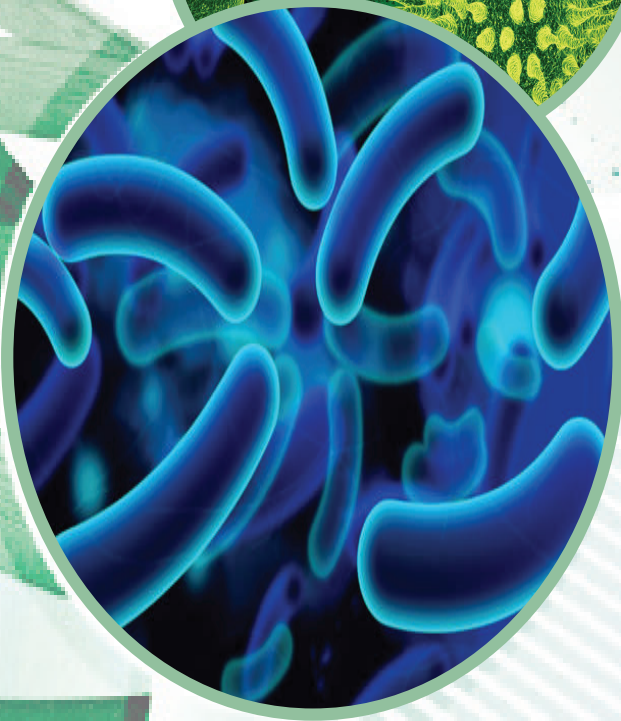
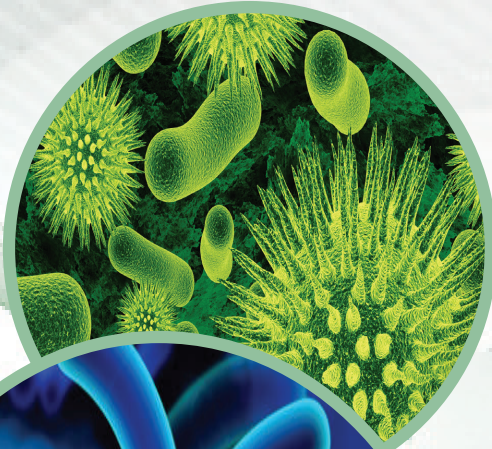


Technical Dossier



ability natural rowant technology Activity
sustainability benefits Ecocert leuconostoc
moisture COSMOS condition peptide
Improving solar choice antimicrobial

PhytoCide Black Currant Powder

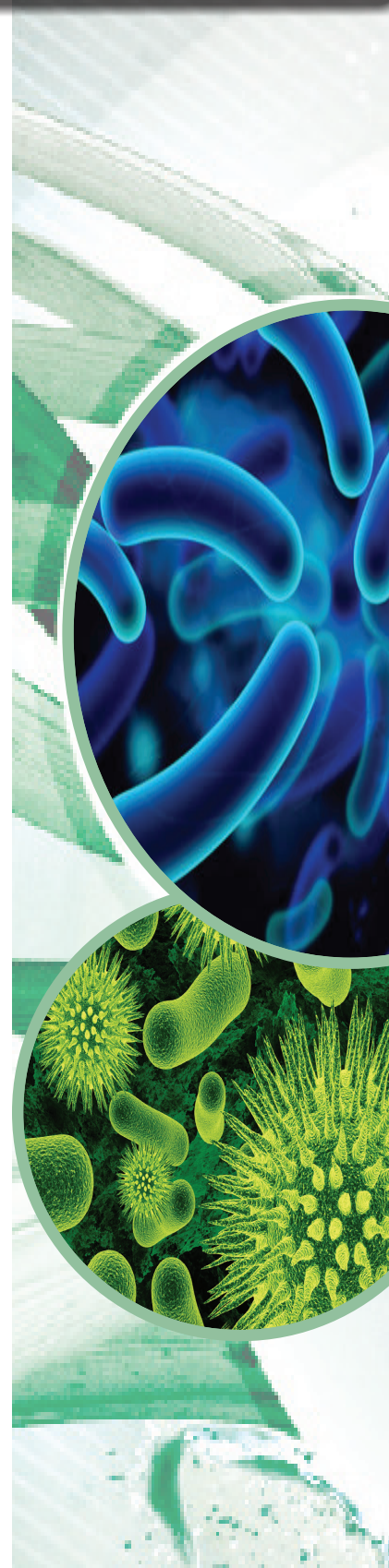
Code Number: M16001

INCI Name: Ribes nigrum (Black Currant) Fruit Extract

Table of Contents

Click on the logo to return
to the Table of Contents

- I. Technical Data Sheet**
- II. Specification Sheet**
- III. Compositional Breakdown**
- IV. Efficacy Tests**
 - a. Oxygen Radical Absorbance Capacity Assay
 - b. Minimum Inhibition Concentration (MIC) Data
 - c. Zone of Inhibition Data
 - d. Challenge Test with 2.0% PhytoCide Black Currant Powder
 - e. Time Kill Test
- V. Safety Information**
 - a. Safety Statement
 - b. *in-vitro* Dermal and Ocular Irritation Tests
 - c. Direct Peptide Reactivity Assay
 - d. OECD 442D TG *in-vitro* Skin Sensitization
 - e. Bacterial Reverse Mutation Test
 - f. Phototoxicity Test
 - g. OECD 202 Acute Daphnia Assay
 - h. OECD 301B Ready Biogradability Assay
 - i. Allergen Statement
 - j. Heavy Metals Statement
- VI. Certificate of Origin**
- VII. Material Safety Data Sheet (GHS SDS)**
- VIII. Additional Documentation**
 - a. Manufacturing Flow Chart
 - b. Certificate of Compliance
 - c. ECOCERT and COSMOS



PhytoCide Black Currant Powder

Technical Data Sheet

BACKGROUND

Over the past several decades, there has been growing public pressure, increasingly strict chemical regulations, preservative sensitization issues, and the potential for developing microbial resistance to the chemical preservative products typically used in cosmetic and personal care formulations. These factors have resulted in numerous preservation chemicals being pulled from the marketplace, despite being the products of choice at one time. To offer a solution to this preservation paradigm, **Active Micro Technologies (AMT)** has developed a line of products based on naturally occurring compounds that provide active cosmetic properties, but by their very nature are also capable of providing product preservation. This antimicrobial capability is due to natural mechanisms developed by plants and microorganisms by which they protect themselves from their environment and other competing organisms.

