Active Concepts

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

Dermal Irritation Test

Sample: ACB Willow Bark Extract 20%

Code: 20200

CAS #: 7732-18-5 & 84650-64-6

Test Request Form/Submission #: 3570

Lot #: 54313P

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Jennifer Goodman*

Test Performed:
In Vitro EpiDerm™ Dermal Irritation Test (EPI-200-SIT)

SUMMARY

An *In vitro* dermal irritation study was conducted to evaluate whether **ACB Willow Bark Extract 20% (20200)** in comparison with salycilic acid would induce dermal irritation in the EpiDerm™ model assays. Both of these test substances are industrialy known for their antimicrobial properties

The product was tested according to the manufacture's protocol. The test article solution **ACB Willow Bark Extract 20%** was found to be non-irritating while the comparable concentrations of salycilic acid were found to have greater irritation under the same testing conditions. Reconstructed human epidermis models 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 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 **ACB Willow Bark Extract 20%** was considered to be **non-irritant**. The negative and positive controls performed as anticipated. The comparative treatment sample salycilic acid was found to induce greater irritation at comparable concentrations.

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

I. Introduction

A. Purpose

An *In vitro* dermal study was conducted to evaluate whether a test article would induce dermal irritation in the EpiDerm™ model assay. MatTek Corporation's reconstructed human epidermal 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

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™, consists of normal human-derived epidermal keratinocytes which have been cultured to form a multilayer, highly differentiated model of the human epidermis. The EpiDerm™ system consists of organized basal, spinous, granular layers, and a multilayer stratum corneum containing intercellular lamellar lipid layers. The EpiDerm™ tissues are cultured on specially prepared cell culture inserts.

B. Negative Control

Sterile DPBS is used as the negative control for the EpiDerm™ assay.

C. Positive Control

5% SDS solution is a known dermal irritant and it used as the positive control for the EpiDerm™ assay.

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%.

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

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.

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).

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.

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™).

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%.

C. Standard Deviation

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

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

VI. Results

A. Tissue Characteristics
The tissue inserts included in the MatTek EpiDerm™ assay kit were in good condition, intact, and viable.

B. Tissue Viability Assay
The results are summarized in Figure 1. In no case was the tissue viability of the EpiDerm™ ≤ 50% in the presence of the test substance **ACB Willow Bark Extract 20%**. In the presence of the test substance salicylic acid at both 0.5% in DPBS unadjusted pH 6.59 and 0.2% in DI Water adjusted to pH 4.68 with NaOH the viability of the EpiDerm™ tissue was ≤ 50%. These test solutions in terms of salicylate content are comparable to **ACB Willow Bark Extract 20%** in the 2.5% solutions which faired very well in tissue viabilty. The negative control mean exhibited acceptable relative tissue viability while the positive control exhibited substantial loss of tissue viability and cell death.

Both DPBS and DI water were used as vehicles for treatment to ensure the buffer system did not have an effect on the outcome of treatment. Salycilic acid was is soluble in water at room temperature up to 2g/L allowing for a 0.2% DI water test solution and slightly increase solubility in DPBS for a 0.5% test solution.

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

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

VII. Conclusion

The negative and positive controls performed as anticipated. Under the conditions of this assay, the test article substance **ACB Willow Bark Extract 20% (20200)** was considered to be **non-irritating** and out performed all correlating salicylic acid test samples. These results suggest the ability of **ACB Willow Bark Extract 20% (20200)** to maintain antimicrobial efficacy while providing significantly highter tissue viability rates and reduced irritation when compared to salicylic acid. As reported at the Society of Investigative Dermatology, Willow Bark Extract provides the benefits of salicylic acid such as exfoliation, and anti-microbial action, without any of the typically associated irritation from the active alone.

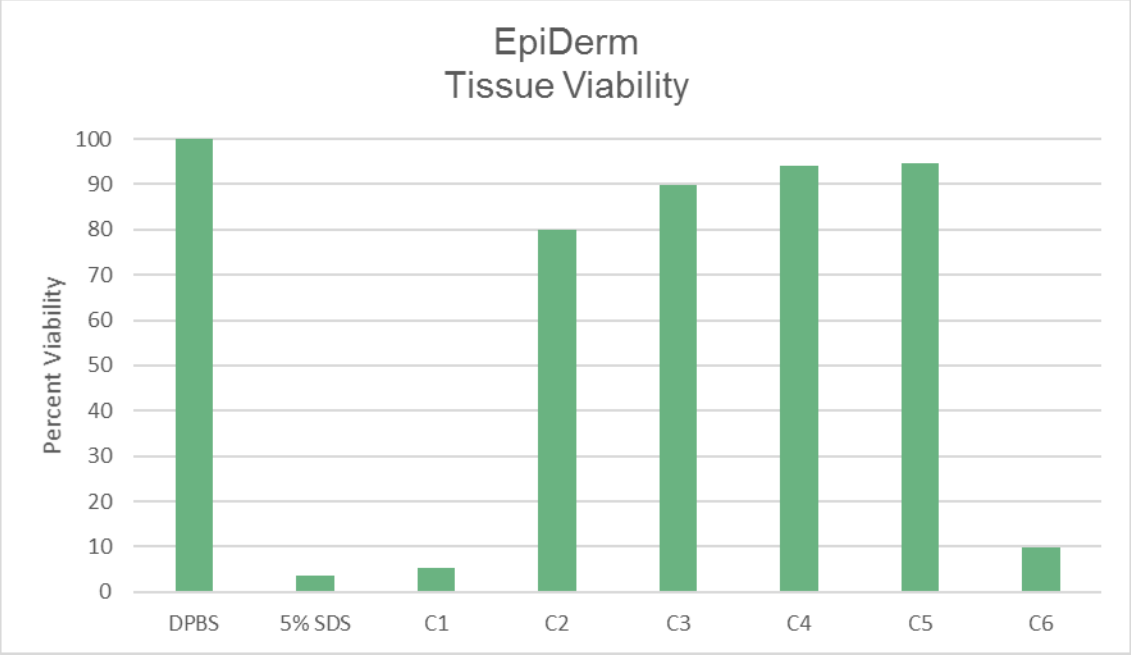

Figure 1: EpiDerm tissue viability

Sample Key:

- Negative Control- DPBS
- Positive Control- 5% SDS
- C1 Salicylic Acid @ 0.5% in DPBS unadjusted pH 6.59
- C2 Salicylic Acid @ 0.1% in DPBS unadjusted pH 6.21
- C3 20200 ACB Willow Bark Extract 20% @ 2.5% in DPBS unadjusted pH 6.77
- C4 20200 ACB Willow Bark Extract 20% @ 1.0% in DPBS unadjusted pH 6.86
- C5 20200 ACB Willow Bark Extract 20% @ 2.5% in DI Water unadjusted pH 4.68
- C6 Salicylic Acid @ 0.2% in DI Water adjusted to pH 4.68 with NaOH