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ACB Olive Leaf Extract PF Toxicology Data

Code: 20349PF
INCI Name: Lactobacillus/Olive Leaf Ferment Extract
CAS #: 8003-25-0
EINECS #: 232-277-0

Name of Study	Type of Study	Results
Dermal & Ocular Irritation Tests	*In-vitro*	Both the dermal and ocular assays reveal that **ACB Olive Leaf Extract PF** is non-irritating and should not cause any of the aforementioned conditions.
AMES Test	*In-vitro*	The results of the Bacterial Reverse Mutation Assay indicate that under the conditions of this assay, that **ACB Olive Leaf Extract PF** was considered to be Non-Mutagenicto Salmonella typhimurium testerstrains TA98, TA100, TA1537, TA1535 and Escherichia coli WP2uvrA

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Dermal and Ocular Irritation Tests

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Sample: ACB Olive Leaf Extract PF

Code: 20349PF

CAS #: 8001-25-0

Test Request Form/Submission #: 119

Lot #: NC120917-F

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Erica Segura*
Principle Investigator: *Meghan Darley*

Test Performed:
In Vitro EpiDerm™ Dermal Irritation Test (EPI-200-SIT)
EpiOcular™ Eye Irritation Test (OCL-200-EIT)

SUMMARY

In vitro dermal and ocular irritation studies were conducted to evaluate whether **ACB Olive Leaf Extract PF** would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays.

The product was tested according to the manufacture's protocol. The test article solution was found to be **non-irritating**. Reconstructed human epidermis and cornea epithelial model were incubated in growth media overnight to allow for tissue equilibration after shipping from MatTek Corporation, Ashland, MA. Test substances were applied to the tissue inserts and incubated for 60 minutes for liquid and solid substances in the EpiDerm™ assay and 30 minutes for liquid substances and 90 minutes for solid substances in the EpiOcular™ assay at 37°C, 5% CO_2, and 95% relative humidity (RH). Tissue inserts were thoroughly washed and transferred to fresh plates with growth media. After post substance dosing incubation is complete, the cell viability test begins. Cell viability is measured by dehydrogenase conversion of MTT [(*3-4,5-dimethyl thiazole 2-yl*)], present in the cell mitochondria, into blue formazan salt that is measured after extraction from the tissue. The irritation potential of the test chemical is dictated by the reduction in tissue viability of exposed tissues compared to the negative control.

Under the conditions of this assay, the test article was considered to be **non-irritant**. The negative and positive controls performed as anticipated.

I. Introduction

A. Purpose

In vitro dermal and ocular irritation studies were conducted to evaluate whether a test article would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays. MatTek Corporation's reconstructed human epidermal and human ocular models are becoming a standard in determining the irritancy potential of test substances. They are able to discriminate between irritants and non-irritants. The EpiDerm™ assay has accuracy for the prediction of UN GHS R38 skin irritating and no-label (non-skin irritating) test substances. The EpiOcular™ assay can differentiate chemicals that have been classified as R36 or R41 from the EU classifications based on Dangerous Substances Directive (DSD) or between the UN GHS Cat 1 and Cat 2 classifications.

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II. Materials

A. Incubation Conditions: 37°C at 5% CO_2 and 95% relative humidity

B. Equipment: Forma humidified incubator, ESCO biosafety laminar flow hood, Synergy HT Microplate reader; Pipettes

C. Media/Buffers: DMEM based medium; DPBS; sterile deionized H_2O

D. Preparation: Pre-incubate (37°C) tissue inserts in assay medium; Place assay medium and MTT diluent at 4°C, MTT concentrate at -20°C, and record lot numbers of kit components

E. Tissue Culture Plates: Falcon flat bottom 96-well, 24-well, 12-well, and 6-well tissue culture plates

F. Reagents: MTT (1.0mg/mL); Extraction Solution (Isopropanol); SDS (5%); Methyl Acetate

G. Other: Nylon Mesh Circles (EPI-MESH); Cotton tip swabs; 1mL tuberculin syringes; Ted Pella micro-spatula; 220mL specimen containers; sterile disposable pipette tips; Parafilm