SCIENCE

Ribes nigrum, or black currant, is a small fruiting shrub native to Europe and northern Asia. The edible fruit is a dark purple color and typically grows 1 cm in diameter. Often used in jam, candy and juices, the popularity of black currants has recently increased due to its high concentration of antioxidants and vitamins, such as vitamin C. Interestingly, black currants are also an excellent source of phytochemicals that exhibit antimicrobial

properties. These phytochemicals can be isolated from the fruit for use as antimicrobial agents in cosmetic and personal care products. The desire for alternatives to traditional preservatives is not novel, however the notion of an antimicrobial with a secondary active that provides value-added benefits is relatively new.

Page 1 of 4

Code Number: M16001

INCI Nomenclature:

Ribes nigrum (Black Currant) Fruit Extract

INCI Status: Approved

REACH Status: Fully Compliant

CAS Number: 68606-81-5

EINECS Number: 271-749-0

Origin: Botanical: *Ribes nigrum* fruit

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, Light Pink with Darker Specks Powder

Soluble/Miscible: Water Soluble at suggested use levels

Suggested Use Levels: 1.0 - 3.0%

Suggested Applications: Antioxidant, Antimicrobial



PhytoCide Black Currant Powder

Active Micro Technologies developed **PhytoCide Black Currant Powder**, an extract rich in phytochemicals that not only inhibits microbial growth, but also have anti-inflammatory benefits.

Black currants are rich in both ellagitannins and anthocyanins which are polyphenols with anti-inflammatory properties. Useful in an array of aqueous systems and emulsions specifically designed for sensitive skin, **PhytoCide Black Currant Powder** is an effective antimicrobial that can also act to preserve systems. This incorporation will either reduce the level of traditional preservatives being used or help create a blend of natural antimicrobials. Additionally, the anti-inflammatory properties of the black currant may help quell mild irritation associated with both sensitive and problem skin.

BENEFITS

An Oxygen Radical Absorbance Capacity (ORAC) assay was conducted to assess the antioxidant capacity of **PhytoCide Black Currant Powder**. As shown in Figure 1, **PhytoCide Black Currant Powder** exhibited antioxidant activity comparable to 200 μ M Trolox[®]. The antioxidant capacity of **PhytoCide Black Currant Powder** increased as the concentration increased. As a result, we can assure that its ability to minimize oxidative stress is dose dependent and that **PhytoCide Black Currant Powder** is capable of providing antioxidant properties. **PhytoCide Black Currant Powder** was designed to be a skin conditioner and provide antimicrobial properties. With the present study we can confirm that this unique ingredient is not only capable of providing functional benefits, but **PhytoCide Black Currant Powder** is also capable of providing antioxidant benefits when added to cosmetic applications.

ORAC Assay

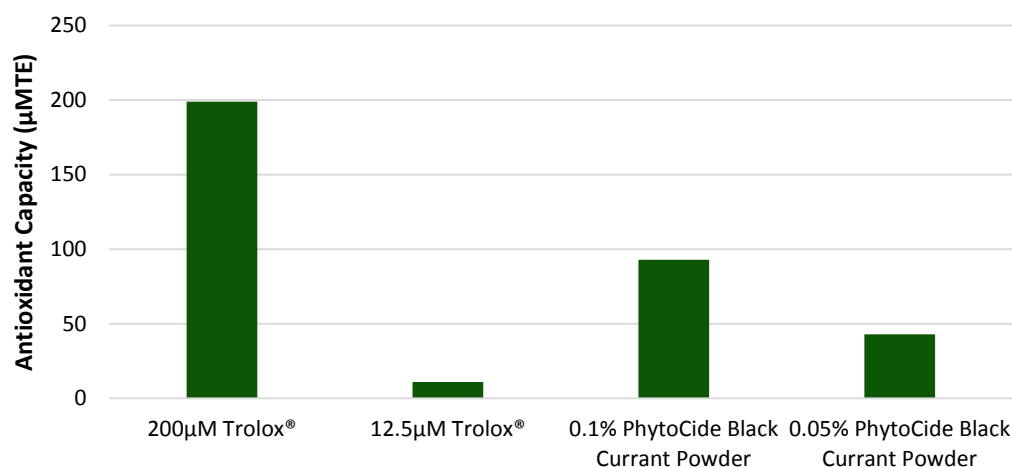


Figure 1. Antioxidant capacities of **PhytoCide Black Currant Powder**.

PhytoCide Black Currant Powder

Minimum Inhibitory Concentrations (MIC), using a standard growth media dilution method in addition to both bacterial and fungal cultures, were determined in order to evaluate the ability of **PhytoCide Black Currant Powder** to protect against microbial proliferation. The results shown in Figure 2 indicate **PhytoCide Black Currant Powder** can provide effective protection for cosmetic formulations.

Microorganism Tested	MIC (%)
<i>E. coli</i>	1.00
<i>P. aeruginosa</i>	0.50
<i>S. aureus</i>	1.00
<i>C. albicans</i>	1.00
<i>A. brasiliensis</i>	2.00

Figure 2. MIC data for **PhytoCide Black Currant Powder**.

The positive MIC screening results warranted further testing to confirm its ability to provide product preservation. A Double Challenge Test was completed using 2.0% **PhytoCide Black Currant Powder** in a generic cream base formulation at pH 5. Samples were inoculated with *E. coli*, *P. aeruginosa*, *S. aureus*, *C. albicans*, and *A. brasiliensis*. 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. Figure 3 shows the positive preservation results for 2.0% **PhytoCide Black Currant Powder**.

