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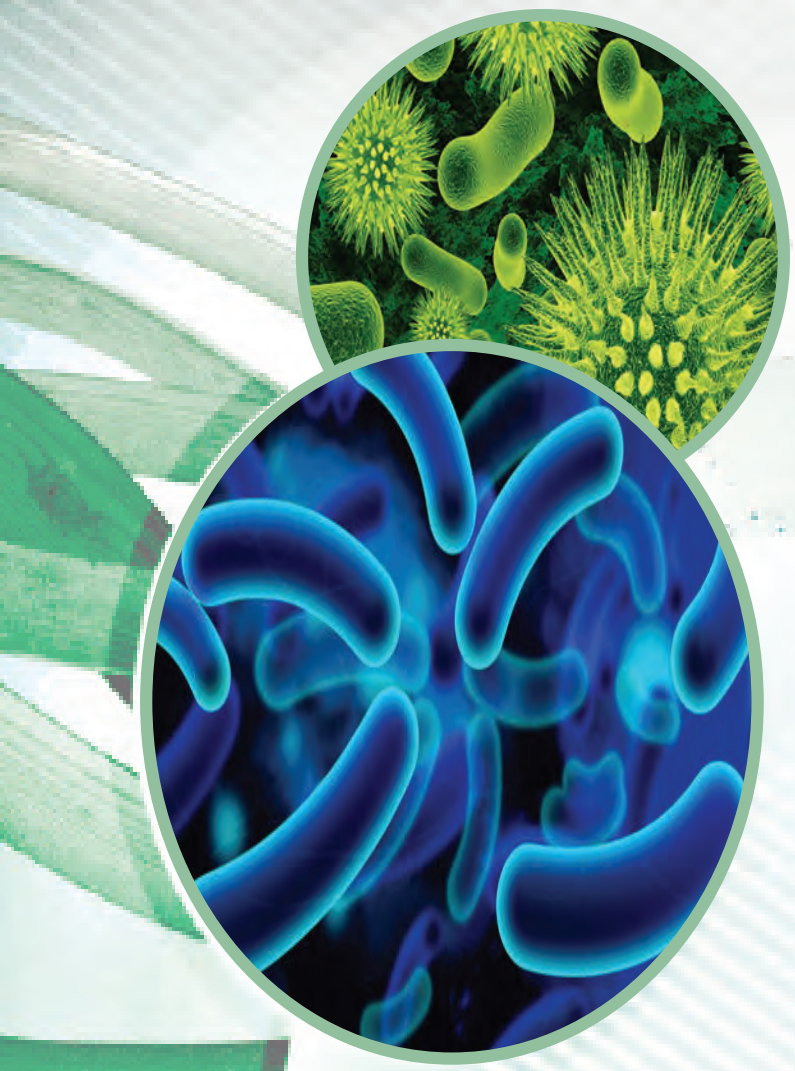
Technical Dossier



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PhytoCide Aspen Bark Extract Powder

Code Number: M16002

INCI Name: Populus tremuloides Bark Extract

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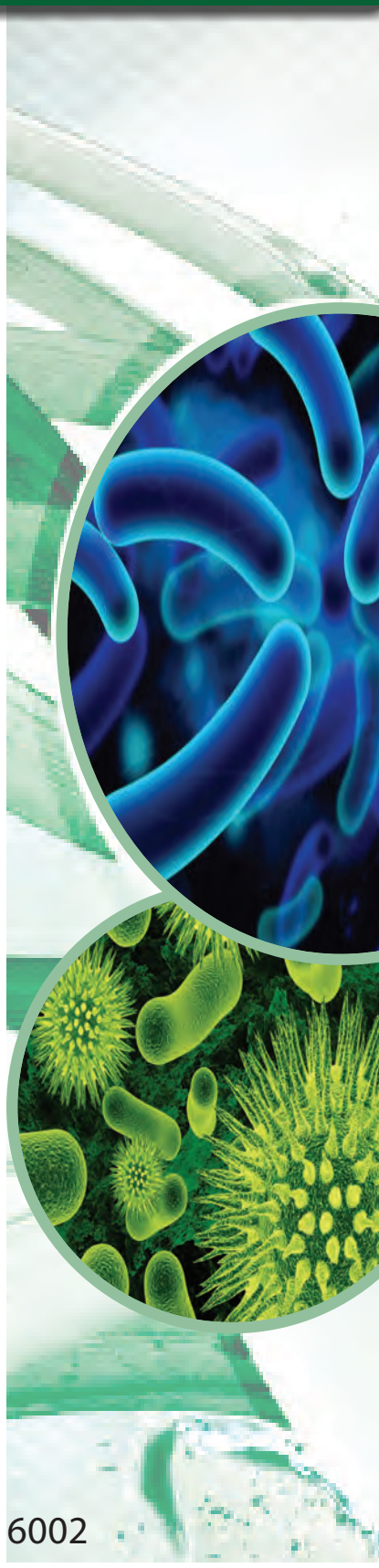

PhytoCide Aspen Bark Extract Powder Code Number: M16002
INCI Name: Populus tremuloides Bark Extract

PhytoCide Aspen Bark Extract Powder

Technical Data Sheet

BACKGROUND

Populus tremuloides also referred to as Quaking, Trembling or American Aspen originates in the northern and western areas of North America. The trees typically grow in vast colonies that originate from a single seedling. The trees' primary means of reproduction is via the sprouting root suckers (new stems) from the roots. Although the trees live on average between 40 and 150 years, the root system can survive for a considerably longer period of time. In fact an Aspen colony in Utah referred to as 'Pando' is said to be 80,000 years old, which would make this colony the oldest living colony. The Aspen's root system is capable of spreading approximately one meter per year and is therefore capable of expanding to cover an area of several hectares. One reason for the tree's success is due to the ability of the colony to withstand fires. Although the trees may burn, the roots that are capable of sprouting new trees are safely below the fire's reach.

SCIENCE

The bark of the Aspen tree is rich in salicylates that may function as the plant's natural defense mechanism against invading parasites. The salicylates in Aspen bark may also be used for medicinal purposes. Medicinal barks have been used as analgesics and to reduce fevers for centuries. Today, doses of 1 to 3 g of bark with concentrations ranging between 60 to 240 mg of salicylates is recommended as an analgesic for low back pain.

Code Number: M16002
INCI Nomenclature:
Populus tremuloides Bark Extract
INCI Status: Approved
REACH Status: Fully Compliant
CAS Number: 90083-05-9
EINECS Number: 290-199-2
Origin: Botanical:
Populus tremuloides
Processing:
GMO Free
No Ethoxylation
No Irradiation
No Sulphonation
No Ethylene Oxide treatment
No Hydrogenation
Additives: None
-Preservatives: None
-Antioxidants: None
Other additives: None
Solvents used: Water
Appearance: Free Flowing Powder
Soluble/Miscible: Fully Water Soluble
Suggested Use Levels: 0.2 - 3.0%
Suggested Applications:
Skin Conditioning & Antimicrobial

BENEFITS

These salicylates may be isolated from Aspen Bark and applied to cosmetic and personal care products as a natural alternative to traditional chemical preservative systems. Our manufacturing method allows us to consistently isolate a salicylate content between 54.0 – 60.0%, which makes **PhytoCide Aspen Bark Extract Powder** an ideal antimicrobial. As an additional benefit **PhytoCide Aspen Bark Extract Powder** may also impart a smooth feel to the skin.

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Version 9 - 2.20.14

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PhytoCide Aspen Bark Extract Powder

A Double Challenge Test was completed using 2% **PhytoCide Aspen Bark Extract Powder** in a generic cream base formulation. Samples were inoculated with *E. coli, P. aeruginosa, S. aureus, A. brasiliensis* and *C. albicans*. During the first 28-day incubation period, samples were periodically collected and tested for the presence of these microorganisms. Following this initial 28 days of incubation, the cream samples were then re-inoculated with the microbial cultures and sampled over an additional 28-day period. Table 1 shows the positive preservation results for **PhytoCide Aspen Bark Extract Powder**.

	E. coli	*P. aeruginosa*	*S. aureus*	*A. brasiliensis*	*C. albicans*
Inoculum (initial)	$1.2x10^6$	$2.1x10^4$	$8.0x10^5$	$1.3x10^4$	$1.8x10^4$
Day 0	99.967%	>99.999%	99.525%	99.462%	95.444%
Day 7	>99.999%	>99.999%	99.998%	99.385%	99.889%
Day 14	>99.999%	>99.999%	>99.999%	99.462%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	99.846%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	99.769%	>99.999%
Inoculum (re-inoculated)	$1.7x10^6$	$9.9x10^4$	$2.1x10^6$	$2.0x10^5$	$1.1x10^5$
Day 7	>99.999%	>99.999%	99.995%	98.500%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	98.500%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	99.000%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	99.850%	>99.999%

Table 1. Challenge Test results for 2% **PhytoCide Aspen Bark Extract Powder** in a cream formulation inoculated on day 0 and re-inoculated on day 28. Results show % reduction in viable organisms.

USE RECOMMENDATIONS

PhytoCide Aspen Bark Extract Powder is water soluble and may therefore be added to aqueous systems, as well as the aqueous phase of emulsions. When using **PhytoCide Aspen Bark Extract Powder** it is recommended to maintain the formulation pH between 3 and 9. We also suggest incorporating the product at temperatures of 60°C or below.

Microorganism Tested	MIC (%)
E. coli	1.00
P. aeruginosa	1.00
S. aureus	2.00
C. albicans	1.00
A. brasiliensis	1.00

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Specification

Product Name: PhytoCide Aspen Bark Extract Powder
Code Number: M16002
CAS #'s: 90083-05-9
EINECS #'s: 290-199-2
INCI Name: *Populus tremuloides* Bark Extract

Specification	Parameter
Appearance	Free Flowing Powder*
Color	White to Light Yellow
Odor	Characteristic
Solubility (in Water)	Soluble
Solids (1g-105°C-1hr)	92.0% Minimum
pH (3% solution in Water)	3.8 – 6.2
Phenolics (tested as Salicylic Acid)[1]	54.0 – 60.0%
Heavy Metals	< 20 ppm
Arsenic	< 3 ppm

*HYGROSCOPIC

Note:
1) Phenolic compounds of natural origin, tested as Salicylic acid via USP HPLC method.

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Compositional Breakdown

PhytoCide Aspen Bark Extract Powder
Code: M16002

Compositional Breakdown:

Ingredient	%
Populus Tremuloides Bark Extract	100.00

- **To our knowledge the above material is free of the following list of heavy metals:**
  - **Heavy Metals < 20 ppm (Max.)**
  - **Lead < 10 ppm (Max.)**
  - **Antimony < 5 ppm (Max.)**
  - **Arsenic < 3 ppm (Max.)**
  - **Mercury < 1 ppm (Max.)**
  - **Cadmium < 1 ppm (Max.)**

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Compositional Breakdown

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This is to certify that the following allergens were not detected in PhytoCide Aspen Bark Extract Powder:

ALLERGENS Dir 2003 15 CEE	
INCI NAME	CAS NUMBER
Alpha-IsoMethyl Ionone	127-51-5
Amyl Cinnamal	122-40-7
Anise Alcohol	105-13-5
Benzyl Alcohol	100-51-69
Benzyl Benzoate	120-51-4
Benzyl Cinnamate	103-41-3
Benzyl Salicylate	118-58-1
Butylphenyl Methylpropional	80-54-6
Cinnamal	104-55-2
Cinnamyl Alcohol	104-54-1
Citral	5392-40-5
Citronellol	106-22-9
Coumarin	91-64-5
Eugenol	97-53-0
Farnesol	4602-84-0
Geraniol	106-24-1
Hexyl Cinnamal	101-86-0
Hydroxycitronellal	107-75-5
Hydroxymethylpentyl 3-Cyclohexene carboxaldehyde	31906-04-4
Isoeugenol	97-54-1
Limonene	5989-27-5
Linalool	78-70-6
Methyl 2 Octynoate	111-12-6
Evernia prunastri	90028-68-5
Evernia furfuracea	90028-67-4
Amylcinnamyl Alcohol	101-85-9

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Compositional Breakdown

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This is to certify that PhytoCide Aspen Bark Extract Powder does not contain pesticide levels exceeding the following:

EPA Pesticide Levels	
INCI NAME	LIMIT (mg/kg)
Alachlor	0.02
Aldrin and Dieldrin	0.05
Azinphos-methyl	1.00
Bromopropylate	3.00
Chlordane(cis and trans)	0.05
Chlorfenvinphos	0.50
Chlorpyrifos	0.20
Chlorpyrifos-methyl	0.10
Cypermethrin	1.00
DDT	1.00
Deltamethrin	0.50
Diazinon	0.50
Dichlorvos	1.00
Dithiocarbamates	2.00
Endosulfan	3.00
Endrin	0.05
Ethion	2.00
Fenitrothion	0.50
Fenvalerate	1.50
Fonofos	0.05
Heptachlor	0.05
Hexachlorobenzene	0.10
Hexachlorocyclohexane	0.30
Lindane	0.60
Malathion	1.00
Methidathion	0.20
Parathion	0.50

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Compositional Breakdown

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Parathion-methyl	0.20
Permethrin	1.00
Phosalone	0.10
Piperonyl butoxide	3.00
Pirimiphos-methyl	4.00
Pyrethrins	3.00
Quintozene(sum of 3 items)	1.00

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Cellular Renewal Assay

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Tradename: PhytoCide Aspen Bark Extract Powder

Code: M16002

Test Request Form #: 975

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

Abstract

PhytoCide Aspen Bark Extract Powder Powder was evaluated for its ability to accelerate cell renewal by means of a traditional Dansyl Chloride protocol.

Methods & Materials

A 5% Dansyl Chloride was prepared by dispersing Dansyl Chloride 95% (Sigma) in petrolatum. Approximately 0.2 g of the ointment was applied to three 2cm x 2cm locations on the volar forearm of 12 (M/F) subjects between the ages of 19 and 43. The material was allowed to remain in place for 24 hours at which time any excess ointment was removed.

Two products were tested, with the remaining untreated site serving as the biological control. The products were applied in a randomized fashion. Approximately 50 μl of product was applied to the appropriate test site once per day. The sites were then examined daily under ultraviolet light (SL-3660 Long Wave Ultra Violet, Black Light Eastern Corp., Westbury, Long Island, NY) for fluorescence. The test was continued until no fluorescence was detectable at any site. The values listed reflect the average time for each product.

Results:

Material	Concentration	% Cell Renewal
Salicylic Acid	1%	22.5%
Untreated Control	N/A	0
PhytoCide Aspen Bark Extract Powder	2%	26.0%

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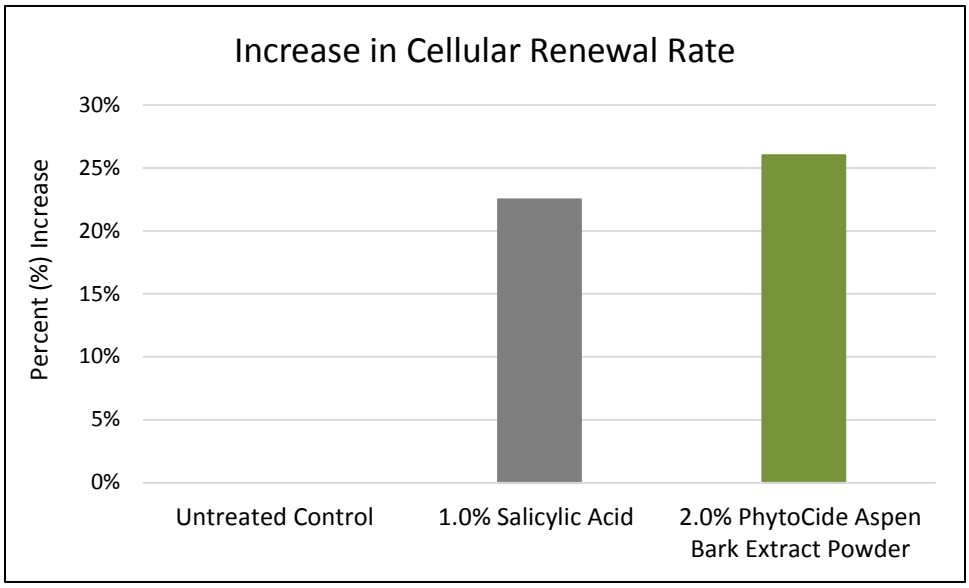

Discussion

The results indicate that **PhytoCide Aspen Bark Extract Powder** is capable of increasing cellular renewal by 26% when compared to the untreated biological control and increases cellular renewal 13.5% faster than using 1.0% Salicylic Acid in a solution. Therefore, we can assume that products incorporating PhytoCide Aspen Bark Extract Powder are capable of exfoliating the skin for a smoother surface aesthetic.

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Inhibition Activity Data

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Product Name: PhytoCide Aspen Bark Extract Powder
Code Number: M16002
Lot Number: 4887P
Test Request Number: 1495
CAS #'s: 90083-05-9
EINECS #'s: 290-199-2
INCI Name: *Populus tremuloides* Bark Extract

Organism (ATCC #)	Minimum Inhibitory Concentration (%)
E.coli #8739	1.0
S. aureus #6538	2.0
P. aeruginosa #9027	1.0
C. albicans #10231	1.0
A. brasiliensis #16404	1.0

QA Signature Monica Beltran

Date 09-10-2015

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Zone of Inhibition Test

Product Name: PhytoCide Aspen Bark Extract Powder
Code Number: M16002
Lot Number: 4703P
Test Request Number: 1142
CAS #'s: 90083-05-9
EINECS #'s: 290-199-2
INCI Name: *Populus tremuloides* Bark Extract

Organism (ATCC #)	Zone of Inhibition (mm)
E.coli #8379	8.0
S. aureus #6538	8.0
P. aeruginosa #9027	8.0
C. albicans #10231	8.0
A. brasiliensis #16404	8.0

QA Signature Monica Beltran

Date 03-09-2015

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Antimicrobial Efficacy Test
PCPC Section 20
Method 3

Determination of Preservation Adequacy of Water- Miscible Personal Care Products

Test Product

PhytoCide Aspen Bark Extract Powder
Code: M16002

Purpose

This study was initiated to determine the efficacy of a cosmetic ingredient with antimicrobial properties in a cream formulation against bioburden as a function of time.