III. Test Assay

A. Test System

The reconstructed human epidermal model, EpiDerm™, and cornea epithelial model, EpiOcular™, consist of normal human-derived epidermal keratinocytes which have been cultured to form a multilayer, highly differentiated model of the human epidermis and cornea epithelium. These models consist of organized basal, spinous, and granular layers, and the EpiDerm™ systems also contains a multilayer stratum corneum containing intercellular lamellar lipid layers that the EpiOcular™ system is lacking. Both the EpiDerm™ and EpiOcular™ tissues are cultured on specially prepared cell culture inserts.

B. Negative Control

Sterile DPBS and sterile deionized water are used as negative controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

C. Positive Control

Known dermal and eye irritants, 5% SDS solution and Methyl Acetate, were used as positive controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

D. Data Interpretation Procedure

a. EpiDerm™

An irritant is predicted if the mean relative tissue viability of the 3 tissues exposed to the test substance is reduced by 50% of the mean viability of the negative controls and a non-irritant's viability is > 50%.

b. EpiOcular™

An irritant is predicted if the mean relative tissue viability of the 2 tissues exposed to the test substance is reduced by 60% of the mean viability of the negative controls and a non-irritant's viability is > 40%.

IV. Method

A. Tissue Conditioning

Upon MatTek kit arrival at Active Concepts, LLC the tissue inserts are removed from their shipping medium and transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO_2 and 95% relative humidity for 60 minutes. After those 60 minutes the inserts are transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO_2 and 95% relative humidity for an additional 18 to 21 hours.

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B. Test Substance Exposure

a. EpiDerm™

30µL (liquid) or 25mg (solid) of the undiluted test substance is applied to 3 tissue inserts and allowed to incubate for 60 minutes in a humidified incubator (37°C, 5% CO_2, 95% RH).

b. EpiOcular™

Each tissue is dosed with 20µL DPBS prior to test substance dosing. 50µL (liquid) or 50mg (solid) of the undiluted test substance is applied to 2 tissue inserts and allowed to incubate for 90 minutes in a humidified incubator (37°C, 5% CO_2, 95% RH).

C. Tissue Washing and Post Incubation

a. EpiDerm™

All tissue inserts are washed with DPBS, dried with cotton tipped swab, and transferred to fresh media and culture plates. After 24 hours the inserts are again transferred into fresh media and culture plates for an additional 18 to 20 hours.

b. EpiOcular™

Tissue inserts are washed with DPBS and immediately transferred into 5mL of assay medium for 12 to 14 minutes. After this soak the inserts are transferred into fresh media and tissue culture plates for 120 minutes for liquid substances and 18 hours for solid substances.

D. MTT Assay

Tissue inserts are transferred into 300µL MTT media in pre-filled plates and incubated for 3 hours at 37°C, 5% CO_2, and 95% RH. Inserts are then removed from the MTT medium and placed in 2mL of the extraction solution. The plate is sealed and incubated at room temperature in the dark for 24 hours. After extraction is complete the tissue inserts are pierced with forceps and 2 x 200µL aliquots of the blue formazan solution is transferred into a 96 well plate for Optical Density reading. The spectrophotometer reads the 96-well plate using a wavelength of 570 nm.

V. Acceptance Criterion

A. Negative Control

The results of this assay are acceptable if the mean negative control Optical Density (OD_{570}) is ≥ 1.0 and ≤ 2.5 (EpiDerm™) or ≥ 1.0 and ≤ 2.3 (EpiOcular™).

B. Positive Control

a. EpiDerm™

The assay meets the acceptance criterion if the mean viability of positive control tissues expressed as a % of the negative control is ≤ 20%.

b. EpiOcular™

The assay meets the acceptance criterion if the mean viability of positive control tissues is < 60% of control viability.

C. Standard Deviation

Since each irritancy potential is predicted from the mean viability of 3 tissues for EpiDerm™ and 2 tissues for EpiOcular™, the variability of the replicates should be < 18% for EpiDerm™ and < 20% EpiOcular™.