2.0% PhytoCide Black Currant Powder in Cream Formula Challenge Test - pH 5

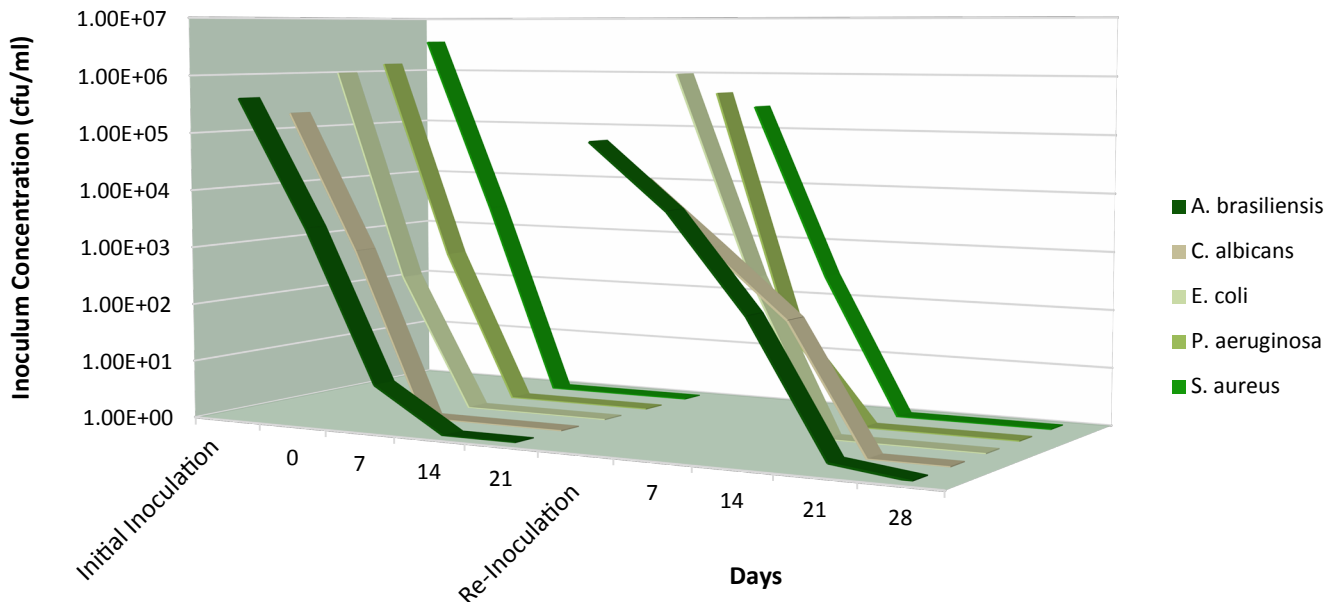


Figure 3. Challenge Test results for Generic Cream Formula pH 5 with 2.0% **PhytoCide Black Currant Powder** inoculated on Day 0 and re-inoculated on Day 28. Results show log reduction in viable organisms.

PhytoCide Black Currant Powder

A Time Kill Test was performed to determine the change in population of aerobic microorganisms within a specified sampling time when tested against 3.0% **PhytoCide Black Currant Powder** solution. 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. As shown in Figure 4, 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.

3.0% PhytoCide Black Currant Powder Time Kill Test

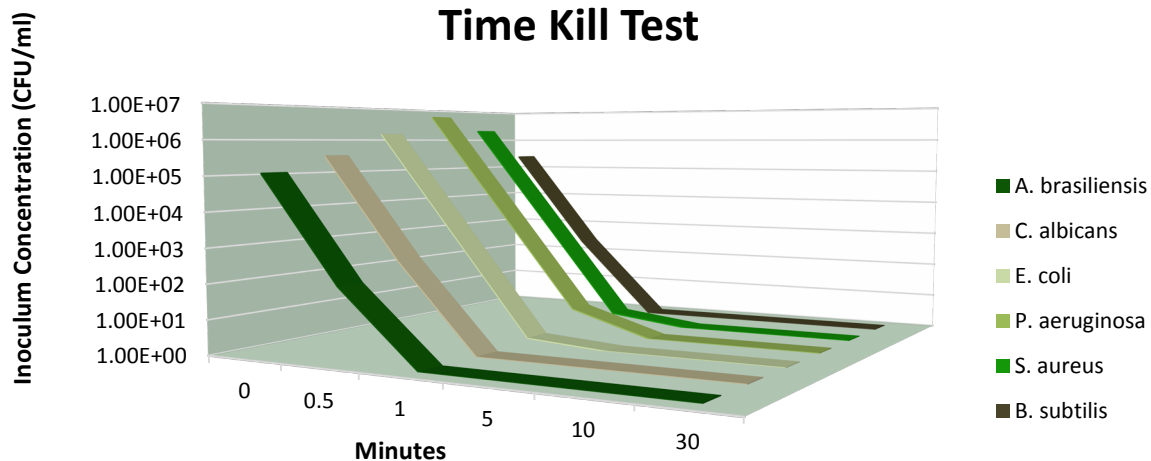


Figure 4. Time Kill Test results for 3.0% **PhytoCide Black Currant Powder**.

USE RECOMMENDATIONS

PhytoCide Black Currant Powder is stable and efficacious at temperatures up to 75°C and a pH between 3 and 8. Not only can this unique multifunctional ingredient be used to deliver the numerous benefits associated with black currants, but it can also be used as a natural antimicrobial agent in a wide variety of cosmetic and personal care applications.