Study Dates

The study was started on March 24th, 2014 and was completed on May 30th, 2014.

Test Organisms

1. *Escherichia coli:* ATCC #8739
2. *Pseudomonas aeruginosa:* ATCC #9027
3. *Staphylococcus aureus:* ATCC #6538
4. *Aspergillus niger:* ATCC #16404
5. *Candida albicans:* ATCC #10231

Neutralization:

Verification of neutralization of the antimicrobial properties of the product was demonstrated prior to performing the test for microbial content by inoculating the product dilution with a low level of challenge microorganisms (100 CFU) and verifying recovery of this viable inoculum. This provides evidence that the antimicrobial has been neutralized and there are no false positive results during the Challenge Test.

Test Method

Fifty grams of Generic Cream pH 5 with 2% PhytoCide Aspen Bark Extract Powder was weighed into five individual containers. Each container was inoculated with one of the five test organisms. The inoculum concentration for each organism was standardized using the 0.5 McFarland turbidity standard and further diluted to yield approximately 10^6 to 10^8 microorganisms/ml. The amount of each inoculum added to each sample was no more than 1% of the product weight, as to not alter the product composition.

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The inoculated samples were evaluated 0, 7, 14, 21, and 28 days after the initial inoculation to determine quantitatively the number of viable microorganisms remaining. On the 28th day of testing the samples were re-inoculated and evaluated 7, 14, 21, and 28 days after the second exposure to determine the number of viable microorganisms. The table below represents the percent reduction of viable organisms after being introduced into the test formulation.

	Organisms				
Inoculum (initial) CFU/ml	*E. coli*	*P. aeruginosa*	*S. aureus*	*A. brasiliensis*	*C. albicans*
	1.2×10^6	2.1×10^4	8.0×10^5	1.3×10^4	1.8×10^4
Day 0	99.967%	>99.999%	99.525%	99.462%	95.444%
Day 7	>99.999%	>99.999%	99.998%	99.385%	99.889%
Day 14	>99.999%	>99.999%	>99.999%	99.462%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	99.846%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	99.769%	>99.999%
Inoculum (re-inoculated) CFU/ml	1.7×10^6	9.9×10^4	2.1×10^6	2.0×10^5	1.1×10^5
Day 7	>99.999%	>99.999%	99.995%	98.500%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	92.500%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	99.000%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	99.850%	>99.999%

Table 1. Challenge Test results for Generic Cream pH 5 with 2% PhytoCide Aspen Bark Extract Powder inoculated on Day 7, 14, 21 and 28 then re-inoculated and tested on Day 7, 14, 21 and 28.

* The days listed in the first column refer to the inoculum/plating day. Bacteria results are read 2 days after plating day, and mold and yeast results are read 5 days after plating day.

Results & Discussion

The results obtained from the Neutralization Test of each product using Dey/Engley (D/E) broth, indicate that the neutralization steps conducted prior to performing the Challenge Test are indeed effective for avoiding false positive Challenge Test results.

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The results of this Challenge Test demonstrate the effectiveness of the preservation system used in Generic Cream pH 5 with 2% PhytoCide Aspen Bark Extract Powder. The recommendations stated in Section 13, Determination of Preservative Adequacy in Cosmetic Formulations, in the PCPC Microbiology Guidelines are as follows:

Bacteria – There should be at least a 99.9% (3 log) reduction of vegetative bacteria within 7 days following each challenge and no increase for the duration of the test period.

Yeasts and Molds – There should be at least a 90% (1 log) reduction of yeasts and molds within 7 days following each challenge and no increase for the duration of the test period.

The Gram positive and Gram negative bacteria as well as the yeast were reduced by greater than 99.9% within 7 days of each challenge. The mold was reduced by greater than 90% within 7 days of each challenge. By the end of each 28-day test period Gram positive and Gram negative bacteria as well as the yeast were reduced by 99.999% or greater. Mold was reduced by 99.0% or greater.

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Phase	Ingredient	Supplier	%
I	Water	-	85.5
	Glycerin	PT. Musim Mas	5.0
	Stearic Acid	Acme Hardesty Oleochemicals	2.5
II	Mineral Oil	RITA	5.0
	Lanolin	RITA	0.5
	Petrolatum	RITA	0.5
	Sepigel 305	Seppic	1.0

Manufacturing Process:

1. Phase I:
Charge water into main beaker and begin propeller mixing. A vortex should form. Begin heating to 80^0C while adding the ingredients.

2. Phase II:
In a separate beaker, combine ingredients and heat to 80^0C while mixing. Mix until homogenous. Then add to the main beaker with high-speed mixing. Maintain temperature at 80^0C and mix for 15 minutes. Begin force cooling to 25^0C.

3. Check the pH and adjust it if necessary.

Specifications:

Appearance: White to Off-White Emulsion
pH: 5.0 – 8.0
*If a different pH is desired, adjust using Citric Acid (50%) or NaOH (25%). Formula is stable in the 3.0 – 7.0 pH range.

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Antimicrobial Efficacy (Challenge) Testing

The intent of performing an Antimicrobial Efficacy or Challenge test is to evaluate whether an antimicrobial agent or preservation system in a given cosmetic formulation has the ability to prevent the growth of test microorganisms. The test methodology employed by Active Micro Technologies (AMT) is based on the methods published in the CTFA Microbiology Guidelines. AMT's goal is to assist our customers by providing a screening test of a product formulation that is approaching finalization. It is expected that the formulation(s) submitted for Challenge testing contain AMT antimicrobials and have already passed the customer's internal stability tests. It is also anticipated that formal challenge testing of the final formulation will subsequently be performed by the customer at an outside lab of their choosing.

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PhytoCide Aspen Bark Extract Powder Efficacy vs. *Propionibacterium acnes*

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Abstract

Propionibacterium acnes is a gram positive, non-spore-forming, microaerophilic, rod-shaped bacterium that is a common inhabitant of human skin. This microorganism metabolizes fatty acids created by sebaceous glands. The combination of fatty acid metabolites and antigens produced by the bacteria can create intense localized areas of inflammation that can fracture hair follicles. As a consequence, lesions develop on the surface of the skin in the form of pustules. This condition is commonly known as acne.

The purpose of this study was to determine the bactericidal efficacy of **PhytoCide Aspen Bark Extract Powder** against *P. acnes* by establishing the minimum inhibitory concentration (MIC) required to inhibit its growth and proliferation. For comparative purposes, an over-the-counter acne treatment product was used as a benchmark. According to the MIC results, **PhytoCide Aspen Bark Extract Powder** is capable of effectively inhibiting the growth of *P. acnes* at a significantly lower concentration than that of the benchmark product.

Materials and Methods

The products tested were a 35% solution of **PhytoCide Aspen Bark Extract Powder** and an over-the-counter, deep cleaning astringent that contains 2% salicylic acid (Benchmark). Each product was tested by preparing a serial dilution in a growth medium, beginning with an initial product concentration of 100%.

To determine the Minimum Inhibitory Concentration (MIC) of each product against *P. acnes,* a standard 9% saline solution was added to a test tube using a sterile pipette. Enough bacteria were added to the saline solution using a sterile loop to match the turbidity of a 0.5 McFarland standard. Two milliliters of this bacterial suspension were then transferred to one additional milliliter of 9% saline solution. Afterwards, 300 µL of the diluted mixture were added to 30 ml of sterile water yielding a final bacterial concentration of approximately 10^6 colony forming units (cfu)/ml. Using an 8-tip pipettor, 150 µL of double strength Tryptic Soy Broth (TSB) were added to the first row of wells in a sterile microwell plate. Then, 150 µL of single strength TSB were pipetted into the remaining rows of the plate.

150 µL of the **PhytoCide Aspen Bark Extract Powder** solution was pipetted into the first row of wells containing the double-strength TSB and mixed 5 times. 150 µL of this mixed material from the first row were then transferred via pipettor into the second row of wells and mixed 5 times. This procedure was repeated for each subsequent row, creating a serial dilution of the **PhytoCide Aspen Bark Extract Powder** solution ranging from 50% to 0.05% concentration through the first 11 rows of the plate. The last row did not receive any of the serially diluted **PhytoCide Aspen Bark Extract Powder** solution. This twelfth row, containing only single strength TSB, served as a positive control to demonstrate the viability of the diluted bacterial culture used to inoculate the plate. This same procedure was then repeated using the over-the-counter benchmark product.

Each plate was inoculated using an inoculating plate that had been dipped in the *P. acnes* inoculum suspension, prepared as previously described. The plates were incubated for 48 hours at 35 +/- 2°C. After the 48-hour incubation period the plates were examined for microbial growth, indicated by turbidity in the wells. The row of wells with the lowest concentration of tested product that remained clear (i.e., inhibited growth) was used to establish the MIC value.

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The following formula was used to calculate the MIC values:

$$\% \text{ MIC} = \frac{\text{Initial product concentration (\%) in Row 1}}{2^{(\text{last no growth row})}}$$

Results

MIC Results		
Product Tested	**Last Clear Row**	**% MIC**
PhytoCide Aspen Bark Extract Powder Solution	6	1.563
Benchmark product	3	12.500

Table 1. MIC Results

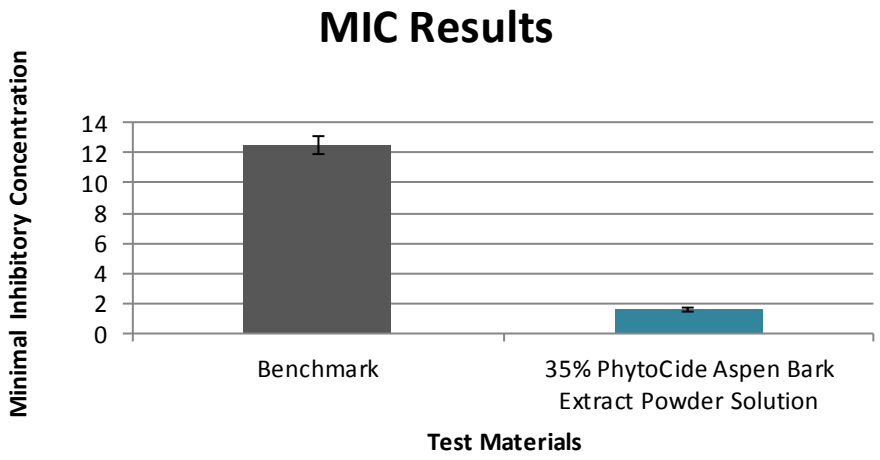

Figure 1. MIC Results

Discussion

Based on these results, we can confirm that **PhytoCide Aspen Bark Extract Powder** is capable of inhibiting the growth of *Propionibacterium acnes* when used at a concentration of approximately 1.5%. This concentration is significantly lower than the 12.5% concentration that is required to equally inhibit growth when using the benchmark product containing 2% salicylic acid.

P. acnes has been identified as a primary factor that causes acne. By inhibiting the proliferation of this bacterium, one may significantly minimize acne formation. **PhytoCide Aspen Bark Extract Powder** is a broad-spectrum antimicrobial that has been shown to be effective against the acne-causing bacterium *Propionibacterium acnes.* These properties make **PhytoCide Aspen Bark Extract Powder** an effective ingredient for formulations developed to address problem skin.

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Time Kill Test
E2315
Assessment of Antimicrobial Activity Using a Time Kill Procedure

Product

PhytoCide Aspen Bark Extract Powder

Test Request #:

1884

Purpose

This study was initiated to measure the change in population of aerobic microorganisms within a specified sampling time when tested against a cosmetic ingredient.

Study Dates

The study was started on April 6th, 2016 and was completed on April 11th, 2016.

Test Organisms

1. *Escherichia coli:* ATCC #8739
2. *Pseudomonas aeruginosa:* ATCC #9027
3. *Staphylococcus aureus:* ATCC #6538
4. *Bacillus subtilis* ATCC #6051
5. *Aspergillus brasiliensis:* ATCC #16404
6. *Candida albicans:* ATCC #10231

Neutralization:

Inactivation of the antimicrobial activity of the test material is achieved through the dilution of the test material during the sampling time at specified sampling intervals.

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Test Method

Ten grams of 4% PhytoCide Aspen Bark Extract Powder solution was weighed into six individual containers. Each container was inoculated with one of the six test organisms. The inoculum concentration for each organism was standardized using the 0.5 McFarland turbidity standard and further diluted to yield approximately 10^6 microorganisms/ml. The amount of each inoculum added to each sample was no more than 1% of the product weight, as to not alter the product composition. Serial dilutions from each container were performed to enumerate the surviving microorganisms using the Plate Count Technique.

The activity of the test material inoculated was evaluated at determine time intervals of 30 seconds, 1, 5, 10 and 30 minutes after the inoculation to determine quantitatively the number of viable microorganisms remaining after the incubation time.

Organisms	Inoculum Concentration CFU/ml	Percentage of Reduction				
		30 seconds	1 minute	5 minute	10 minute	30 minutes
E.coli * ATCC# 8739	2.6×10^6	99.9%	99.9%	99.9%	99.9%	99.9%
S.aureus ATCC# 6538	2.1×10^6	99.9%	99.9%	99.9%	99.9%	99.9%
P.aeruginosa ATCC# 9027	3.2×10^5	99.9%	99.9%	99.9%	99.9%	99.9%
B.subtilis ATCC# 6051	2.5×10^5	99.9%	99.9%	99.9%	99.9%	99.9%
A.brasiliensis ATCC# 16404	1.2×10^5	99.9%	99.9%	99.9%	99.9%	99.9%
C.albicans ATCC# 10231	1.0×10^5	99.9%	99.9%	99.9%	99.9%	99.9%

Table 1. Time Kill Test results for 4% PhytoCide Aspen Bark Extract Powder inoculated with 10^6 microorganisms' population. Results show % reduction in viable organisms after inoculation and sampling time intervals.

*Bacteria results are read 2 days after plating day, and mold and yeast results are read 5 days after plating day.

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Results & Discussion

The results of this Time Kill Test determine the changes in population of aerobic microorganisms within a specified sampling time when tested against 4% PhytoCide Aspen Bark Extract Powder solution.

The Gram positive and Gram negative bacteria as well as the yeast and mold were reduced by 99.9% within 30 seconds interval of the test after the inoculation.

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Safety Statement

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Product Name: PhytoCide Aspen Bark Extract Powder

Code: M16002

INCI Name: *Populus tremuloides* Bark Extract

Aspen Bark contains a variety of phenolic compounds, but is notably rich in salicylates.[1] PhytoCide Aspen Bark Extract Powder contains a minimum of 54.0% of salicylates with an average content of 56.7%. This salicylate content is measured using HPLC (High Performance Liquid Chromatography). Due to the required test method and its associated mobile phase, the pH of the test sample is lowered from its natural pH. When salicylates are exposed to an acidic environment, they convert into salicylic acid. Therefore when testing for salicylate content of PhytoCide Aspen Bark Extract Powder using HPLC, the value is quantified as the percent of salicylic acid as an artifact of the test method; although no salicylic acid actually exists in the product.

Aspen bark extracts and willow bark extracts are very common cosmetic ingredients as they share similar phenolic glycoside compositions and functionality. Similarly, both species of woody trees contain high natural salicylate distribution within their bark and leaves.[1,2] Because *Salix* bark extracts have been long used in the cosmetic industry and they are know to have a high natural salicylate content similar to the *Populus* species, we are able to use existing toxicological information to infer the safety of aspen bark extracts.

The European Medicines Agency has completed an *Assessment Report on Salicis Cortex (Willow Bark) and Herbal Preparation (s) Thereof with Well-Established Use and Traditional Use* which outlines a wide variety of pharmacological and toxicological findings when taken orally. When comparing the *Populus* and *Salix* species, we can expect that this report would classify PhytoCide Aspen Bark Extract Powder as a quantifiable herbal preparation as it would willow bark extracts.[3] Willow bark extracts must contain a minimum 5.0% of total salicylic derivatives to be considered for this type of use as determined by European Pharmacopoeia (04/2008:2312). PhytoCide Aspen Bark Extract Powder is quantified to contain a minimum of 54% salicylates which is well within the parameters of this classification.[3]

PhytoCide Aspen Bark Extract Powder is a natural antimicrobial used for topical applications. A Human Subject Repeat Insult Patch Test (HRIPT) evaluation was completed to determine if PhytoCide Aspen Bark Extract Powder would be classified as an irritant or sensitizing agent. The powder was diluted to a 5% solution in water and 0.2 grams was applied to an area of $1cm^2$. The procedure was repeated until a series of nine consecutive 'open patch' applications were made every Monday, Wednesday, and Friday for three consecutive weeks. Under the reported testing conditions, results indicated that PhytoCide Aspen Bark Extract Powder was not a primary sensitizer and a non-irritating material. Please find attached a copy of these results.

---

[1] Abreu, Ilka N., Ahnlund, Maria., Moritz, Thomas., and Albrectsen, Benedicte R. *UHPLC-ESI/TOFMS Determiniation of Salicylate-Like Phenolic Glycosides in Populus tremula Leaves.* Journal of Chemical Ecology. 2011. 37:8. 857-870.Palo, R.T., *Distribution of Birch (Betula SPP.) Willow (Salix SPP.), and Poplar (Populus SPP.) Secondary Metabolites and Their Potential Role As Chemical Defence against Herbivores.* Journal of Chemical Ecology. 1984. 10:3.

[2] Palo, R.T., *Distribution of Birch (Betula SPP.) Willow (Salix SPP.), and Poplar (Populus SPP.) Secondary Metabolites and Their Potential Role As Chemical Defence against Herbivores.* Journal of Chemical Ecology. 1984. 10:3..

