		Route of Administration	Dose volume	Observations	Body Weights	Gross Necropsy
		Test	Control					
1	M	5	5	Oral Gavage	6.4 ml/kg	Immediately; 4 hours ± 15 minutes after dosing; daily	Prior to Dose 1, Dose 7, and Dose 14	Yes
2	F	5	5	Oral Gavage	6.4 ml/kg	Immediately; 4 hours ± 15 minutes after dosing; daily	Prior to Dose 1, Dose 7, and Dose 14	Yes

Change (1) To:

Table 1. Study Design

Group	Sex	Number of Animals (n)		Route of Administration	Dose volume	Observations	Body Weights	Gross Necropsy
		Test	Control					
1	M	5	5	Oral Gavage	0.1 ml	Immediately; 4 hours ± 15 minutes after dosing; daily	Prior to Dose 1, Dose 7, and Dose 14	Yes
2	F	5	5	Oral Gavage	0.1 ml	Immediately; 4 hours ± 15 minutes after dosing; daily	Prior to Dose 1, Dose 7, and Dose 14	Yes

Justification: Updated Dose volume

APPROVAL SIGNATURES

William Denizard — June 22, 2023

Sponsor — Date

Simret Beraki — 22Jun2023

Study Director
Pacific BioLabs — Date

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PROTOCOL

AMENDMENT 2

Sponsor:	Immune & Genetics Protocols, LLC 5750 SW 6th Place Ocala, FL 34474 United States
Sponsor Representative:	William Denizard Phone: 407-616-2970 Email: billydenizard@gmail.com
Test Facility:	Pacific BioLabs 551 Linus Pauling Drive Hercules, CA 94547 United States Phone: (510) 964-9000
Study Director:	Simret Beraki, Ph.D. Phone: (510) 964-9000 E-mail: simretberaki@pacificbiolabs.com
Pacific BioLabs Study Number:	23E0241G-X01G

Page 1 of *3*

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Change (1) From:

3.3. Experimental Design

Test Article Preparation. The test article will be prepared per Sponsor instructions. The test article is a diluted plant extraction a liquid form and will be tested as received. Physiological saline will be used as a control.

Procedures. A total of twenty albino mice (male/female; ten five for test article and five for corresponding control) will be used. Animals will be randomly assigned to test or control group.

Each animal will be administered 0.1ml of the test article or control according to Table 1. Each animal will be administered approximately 5ml/kg instead of 6.4 ml/kg.

Change (1) To:

3.3. Experimental Design

Test Article Preparation. The test article will be prepared per Sponsor instructions. The test article is a diluted plant extraction in liquid form and will be tested as received. Physiological saline will be used as a control.

Procedures. A total of twenty albino mice (ten male and ten female; Five males and five females for test article and five males and five females for corresponding control) will be used. Animals will be randomly assigned to test or control group.

Each animal will be administrated with 0.1 ml (approximately 5 ml/kg as per average mice weight) of the test article or control according to Table 1. The doses will be adjusted based on last obtained body weight. The doses will be rounded to the nearest 0.1 ml.

Justification: Updated Test Procedures

Change (2) From:

7. REFERENCES

PBL ACUP 17C-09, rev. IACUC 9.0, *Toxicity Study in Mice*
Good Laboratory Practice Regulations; Food and Drug Administration: 21 CFR Part 58.
Good Laboratory Practice Regulations; Environmental Protection Agency: 40 CFR Part 160
National Institutes of Health. Position statement on the Use of Animals in Research, NIH Guide 22(8), Feb 26, 1993.

Change (2) To:

7. REFERENCES

ISO 10993-11:2017 (E), *Biological Evaluation of Medical Devices* – Part 11: *Tests for Systemic Toxicity, Acute Systemic Toxicity*
ISO 10993-12:2021, *Biological Evaluation of Medical Devices – Sample Preparation and Reference Materials*
PBL SOP 16G-63, rev. 3G.00, ISO Acute Systemic Toxicity Test (Mice/Rats)
PBL ACUP 17C-09, rev. IACUC 9.0, *Toxicity Study in Mice*
Good Laboratory Practice Regulations; Food and Drug Administration: 21 CFR Part 58.
Good Laboratory Practice Regulations; Environmental Protection Agency: 40 CFR Part 160
National Institutes of Health. Position statement on the Use of Animals in Research, NIH Guide 22(8), Feb 26, 1993.

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Justification: References section updated

APPROVAL SIGNATURES

William Denizard

Sponsor

June 30, 2023

Date

Siwret Berahi

Study Director
Pacific BioLabs

30 Jun 2023

Date

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