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Specification

Product Name: PhytoCide Black Currant Powder
Code Number: M16001
CAS #'s: 68606-81-5
EINECS #'s: 271-749-0
INCI Name: *Ribes nigrum* (Black Currant) Fruit Extract

Specification	Parameter
Appearance	Free Flowing Powder
Color	Light Pink with Darker Specks
Odor	Characteristic
pH (1% Solution in Water)	3.0 – 6.0
Loss on Drying (1g-1hr-105 ⁰ C)	8.0% Maximum
Solubility (in Water)	Partially Water Soluble
Citric Acid	1.5 – 4.0%
Heavy Metals (Total)	< 20 ppm
Lead	< 10 ppm
Arsenic	< 3 ppm
Mercury	< 1 ppm

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Compositional Breakdown

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PhytoCide Black Currant Powder Code: M16001

Compositional Breakdown:

Ingredient	%
Ribes nigrum (Black Currant) Fruit 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.)**



Compositional Breakdown

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This is to certify that PhytoCide Black Currant Powder does not contain allergen levels exceeding the following (Gas Chromatography-Mass Spectrometer Coupled):

ALLERGENS Dir 2003 15 CEE		
INCI NAME	CAS NUMBER	Limit (ppm)
Alpha-IsoMethyl Ionone	127-51-5	< 0.02
Amyl Cinnamal	122-40-7	< 0.10
Anise Alcohol	105-13-5	< 0.00
Benzyl Alcohol	100-51-69	< 0.01
Benzyl Benzoate	120-51-4	< 0.09
Benzyl Cinnamate	103-41-3	< 0.30
Benzyl Salicylate	118-58-1	< 0.06
Butylphenyl Methylpropional	80-54-6	< 0.50
Cinnamal	104-55-2	< 0.01
Cinnamyl Alcohol	104-54-1	< 0.30
Citral	5392-40-5	< 1.00
Citronellol	106-22-9	< 1.00
Coumarin	91-64-5	< 0.00
Eugenol	97-53-0	< 0.70
Farnesol	4602-84-0	< 0.04
Geraniol	106-24-1	< 0.08
Hexyl Cinnamal	101-86-0	< 0.40
Hydroxycitronellal	107-75-5	< 1.00
Hydroxymethylpentyl 3-Cyclohexene carboxaldehyde	31906-04-4	< 0.30
Isoeugenol	97-54-1	< 0.06
Limonene	5989-27-5	< 0.05
Linalool	78-70-6	< 0.00
Methyl 2 Octynoate	111-12-6	< 0.20
Evernia prunastri	90028-68-5	< 0.02
Evernia furfuracea	90028-67-4	< 0.00
Amylcinnamyl Alcohol	101-85-9	< 1.00

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This is to certify that PhytoCide Black Currant Powder does not contain pesticide levels exceeding the following (Reverse Phase High Performance Liquid Chromatography-Mass Spectrometer Coupled):

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

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Parathion	< 0.50
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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Oxygen Radical Absorbance Capacity (ORAC) Assay

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Tradename: Phytocide Black Currant Powder

Code: M16001

CAS #: 68606-81-5

Test Request Form #: 59

Lot #: 25297

Sponsor: Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092

Study Director: Erica Segura

Principle Investigator: Meghan Darley

Test Performed:

Oxygen Radical Absorbance Capacity (ORAC)

Introduction

Reactive oxygen species (ROS) are generated by normal cellular processes, environmental stresses, and UV irradiation. ROS are dangerous to cellular structures and functional molecules (i.e., DNA, proteins, lipids) as they act as strong oxidizing agents or free radicals. The oxygen radical absorbance capacity (ORAC) assay is a standard method used to assess antioxidant capacity of physiological fluids, foods, beverages, and natural products. The assay quantitatively measures a sample's ability to quench free radicals that have the potential to react with and damage cellular components.

Oxygen Radical Absorbance Capacity (ORAC) assay was conducted to assess the antioxidant capacity of **Phytocide Black Currant Powder**.

Assay Principle

This assay is based upon the effect of peroxy radicals generated from the thermal decomposition of 2, 2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH) on the signal intensity from the fluorescent probe, fluorescein, in the presence of an oxygen radical absorbing substance. The degree of change is indicative of the amount of radical damage and the presence of antioxidants results in an inhibition in the free radical damage to the fluorescein. The antioxidant protection of the sample can be calculated by comparing it to a set of known standards. Trolox®, a water soluble vitamin E analog, with known antioxidant capabilities is used in this ORAC assay as the standard for measuring the antioxidant capacity of unknown substances. ORAC values, expressed in μM of Trolox® equivalents (TE), are calculated using the area under the curves (AUC) of the test product, Trolox®, and the control materials. Trolox equivalency is used as the benchmark for antioxidant capacity of mixtures since it is difficult to measure individual components.

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Materials

- A. Equipment:** Synergy H1 Microplate reader (BioTek Instruments, Winooski, VT); Gen5 software (BioTek Instruments, Winooski, VT); Pipettes
- B. Buffers:** 75mM Potassium Phosphate (pH 7.4); Deionized H₂O
- C. Reagents:** 2,2'-Azobis(2-methylpropionamide) dihydrochloride (AAPH) (153mM); 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox®); Fluorescein Sodium Salt (4nM)
- D. Preparation:** Pre-heat (37°C) Synergy H1 Microplate reader; Prepare Trolox® standards, sample dilutions, fluorescein solution, and AAPH.
- E. Microtitre Plates:** Corning 96 Well Black Side/Clear Bottom Microplates

Methods

Solutions of **Phytocide Black Currant Powder** and Trolox® (positive control) were prepared in 75mM potassium phosphate buffer. Materials were prepared at three different concentrations/dilutions. Trolox® was used as a reference for antioxidant capacity and prepared a concentrations ranging from 12.5µM to 200µM in 75mM potassium phosphate buffer.

For the ORAC assay, 25µL of test material and Trolox® were combined with 150µL of fluorescein in 75mM potassium phosphate buffer and incubated in the Synergy HT Microplate reader at 37°C for 30 minutes. At the end of the incubation period, 25µL of AAPH were pipetted into each well. Fluorescent measurements were then taken every 2 minutes for approximately 2 hours at an excitation wavelength of 485nm and an emissions wavelength of 520nm.