[3] European Medicines Agecny: Evaluation of Medicines for Human Use. *Assessment report on Salicis Cortex (Willow Bark) and Herbal Preparations Thereof with Well Established use and Traditional Use.* London, 26, September 2009. Doc Ref: EMEA/HMPC/295337/2007

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Additionally, an *In vitro* ocular irritation study was conducted to evaluate whether PhytoCide Aspen Bark Extract Powder would induce ocular irriation in the EpiOcular™ model assay. Test substances were applied to the tissue inserts and incubated. Cell viability was measured by dehydrogenase conversion of MTT, present in cell mitochondria, into blue formazan salt that is measured after extraction from the tissue. The irritation potential of the test chemical was dictated by the reduction in tissue viability of exposed tissues compared to the negative control. Under conditions of this assay, the test article was considered to be non-irritating. Please find attached a copy of those results.

As a result, this knowledge combined with the completed HRIPT study and ocular irritation study, allows us to support the safety of PhytoCide Aspen Bark Extract Powder in cosmetic applications when used at suggested levels of 0.2 – 3.0%. No further testing is required.

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Dermal and Ocular Irritation Tests

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Tradename: PhytoCide Aspen Bark Extract Powder

Code: M16002

CAS #: 90083-05-9

Test Request Form #: 279

Lot #: 4330

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 **PhytoCide Aspen Bark Extract Powder** 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 a **non-irritant**. 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-irritating**. 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 Figures 1 and 2. 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

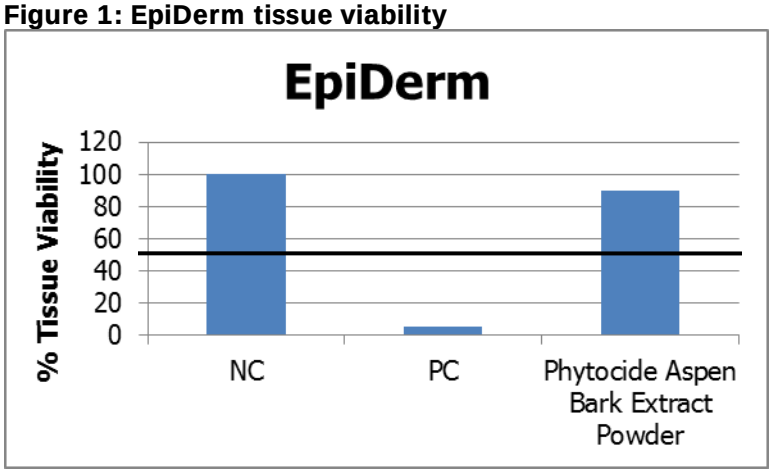
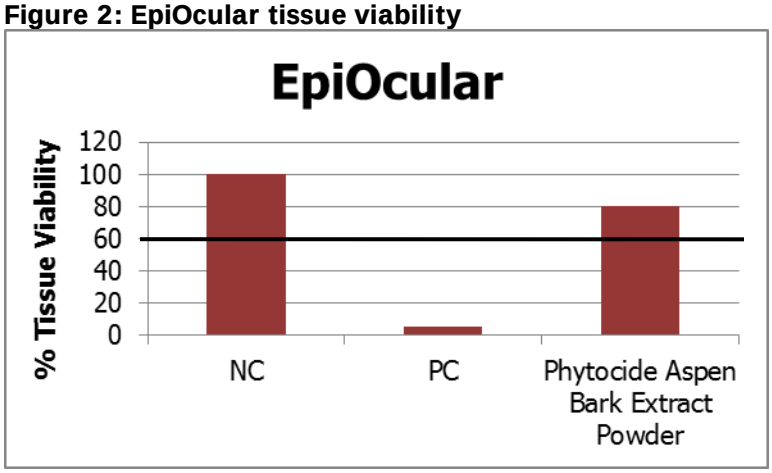


Figure 2: EpiOcular tissue viability



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50 HUMAN SUBJECT REPEAT INSULT PATCH TEST SKIN IRRITATION/SENSITIZATION EVALUATION (Occlusive Patch)

AMA Ref. No.: MS08.RIPT.L2091O.50.ACTC

Date: February 18, 2008

Sponsor: Active Concepts, LLC
121 Ethel Road West, Suite 3
Piscataway, New Jersey 08854

1.0 Objective:

Consumer products or raw materials designed for consistent reapplication to areas of the skin may, under proper conditions, prove to be contact sensitizers or irritants in certain individuals. It is the intention of a Repeat Insult Patch Test (RIPT) to provide a basis for evaluation of this irritation/sensitization potential if such exists.

2.0 Test Material:

2.1 Test Material Description:

On January 11, 2008 one test sample labeled EN080110-F was received from Active Concepts, LLC and assigned AMA Lab No. L-2091.

2.2 Handling:

Upon arrival at AMA Laboratories, Inc., the test material is assigned a unique laboratory code number and entered into a daily log identifying the lot number, sample description, sponsor, date received and tests requested.

Samples are retained for a period of three months beyond submission of final report unless otherwise specified by the sponsor or, if sample is known to be in support of governmental applications, representative retained samples are kept two years beyond final report submission.

Sample disposition is conducted in compliance with appropriate federal, state and local ordinances.

2.3 Test Material Evaluation Prerequisite:

Prior to induction of a human test panel, toxicology, microbiology or in-vitro performance spectra may be required to assess the feasibility of commencement as dictated by an Institutional Review Board (IRB) described in Section 3.0.

Sponsor purports that prior to sample submission the following tests were conducted with no adverse results and that the test data are on file on their premises and have not been made available to AMA personnel:

- USP or CTFA Preservative Efficacy Test or equivalent
- 90 Day Accelerated Stability and Container Compatibility Study

3.0 Institutional Review Board:

Reference: CFR Title 21 Part 56, Subparts A, B, C, and D. The IRB of AMA Laboratories, Inc., consists of five or more individuals, chosen from within the company for technical expertise and from the local community for lay interaction. The list of IRB members is kept on file at AMA Laboratories, Inc. and is available for inspection during the hours of operation.

4.0 Panel Selection:

4.1 Standards for Inclusion in a Study:

- Individuals who are not currently under a doctor's care.
- Individuals free of any dermatological or systemic disorder which would interfere with the results, at the discretion of the Investigator.
- Individuals free of any acute or chronic disease that might interfere with or increase the risk of study participation.
- Individuals who will complete a preliminary medical history form mandated by AMA Laboratories, Inc. and are in general good health.
- Individuals, who will read, understand and sign an informed consent document relating to the specific type of study they are subscribing. Consent forms are kept on file and are available for examination on the premises of AMA Laboratories, Inc. only.
- Individuals able to cooperate with the Investigator and research staff, willing to have test materials applied according to the protocol, and complete the full course of the study.

4.2 Standards for Exclusion from a Study:

- Individuals under 18 years of age.
- Individuals who are currently under a doctor's care.
- Individuals who are currently taking any medication (topical or systemic) that may mask or interfere with the test results.
- Subjects with a history of any acute or chronic disease that might interfere with or increase the risk associated with study participation.
- Individuals diagnosed with chronic skin allergies.
- Female volunteers who indicate that they are pregnant or lactating.

4.3 Recruitment:

Panel selection is accomplished by advertisements in local periodicals, community bulletin boards, phone solicitation, electronic media or any combination thereof.

4.4 Informed Consent and Medical History Forms:

An informed consent was obtained from each volunteer prior to initiating the study describing reasons for the study, possible adverse effects, associated risks and potential benefits of the treatment and their limits of liability. Panelists signed and dated the informed consent document to indicate their authorization to proceed and acknowledge their understanding of the contents. Each subject was assigned a permanent identification number and completed an extensive medical history form. These forms along with the signed consent forms, are available for inspection on the premises of AMA Laboratories, Inc. only. Reference 21 CFR Ch. 1 Part 50, Subpart B.

The parties agree to comply with applicable state and federal privacy laws for the use and disclosure of a subject's personal health information by taking reasonable steps to protect the confidentiality of this information. This obligation shall survive the termination or expiration of this Agreement.

5.0 Population Demographics:

Number of subjects enrolled		52
Number of subjects completing study		50
Age Range		26-64
Sex	Male	7
	Female	45
Race	Caucasian	42
	Hispanic	9
	Asian	1

6.0 Equipment:

- Patch Description: Parke-Davis Hypoallergenic Readi Bandages or the equivalent.
- 1ml volumetric syringe without a needle.

7.0 Procedure:

- Subjects are requested to bathe or wash as usual before arrival at the facility.
- 0.2 ml or 0.2g of the test material is dispensed onto the occlusive, hypoallergenic patch.
- The patch is then applied directly to the skin of the infrascapular regions of the back, to the right or left of the midline and the subject is dismissed with instructions not to wet or expose the test area to direct sunlight.
- After 24 hours the patch is removed by the panelist at home.
- This procedure is repeated until a series of nine consecutive 24 hour exposures have been made for every Monday, Wednesday, and Friday for three consecutive weeks.
- In the event of an adverse reaction, the area of erythema and edema is measured. The edema is estimated by the evaluation of the skin with respect to the contour of the unaffected normal skin. Reactions are scored just before applications two through nine and the next test date following application nine. In most instances this is approximately 24 hours after patch removal. Clients are notified immediately in the case of adverse reaction and determination is made as to treatment program if necessary.
- Subjects are then given a 10 - 14 day rest period after which a challenge or retest dose is applied once to a previously unexposed test site. The retest dose is equivalent to any one of the original nine exposures. Reactions are scored 24 and 48 hours after application.
- Comparison is made between the nine inductive responses and the retest dose.

8.0 Results:

Please refer to attached Table.

9.0 Observations:

No adverse reactions of any kind were noted during the course of this study.

10.0 Archiving:

All original samples, raw data sheets, technician's notebooks, correspondence files and copies of final reports and remaining specimens are maintained on premises of AMA Laboratories, Inc. in limited access storage files marked "Archive". A duplicate disk copy of final reports is separately archived in a bank safe deposit vault.

11.0 Reference:

Appraisal of the Safety of Chemicals in Food, Drugs and Cosmetics, published by The Association of Food and Drug Officials of The United States, 1965 (modified).

12.0 Conclusions:

The test material (AMA Lab. No.: L-2091; Client No.: EN080110-F) when tested under occlusion as described herein, may be considered:
a **NON-PRIMARY IRRITANT** and **NON-PRIMARY SENSITIZER** to the skin according to the reference.

Mayya Tatsene, M.D.
Study Director

Patrycja Bienias, M.S.
Technician

David R. Winne, B.S.
Technical Director

2/13/08
Date

TABLE
SUMMARY OF RESULTS
(Occlusive Patch)

AMA Lab No.: L-2091
Client No.: EN080110-F

No.	Subject ID	RACE	SEX	Response									Chall.		Score
				1	2	3	4	5	6	7	8	9	24 HR	48 HR	
1	25 0215	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
2	28 0971	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
3	34 4672	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
4	36 2168	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
5	36 7304	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
6	36 7970	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
7	36 8248	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
8	40 6489	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
9	42 1835	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
10	42 1837	C	F	0	0	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	N/A
11	44 9258	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
12	46 4172	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
13	48 4004	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
14	50 1699	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
15	50 1729	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
16	50 3800	A	M	0	0	0	0	0	0	0	0	0	0	0	0.0
17	50 5772	C	M	0	0	0	0	0	0	0	0	0	0	?	0.0
18	50 8253	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
19	52 4898	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
20	52 5000	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
21	54 0763	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
22	54 1935	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
23	54 2951	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
24	54 4408	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
25	54 6357	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
26	56 0719	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
27	56 3659	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
28	56 4962	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
29	56 5529	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0

TABLE (CONT'D)
SUMMARY OF RESULTS
(Occlusive Patch)

AMA Lab No.: L-2091
Client No.: EN080110-F

No.	Subject ID	RACE	SEX	Response									Chall.		Score
				1	2	3	4	5	6	7	8	9	24 HR	48 HR	
30	58 3087	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
31	58 3965	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
32	58 7412	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
33	58 9750	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
34	60 0082	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
35	60 1825	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
36	60 2888	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
37	60 3135	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
38	60 6328	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
39	60 9336	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
40	62 3596	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
41	62 5624	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
42	62 8070	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
43	64 2464	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
44	64 4340	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
45	64 6653	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
46	64 8003	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
47	66 1927	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
48	70 5391	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
49	72 2318	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
50	76 2719	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
51	82 4417	H	M	0	0	0	0	0	0	0	0	0	0	0	0.0
52	90 3845	H	F	0	0	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	N/A

Evaluation Period:

This study was conducted from January 14, 2008 through February 15, 2008.

Scoring Scale and Definition of Symbols Shown in Table:

0 - No evidence of any effect

? - (Barely perceptible) minimal faint (light pink) uniform or spotty erythema

1 - (Mild) pink uniform erythema covering most of contact site

2 - (Moderate) pink\red erythema visibly uniform in entire contact area

3 - (Marked) bright red erythema with accompanying edema, petechiae or papules

4 - (Severe) deep red erythema with vesiculation or weeping with or without edema

D - Patch eliminated due to reaction

Dc - Discontinued due to absence of subject on application date

M - Patch applied to an adjacent site after strong test reaction

N/A - Score is not calculated for subjects discontinued before challenge

S - Skin stained from pigment in product

T - Tan

NOTE: All technical employees of AMA LABORATORIES, INC. are required to take and pass a visual discrimination examination conducted by a Board Certified Ophthalmologist using the Farnsworth-Munsell 100 Hue Test as published; which determines a person's ability to discern color against a black background. This test was additionally modified to include a flesh tone background more nearly approaching actual use conditions, wherein erythematous skin is graded according to intensity.

13.0 Quality Assurance Statement:

This study was inspected in accordance with the Standard Operating Procedures of AMA Laboratories, Inc. To assure compliance with the study protocol, the Quality Assurance Unit completed an audit of the study records and report.

Report reviewed by:

Kamil Wojtowicz

Kamil Wojtowicz, M.S.
Quality Assurance Supervisor

2/18/08
Date

ACTIVE MICRO TECHNOLOGIES

OECD TG 442C: *In Chemico* Skin Sensitization

107 Technology Drive • Lincolnton, NC 28092
(704) 276-7100 • Fax (704) 276-7101

Tradename: PhytoCide Aspen Bark Extract Powder

Code: M16002

CAS #: 90083-05-9

Test Request Form #: 1418

Lot #: 4854P

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

Test Performed:
OECD TG 442C: *In Chemico* Skin Sensitization
Direct Peptide Reactivity Assay (DPRA)

Introduction

A skin sensitizer is a substance that will lead to an allergic response following skin contact[1]. Haptenation is the covalent binding of a hapten, or low-molecular weight substance or chemical, to proteins in the skin. This is considered the prominent mechanism which defines a chemical as a sensitizer. Haptenation is described as a "molecular initiating event" in the OECD Adverse Outcome Pathway (AOP) for skin sensitization which summarizes the key events known to be involved in chemically-induced allergic contact dermatitis[2]. The direct peptide reactivity assay (DPRA) is designed to mimic the covalent binding of electrophilic chemicals to nucleophilic centers in skin proteins by quantifying the reactivity of chemicals towards the model synthetic peptides containing cysteine and lysine. The DPRA is able to distinguish sensitizers from non-sensitizer with 82% accuracy (sensitivity of 76%; specificity of 92%)[3].

This assay was conducted to determine skin sensitization hazard of **PhytoCide Aspen Bark Extract Powder** in accordance with European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) and OECD Test Guideline 442C.

Assay Principle

The DPRA is an *in chemico* method which addresses peptide reactivity by measuring depletion of synthetic heptapeptides containing either cysteine or lysine following 24 hours incubation with the test substance. The peptide is a custom material containing phenylalanine to aid in detection. Depletion of the peptide in the reaction mixture is measured by HPLC with gradient elution and UV detection at 220 nm. Cysteine and lysine peptide percent depletion values are then calculated and used in a prediction model which allows assigning the test chemical to one of four reactivity classes used to support the discrimination between sensitizers and non-sensitizers.

1. United Nations Economic Commission (UNECE) (2013) Global Harmonized System of Classification and Labelling of Chemicals (GHS) 5th Revised Edition
2. OECD (2012). The Adverse Outcome Pathway for Skin Sensitization Initiated by Covalent Binding to Proteins. Part 1: Scientific Evidence. Series on Testing and Assessment No. 168
3. EC EURL ECVAM (2012) Direct peptide reactivity assay (DPRA) validation study report; pp 1 -74.

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Materials

A.	**Equipment:**	HPLC-UV (Waters Breeze - Waters 2998 Photodiode Array Detector); Pipettes; Analytical balance
B.	**HPLC/Guard Columns:**	Agilent Zorbax SB-C18 2.1mm x 100mm x 3.5µm; Phenomenex Security Guard C18 4mm x 2mm
C.	**Chemicals:**	Trifluoroacetic acid; Ammonium acetate; Ammonium hydroxide; Acetonitrile; Cysteine peptide (Ac-RFAA**C**AA-COOH); Lysine peptide (Ac-RFAA**K**AA-COOH); Cinnamic aldehyde
D.	**Reagents/Buffers:**	Sodium phosphate buffer (100mM); Ammonium acetate buffer (100mM)
E.	**Other:**	Sterile disposable pipette tips

Methods

Solution Preparation:

- 0.667mM Cysteine Peptide in 100mM Phosphate Buffer (pH 7.5)
- 0.667mM Lysine Peptide in 100mM Ammonium Acetate Buffer (pH 10.2)
- 100mM Cinnamic Aldehyde in Acetonitrile
- 100mM **PhytoCide Aspen Bark Extract Powder** in Acetonitrile

Reference Controls:

- Reference Control A: For calibration curve accuracy
- Reference Control B: For peptide stability over analysis time of experiment
- Reference Control C: For verification that the solvent does not impact percent peptide depletion

Sample, Reference Control, and Co-Elution Control Preparation:

- Once these solutions have been made they should be incubated at room temperature, protected from light, for 24±2 hours before running HPLC analysis.
- Each chemical should be analyzed in triplicate.