VI. Results

A. Tissue Characteristics

The tissue inserts included in the MatTek EpiDerm™ and EpiOcular™ assay kits were in good condition, intact, and viable.

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B. Tissue Viability Assay

The results are summarized in Figure 1. In no case was the tissue viability ≤ 50% for EpiDerm™ or ≤ 60% for EpiOcular™ in the presence of the test substance. The negative control mean exhibited acceptable relative tissue viability while the positive control exhibited substantial loss of tissue viability and cell death.

C. Test Validity

The data obtained from this study met criteria for a valid assay.

VII. Conclusion

Under the conditions of this assay, the test article substance was considered to be **non-irritating**. The negative and positive controls performed as anticipated.

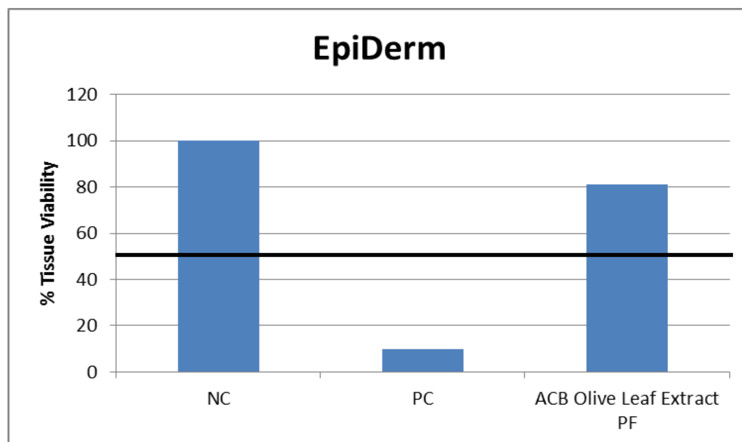

Figure 1: EpiDerm tissue viability

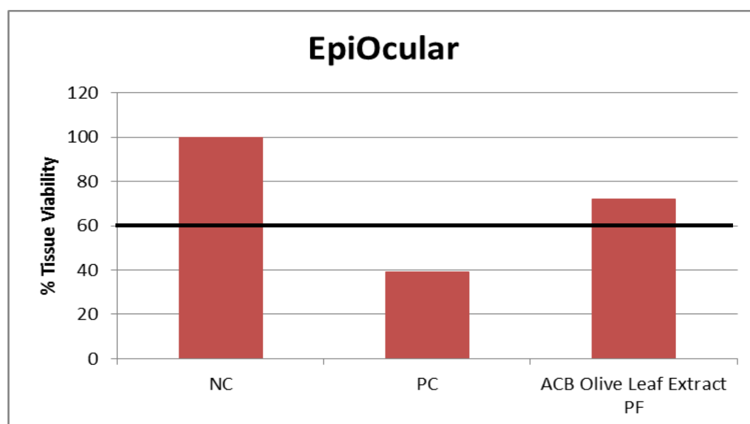

Figure 2: EpiOcular tissue viability

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Bacterial Reverse Mutation Test

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Sample: ACB Olive Leaf Extract PF
CAS #: N/A

Sponsor:
Active Concepts, LLC
107 Technology Drive
Lincolnton, NC 28092
Study Director: *Erica Babson*
Principle Investigator: *Dale Hanna*

Test Performed:
Genotoxicity: Bacterial Reverse Mutation Test

Reference:
OECD471/ISO10993.Part3

SUMMARY

A *Salmonella typhimurium* reverse mutation standard plate incorporation study was conducted to evaluate whether a test article solution **ACB Olive Leaf Extract PF** would cause mutagenic changes in the average number of reveratants for histidine-dependent *Salmonella typhimurium* strains TA97a, TA98, TA100, TA1537 in the presence and absence of S9 metabolic activation. This study was conducted to satisfy, in part, the Genotoxicity requirement of the International Organization for Standardization: Biological Evaluation of Medical Devices, Part 3: Tests for Genotoxicity, Carcinogenicity and Reproductive Toxicity.