The AUC and Net AUC values of the standards and samples were determined using Gen5 2.0 Data Reduction Software using the below equations:

$$AUC = 0.5 + \frac{R_2}{R_1} + \frac{R_3}{R_1} + \frac{R_4}{R_1} + \dots + \frac{R_n}{R_1} \rightarrow \text{Where } R \text{ is fluorescence reading}$$

$$Net\ AUC = AUC_{sample} - AUC_{blank}$$

The standard curve was obtained by plotting the Net AUC of different Trolox® concentrations against their concentration. ORAC values of samples were then calculated automatically using the Gen5 software to interpolate the sample's Net AUC values against the Trolox® standard curve. ORAC measurements for the test material were expressed in micro moles Trolox® equivalents (µMTE), where 1 ORAC unit is equal to 1 µMTE.

Results

Phytocide Black Currant Powder exhibited potent antioxidant activity at a 0.1% concentration.

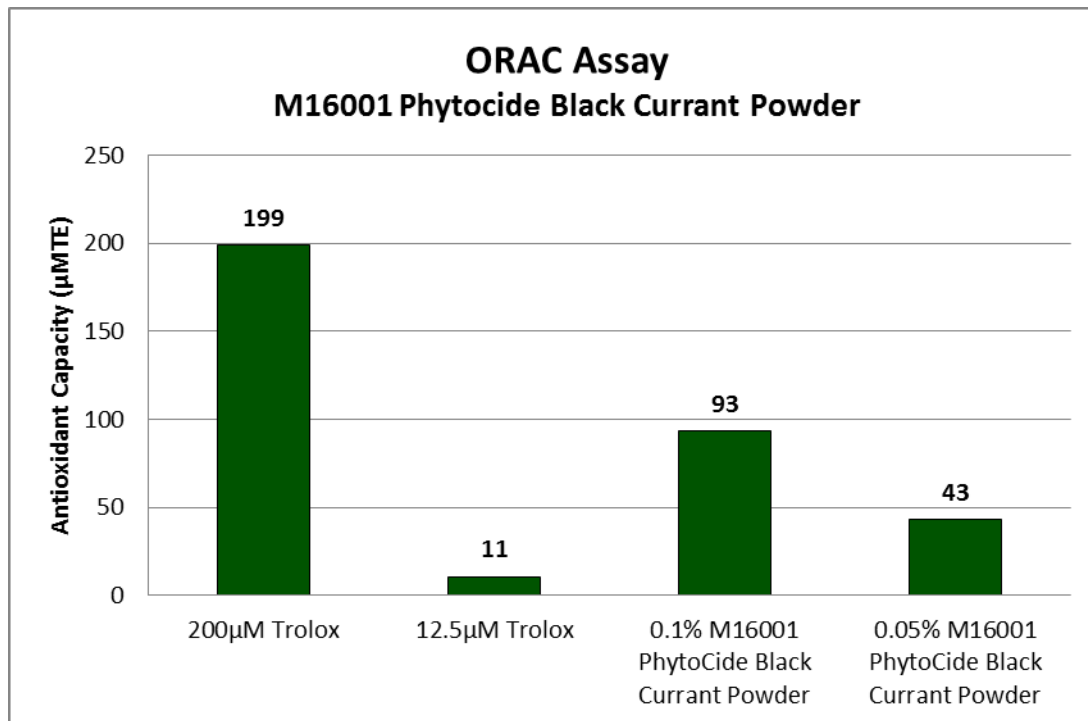


Figure 1: Antioxidant capacities

Discussion

As shown in figure 1, **Phytocide Black Currant Powder** (code M16001) exhibited antioxidant activity comparable to 50µM Trolox®. The antioxidant capacity of **Phytocide Black Currant Powder** increased as the concentration increased. As a result we can assure that its ability to minimize oxidative stress is dose dependent. It can therefore be concluded that **Phytocide Black Currant Powder** is capable of providing antioxidant properties.



Inhibition Activity Data

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Product Name: PhytoCide Black Currant Powder
Code Number: M16001
Lot Number: 43436P
Test Request Number: 1496
CAS #'s: 68606-81-5
EINECS #'s: 271-749-0
INCI Name: *Ribes nigrum* (Black Currant) Fruit Extract

Organism (ATCC #)	Minimum Inhibitory Concentration (%)
<i>E.coli</i> #8739	0.5
<i>S. aureus</i> #6538	0.5
<i>P. aeruginosa</i> #9027	0.5
<i>C. albicans</i> #10231	0.5
<i>A. brasiliensis</i> #16404	0.5

QA Signature _____ Monica Beltran _____

Date _____ 09-10-2015 _____

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Zone of Inhibition Test

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Product Name: PhytoCide Black Currant Powder
Code Number: M16001
Lot Number: 40785P
Test Request Number: 1086
CAS #'s: 68606-81-5
EINECS #'s: 271-749-0
INCI Name: *Ribes nigrum* (Black Currant) Fruit Extract

Organism (ATCC #)	Zone of Inhibition (mm)
<i>E.coli</i> #8379	8.0
<i>S. aureus</i> #6538	8.0
<i>P. aeruginosa</i> #9027	8.0
<i>C. albicans</i> #10231	8.3
<i>A. brasiliensis</i> #16404	8.0

QA Signature _____ Monica Beltran _____

Date _____ 03-09-2015 _____

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Challenge Test with 2.0% PhytoCide Black Currant Powder

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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 Black Currant Powder
Code: M16001

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 27th, 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 Black Currant 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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Challenge Test with 2.0% PhytoCide Black Currant Powder