1:10 Ratio, Cysteine Peptide 0.5mM Peptide, 5mM Test Chemical	1:50 Ratio, Lysine Peptide 0.5mM Peptide, 25mM Test Chemical
• 750µL Cysteine Peptide Solution (or 100mM Phosphate Buffer, pH 7.5, for Co-Elution Controls) • 200µL Acetonitrile • 50µL Test Chemical Solution (or Acetonitrile for Reference Controls)	• 750µL Lysine Peptide Solution (or 100mM Ammonium Acetate Buffer, pH 10.2, for Co-Elution Controls) • 250µL Test Chemical Solution (or Acetonitrile for Reference Controls)

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Calibration Curve:

- Standards are prepared in a solution of 20% Acetonitrile:Buffer
  - For the Cysteine peptide using the phosphate buffer, pH 7.5
  - For the Lysine peptide using the ammonium acetate buffer, pH 10.2

	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6	Standard 7
mM Peptide	0.534	0.267	0.1335	0.0667	0.0334	0.0167	0.000

HPLC Analysis:

- HPLC-UV system should be equilibrated at 30°C with 50% Mobile Phase A (0.1% (v/v) trifluoroacetic acid in water) and 50% Mobile Phase B (0.085% (v/v) trifluoroacetic acid in acetonitrile) for 2 hours
- Absorbance is measured at 220nm
- Flow Conditions:

Time	Flow	%A	%B
0 minutes	0.35 mL/min	90	10
10 minutes	0.35 mL/min	75	25
11 minutes	0.35 mL/min	10	90
13 minutes	0.35 mL/min	10	90
13.5 minutes	0.35 mL/min	90	10
20 minutes	End Run		

Data and Reporting

Acceptance Criteria:

1. The following criteria must be met for a run to be considered valid:
   a. Standard calibration curve should have an $r^2 > 0.99$.
   b. Mean percent peptide depletion values of three replicates for the positive control cinnamic aldehyde should be between 60.8% and 100% for the cysteine peptide and between 40.2% and 69% for the lysine peptide and the maximum standard deviation should be <14.9 for the percent cysteine depletion and <11.6 for the percent lysine depletion.
   c. Mean peptide concentration of reference controls A should be 0.50±0.05mM and the coefficient of variable of the peptide peak areas for reference B and C in acetonitrile should be <15.0%.

2. The following criteria must be met for a test chemical's results to be considered valid:
   a. Maximum standard deviation should be <14.9 for percent cysteine depletion and <11.6 for percent lysine depletion.
   b. Mean peptide concentration of the three reference control C should be 0.50±0.05mM.

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Prediction Model:

Cysteine 1:10/Lysine 1:50 Prediction Model		
Mean of Cysteine and Lysine % Depletion	**Reactivity Class**	**Prediction**
0% < Mean % Depletion < 6.38%	Minimal Reactivity	Non-sensitizer
6.38% < Mean % Depletion < 22.62%	Low Reactivity	Sensitizer
22.62% < Mean % Depletion < 42.47%	Moderate Reactivity	Sensitizer
42.47% < Mean % Depletion < 100%	High Reactivity	Sensitizer

If co-elution occurs with the lysine peptide, than the cysteine 1:10 prediction model can be used:

Cysteine 1:10 Prediction Model		
Mean of Cysteine and Lysine % Depletion	**Reactivity Class**	**Prediction**
0% < Cys % Depletion < 13.89%	Minimal Reactivity	Non-sensitizer
13.89% < Cys % Depletion < 23.09%	Low Reactivity	Sensitizer
23.09% < Cys % Depletion < 98.24%	Moderate Reactivity	Sensitizer
98.24% < Cys % Depletion < 100%	High Reactivity	Sensitizer

Results and Discussion

The data obtained from this study met criteria for a valid assay and the controls performed as anticipated.

Percent peptide depletion is determined by the following equation:

$$Percent\ Peptide\ Depletion = \left[1 - \left(\frac{Peptide\ Peak\ Area\ in\ Replicate\ Injection}{Mean\ Peptide\ Peak\ Area\ in\ Reference\ Controls\ C}\right)\right] \times 100$$

Based on HPLC-UV analysis of **PhytoCide Aspen Bark Extract Powder (code M16002)** we can determine that this product is not a sensitizer and will not cause allergic contact dermatitis. The Mean Percent Depletion of Cysteine and Lysine was 2.82% causing minimal reactivity in the assay giving us the prediction of a non-sensitizer.

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ACTIVE MICRO TECHNOLOGIES

107 Technology Drive • Lincolnton, NC 28092
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OECD TG 442D: *In Vitro* Skin Sensitization

Tradename: Phytocide Aspen Bark Extract Powder

Code: M16002

CAS #: 90083-05-9

Test Request Form #: 1417

Lot #: 4854P

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

Test Performed:
OECD TG 442D: In Vitro Skin Sensitization
ARE-Nrf2 Luciferase Test Method

Introduction

Skin sensitization refers to an allergic response following skin contact with the tested chemical, as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals[1]. Substances are classified as skin sensitizers if there is evidence in humans that the substance can lead to sensitization by skin contact or positive results from appropriate tests, both *in vivo* and *in vitro*. Utilization of the KeratinoSens™ cell line allows for valid *in vitro* testing for skin sensitization.

This assay was conducted to determine skin sensitization potential of **Phytocide Aspen Bark Extract Powder** in accordance with the UN GHS.

Assay Principle

The ARE-Nrf2 luciferase test method addresses the induction of genes that are regulated by antioxidant response elements (ARE) by skin sensitizers. The Keap1-Nrf2-ARE pathways have been shown to be major regulator of cytoprotective responses to oxidative stress or electrophilic compounds. These pathways are also known to be involved in the cellular processes in skin sensitization. Small electrophilic substances such as skin sensitizers can act on the sensor protein Keap1 (Kelch-like ECH-associated protein 1), by covalent modification of its cysteine residue, resulting in its dissociation from the transcription factor Nrf2 (nuclear factor-erythroid 2-related factor 2). The dissociated Nrf2 can then activate ARE-dependent genes such as those coding for phase II detoxifying enzymes.

The skin sensitization assay utilizes the KeratinoSens™ method which uses an immortalized adherent human keratinocyte cell line (HaCaT cell line) that has been transfected with a selectable plasmid to quantify luciferase gene induction as a measure of activation of Keap1-Nrf2-antioxidant/electrophile response element (ARE). This test method has been validated by independent peer review by the EURL-ECVAM. The addition of a luciferin containing reagent to the cells will react with the luciferase produced in the cell resulting in luminescence which can be quantified with a luminometer.

1. United Nations (UN) (2013). Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Fifth revised edition, UN New York and Geneva, 2013

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Materials

A. Incubation Conditions:	37°C at 5% CO_2 and 95% relative humidity (RH)
B. Equipment:	Humidified incubator; Biosafety laminar flow hood; Microplate Reader; Pipettes
C. Cell Line:	KeratinoSens™ by Givaudan Schweiz AG
D. Media/Buffers:	Dulbecco's Modified Eagle Medium (DMEM); Fetal Bovine Serum (FBS); Phosphate Buffered Saline (PBS); Geneticin
E. Culture Plate:	Flat bottom 96-well tissue culture treated plates
F. Reagents:	Dimethyl Sulfoxide (DMSO); Cinnamic Aldehyde; ONE-Glo Reagent; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); sodium lauryl sulfate (SLS)
G. Other:	Sterile disposable pipette tips; wash bottles

Methods

KeratinoSens™ were into seeded four 96-well tissue culture plates and allowed to grow to 80 – 90% confluency in DMEM containing 10% FBS and 500µg/mL G418 geneticin. Twelve test concentrations of **Phytocide Aspen Bark Extract Powder** were prepared in DMSO with a concentration range from 0.098 – 200µM. These 12 concentrations were assayed in triplicate in 2 independently performed experiments. The positive control was cinnamic aldehyde for which a series of 5 concentrations prepared in DMSO had final test concentrations of 4 – 64µM. The negative control was a 1% test concentration of DMSO.

24 hour post KeratinoSens™ seeding, the culture media was removed and replaced with fresh media containing 10% FBS without G418 geneticin. 50 µL of the above described test concentrations was added to the appropriate wells. The treated plates were then incubated for 48 hours at 37°C in the presence of 5% CO_2 and 95% relative humidity. After treatment incubation was complete the media was removed and the wells were washed with PBS 3 times.

One of the four plates was used for a cytotoxicity endpoint, where MTT was added to the wells and incubated for 4 hours at 37°C in the presence of 5% CO_2. SLS was then added to the wells and incubated overnight at room temperature. A spectrometer measured the absorbance at 570 nm. The absorbance values (optical density) were then used to determine the viability of each well by comparing the optical density of each test material treated well to that of the solvent control wells to determine the IC_{50} and IC_{30} values.

The remaining 3 plates were used in the luciferase induction endpoint of the assay. 100 µL of Promega's ONE-Glo Reagent was added to 100 µL of fresh media containing 10% FBS without geneticin. Cells were incubated for 5 minutes to induce cell lysis and release luciferin into the media. Plates were read with a luminometer and $EC_{1.5}$ and maximum response (I_{max}) values were obtained.

Data and Reporting

Acceptance Criteria:

1. Gene induction obtained with the positive control, cinnamic aldehyde, should be statistically significant above the threshold of 1.5 in at least one of the tested concentrations (from 4 to 64 µM).
2. The EC1.5 value should be within two standard deviations of the historical mean and the average induction in the three replicates for cinnamic aldehyde at 64 µM should be between 2 and 8.
3. The average coefficient of variability of the luminescence reading for the negative (solvent) control DMSO should be below 20% in each experiment.

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A KeratinoSens™ prediction is considered positive if the following conditions are met:

1. The Imax is higher than 1.5-fold and statistically significantly higher as compared to the solvent (negative) control
2. The cellular viability is higher than 70% at the lowest concentration with a gene induction above 1.5 fold (i.e., at the EC1.5 determining concentration)
3. The $EC_{1.5}$ value is less than 1000 µM (or < 200 µg/ml for test chemicals with no defined MW)
4. There is an apparent overall dose-response for luciferase induction

Results

Compound	Classification	$EC_{1.5}$ (µM)	IC_{50}	I_{max}
Cinnamic aldehyde	Sensitizer	19	289.19 µM	31.6
DMSO	Non-Sensitizer	No Induction	243.24 µM	1.2
Phytocide Aspen Bark Extract Powder	Non-Sensitizer	No Induction	> 1000 µM	0.5

Table 1: Overview of KeratinoSens™ Assay Results

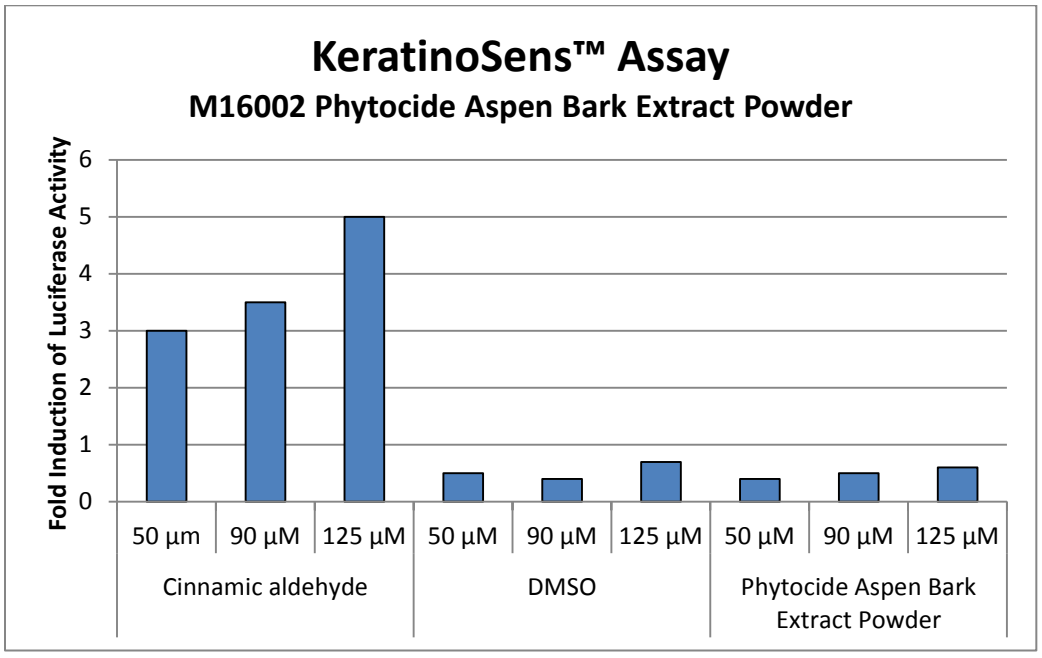

Figure 1: Fold Induction of Luciferase

Discussion

As shown in the results, **Phytocide Aspen Bark Extract Powder (code M16002)** was not predicted to be a skin sensitizer based on the KeratinoSens™ ARE-Nrf2 Luciferase Test Method as there was not a significant increase in luciferase expression. It can be concluded that **Phytocide Aspen Bark Extract Powder** can be safely used in cosmetics and personal care products at typical use levels.

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ACTIVE MICRO TECHNOLOGIES

Bacterial Reverse Mutation Test

107 Technology Drive • Lincolnton, NC 28092
(704) 276-7100 • Fax (704) 276-7101

Test Article: PhytoCide Aspen Bark Extract Powder
Code Number: M16002
CAS #: 90083-05-9

Sponsor:
Active Micro Technologies, LLC
107 Technology Drive
Lincolnton, NC 28092

Study Director: *Erica Segura*
Principle Investigator: *Monica Beltran*

Test Performed:
Genotoxicity: Bacterial Reverse Mutation Test

Reference:
OECD471/ISO10993.Part 3

Test Request Number: 1019

SUMMARY

A *Salmonella typhimurium/Escherichia coli* reverse mutation standard plate incorporation study described by Ames *et al.* (1975) was conducted to evaluate whether a test article in solution **PhytoCide Aspen Bark Extract Powder** would cause mutagenic changes in the average number of reveratants for histidine-dependent *Salmonella typhimurium* strains TA98, TA100, TA1537, TA1535 and tryptophan-dependent *Escherichia coli* strain WP2*uvr*A in the presence and absence of Aroclor-induced rat liver S9. 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 tested at eight doses levels along with appropriate vehicle control and positive controls with overnight cultures of tester strains. The test article in solution was found to be noninhibitory to growth of tester strain TA98, TA100, TA1537, TA1535 and WP2*uvr*A after Sport Inhibition Screen.

Separate tubes containing 2 ml of molten top agar at 45^0C supplemented with histidine-biotin solution for the *Salmonella typhimurium* strains and supplemented with tryptophan for *Escherichia coli* strain were inoculated with 100 µl of tester strains, 100 µl of vehicle or test article dilution were added and 500 µl aliquot of S9 homogenate, simulating metabolic activation, was added when necessary. After vortexing, the mixture was poured across the Minimal Glucose Agar (GMA) plates. Parallel testing was also conducted with positive control correspond to each strain, replacing the test article aliquot with 50µl aliquot of appropriate positive control. After the overlay had solidified, the plates were inverted and incubated for 48 hours at 37^0C. The mean numbers of revertants of the test plates were compared to the mean number of revertants 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 in solution was considered to be Non-Mutagenic to *Salmonella typhimurium* tester strains TA98, TA100, TA1537, TA1535 and *Escherichia coli* tester strain WP2*uvr*A. 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/Escherichia coli* 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 TA98, TA100, TA1537, TA1535 and *Escherichia coli* WP2*uvr*A 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.

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II. Materials

A. **Storage Conditions:** Room temperature (23-25C).
B. **Vehicle:** Sterile DI Water.
C. **Preparation:** Eight different doses level were prepared immediately before use with sterile DI water.
D. **Solubility/Stability:** 100% Soluble and Stable.
E. **Toxicity:** No significant inhibition was observed.

III. Test System

A. Test System

Each *Salmonella typhimurium* and *Escherichia coli* tester strain contains a specific deep rough mutation (*rfa)*, the deletion of *uvr*B gene and the deletion in the *uvr*A gene that increase their ability to detect mutagens, respectively. These genetically altered *Salmonella typhimurium* strains (TA98, TA100, TA1537 and TA1535) and *Escherichia coli* strain (WP2*uvr*A) cannot grow in the absence of histidine and tryptophan, respectively. When placed in a histidine-tryptophan free medium, only those cells which mutate spontaneously back to their wild type states are able to form colonies. The spontaneous mutation rate (or 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
TA98	hisD3052, Dgal chlD bio *uvr*B *rfa* pKM101
TA100	hisG46, Dgal chlD BIO *uvr*B *rfa* pKM101
TA1537	hisC3076, *rfa*, Dgal chlD bio *uvrB*
TA 1535	hisG46, Dgal chlD bio *uvr*B *rfa*
WP2*uvr*A	trpE, *uvr*A

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)
pKM101	=	plasmid confers ampicillin resistance (R-factor) and enhances sensitivity to mutagens.
*uvr*A	=	All possible transitions and transversions, small deletions.

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, Molecular Toxicology, Inc.

C. Preparation of Tester strains

Cultures of *Salmonella typhimurium* TA98, TA100,TA1537, TA1535 and *Escherichia coli* WP2uvrA were inoculated to individual flasks containing Oxoid broth No.2. 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 for each strain was used as a positive control to demonstrate that tester strains were sensitive to mutation to the wild type state. The positive controls are tested with and without the presence of S9 homogenate.

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F. Titer of the Strain Cultures:
Fresh cultures of bacteria were grown up to the late exponential or early stationary phase of growth; to confirm this, serial dilutions from each strain were conducted, indicating that the initial population was in the range of 1 to $2x10^9$/ml.

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* and tryptophan for *Escherichia coli* were inoculated with 100 µl of culture for each strain and 100 µl of testing solution or vehicle without test material. 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 (+/-). When plating the positive controls, the test article aliquot was replaced by 50µl aliquot of appropriate positive control. The test was conducted per duplicate. 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 numbers of revertants of the test plates were compared to the mean number of reverants of the negative control of each strain used.