The stock test article was diluted with sterile deionoized (DI) water to a concentration of 50µl/ml for testing. The test article solution was found to be noninhibitory to growth of tester strains TA97a, TA98, TA100, and TA1537. Separate tubes containing 2ml of molten top agar supplemented with histidine-biotin solution for the *S. typhimurium* strains were inoculated with 100µl of culture for each of the tester strains and 100 µl of the test solution. A 500 µl aliquot of S9 homogenate, simulating metabolic activation, was added when necessary. The mixture was poured across the Minimal Glucose Plates. Parallel testing was also conducted with a negative control and one positive control. The mean numbers of reveratants of the test plates were compared to the mean number of reveratants of the negative control plates for each of the strains tested. The means obtained for the positive controls were used as points of reference.

Under the conditions of this assay, the test article solution was considered to be nonmutagenic to *Salmonella typhimurium* tester strains TA97a, TA98, TA100, and TA1537. The negative and positive controls performed as anticipated. The results of this study should be evaluated in conjunction with other required tests as listed in ISO 100993, Part 3: Tests for Genotoxicity, Carcinogenicity, and Reproductive Toxicology.

I. Introduction

A. **Purpose**

A *Salmonella typhimurium* reverse mutation standard plate incorporation study was conducted to evaluate whether a test article solution would cause mutagenic changes in the average number of revertants for *Salmonella typhimurium* tester strains TA97a, TA98, TA100, and TA1537 in the presence and absences of the S9 metabolic activation. Bacterial reverse mutation tests have been widely used as rapid screening procedures for the determination of mutagenic and potential carcinogenic hazards.

II. Materials

A. **Storage Conditions:** Room temperature (23-25C)
B. **Vehicle:** Sterile DI Water
C. **Preparation:** A 50 µl/ml solution was prepared with sterile DI water. A negative control (vehicle with out test material) was similarly prepared
D. **Solubility:** 100% Soluble and Stable

III. Test System

A. Test System

Each *Salmonella typhimurium* tester strain contains a specific mutation in the histidic operon and other mutations that increase their ability to detect mutagens. These genetically altered *S. typhimurium* strains (TA97a, TA98, TA100, and TA1537) cannot grow in the absence of histidine, respectively. When placed in a histidine-free medium, only those cells which mutate spontaneously back to their wild type states (histidine independent by manufacturing their own histidine) are able to form

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colonies. The spontaneous mutation rate (ore reversion rate) for any one strain is relatively constant, but if a mutagen is added to the test system, the mutation rate is significantly increased.

Tester strain	Mutations/Genotypic Relevance
TA97a	hisO1242
TA98	hisD3052, *rfa, uvrB*, frame shift, pKM101
TA100	hisG46, *rfa, uvrB*, missense, pKM101
TA1537	hisC3076, *rfa*, *uvrB*, frame shift

rfa	=	causes partial loss of the lip polysaccharide wall which increases permeability of the cell to large molecules.
uvrB	=	deficient DNA excision-repair system (i.e., ultraviolet sensitivity)
frame shift	=	base-pair addition/deletion
missense	=	base-pair substation
pKM101	=	plasmid confers ampicillin resistance (R-factor) and enhances sensitivity to mutagens.

B. Metabolic Activation
Aroclor induced rat liver (S9) homogenate was used as metabolic activation. The S9 homogenate is prepared from male, Sprague Dawley rats. Material is supplied by Moltox, Inc.

C. Preparation of Tester strains
Cultures of *Salmonella typhimurium* TA97a, TA98, TA100, and TA1537 were inoculated to individual flasks containing oxoid broth. The inoculated broth cultures were incubated at 37°C in an incubator shaker operating at 140-150 rpm for 12-16 hours.
D. Negative Control
Sterile DI water (vehicle without test material) was tested with each tester strain to determine the spontaneous reversion rate. Each strain was tested with and without S9 activation. These data represented a base rate to which the number of reveratants colonies that developed in each test plate were compared to determine whether the test material had significant mutagenic properties.
E. Positive Control
A known mutagen, Sodium Azide, was used as a positive control to demonstrate that tester strain TA100 was sensitive to mutation to the wild type state. The positive control is tested with and without the presence of S9 homogenate.
F. Strain Characteristics and Strain Standard Plate Counts
Strain characteristics were verified and viable counts were determined.