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The inoculated samples were evaluated 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	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>C. albicans</i>	<i>A. brasiliensis</i>
	1.0 x 10 ⁶	1.4 x 10 ⁶	3.6 x 10 ⁶	2.0 x 10 ⁷	4.0 x 10 ⁵
Day 0*	99.980%	99.971%	99.932%	99.615%	99.400%
Day 7	>99.999%	>99.999%	>99.999%	99.856%	99.760%
Day 14	>99.999%	>99.999%	>99.999%	99.896%	99.775%
Day 21	>99.999%	>99.999%	>99.999%	99.904%	99.782%
Day 28	>99.999%	>99.999%	>99.999%	99.909%	99.782%
Inoculum (re-inoculated) CFU/ml	1.1 x 10 ⁶	4.9 x 10 ⁵	2.6 x 10 ⁵	2.0 x 10 ⁴	1.0 x 10 ⁵
Day 7	99.961%	99.992%	99.900%	91.500%	91.500%
Day 14	99.982%	>99.999%	99.946%	92.000%	97.400%
Day 21	>99.999%	>99.999%	>99.999%	99.991%	99.350%
Day 28	>99.999%	>99.999%	>99.999%	99.991%	99.955%

Table 1. Challenge Test results for Generic Cream pH 5 with 2% PhytoCide Black Currant 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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Challenge Test with 2.0% PhytoCide Black Currant Powder

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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 Black Currant 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.



Challenge Test with 2.0% PhytoCide Black Currant Powder

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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°C while adding the ingredients.

2. Phase II:

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

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.



Challenge Test with 2.0% PhytoCide Black Currant Powder

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

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.

The information contained in this report is provided by Active Micro Technologies after the exercise of all reasonable care and skill in its compilation, preparation, and issue. It is provided without liability regarding its subsequent application and use. This type of screening test will be conducted only for validation of the efficacy of the antimicrobial agent or preservative system in the specific formulation tested. It does not address the suitability of the overall formula, nor does it address the regulatory status of any component therein. This testing does not account for the possibility of environmental microorganisms and cannot be relied upon as sufficient to justify commercialization of the product tested. By submitting samples for testing, the customer acknowledges that they will not hold Active Micro Technologies responsible for products launched based solely on the support of these studies.

Time Kill Test

E2315

Assessment of Antimicrobial Activity Using a Time Kill Procedure

Product

PhytoCide Black Currant Powder

Test Request #:

1883

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 4th, 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.

Test Method

Ten grams of 4% PhytoCide Black Currant 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
<i>E.coli</i> * ATCC# 8739	1.5×10^6	99.9%	99.9%	99.9%	99.9%	99.9%
<i>S.aureus</i> ATCC# 6538	2.0×10^6	99.9%	99.9%	99.9%	99.9%	99.9%
<i>P.aeruginosa</i> ATCC# 9027	5.5×10^6	99.9%	99.9%	99.9%	99.9%	99.9%
<i>B.subtilis</i> ATCC# 6051	2.5×10^5	99.9%	99.9%	99.9%	99.9%	99.9%
<i>A.brasiliensis</i> ATCC# 16404	1.2×10^5	99.9%	99.9%	99.9%	99.9%	99.9%
<i>C.albicans</i> ATCC# 10231	3.4×10^5	99.9%	99.9%	99.9%	99.9%	99.9%

Table 1. Time Kill Test results for 4% PhytoCide Black Currant 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 Black Currant 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

107 Technology Drive • Lincolnton, NC 28092
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Product Name: PhytoCide Black Currant Powder

Product Code: M16001

INCI Name: *Ribes nigrum* (Black Currant) Fruit Extract

INCI Status: Approved

PhytoCide Black Currant Powder is manufactured by aqueous extraction of black currant fruit under controlled conditions. The extract is then filtered.

The FDA (Food and Drug Administration) states in sections 201 and 409 of the Federal Food, Drug and Cosmetic Act that "any substance that is intentionally added to food is a food additive, that is subject to review and approval by FDA, unless the substance is generally recognized, among qualified experts, as having been adequately shown to be safe under conditions of its use or unless the use of the substance is otherwise excluded for the definition of a food additive."¹

Ribes nigrum is widely used in culinary preparations, and is a common ingredient in desserts and beverages especially. The fruit is also utilized in nutritional supplements due to its phytochemical content. Therefore, the Federal Food, Drug and Cosmetic Act classifies materials such as PhytoCide Black Currant Powder as Generally Recognized as Safe (GRAS). This knowledge combined with the toxicity data provided allows us to support the safety of PhytoCide Black Currant Powder in cosmetic applications at the recommended use level of 1 - 3%.

Due to the restriction placed on the animal testing of cosmetic raw materials, and our internal non-animal testing policy Active Micro Technologies, LLC does not test for NOAEL.

1. Federal Food, Drug and Cosmetic Act. U.S Food and Drug Administration. www.fda.gov.



Dermal and Ocular Irritation Test

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

Tradename: Phytocide Black Currant Powder

Code: M16001

CAS #: 84603-58-7

Test Request Form #: 231

Lot #: SN120626-7

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 Black Currant 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% CO₂, 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% CO₂ 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 H₂O
- 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% CO₂ 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% CO₂ and 95% relative humidity for an additional 18 to 21 hours.

B. Test Substance Exposure

a. EpiDerm™

30µL (liquid) or 25mg (solid) of the undiluted test substance is applied to 3 tissue inserts and allowed to incubate for 60 minutes in a humidified incubator (37°C, 5% CO₂, 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% CO₂, 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% CO₂, 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 (OD₅₇₀) 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 $\leq 20\%$.

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.