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 and Discussion

A. Solubility:
Water was used as a solvent. Solutions from the test article were made from 0.015 to 50mg/ml.

B. Dose levels tested:
The maximum dose tested was 5000 µg per plate. The dose levels tested were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg per plate.

C. Titer (Organisms/ml):
5×10^8 UFC/ml plate count indicates that the initial population was in the range of 1 to 2×10^9 UFC/ml.

C. Standard Plate Incorporation Assay
In no case was there a 2-fold or greater increase in the mean number of revertant testing strains TA98, TA100, TA1537, TA1535 and WP2*uvr*A in the presence of the test solution compared with the mean of vehicle control value. The positive controls mean exhibited at least a 3-fold increase over the respective mean of the *Salmonella typhimurium* and *Escherichia coli* tester strains used. The results are summarized in Appendix 2.

VII. Conclusion

All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that under the conditions of this assay, the test article solution was considered to be Non-Mutagenic to *Salmonella typhimurium* tester strains TA98, TA100, TA1537, TA1535 and *Escherichia coli* WP2*uvr*A.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.

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Appendix 2:

Bacterial Mutation Assay
Plate Incorporation Assay Results

	Concentration µg per Plate	*TA98*		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	18	22	20
	1500	24	33	29
	500	38	48	43
	150	30	57	44
	50	41	36	39
	15	39	56	48
	5.0	37	39	38
	1.5	29	46	38
Test Solution w/o S9	5000	9	19	14
	1500	38	29	34
	500	48	28	38
	150	46	44	45
	50	29	36	33
	15	42	36	39
	5.0	67	47	57
	1.5	47	33	40
DI Water w/S9		48	34	41
DI Water w/o S9		44	39	42
2-aminoanthracen w/ S9		380	347	364
2-nitrofluorene w/o S9		178	137	158
Historical Count Positive w/S9		**43-1893**		
Historical Count Positive w/o S9		**39-1871**		
Historical Count Negative w/S9		**4-69**		
Historical Count Negative w/o S9		**3-59**		

*CFU = Colony Forming Units
*Mean = Average of duplicate plates

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	Concentration µg per Plate	***TA100***		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	28	27	28
	1500	105	107	106
	500	100	95	98
	150	104	105	105
	50	86	93	90
	15	81	84	83
	5.0	91	99	95
	1.5	105	124	115
Test Solution w/o S9	5000	11	10	11
	1500	48	69	59
	500	91	97	94
	150	94	102	98
	50	86	107	97
	15	100	84	92
	5.0	100	84	92
	1.5	102	89	96
DI Water w/S9		94	124	109
DI Water w/o S9		85	108	97
2-aminoanthracen w/ S9		812	813	813
Sodium azide w/o S9		688	634	661
Historical Count Positive w/S9		**224-3206**		
Historical Count Positive w/o S9		**226-1837**		
Historical Count Negative w/S9		**55-268**		
Historical Count Negative w/o S9		**47-250**		

*CFU = Colony Forming Units
*Mean = Average of duplicate plates

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	Concentration µg per Plate	***TA1537***		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	6	0	3
	1500	8	14	11
	500	10	4	7
	150	6	6	6
	50	11	5	8
	15	4	4	4
	5.0	8	6	7
	1.5	4	3	4
Test Solution w/o S9	5000	4	1	3
	1500	4	1	3
	500	1	10	6
	150	11	6	9
	50	1	6	4
	15	8	6	7
	5.0	5	5	5
	1.5	5	4	5
DI Water w/S9		4	6	5
DI Water w/o S9		5	5	5
2-aminoanthracen w/ S9		58	51	55
2-aminoacridine w/o S9		530	471	501
Historical Count Positive w/S9		**13-1934**		
Historical Count Positive w/o S9		**17-4814**		
Historical Count Negative w/S9		**0-41**		
Historical Count Negative w/o S9		**0-29**		

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	Concentration µg per Plate	***TA1535***		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	3	3	3
	1500	19	18	19
	500	19	18	19
	150	11	14	13
	50	11	19	15
	15	9	10	10
	5.0	14	19	17
	1.5	10	20	15
Test Solution w/o S9	5000	8	0	4
	1500	0	4	2
	500	10	6	8
	150	11	20	16
	50	9	5	7
	15	5	14	10
	5.0	4	14	9
	1.5	5	6	6
DI Water w/S9		13	15	14
DI Water w/o S9		6	11	9
2-aminoanthracen w/ S9		86	95	91
Sodium azide w/o S9		541	601	571
Historical Count Positive w/S9		**22-1216**		
Historical Count Positive w/o S9		**47-1409**		
Historical Count Negative w/S9		**1-50**		
Historical Count Negative w/o S9		**1-45**		

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*Mean = Average of duplicate plates

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	Concentration µg per Plate	***WP2uvrA***		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	27	30	29
	1500	41	29	35
	500	20	33	27
	150	48	33	41
	50	33	20	27
	15	32	32	32
	5.0	37	41	39
	1.5	37	23	30
Test Solution w/o S9	5000	19	32	26
	1500	18	36	27
	500	22	20	21
	150	22	27	25
	50	30	24	27
	15	29	19	24
	5.0	33	25	29
	1.5	28	33	31
DI Water w/S9		17	25	21
DI Water w/o S9		30	24	27
2-aminoanthracen w/ S9		130	11	121
Methylmethanesulfonate w/o S9		258	271	265
Historical Count Positive w/S9		**44-1118**		
Historical Count Positive w/o S9		**42-1796**		
Historical Count Negative w/S9		**8-80**		
Historical Count Negative w/o S9		**8-84**		

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*Mean = Average of duplicate plates

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Phototoxicity Assay Analysis

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Tradename: PhytoCide Aspen Bark Extract Powder

Code: M16002

CAS #: 90083-05-9

Test Request Form #: 1096

Lot #: 4728P

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

Test Performed:
In Vitro EpiDerm™ Model (EPI-200-SIT) Phototoxicity

SUMMARY

In vitro phototoxicity irritation studies were conducted to evaluate whether **PhytoCide Aspen Bark Extract Powder** would induce phototoxic irritation in the EpiDerm™ model assay.

The product was tested according to the manufacturer's protocol. The test article solution was found to be a **non-photoirritant** at concentrations of 0.5%, 1.3%, and 4.5%. Reconstructed human epidermis was incubated in growth media for one hour to allow for tissue equilibration after shipping from MatTek Corporation, Ashland, MA. Test substance was applied to the tissue inserts in five varying concentrations and incubated overnight at 37°C, 5% CO_2, and 95% relative humidity (RH). The following day, the appropriate tissue inserts were irradiated (UVA) for 60 minutes with 1.7 mW/cm^2 (=6 J/cm^2). After substance incubation, irradiation, and washing was completed, the cell viability test was conducted. Cell viability was measured by dehydrogenase conversion of MTT [(*3-4,5-dimethyl thiazole 2-yl*)], present in the cell mitochondria, into blue formazan salt that was measured after extraction from the tissue. The photoirritation potential of the test chemical was dictated by the reduction in tissue viability of UVA exposed tissues compared to non-UVA exposed tissues.

Under the conditions of this assay, the test article was considered to be **non-phototoxic** at concentrations of 0.5%, 1.3%, and 4.5%. The negative and positive controls performed as anticipated.

I. Introduction

A. Purpose

In vitro dermal phototoxicity study was conducted to evaluate whether a test article would induce photoirritation in the EpiDerm™ model assay. MatTek Corporation's reconstructed human epidermal model is becoming a standard in determining the phototoxicity potential of a test substance. This assay is able to discriminate between photoirritants and non-photoirritants at varying concentrations.

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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; UVA-vis Irradiation Equipment; UVA meter; Pipettes
C. Media/Buffers:	Dulbecco's Modified Eagle Medium (DMEM) based medium; Dulbecco's Phosphate-Buffered Saline (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, and 6-well tissue culture plates
F. Reagents:	MTT (*3-4,5-dimethyl thiazole 2-yl*) (1.0mg/mL); Extraction Solution (Isopropanol); Chlorpromazine; Triton X-100 (1%)
G. Other:	Wash bottle; sterile disposable pipette tips; Parafilm; forceps

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. This model consists of organized basal, spinous, and granular layers, and contains a multilayer stratum corneum containing intercellular lamellar lipid layers. The EpiDerm™ tissues are cultured on specially prepared cell culture inserts.

B. Negative Control

Sterile deionized water is used as the negative controls for the EpiDerm™ Phototoxicity assay.

C. Positive Control

Concentrations of chloropromazine, ranging from 0.001% to 0.1%, were used as positive controls for the EpiDerm™ Phototoxicity assay.

D. Data Interpretation Procedure

A photoirritant is predicted if the mean relative tissue viability of the 2 tissues exposed to the test substance and 60 minutes of 6 J/cm^2 is reduced by 20% compared to the non-irradiated control tissues.

IV. Method

A. Tissue Conditioning

Upon MatTek kit arrival at Active Micro Technologies, 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 tissue insert dosing begins.

B. Test Substance Exposure

50µL of the diluted test substance in their respective concentrations are applied to 2 tissue inserts and allowed to incubate for overnight in a humidified incubator (37°C, 5% CO_2, 95% RH).

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C. Tissue Irradiation

Tissue inserts in their 6-well plates are UVA-irradiated for 60 minutes with 6 J/cm^2 at room temperature. The non-irradiated tissue inserts are incubated at room temperature in the dark.

D. Tissue Washing and Post Incubation

After UVA-irradiation and dark incubation is complete the tissue inserts are washed using sterile DPBS and transferred to fresh 6-well plates and media for overnight incubation at 37°C, 5% CO_2, 95% RH.

E. 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 ≥ 0.8.

B. Positive Control

The assay meets the acceptance criterion if a dose dependent reduction in cell viability in the UVA-irradiated tissues is between 0.00316% and 0.0316%.

C. Standard Deviation

Since the phototoxicity potential is predicted from the mean viability of 2 tissues for the EpiDerm™ Phototoxicity Protocol, the variability of the replicates should not exceed 30%.

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. Cell viability is calculated for each tissue as a percentage of the corresponding vehicle control either irradiated or non-irradiated. Tissue viability was not reduced by 20% in the presence of the test substance and UVA-irradiation at concentrations of 0.5%, 1.3%, and 4.5%. The negative control mean exhibited acceptable relative tissue viability while the positive control exhibited dose dependent loss of tissue viability and cell death.

C. Test Validity

The data obtained from this study met criteria for a valid assay. The negative and positive controls performed as anticipated.

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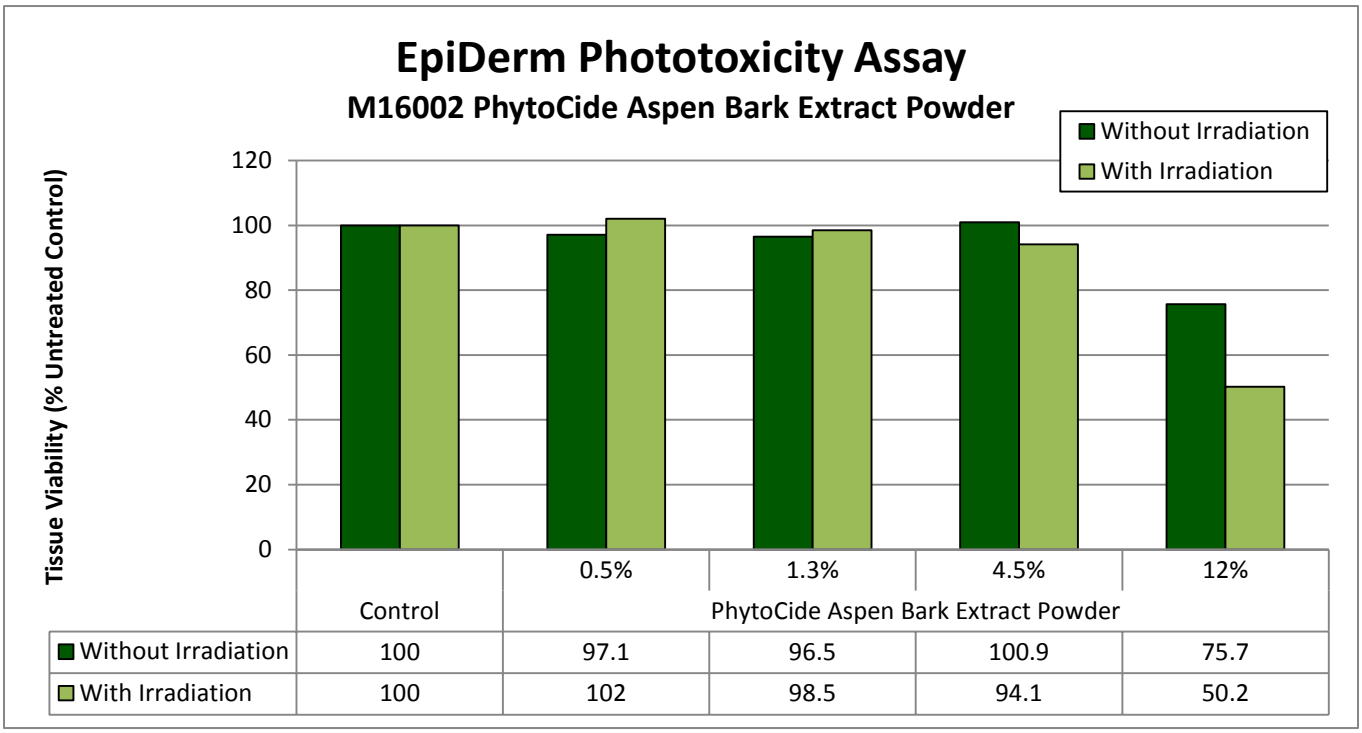
VII. Conclusion

Phototoxicity (photoirritation) is defined as an acute toxic response that is elicited after exposure of the skin to certain chemicals and subsequent exposure to light. Under the conditions of this assay, the test article substance was considered to be **non-phototoxic** at concentrations of 0.5%, 1.3%, and 4.5%. There is a decrease in viability at the 12% test concentration with and without irradiation but this concentration is significantly higher than the suggested use levels. We can safely say that **Phytocide Aspen Bark Extract Powder** is not a photoirritant when used at the suggested use levels of 0.2 – 3%.



	Control	0.5%	1.3%	4.5%	12%
Without Irradiation	100	97.1	96.5	100.9	75.7
With Irradiation	100	102	98.5	94.1	50.2

Figure 1: EpiDerm Phototoxicity Graph

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OECD 202 Acute Daphnia Assay

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Tradename: PhytoCide Aspen Bark Extract Powder

Code: M16002

CAS #: 90083-05-9

Test Request Form #: 1049

Lot #: 4728P

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

Test Performed:
OECD 202
Daphnia spp. Acute Immobilization Test

Introduction

The purpose of the present study is to determine the toxicity of **PhytoCide Aspen Bark Extract Powder** by exposing Daphnia spp. to the test substance for 48 hours and measuring the immobilization rate against the control. The present study defines an organism as being immobilized when it does not move for 15 seconds after the test vessel is gently shaken.

OECD Guideline 202 on "*Daphnia* spp., Acute Immobilization Test and Reproduction Test", adopted in 1984, included two parts: Part I – the 24 hour EC_{50} acute immobilization test and Part II – the reproduction test (at least 14 days). Revision of the reproduction test resulted in the adoption and publication of Test Guideline 211 on "*Daphnia magna* Reproduction Test" in September 1998. Consequently, the new version of Guideline 202 is restricted to the acute immobilization test.

Assay Principle

Young daphnids, aged less than 24 hours at the start of the test, are exposed to the test substance at a range of concentrations for a period of 48 hours. Immobilization is recorded at 24 hours and 48 hours and compared with control values. The results are analyzed in order to calculate the EC_{50} at 48 hours. EC_{50} is the concentration estimated to immobilize 50% of the daphnids within a stated exposure period. Immobilization refers to those animals that are not able to swim within 15 seconds after gentle agitation of the test vessel, even if they can still move their antennae.

The water solubility and vapor pressure of the test substance should be known. A reliable analytical method for the quantification of the substance in the test solutions with reported recovery efficiency and limit of determination should also be available.

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A reference substance may be tested for EC_{50} as a means of assuring that the test conditions are reliable.

For this assay to be valid, the following performance criteria apply:

- In the control, not more than 10% of the daphnids should have been immobilized.
- The dissolved oxygen concentration at the end of the test should be at least 3 mg/L in control and test vessels.

Materials

- Glass Test Tubes and/or Beakers
- Dissolved Oxygen Meter
- pH Meter
- Temperature Control Apparatus
- Total Organic Carbon (TOC) Analyzer
- Chemical Oxygen Demand (COD) Analyzer
- *Daphnia magna* Straus
  - Use organisms less than 24 hours old. Do not use first offspring of parents.
- Water
  - Use water suitable for culturing and testing Daphnia spp. It can be natural water (surface water or groundwater), dechlorinated tap water, or artificially prepared water (Table 1), but must satisfy the conditions listed in Table 2. Do not use Elendt M4 or M7 media or water containing chelating agents for testing metal-containing substances. The water hardness should be 250 mg/L or smaller in terms of calcium carbonate concentration, and the pH should be 6-9. Aerate the material water before using it for the test.