IV. Method
A. Standard Plate Incorporation Assay
Separate tubes containing 2 ml of molten top agar supplemented with histidine-biotin solution for the *Salmonella typhimurium* were inoculated with 100 µl of culture for each strain and 100 µl of testing solution. A 500 µl aliquot of S9 homogenate, simulating metabolic activation, was added when necessary. The mixture was poured across Minimal Glucose Agar plates labeled with strain number and S9 activation (+/-).

Histidine free media plates were prepared as follows:
1. Test solution with and without S9 activation
2. Negative control with and without S-9 activation
3. Positive control, Sodium Azide (a known mutagen), with and without S9 activation for TA100.

The plates were incubated for 37°C for 2 days. Following the incubation period, the revertant colonies on each plate were recorded. The mean number of reveratnts was determined. The mean number of revertants of the test plates were compared to the mean number of reverants of the negative control of each strain used.

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Bacterial Reverse Mutation Test

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V. Evaluation

For the test solution to be evaluated as a test failure or "potential mutagen" there must have been a 2-fold or greater increase in the number of mean revertants over the means obtained from the negative control for any or all strains. Each positive control mean must have exhibited at least a 3-fold increase over the respective negative control mean of the *Salmonella* tester strain used.

VI. Results

A. Strain Characteristics and Strain Standard Plate Count

Salmonella typhimurium strains TA97a, TA98, TA100, and TA1537 exhibited appropriate genetic characteristics pertaining to this assay.

Titer (Organisms/ml): 5.0x10^8

B. Standard Plate Incorporation Assay

The results are summarized in Appendix 1. In no case was there a 2-fold or greater increase in the mean number of revertant testing strains TA97a, TA98, TA100, and TA1537 in the presence of the test solution. The positive control mean exhibited at least a 3-fold increase over the respective mean of the *S. typhimurium* tester strain used.

1. Test Validity

The data obtained from this study met criteria for a valid assay.

VII. Conclusion

Under the conditions of this assay, the test article solution was considered to be nonmutagenic to *Salmonella typhimurium* tester strains TA97a, TA98, TA100, and TA1537. The negative and positive controls performed as anticipated. The results of this study should be evaluated in conjunction with other required tests as listed in ISO 100993, Part 3: Tests for Genotoxicity, Carcinogenicity, and Reproductive Toxicology.

	TA97a		TA98		TA100		TA1537	
	CFU	Mean	CFU	Mean	CFU	Mean	CFU	Mean
DI Water w/o S9 negative control	7	7	3	2	8	6	37	41
DI Water w/o S9 negative control	7		1		4		45	
DI Water w/ S9 negative control	213	199	150	162	114	116	133	131
DI Water w/ S9 negative control	186		175		118		130	
Test Solution w/o S9	11	11	5	6	13	18	56	44
Test Solution w/o S9	11		7		24		32	
Test Solution w/ S9	150	138	120	115	191	200	145	142
Test Solution w/ S9	127		110		210		140	
Sodium Azide w/o S9					TNTC	TNTC		
Sodium Azide w/o S9					TNTC			
Sodium Azide w/ S9					TNTC	TNTC		
Sodium Azide w/ S9					TNTC			
Historical Count w/o S9[1]	75-200		20-50		75-200		20-50	
Historical Count w/ S9[1]	100-200		20-50		75-200		20-50	

Table 1. Standard Plate Count Results and Historical Count Values.
CFU = Colony Forming Units Mean= Average of duplicate plates

[1] Mortelmans. K and Zeiger. E. *The Ames Salmonella/microsome mutagenicity assay.* Mutation Research. 455: 29-60

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