B. Tissue Viability Assay

The results are summarized in Figures 1 and 2. In no case was the tissue viability $\leq 50\%$ for EpiDerm™ or $\leq 60\%$ 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

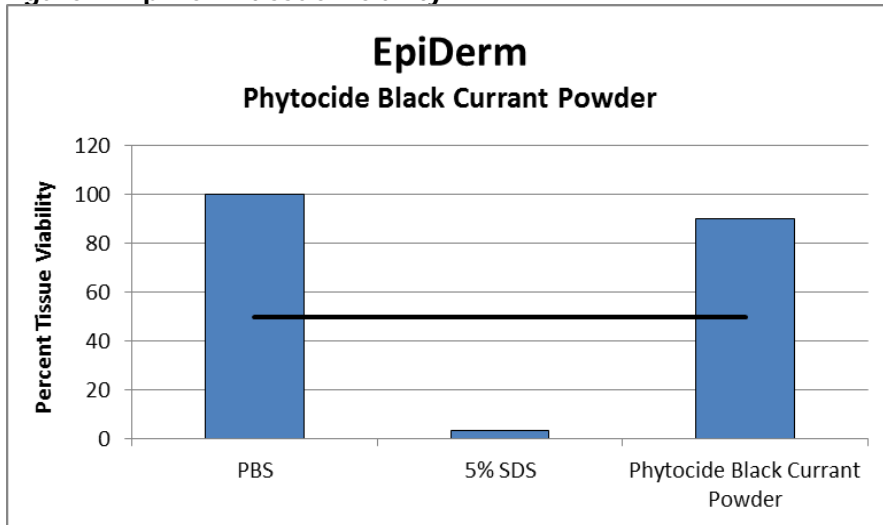
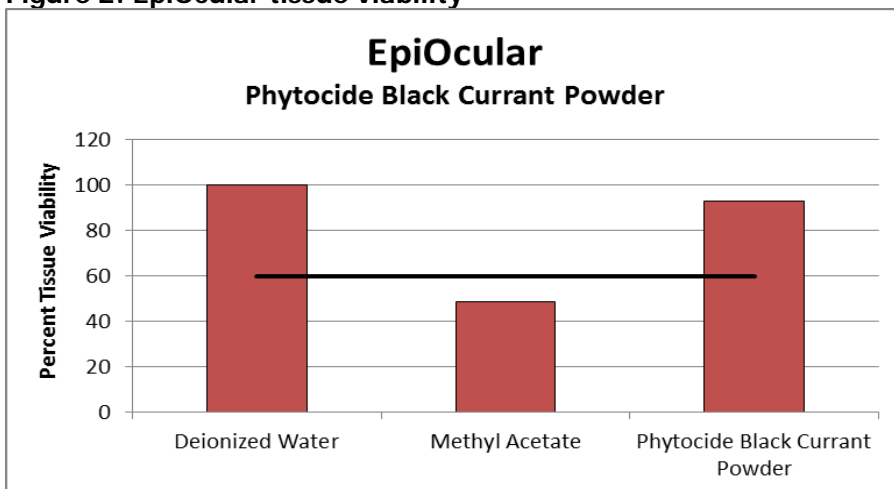


Figure 2: EpiOcular tissue viability



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OECD TG 442C: *In Chemico* Skin Sensitization

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(704) 276-7100 • Fax (704) 276-7101

Tradename: PhytoCide Black Currant Powder

Code: M16001

CAS #: 68606-81-5

Test Request Form #: 1423

Lot #: 41416P

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 contact¹. 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 dermatitis². 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 Black Currant 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-RFAACAA-COOH); Lysine peptide
(Ac-RFAAKAA-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 Black Currant 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
<ul style="list-style-type: none"> • 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) 	<ul style="list-style-type: none"> • 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 ± 0.05 mM 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 ± 0.05 mM.

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OECD TG 442C: In Chemico Skin Sensitization

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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:

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

Based on HPLC-UV analysis of **PhytoCide Black Currant Powder (code M16001)** 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 3.39% causing minimal reactivity in the assay giving us the prediction of a non-sensitizer.

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OECD TG 442D: *In Vitro* Skin Sensitization

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

Tradename: PhytoCide Black Currant Powder

Code: M16001

CAS #: 68606-81-5

Test Request Form #: 1424

Lot #: 41416P

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 Chemicals¹. 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 Black Currant 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% CO₂ 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 Black Currant Powder** were prepared in DMSO with a concentration range from 0.98 – 2000 µM. 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% CO₂ 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% CO₂. 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 IC₅₀ and IC₃₀ 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 EC_{1.5} 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 I_{max} 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 EC_{1.5} 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 Black Currant Powder	Non-Sensitizer	No Induction	> 1000 μM	0.5

Table 1: Overview of KeratinoSens™ Assay Results

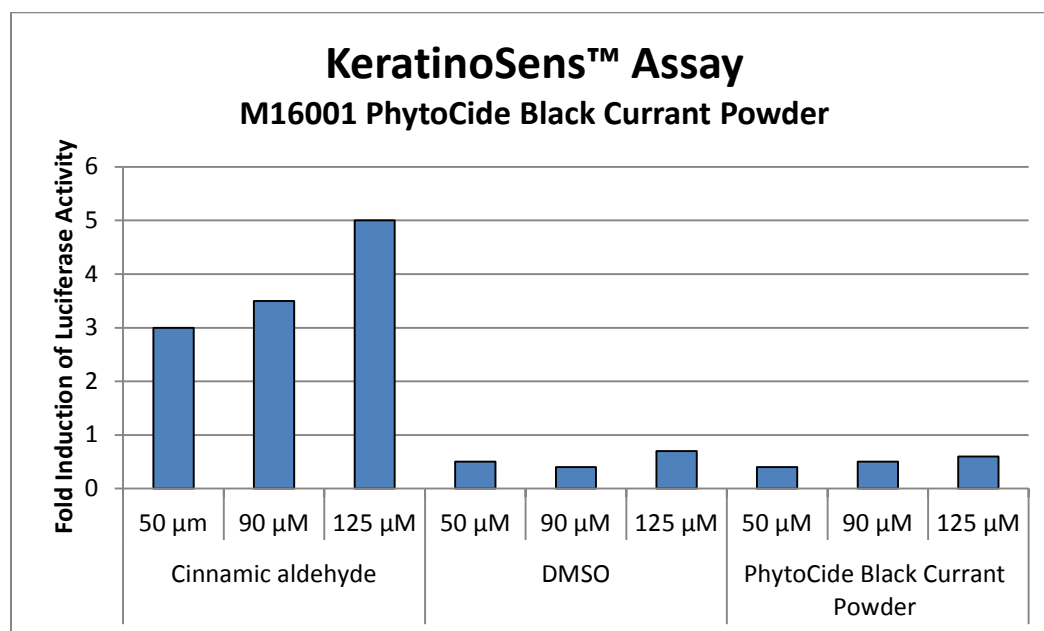


Figure 1: Fold Induction of Luciferase

Discussion

As shown in the results, **PhytoCide Black Currant Powder (code M16001)** 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 Black Currant Powder** can be safely used in cosmetics and personal care products at typical use levels.