Substance	Concentration
Particulate Matter	<20 mg/L
Total Organic Carbon	<2 mg/L
Unionized Ammonia	<1 ug/L
Residual Chlorine	<10 ug/L
Total Organophosphorus Pesticides	<50 ng/L
Total Organochlorine Pesticides plus Polychlorinated Biphenyls	<50 ng/L
Total Organic Chlorine	<25 ng/L

Table 1: Chemical Characteristics of Suitable Water

Substance	Amount Added to 1 Liter Water	To prepare the reconstituted water, add the following volumes of stock solutions to 1 liter water
Calcium Chloride	11.76 grams	25 mL
Magnesium Sulfate	4.93 grams	25 mL
Sodium Bicarbonate	2.59 grams	25 mL
Potassium Chloride	0.23 grams	25 mL

Table 2: Examples of Suitable Reconstituted Test Water

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Methods

Test Conditions

- Test Method
  - Test is performed under a static, semi-static, or flow-through condition. If test substance is unstable, a semi-static or flow-through test is recommended.
- Exposure Period
  - 48 hours
- Test Volume
  - At least 2 milliliters
- Number of Test Organisms
  - At least 20 organisms for each test concentration and the control.
- Test Concentration
  - Adopt a concentration range of at least 5 concentrations, with the highest concentration inducing 100% immobilization and no effect at the lowest concentration.
- Culture Method
  - Illumination: The photoperiod is set to 16 hours light and 8 hours dark
  - Temperature: The temperature is between 18°C to 22°C
  - Dissolved Oxygen Concentration: Must be kept at 3mg/L or higher
  - Feeding: Do not feed test organisms

Observation

- Observe mobility of the organisms at least twice (i.e., at 24 and 48 hours after exposure).
- The organisms are considered immobilized when they do not move for 15 seconds after test vessel is gently shaken.

Measurement of Test Substance Concentrations

- At the beginning and end of exposure, measure test substance concentrations at the lowest and highest test concentration groups.
  - For volatile or adsorptive substances, additional measurements are recommended at 24 hours intervals during exposure period.

Test Condition Measurements

- Measure dissolved oxygen in the control and at the highest test concentration at the beginning and end of the exposure period.
- Measure pH in the control and at the highest test concentration at the beginning and end of the exposure period.
- Water temperature should be measured at the beginning and end of the exposure period.

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Data and Reporting

I. Data
   a. Data should be summarized in tabular form, showing for each treatment group and control, the number of daphnids used, and immobilization at each observation. The percentages immobilized at 24 and 48 hours are plotted against test concentrations. Data are analyzed by appropriate statistical methods (e.g. probit analysis, etc.) to calculate the slopes of the curves and the EC_{50} with 95% confidence limits (p = 0.95).
   b. Where the standard methods of calculating the EC_{50} are not applicable to the data obtained, the highest concentration causing no immobility and the lowest concentration producing 100% immobility should be used as an approximation for the EC_{50} (this being considered the geometric mean of these two concentrations).

II. Test Report
   a. The test report must include the following:
      i. Test substance:
         1. Physical nature and relevant physical-chemical properties
         2. Chemical identification data, including purity
      ii. Test species:
         1. Source and species of *Daphnia*, supplier of source (if known), and the culture conditions (including source, kind and amount of food, feeding frequency)
      iii. Test conditions:
         1. Description of test vessels: type and volume of vessels, volume of solution, number of daphnids per test vessel, number of test vessels (replicates) per concentration
         2. Methods of preparation of stock and test solutions including the use of any solvent or dispersants, concentrations used
         3. Details of dilution water: source and water quality characteristics (pH, hardness, Ca/Mg ratio, Na/K ratio, alkalinity, conductivity, etc); composition of reconstituted water if used
         4. Incubation conditions: temperature, light intensity and periodicity, dissolved oxygen, pH, etc.
      iv. Results:
         1. The nominal test concentrations and the result of all analyses to determine the concentration of the test substance in the test vessels; the recovery efficiency of the method and the limit of determination should also be reported
         2. All physical-chemical measurements of temperature, pH and dissolved oxygen made during the test
         3. The EC_{50} at 48 hours for immobilization with confidence intervals and graphs of the fitted model used for calculation, the slopes of the dose-response curves and their standard error; statistical procedures used for determination of EC_{50}

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Results

General Information:

Name of new chemical substance	PhytoCide Aspen Bark Extract Powder		
INCI Nomenclature	*Populus tremuloides Bark Extract*		
CAS number	90083-05-9		
Structural or rational formula (if neither is available, summarize its formulation method)	Botanical: *Populus tremuloides*		
Molecular weight	106.2 Daltons		
Purity of the new chemical substance used for the test (%)	100%		
Lot number of the new chemical substance used for the test	4728P		
Names and contents of impurities	n/a		
Solubility in water	Fully soluble		
Melting point	n/a		
Boiling point	n/a		
Properties at room temperature	Free flowing white powder		
Stability	Stable under ordinary conditions		
Solubility in solvents, etc.	**Solvent**	**Solubility**	**Stability in solvent**
	n/a	n/a	n/a

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Test Materials and Methods:

Items			Contents
Test Organisms	Species		*Daphnia magna*
	Source		Carolina Biological Supply Company
	Susceptibility to reference substance (EC_{50})		Potassium dichromate (0.94 mg/L)
Culture	Kind of Medium		Elendt Medium M4
	Conditions (Temperature/Photoperiod)		20°C/16 Hour Light-8 Hour Dark
Test Conditions	Test Vessel		Glass
	Material Water	Kind	Elendt Medium M4
		Hardness	250 mg/L
		pH	7.4
	Date of Exposure		1/20/2015
	Test Concentrations		200, 90.1, 43.2, 10.8, 6.9 mg/L
	Number of organisms		120
	Number of Replicates	Exposure Group	4
		Control Group	4
	Test Solution Volume		2 mL
	Vehicle	Use or Not	N/A
		Kind	N/A
		Concentration	N/A
		Number of Replicates	N/A
	Culture Method (Static, Semi-Static, Flow-Through)		Static
	Water Temperature		20°C ± 2°C
	Dissolved Oxygen Concentration (DO)		3 mg/L
	Photoperiod		16 Hour Light-8 Hour Dark
Calculation of Results	Statistical Method		Probit Analysis

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Test Results:

Items		Contents
Toxicity Value	48hr EC50	136.0 mg/L
Exposure Concentrations Used for Calculation	Nominal Values	200, 90.1, 43.2, 10.8, 6.9 mg/L
Remarks		Not harmful to aquatic organisms

Discussion

After 48 hours, the EC50 value for **PhytoCide Aspen Bark Extract Powder** was determined to be 136.0 mg/L. The conditions of OECD guideline 202 for the validity of the test were adhered to: The immobility of controls in purified drinking water (dilution water) did not exceed 10%. According to the EU Directive 93/67/EEC, this product is not classified and therefore not harmful to aquatic organisms.

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OECD 301B Ready Biodegradability Assay

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Tradename: PhytoCide Aspen Bark Extract Powder

Code: M16002

CAS #: 90083-05-9

Test Request Form #: 1050

Lot #: 4728P

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

Test Performed:
OECD 301 B
Ready Biodegradability: CO_2 Evolution (Modified Sturm Test)

Introduction

A study was conducted to assess the ready biodegradability of **PhytoCide Aspen Bark Extract Powder** in an aerobic aqueous medium. In the OECD guideline 301 for ready biodegradability, six methods are provided as options. This report uses method B, CO_2 Evolution, also known as a Modified Sturm Test. This method was chosen based on the solubility, volatility, and adsorbing capabilities of the test sample.

Assay Principle

A solution or suspension of the test substance in a mineral medium is inoculated and incubated under aerobic conditions in the dark or in diffuse light. The amount of DOC (Dissolved Organic Carbon) in the test solution due to the inoculum should be kept as low as possible compared to the amount of organic carbon due to the test substance. Allowance is made for the endogenous activity of the inoculum by running parallel blanks with inoculum but without test substance. A reference compound is run in parallel to check the procedures' operation.

In general, degradation is followed by the determination of parameters such as DOC, carbon dioxide production, and oxygen uptake. Measurements are taken at sufficiently frequent intervals to allow the identification of the beginning and end of biodegradation.

Normally this test lasts for 28 days, but it may be ended before that time if the biodegradation curve reaches a plateau for at least three determinations. Tests may also be prolonged beyond 28 days when the curve shows that biodegradation has started but the plateau has not yet been reached. In such cases the test substance would not be classified as readily biodegradable.

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The pass levels for ready biodegradability are 70% removal of DOC and 60% of ThOD (Theoretical Oxygen Demand) or $ThCO_2$ (Theoretical Carbon Dioxide) production for respirometric methods. They are lower in the respirometric methods since, as some of the carbon from the test chemical is incorporated into new cells, the percentage of CO_2 produced is lower than the percentage of carbon being used. These pass values have to be reached in a 10-day window within the 28-day period of the test. The 10-day window begins when the degree of biodegradation has reached 10% DOC, ThOD, or $ThCO_2$ and must end before day 28 of the test. Test substances which reach the pass levels after the 28-day period are not deemed to be readily biodegradable.

In order to check the procedure, reference compounds which meet the criteria for ready biodegradability are tested by setting up an appropriate vessel in parallel as part of normal test runs. Suitable compounds are freshly distilled aniline, sodium acetate, and sodium benzoate. These compounds all degrade in this method even when no inoculum is deliberately added.

Because of the nature of biodegradation and of the mixed bacterial populations used as inocula, determinations should be carried out at least in duplicate. It is usually found that the larger the concentration of microorganisms initially added to the test medium, the smaller the variation between replicates.

Materials

- Water
  - Deionized or distilled, free from inhibitory concentrations of toxic substances
  - Must contain no more than 10% of the organic carbon content introduced by the test material
  - Use only one batch of water for each series of tests
- Mineral media
  - To prepare the mineral medium, mix 10 mL of solution A with 800 mL water. Then add 1 mL each of solutions B, C, and D and make up to 1 liter with water.
  - Solution A (Dissolve in water and make up to 1 liter; pH 7.4)
    - Potassium dihydrogen orthophosphate, KH_2PO……....8.5g
    - Dipotassium hydrogen orthophosphate, K_2HPO_4……21.8g
    - Disodium hydrogen orthophosphate dehydrate, $Na_2HPO_4.2H_2O$.....33.4g
    - Ammonium chloride, NH_4Cl……....0.5g
  - Solution B (Dissolve in water and make up to 1 liter)
    - Calcium chloride, anhydrous, $CaCl_2$……27.50g
      Or
    - Calcium chloride dehydrate, $CaCl_2.2H_2O$……36.40g
  - Solution C (Dissolve in water and make up to 1 liter)
    - Magnesium sulphate heptahydrate, $MgSO_4.7H_2O$…… 22.50g
  - Solution D (Dissolve in water and make up to 1 liter.)
    - Iron (III) chloride hexahydrate, $FeCl_3.6H_2O$……...0.25g
  - Flasks, 2-5 liters each, fitted with aeration tubes reaching nearly to the bottoms of the vessels and an outlet
  - Magnetic stirrers
  - Gas absorption bottles
  - Device for controlling and measuring air flow
  - Apparatus for carbon dioxide scrubbing, for preparation of air which is free from carbon dioxide; alternatively, a mixture of CO_2-free oxygen and CO_2-free nitrogen from gas cylinders in the correct proportions (20% O_2 : 80% N_2)
  - Device for determination of carbon dioxide, either titrimetrically or by some form of inorganic carbon analyzer

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- Stock solutions of test substances
  - When solubility of the substance exceeds 1 g/L, dissolve 1-10 g, as appropriate, of test or reference substance in water and make up to 1 liter. Otherwise, prepare stock solutions in mineral medium or add the chemical directly to the mineral medium, making sure it
- Inoculum
  - The inoculum may be derived from the following sources
    - Activated sludge
    - Sewage effluents
    - Surface waters
    - Soils
    - Or from a mixture of these.
  - Inoculum may be pre-conditioned to the experimental conditions, but not pre-adapted to the test substance. Pre-conditioning consists of aerating activated sludge in mineral medium or secondary effluent for 5-7 days at the test temperature. Pre-conditioning sometimes improves the precision of the test method by reducing blank values.

Methods

I. Preparation of flasks: As an example, the following volumes and weights indicate the values for 5-liter flasks containing 3 liters of suspension. If smaller volumes are used, modify the values accordingly.
   a. To each 5-liter flask, add 2,400 mL mineral medium.
   b. Add an appropriate volume of the prepared activated sludge to give a concentration of suspended solids of not more than 30 mg/L in the final 3 liters of inoculated mixture. Alternatively, first dilute the prepared sludge to give a suspension of 500-1000 mg/L in the mineral medium before adding an aliquot to the contents of the 5-liter flask to attain a concentration of 30 mg/L.
   c. Aerate these inoculated mixtures with CO_2-free air overnight to purge the system of carbon dioxide.
   d. Add the test material and reference compound, separately, as known volumes of stock solutions, to replicate flasks to yield concentrations, contributed by the added chemicals, of 10 – 20 mg DOC or TOC per liter. Leave some flasks without addition of chemicals as inoculum controls. Add poorly soluble test substances directly to the flasks on a weight or volume basis. Make up the volumes of suspensions in all flasks to 3 liters by the addition of mineral medium previously aerated with CO_2-free air.
   e. If required, use one flask to check the possible inhibitory effect of the test substance by adding both the test and reference substances at the same concentrations as present in the other flasks.
   f. If required, check whether the test substance is degraded abiotically by using a sterilized uninoculated solution of the chemical. Sterilize by the addition of a toxic substance at an appropriate concentration.
   g. If barium hydroxide is used, connect three absorption bottles, each containing 100 mL of 0.0125M barium hydroxide solution, in series to each 5-liter flask. The solution must be free of precipitated sulfate and carbonate and its strength must be determined immediately before use.
   h. If sodium hydroxide is used, connect two traps, the second acting as a control to demonstrate that all the carbon dioxide was absorbed in the first. Absorption bottles fitted with serum bottle closures are suitable. Add 200 mL 0.05M sodium hydroxide to each bottle. This is sufficient to absorb the total quantity of carbon dioxide evolved when the test substance is completely degraded.
   i. In a typical run, the following flasks are used:
      i. Flasks 1 & 2: containing test substance and inoculum (test suspension)
      ii. Flasks 3 & 4: containing only inoculum (inoculum blank)
      iii. Flask 5: containing reference compound and inoculum (procedure control)
      iv. Flask 6: containing test substance and sterilizing agent (abiotic sterile control)
      v. Flask 7: containing test substance, reference compound and inoculum (toxicity control)

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II. Start the test by bubbling CO_2-free air through the suspensions at a rate of 30-100 mL/minute.

III. CO_2 Determination
   a. It is mandatory to follow the CO_2 evolution from the test suspensions and inoculum blanks in parallel and it is advisable to do the same for the other test vessels.
   b. During the first ten days it is recommended that analyses of CO_2 should be made every second or third day and then at least every fifth day until the 28th day so that the 10-day window period can be identified. On the days of CO_2 measurement, disconnect the barium hydroxide absorber closest to the test vessel and titrate the hydroxide solution with 0.05M HCl using phenolphthalein as the indicator. Move the remaining absorbers one place closer to the test vessel and place a new absorber containing 100 mL fresh 0.0125M barium hydroxide at the far end of the series. Make titrations are needed (for example, when substantial precipitation is seen in the first trap and before any is evident in the second, or at least weekly). Alternatively, with NaOH as absorbent, withdraw a sample of the sodium hydroxide solution from the absorber nearest to the test vessel using a syringe. The sample volume needed will depend on the carbon analyzer used, but sampling should not significantly change the absorbent volume over the test period. Inject the sample into the IC part of the carbon analyzer for analysis of evolved carbon dioxide directly. Analyze the contents of the second trap only at the end of the test in order to correct for any carry-over of carbon dioxide.
   c. On the 28th day withdraw samples, optionally, for DOC and/or specific chemical analysis. Add 1 mL of concentrated hydrochloric acid to each test vessel and aerate them overnight to drive off the carbon dioxide present in the test suspensions. On day 29 make the last analysis of evolved carbon dioxide.

Data and Reporting

I. Treatment of Results
   a. Data from the test should be entered onto the attached data sheet.
   b. The amount of CO_2 produced is calculated from the amount of base remaining in the absorption bottle. When 0.0125M $Ba(OH)_2$ is used as the absorbent, the amount remaining is assessed by titrating with 0.05M HCl.
   c. Since 1 mmol of CO_2 is produced for every mol of $Ba(OH)_2$ reacted to $BaCl_2$ and 2 mmol of HCl are needed for the titration of the remaining $Ba(OH)_2$ and given that the molecular weight of CO_2 is 44 g, the weight of CO_2 produced (in mg) is calculated by:

$$\frac{0.05 \times (50 - mL\ HCl\ Titrated) \times 44}{2} = 1.1 \times (50 - mL\ HCl\ Titrated)$$

   Therefore, the factor to convert volume of HCl titrated to mg CO_2 produced is 1.1 in this case. Calculate the weights of CO_2 produced from the inoculum alone and from the inoculum plus test substance using the respective titration values. The difference is the weight of CO_2 produced from the test substance alone.

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d. The percentage biodegradation is calculated from:

$$\%\ Degradation = \frac{mg\ CO_2\ Produced}{ThCO_2 \times mg\ Test\ Substance\ Added} \times 100$$

Or

$$\%\ Degradation = \frac{mg\ CO_2\ Produced}{mg\ TOC\ Added\ in\ Test\ \times 3.67} \times 100$$

$$Where\ 3.67\ is\ the\ conversion\ factor\ \left(\frac{44}{12}\right)\ for\ carbon\ to\ carbon\ dioxide$$

e. When NaOH is used as the absorbent, calculate the amount of CO_2 produced after any time interval from the concentration of inorganic carbon and the volume of absorbent used. Calculate the percentage degradation from:

$$\%\ ThCO_2 = \frac{mg\ IC\ from\ Test\ Flask - mg\ IC\ from\ Blank}{mg\ TOC\ Added\ as\ Test\ Substances} \times 100$$

f. Display the course of degradation graphically and indicate the 10-day window. Calculate and report the percentage removal achieved at the plateau, at the end of the test, and/or at the end of the 10-day window, whichever is appropriate.
g. When appropriate, calculate DOC removals using the equation given in 301 A paragraph 27.
h. When an abiotic control is used, calculate the percentage abiotic degradation by:

$$\%\ Abiotic\ Degradation = \frac{CO_2\ Produced\ by\ Sterile\ Flask\ After\ 28\ Days\ (mg)}{ThCO_2\ (mg)} \times 100$$

Validity of Tests

I. The IC content of the test substance suspension in the mineral medium at the beginning of the test must be less than 5% of the TC, and the total CO_2 evolution in the inoculum blank at the end of the test should not normally exceed 40 mg/L medium. If values greater than 70 mg CO_2/L are obtained, the data and experimental technique should be examined critically.

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ACTIVE MICRO TECHNOLOGIES

OECD 301B Ready Biodegradability Assay

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Data Sheet

Laboratory	Active Micro Technologies Tissue Culture Laboratory		
Test Start Date	12/29/2014		
Test Substance	**Name**	PhytoCide Aspen Bark Extract Powder	
	Stock Solution Concentration	2 g/L	
	Initial Concentration in Medium	20 mg/L	
Inoculum	**Source**	Activated Sludge	
	Treatment Given	Centrifugation	
	Pre-conditioning	N/A	
	Suspended Solids Concentration in Reaction Mixture	4 mg/L	
Reference Material	Sodium Benzoate	**Concentration**	20 mg/L
CO_2 Production and Degradability	**Method**	**$Ba(OH)_2$**	0.0125M
		NaOH	N/A
		Other	N/A
Total Contact Time	28 Days		
Total CO_2 Evolved Measurements	**Days**	2, 4, 11, 17, 23, 28	
Degradation Over Time	93.4%		
Remarks	Test material was readily biodegradable		
Conclusion	This test met the criteria for a valid assay		

Discussion

Based on the testing conducted in accordance with the specified method, test **PhytoCide Aspen Bark Extract Powder** achieved 93.4% biodegradation after 28 days of testing. The product met method requirements for Readily Biodegradability classification.

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ACTIVE MICRO TECHNOLOGIES

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Date Issued: January 23, 2015

ALLERGEN DECLARATION

RE: *PhytoCide Aspen Bark Extract Powder (M16002)*

Please be advised that this form is to certify that the above referenced product, manufactured at Active Micro Technologies, LLC, does not contain any of the allergens listed below:

Eggs – or egg products

Milk – or milk products (includes whey, lactose, casein, milk, cream)

Peanuts – or peanut products

Fish – (includes fish: surimi, cod, pollack, whitefish)

Shellfish – (shrimp, lobster, crab, clams, etc.)

Soybeans – or soybean products (includes soya powder, protein, oil, lecithin, tofu)

Wheat – or wheat products (includes Gluten)

Tree nuts – (almond, brazil nut, cashew, chestnut, hazelnut, filbert, pine nuts (pinyon, pinon), pistachio, pecan, macadamia, walnut).

Palm Oil – or palm kernel oil

Corn – or corn products

If you have any further questions or concerns, please contact us at: 1-704-276-7100

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Heavy Metals Statement

107 Technology Drive • Lincolnton, NC 28092
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May 10, 2016

To Whom It May Concern,

This letter is to certify that PhytoCide Aspen Bark Extract Powder (M16002) has the following heavy metals profile:

Heavy Metals:	Less than 20 ppm
Lead:	Less than 10 ppm
Antimony:	Less than 5 ppm
Arsenic:	Less than 3 ppm
Mercury:	Less than 1 ppm
Cadmium:	Less than 1 ppm

**Please note: The above levels illustrate the Maximum Limits. Values for Lead, Antimony, Mercury and Cadmium do not appear on the Specification for PhytoCide Aspen Bark Extract Powder.

Best Regards,

Tomorrow's Vision... *Today!*®

Heather Ferguson |R&D Coordinator

107 Technology Drive | Lincolnton, NC 28092

Direct: 704.276.7083|Main: 704.276.7100|Fax: 704.276.7101

Email: hferguson@activeconceptsllc.com

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Certificate of Origin

PhytoCide Aspen Bark Extract Powder
Code: M16002

Active Micro Technologies, LLC certifies that all raw material(s) used to manufacture the above listed ingredient originate in the United States of America.

Active Micro Technologies, LLC certifies that the above listed ingredient is plant derived from non-GMO *Populus tremuloides* and therefore is BSE-Free.

Active Micro Technologies, LLC certifies that the above listed ingredient has never been tested on animals and therefore complies with the European Union cut-off date of September 2004.

Active Micro Technologies, LLC certifies that the *Populus tremuloides* used to manufacture the above listed ingredient is not classified as a threatened or endangered species under any federal or state laws.

Active Micro Technologies, LLC certifies that the above listed ingredient can be classified as Vegan Compliant.

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ACTIVE MICRO TECHNOLOGIES

Safety Data Sheet

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SECTION 1. IDENTIFICATION

Product Name/Identifier	PhytoCide Aspen Bark Extract Powder
Product Code	M16002
Recommended Use	Topical Cosmetic Use; Antimicrobial
Restrictions on Use	Refer to the detailed list of labeling/restrictions (Section 15 Regulatory Information)
Supplier/Manufacturing Site	Active Micro Technologies, LLC
Address	107 Technology Drive Lincolnton, NC 28092, USA
Telephone No. (24hrs)	1-704-276-7100
Fax No.	1-704-276-7101
Emergency Telephone #	1-704-276-7100 (Mon-Fri: 8:00AM – 5:00PM EST)

SECTION 2. HAZARD(S) IDENTIFICATION

Classification:

GHS / CLP
Basis for Classification: Based on present data no classification and labeling is required according to GHS, taking into account the national implementation (United Nations version 2011)

USA
OSHA Regulatory Status: This material is non-hazardous as defined by the American OSHA Hazard Communication Standard (29 CFR 1910.1200).

Europe
Basis for Classification:
-According to present data no classification and labeling is required according to Directives 67/548/EEC or 1999/45/EC.
-This product is not classified as hazardous to health or environment according to the CLP regulation.

Labeling Elements:

Pictograph: No hazard symbol expected

Hazard statements/Signal Word: H320: WARNING: Causes minimal eye irritation

Precautionary statements:
P233: Keep container tightly closed
P281: Use personal protective equipment as required
P402: Store in a dry place
P404: Store in a closed container
P410: Protect from sunlight
P411: Store at temperatures not exceeding 25°C

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Other hazards which do not result in classification:

No particular fire or explosion hazard.
By mechanical effect: No particular hazards.
By hydroscopic effect: No particular hazards.

US NFPA 704 (National Fire Protection Association) Hazard Rating System:

Health hazard: Rating 0; Normal Material
Flammability: Rating 0, Will Not Burn
Reactivity: Rating 0, Stable
Other Hazard Information: None

Results of PBT and vPvB assessment:

-PBT: Not applicable
-vPvB: Not applicable

SECTION 3. COMPOSITION / INFORMATION ON INGREDIENTS

Common Chemical Name: Populus Tremuloides Bark Extract

Generic name:

Chemical Family: Plant Extract

Description: Substance

Substance	CAS Numbers	EC Numbers	Percentage
Populus Tremuloides Bark Extract	90083-05-9	290-199-2	100.00%

Formula: Not applicable

SECTION 4. FIRST-AID MEASURES

General: In all cases of doubt, or when symptoms persist, seek medical attention.

Inhalation: Move to fresh air from exposure area. Get medical attention for any breathing difficulty.

Skin contact: Rinse with soap and water. Get medical advice if irritation develops.

Eye contact: Immediately rinse with water for at least 15 minutes, while keeping the eyes wide open. Consult with a physician.

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Ingestion:	Consult with a physician.
Protection of first-aiders:	No special protection required.

SECTION 5. FIRE-FIGHTING MEASURES

Fire and explosion hazards:	Not considered to be a fire and explosion hazard
Extinguishing media:	
Suitable:	Water, dry chemicals, foam & carbon dioxide.
Not suitable:	None known
Fire fighting:	Move container from fire area if it can be done without risk. Avoid inhalation of material or combustion by-products. Stay upwind and keep out of low area
Protection for fire-fighters:	Boots, gloves, goggles.

SECTION 6. ACCIDENTAL RELEASE MEASURES

Personal precautions:	Avoid contact with eyes. Personal Protective Equipment: -Protective goggles
Environmental precautions:	Prevent entry into sewers and waterways. Do not allow material to contaminate ground water system
Methods for cleaning up:	
Recovery:	Pick up free liquid for recycling or disposal. Residual liquid can be absorbed on an inert material.
Cleaning/Decontamination:	Wash non-recoverable remainder with water.
Disposal:	For disposal of residues refer to sections 8 & 13.

SECTION 7. HANDLING AND STORAGE

Handling

Technical measures:	Labeling: Keep out of the reach of children.
Measures:	For industrial use, only as directed.
Safe handling advice:	Wash hands after use. Avoid storage near feed or food stuff.

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Storage

Technical measures:	Keep container closed.
Recommended Storage Conditions:	Store in a cool, dry place. This product should be stored at room temperature (23 - 25°C). It should not be exposed to excessive heat or cold. Do not freeze.
Incompatible products:	Avoid contact with strong oxidizers. Refer to the detailed list of incompatible materials (Section 10 Stability/Reactivity)
Packaging:	Product may be packaged in normal commercial packaging.
Packaging materials:	Recommended - Polypropylene & High Density Polyethylene

SECTION 8. EXPOSURE CONTROLS / PERSONAL PROTECTION

Precautionary statements: Ensure adequate ventilation

Control parameters

Occupational exposure Limits:

France:	Not Determined
ACGIH:	Not Determined
Korea:	Not Determined
UK:	Not Determined
Surveillance procedures:	Not Determined
Engineering measures:	Not Determined

Personal Protective Equipment:

Respiratory protection:	Local exhaust
Hand protection:	Protective gloves made of rubber or neoprene.
Eye protection:	Safety glasses.
Collective emergency equipment:	Eye fountain.
Skin and Body Protection:	Suitable protective clothing
Hygiene measures:	Handle in accordance with good industrial hygiene and safety practice.
Measures related to the Environment:	No particular measures.

SECTION 9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance:	Free flowing powder - Hygroscopic
Color:	White to light yellow
Odor:	Characteristic

This information is presented in good faith but is not warranted as to accuracy of results. Also, freedom from patent infringement is not implied.
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Solubility (in Water):	Soluble
Solids (1g-105°C-1hr):	92.0% Minimum
pH (3% solution in water):	3.8 – 6.2
Phenolics (tested as Salicylic Acid):	54.0 – 60.0%
Heavy Metals:	< 20 ppm
Arsenic:	< 3 ppm
Specific Gravity (25°C):	Not determined
Vapor density:	Not applicable
Boiling Point:	Not applicable
Freezing Point:	Not applicable
Melting point:	Not determined
Flash point:	Not applicable
Oxidizing properties:	Non oxidizing material according to EC criteria.
Solubility:	
In water:	Soluble
In organic solvents:	Not determined
Log P:	Not determined

SECTION 10. STABILITY AND REACTIVITY

Stability:	Stable under ordinary conditions of use and storage up to one year then re-test to full product specifications to extend shelf life
Hazardous reactions:	None known
Conditions to avoid:	No dangerous reactions known under use of normal conditions. Avoid extreme heat.
Materials to avoid:	No dangerous reaction known with common products.

Hazardous decomposition products: None known

SECTION 11. TOXICOLOGICAL INFORMATION

Ingestion:	Not Determined
Dermal:	Non-Irritant (Dermal Irritection Model)
Ocular:	Minimal Irritant (Ocular Irritection Model)
Inhalation:	Not Determined

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Acute toxicity data: EC50 (Acute Daphnia): 136.0 mg/L - Not harmful to aquatic organisms

Sensitization: Non-Primary Irritant & Non-Primary Sensitizer (RIPT, In-Vitro Skin Sensitization Report & Direct Peptide Reactivity Assay)

Repeated dose toxicity: No known effects
Subacute to chronic toxicity: Not Determined
Mutagenicity/genotoxicity: Non-mutagenic

Additional Toxicological Information: This product is not subject to classification according to the calculation method of the General EU Classification Guidelines for Preparations as issued in the latest version.

Specific effects:

Carcinogenicity: No known effects
Mutagenicity: No known effects
Reproductive toxicity: No known effects
Neuro-toxicity: No known effects

For more information: Does not present any particular risk on handling under normal conditions of good occupational hygiene practice.

This product has not been tested for the following:
-Primary cutaneous and corrosive irritation
-Acute oral toxicity

SECTION 12. ECOLOGICAL INFORMATION

Ecotoxicity
Effects on the aquatic environment: Not Determined

Biodegradability:
Persistence: Readily Biodegradable

Bioaccumulation:
Octanol / water partition coefficient: Not Determined

Mobility:
Precipitation:
Expected behavior of the product: Ultimate destination of the product: Soil & sediment.

Other Adverse Effects: None known

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This information is offered solely for your investigation, verification, and consideration.

SECTION 13. DISPOSAL CONSIDERATIONS

Residues from product

Prohibition:	Do not allow the product to be released into the Environment.
Destruction/Disposal:	Dispose of in accordance with relevant local regulations

Contaminated packaging

Decontamination/cleaning:	Cleaning is not required prior to disposal.
Destruction/Disposal:	

Note: Take all necessary precautions when disposing of this product according to local regulations.

SECTION 14. TRANSPORT INFORMATION

UN Number:	None
UN Shipping Name:	None
Transport Hazard Class:	Not classified as dangerous for transport
Land (rail/road):	Material is not restrictive for land transport and is not regulated by ADR/RID
Sea:	Material is not restrictive for sea transport and is not regulated by IMO/IMDG
Air:	Material is not restrictive for land transport and is not regulated by ICA/IATA
Marine Pollutant:	No
Transport/Additional Information:	Not regulated for US DOT Transport in non-bulk containers This material is not dangerous or hazardous
Special Precautions for User:	None known

The above regulatory prescriptions are those valid on the date of publication of this sheet. However, given the possible evolution of transport regulations for hazardous materials and in the event of the MSDS in your possession dating back more than 12 months, it is advisable to check their validity with your sales office.

SECTION 15. REGULATORY INFORMATION

Labeling/Restrictions:

EC regulations:	Not to be used for children under three years of age
Chinese regulations:	Not to be used for children under three years of age
Brazilian regulations:	Not to be used for children under three years of age
ASEAN regulations:	Not to be used for children under three years of age
Mexico regulations:	Not to be used for children under three years of age

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Further regulations

United Kingdom:	Handle in accordance with relevant British regulation: control of substance Hazardous to Health Regulations Environmental Hygiene Guidance: EH40 Workplace Exposure Limits (revised annually)
Korea regulations:	Industrial safety and hygiene regulation: No Hazardous material control regulation: No Fire prevention regulation: No

Other regulations:

EINECS inventory status:	Populus Temuloides Bark Extract: 290-199-2
TSCA inventory status:	Exempt
AICS inventory status:	Listed: 90083-05-9
Canadian (CEPA DSL) inventory status:	Exempt: Populus Temuloides Bark Extract (90083-05-9)
Japan (MITI list):	Populus Temuloides Bark Extract
Korea:	Populus Temuloides Bark Extract
China inventory status:	Populus Temuloides Bark Extract
Philippines inventory status:	Exempt: Populus Temuloides Bark Extract (90083-05-9)

Note: The regulatory information given above only indicates the principal regulations specifically applicable to the products described in this sheet. The user's attention is drawn to the possible existence of additional provision which complete these regulations. Please refer to all applicable international, national and local regulations and provisions

SECTION 16. OTHER INFORMATION

Prohibited uses:	For specific uses, food industry, ask the manufacturer for more information.
Last Revision Date:	03/18/2015
Preparation Date:	08/13/2015
MSDS summary of changes	- Removed Relative Density & Updated Melting Point Section 9 (Physical & Chemical Properties) - New Logo - Added Precautionary Statements - Section 2 (Hazards Identification) - Added Heavy Metals & Arsenic – Section 9 (Physical & Chemical Properties) - Added Mutagenicity Data – Section 11 (Toxicological Information) & Updated Transport Information – Section 14 (Transport Information) - Added Acute Toxicity Data – Section 11 (Toxicological Information) & Added Biodegradability Data – Section 12 (Ecological Information) - Added Sensitization Data – Section 11 (Toxicological Information)

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The information given is based on our knowledge of this product, at the time of publication in good faith. The attention of the user is drawn to the possible risks incurred by using the product for any other purpose other than which it was intended. This is not in any way excuse the user from knowing and applying all the regulations governing their activity. It is sole responsibility of the user to take all precautions required in handling the product. The purpose of mandatory regulation mentioned is to help the user to fulfill his obligations regarding the use of products. This information is not exhaustive, this is not exonerate the user from ensuring that legal obligations other than those mentioned, relating to the use and storage.

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PhytoCide Aspen Bark Extract Powder Manufacturing Flow Chart

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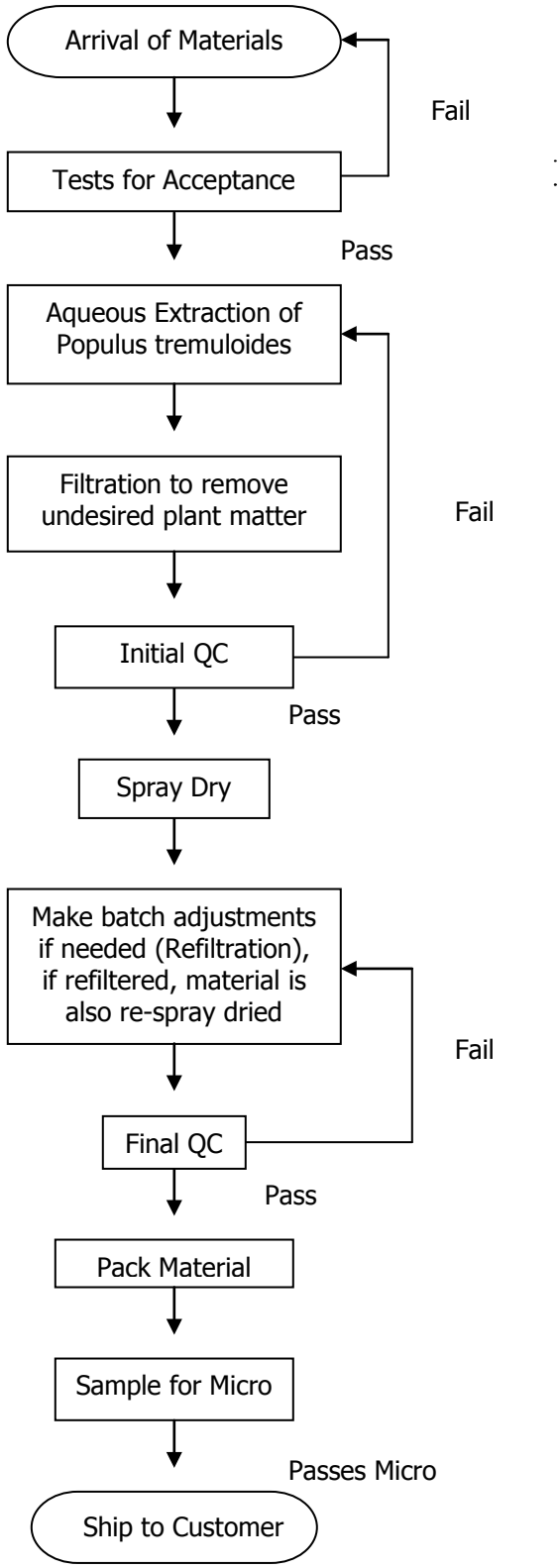

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PhytoCide Aspen Bark Extract Powder
Certificate of Compliance

Code: M16002
INCI Name: Populus Tremuloides Bark Extract
INCI Status: Conforms
CAS #: 90083-05-9
EINECS #: 290-199-2

The following information on regulatory clearances is believed to be accurate and is given in good faith as a guide to a global use of our ingredients in cosmetic applications. No representation or warranty as to its competences or accuracy is made. Information is offered for use in general cosmetic applications and may vary in particular applications. Users are responsible for determining the suitability of these products for their own particular use. All regulatory decisions should be made on the advice of your regulatory group or legal counsel.

Country / Regulatory Body	Status of Product
EU (REACH)	Compliant at Suggested Use Levels Labeling requirements: Not to be used for children under three years of age Restrictions: Not to be used in preparations for children under 3 years of age, except for shampoos
USA (TSCA)	Exempt
Australia (AICS)	Compliant
Japan (METI)	Compliant up to 1.6%
Canada (DSL)	Compliant at Suggested Use Levels
China (IECSC)	Compliant at Suggested Use Levels Labeling requirements: Not to be used for children under three years of age Restrictions: Not to be used in preparations for children under 3 years of age, except for shampoos
Brazil	Compliant at Suggested Use Levels Labeling requirements: Not to be used for children under 3 years of age Restrictions: Not to be used in preparations for children under 3 years of age, except for shampoos
Korea (KECI)	Compliant
Philippines (PICCS)	Compliant at Suggested Use Levels Labeling requirements: Not to be used for children under three years of age Restrictions: Not to be used in preparations for children under 3 years of age, except for shampoos

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PhytoCide Aspen Bark Extract Powder
Code: M16002

Attention must be paid to the use of PhytoCide Aspen Bark Extract Powder in the equivalent of OTC formulations (eg. quasi-drugs in Japan, or therapeutic goods in Australia). Some countries maintain restricted inventories of raw materials that can be used in those applications so more detailed guidance may be required.

PhytoCide Aspen Bark Extract Powder and any components or impurities are in compliance with the rules governing cosmetic products in the European Union (Directive 76/768/ECC & Regulation No. 1223/2009). However, PhytoCide Aspen Bark Extract Powder contains natural phenolics which will test positive for salicylic acid (see also Specification). This should be borne in mind when formulating products containing PhytoCide Aspen Bark Extract Powder. The recommended use levels for PhytoCide Aspen Bark Extract Powder is 0.20 – 3.00%.

PhytoCide Aspen Bark Extract Powder is in compliance with the standardized set of rules developed and approved by the NPA (Natural Products Association).

PhytoCide Aspen Bark Extract Powder is considered a non-hazardous material. All significant toxicological routes of absorption have been considered as well as the systemic effects and margin of safety (MoS) based on a no observed adverse effects level (NOAEL). Due to the restriction placed on animal testing of cosmetic raw materials, and Active Micro Technologies, LLC's internal non-animal testing policy, this product was not tested for NOAEL.

PhytoCide Aspen Bark Extract Powder was tested using *in vitro* dermal and ocular irritation models. This product was found to be non-irritating in both models.

To our knowledge the above material is free of CMR (*) substances, as defined according to Regulation (EC) No 1272/2008 and Cosmetic Regulation (EC) No 1223/2009 as amended.

(*) Carcinogenic, Mutagenic, toxic for Reproduction

Active Micro Technologies, LLC certifies that to the best of our knowledge our product does not contain any material listed on California Proposition 65.

Active Micro Technologies, LLC certifies that PhytoCide Aspen Bark Extract Powder does not contain any materials prohibited by Halal laws.

Active Micro Technologies, LLC certifies that this material has not been manufactured using any of the species listed in the CITES Appendices as of November 20, 2015.

PhytoCide Aspen Bark Extract Powder is REACH Compliant and free of the following:

- BHA & BHT
- Diethylene glycol (DEG)
- Formaldehyde or formaldehyde donors
- Glycol ethers
- Gluten
- Irradiation
- Lactose
- Nanoparticles
- Nitrosamines
- Palm oil/palm kernel oil (or derivatives)
- Parabens
- Paraffin/petroleum products
- Phthalates
- Polyethylene glycol (PEG)
- Residual solvents
- Sulfates
- Volatile organic compounds

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Raw Component Regulations

Please note that the below are global regulations for the raw materials used to manufacture PhytoCide Aspen Bark Extract Powder and are not for the product itself.

PhytoCide Aspen Bark Extract Powder contains 54.0 – 60.0% Phenolics (tested as Salicylic Acid). See below for a list of regulations:

Salicylic Acid and salts:

- **Europe: Maximum Authorized Concentration up to 3.00% (0.50% as acid) when used other than a preservative, depending on the application:**
  a) Rinse-off products: Up to 3.00%
  b) Other products: Up to 2.00%
  *Limitations and requirements: Not to be used in preparations for children under 3, except for shampoos
  *Conditions of use and warnings which must be printed on the label: Not to be used for children under three years of age (1)
  *Note (1): Solely for products which might be used for children under three years of age and which remain in prolonged contact with the skin
  *Intended Purpose must be apparent from the presentation of the final product (e.g. facial toner, anti-acne lotion, peeling gel, etc.)

- **USA: Salicylic Acid is safe when formulated to avoid irritation and to avoid increasing sun sensitivity, or when increased sun sensitivity would be expected, directions for use include the daily use of sun protection.** (Journal Citation: IJT 22(3):1-108)

- **Japan: Maximum Authorized Concentration:**
  *Salicylic Acid: 0.20 (per 100 grams)
  *Salicylic Acid Salts: 1.00 as total (per 100 grams)

- **Canada: Salicylic Acid permitted in concentrations of 2.00% or less**

- **China: Maximum Authorized Concentration of 0.50% (as acid)**
  *Limitations and requirements: Not to be used in products for children under age 3, except for shampoo
  *Warnings: Do not use for children under 3

- **Brazil: Maximum authorized concentration 0.50% (as acid)**:
  *Limitations: Not to be used in children's products under 3 years, except for shampoos
  *Warnings: Not to be used for children under 3 years of age (1)
  *Note (1): Solely for products which might be used for children under three years of age and which remain in prolonged contact with the skin

- **Mexico: Maximum authorized concentration 0.50% (as acid)**:
  *Limitations: Not to be used in preparations for children under 3 years of age

- **ASEAN: Maximum authorized concentration 0.50% (as acid)**:
  *Limitations: Not to be used in preparations for children under 3 years of age, except for shampoos
  *Warnings: Not to be used for children under 3 years of age

- **Mercosur: Maximum authorized concentration 0.50% (as acid)**:
  *Limitations: Not to be used in preparations for children under 3 years of age, except for shampoos
  *Warnings: Not to be used for children under 3 years of age

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Free Protein Residue Statement

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April 16, 2015

To whom it may concern,

This letter is to certify that PhytoCide Aspen Bark Extract Powder (M16002) contains less than 50 ppm free protein residue.

Thank you for your interest in Active Micro Technologies' products. If you have any further questions, feel free to contact us at (704) 276-7100.

Best Regards,

Tomorrow's Vision... Today!®
Heather Ferguson |R&D Coordinator
107 Technology Drive | Lincolnton, NC 28092
Direct: 704.276.7083|Main: 704.276.7100|Fax: 704.276.7101
Email: hferguson@activeconceptsllc.com
www.activeconceptsllc.com

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CEPA Statement

PhytoCide Aspen Bark Extract Powder
Code: M16002

According to the **Canadian Environmental Protection Act, 1999,** any products listed under the **Domestic Substance List** are considered acceptable in Canada for Cosmetic use.

According to Part I 6(a), if a product is not listed on the **Domestic Substance List** and the import amount exceeds 20kg but does not exceed 1000kg per calendar year, **Schedule 1** states that the trade name and the material safety data sheet is acceptable documentation for determining the product's safety and toxicity for use in Canada.

These consist of the following materials:
-*Populus tremuloides* Bark Extract (CAS 90083-05-9): *listed on MSDS*

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Rare Earth Elements

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PhytoCide Aspen Bark Extract Powder
Code: M16002

Active Micro Technologies, LLC certifies that we have not analyzed the above listed ingredient for rare earth elements listed in the Chinese FDA PRC regulatory documents. However, based on the origin of the raw materials and the manufacturing processes utilized in this production, we do not expect any of the below listed rare earth elements to be present in PhytoCide Aspen Bark Extract Powder. These elements include:

Element	Symbol
Cerium	Ce
Dysprosium	Dy
Erbium	Er
Europium	Eu
Gadolinium	Gd
Holmium	Ho
Lanthanum	La
Lutetium	Lu
Neodymium	Nd
Praseodymium	Pr
Samarium	Sm
Terbium	Tb
Thulium	Tm
Yttrium	Y
Ytterbium	Yb

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Nanomaterials Certificate

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PhytoCide Aspen Bark Extract Powder
Code: M16002

We, **Active Micro Technologies, LLC,** hereby certify that the product **PhytoCide Aspen Bark Extract Powder** does not contain nanomaterials as defined by:

- **French Decree N°2012-232**: "Substances as defined in Article 3 of Regulation (EC) No 1907/2006, made intentionally at nanoscale containing free particles, or contained in an aggregate or agglomerate with a minimum proportion of the particles in the distribution size in number, have one or more outer dimensions between 1 nm and 100 nm. By law of 6 August 2012, the minimum proportion of the nanoparticles in the distribution size in number is set up at 50%."

- **Regulation N° 1223/2009**: "insoluble or biopersistant and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm."

Location:	**Lincolnton, NC 28092 (USA)**
Date:	**07/01/2015**
Status of the person in charge:	**R&D Coordinator**
Name of the person:	**Heather N. Ferguson**
SIGNATURE:	Heather N. Ferguson

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REACH Compliance Statement

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Trade Name: PhytoCide Aspen Bark Extract Powder (M16002)

INCI Name: Populus Tremuloides Bark Extract

This is to certify that PhytoCide Aspen Bark Extract Powder is REACH compliant. Populus Tremuloides Bark Extract is exempt as natural and minimally processed in accordance with Annex V.

If you have further questions, please feel free to contact Heather Ferguson at hferguson@activeconceptsllc.com.

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VERIFICATION OF THE RAW MATERIALS CONFORMITY TO THE ECOCERT AND COSMOS COSMETIC STANDARDS

THIS DOCUMENT IS NOT AN ORGANIC CERTIFICATE

Company: **ACTIVE MICRO TECHNOLOGIES LLC** Attestation n° : 5468

The conformity (conf.) is established according to the requirements related to the raw materials contained in the applicable standard(s).

The present document is only valid for ECOCERT until official COSMOS publication of the raw materials on the website: http://www.cosmos-standard-rm.org/

*reference related to the appendices II and/or V of the Cosmos standard.

AMTicide Coconut (M14003) Function: **Skin conditioning, Hair conditioning**

INCI: Lactobacillus (and) Cocos Nucifera (Coconut) Fruit Extract

Conf. ECOCERT: YES **100** % of natural origin (**0** % of physically processed vegetal ingredients)
0 % synthetic

Conf. COSMOS: YES PPAI : **0** % CPAI : **100** % Petrochemical moiety : **0** %
Non natural ingredient : **0** %

Comments:

Leucidal Advanced - Aloe (M15015) Function: **Moisturizing, Skin conditioning, Antimicrobial**

INCI: Water (and) Leuconostoc/Aloe Barbadensis Leaf/Sorbus Aucuparia Fruit Ferment Filtrate

Conf. ECOCERT: YES **100** % of natural origin (**0** % of physically processed vegetal ingredients)
0 % synthetic

Conf. COSMOS: YES PPAI : **0** % CPAI : **18** % Petrochemical moiety : **0** %
Non natural ingredient : **0** %

Comments:

Drawn up in l'Isle Jourdain, valid from 01/01/2016
until 31/12/2016

Matthieu Bouffartigue

Raw Materials Service Manager

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The approval of the raw material(s) listed above is PERSONAL to the beneficiary named herein, and the BUYERS of the raw material(s) ARE IN NO EVENT AUTHORIZED TO MAKE REFERENCE TO THE APPROVAL BY ECOCERT GREENLIFE OR TO USE AN ECOCERT LOGO, whether in its communication or on the packaging or labeling of the raw material(s) or of a finished cosmetic product.

The conformity (conf.) is established according to the requirements related to the raw materials contained in the applicable standard(s).

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*reference related to the appendices II and/or V of the Cosmos standard.

Leucidal Advanced - Rowan (M15018) Function: **Emollient, Skin conditioning, Antimicrobial**

INCI: Water (and) Leuconostoc/Sorbus Aucuparia Fruit Ferment Filtrate

Conf. ECOCERT: YES **100** % of natural origin (**0** % of physically processed vegetal ingredients)
0 % synthetic

Conf. COSMOS: YES PPAI : **0** % CPAI : **16** % Petrochemical moiety : **0** %
Non natural ingredient : **0** %

Comments:

Leucidal Liquid (M15008) Function: **Moisturizing, Skin conditioning, Antimicrobial**

INCI: Leuconostoc/Radish Root Ferment Filtrate

Conf. ECOCERT: YES **100** % of natural origin (**0** % of physically processed vegetal ingredients)
0 % synthetic

Conf. COSMOS: YES PPAI : **0** % CPAI : **52** % Petrochemical moiety : **0** %
Non natural ingredient : **0** %

Comments:

Leucidal Liquid PT (M15021) Function: **Skin conditioning, Antimicrobial**

INCI: Lactobacillus Ferment

Conf. ECOCERT: YES **100** % of natural origin (**0** % of physically processed vegetal ingredients)
0 % synthetic

Conf. COSMOS: YES PPAI : **0** % CPAI : **18** % Petrochemical moiety : **0** %
Non natural ingredient : **0** %

Comments:

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*reference related to the appendices II and/or V of the Cosmos standard.

Leucidal Liquid SF (M15019) Function: **Moisturizing, Skin conditioning, Antimicrobial**

INCI: Lactobacillus Ferment

Conf. ECOCERT: YES **100** % of natural origin (**0** % of physically processed vegetal ingredients)
0 % synthetic

Conf. COSMOS: YES PPAI : **0** % CPAI : **10** % Petrochemical moiety : **0** %
Non natural ingredient : **0** %

Comments:

Leucidal Liquid SF (M15019CHI) Function: **Skin conditioning, Antimicrobial**

INCI: Leuconostoc/Radish Root Ferment Filtrate

Conf. ECOCERT: YES **100** % of natural origin (**0** % of physically processed vegetal ingredients)
0 % synthetic

Conf. COSMOS: YES PPAI : **0** % CPAI : **10** % Petrochemical moiety : **0** %
Non natural ingredient : **0** %

Comments:

PhytoCide Aspen Bark Extract Powder (M16002) Function: **Skin conditioning, Antimicrobial**

INCI: Populus Tremuloides Bark Extract

Conf. ECOCERT: YES **100** % of natural origin (**100** % of physically processed vegetal ingredients)
0 % synthetic

Conf. COSMOS: YES PPAI : **100** % CPAI : **0** % Petrochemical moiety : **0** %
Non natural ingredient : **0** %

Comments:

Drawn up in l'Isle Jourdain, valid from 01/01/2016
until 31/12/2016

Matthieu Bouffartigue

Raw Materials Service Manager

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GROUPE ECOCERT

The conformity (conf.) is established according to the requirements related to the raw materials contained in the applicable standard(s).

The present document is only valid for ECOCERT until official COSMOS publication of the raw materials on the website: http://www.cosmos-standard-rm.org/

*reference related to the appendices II and/or V of the Cosmos standard.

PhytoCide Black Currant Powder (M16001) Function: **Soothing, Skin conditioning, Antimicrobial**

INCI: Ribes Nigrum (Black Currant) Fruit Extract

Conf. ECOCERT: YES **100** % of natural origin (**100** % of physically processed vegetal ingredients)
0 % synthetic

Conf. COSMOS: YES PPAI : **100** % CPAI : **0** % Petrochemical moiety : **0** %
Non natural ingredient : **0** %

Comments:

PhytoCide Elderberry OS (M16003) Function: **Skin conditioning, Antimicrobial**

INCI: Sambucus Nigra Fruit Extract

Conf. ECOCERT: YES **100** % of natural origin (**100** % of physically processed vegetal ingredients)
0 % synthetic

Conf. COSMOS: YES PPAI : **100** % CPAI : **0** % Petrochemical moiety : **0** %
Non natural ingredient : **0** %

Comments:

Drawn up in l'Isle Jourdain, valid from 01/01/2016
until 31/12/2016

Matthieu Bouffartigue
Raw Materials Service Manager

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