Test Article: PhytoCide Black Currant Powder
Code Number: M16001
CAS #: 999999-99-4

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: 1039

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 Black Currant Powder** would cause mutagenic changes in the average number of revertants for histidine-dependent *Salmonella typhimurium* strains TA98, TA100, TA1537, TA1535 and tryptophan-dependent *Escherichia coli* strain WP2uvrA 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 WP2uvrA after Spot Inhibition Screen.

Separate tubes containing 2 ml of molten top agar at 45°C 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°C. 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 WP2uvrA. 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* WP2uvrA 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 *uvrB* gene and the deletion in the *uvrA* 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*uvrA*) 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.

<u>Tester strain</u>	<u>Mutations/Genotypic Relevance</u>
TA98	hisD3052, Dgal chlD bio <i>uvrB rfa</i> pKM101
TA100	hisG46, Dgal chlD BIO <i>uvrB rfa</i> pKM101
TA1537	hisC3076, <i>rfa</i> , Dgal chlD bio <i>uvrB</i>
TA 1535	hisG46, Dgal chlD bio <i>uvrB rfa</i>
WP2 <i>uvrA</i>	trpE, <i>uvrA</i>

<i>rfa</i>	=	causes partial loss of the lip polysaccharide wall which increases permeability of the cell to large molecules.
<i>uvrB</i>	=	deficient DNA excision-repair system (i.e., ultraviolet sensitivity)
pKM101	=	plasmid confers ampicillin resistance (R-factor) and enhances sensitivity to mutagens.
<i>uvrA</i>	=	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* WP2*uvrA* 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 revertants 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.

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 2×10^9 /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 μ l of culture for each strain and 100 μ l of testing solution or vehicle without test material. A 500 μ l 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 50 μ l 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 revertants was determined. The mean numbers of revertants of the test plates were compared to the mean number of revertants 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 μ g per plate. The dose levels tested were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 μ g 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 WP2uvrA 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* WP2uvrA. 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.

Appendix 2:

Bacterial Mutation Assay Plate Incorporation Assay Results

	Concentration µg per Plate	TA98		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	21	25	23
	1500	21	32	27
	500	45	48	47
	150	35	34	35
	50	48	37	43
	15	39	55	47
	5.0	42	44	43
	1.5	32	31	32
Test Solution w/o S9	5000	15	25	20
	1500	32	44	38
	500	29	34	32
	150	36	18	27
	50	20	15	18
	15	37	36	37
	5.0	33	25	29
	1.5	12	16	14
DI Water w/S9		44	36	40
DI Water w/o S9		35	47	41
2-aminoanthracen w/ S9		398	364	381
2-nitrofluorene w/o S9		145	127	136
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	35	32
	1500	111	106	109
	500	102	98	100
	150	103	105	104
	50	92	95	94
	15	87	86	87
	5.0	99	99	96
	1.5	112	115	114
Test Solution w/o S9	5000	26	35	31
	1500	45	44	45
	500	63	65	64
	150	72	64	68
	50	45	65	55
	15	88	84	86
	5.0	87	86	87
	1.5	115	89	102
DI Water w/S9		102	117	110
DI Water w/o S9		95	111	103
2-aminoanthracen w/ S9		822	815	819
Sodium azide w/o S9		645	676	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	4	5	5
	1500	11	15	13
	500	12	14	13
	150	10	11	11
	50	15	15	15
	15	12	14	13
	5.0	10	10	10
	1.5	8	9	9
Test Solution w/o S9	5000	2	8	5
	1500	6	2	4
	500	8	10	9
	150	12	16	14
	50	10	14	12
	15	11	14	13
	5.0	13	15	14
	1.5	9	13	11
DI Water w/S9		10	12	11
DI Water w/o S9		11	13	12
2-aminoanthracen w/ S9		67	74	71
2-aminoacridine w/o S9		512	550	531
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		

*CFU = Colony Forming Units

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	Concentration µg per Plate	TA1535		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	15	13	14
	1500	12	13	13
	500	15	14	15
	150	12	16	14
	50	8	19	14
	15	11	10	11
	5.0	12	21	17
	1.5	15	13	14
Test Solution w/o S9	5000	15	2	9
	1500	12	16	14
	500	11	17	14
	150	18	16	17
	50	10	15	13
	15	17	19	18
	5.0	13	14	14
	1.5	11	16	14
DI Water w/S9		12	17	15
DI Water w/o S9		13	14	14
2-aminoanthracen w/ S9		95	97	96
Sodium azide w/o S9		577	583	580
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		

*CFU = Colony Forming Units

*Mean = Average of duplicate plates

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	Concentration µg per Plate	WP2uvrA		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	35	32	34
	1500	42	25	34
	500	27	28	28
	150	32	34	33
	50	44	16	30
	15	28	31	30
	5.0	38	45	42
	1.5	35	37	36
Test Solution w/o S9	5000	21	25	23
	1500	26	29	28
	500	44	47	46
	150	25	31	28
	50	35	23	29
	15	26	31	29
	5.0	34	33	34
	1.5	31	36	34
DI Water w/S9		32	30	31
DI Water w/o S9		31	38	35
2-aminoanthracen w/ S9		185	176	181
Methylmethanesulfonate w/o S9		301	312	307
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		

*CFU = Colony Forming Units

*Mean = Average of duplicate plates

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Phototoxicity Assay Analysis

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

Tradename: PhytoCide Black Currant Powder

Code: M16001

CAS #: 68606-81-5

Test Request Form #: 1137

Lot #: 41416P

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 Black Currant 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% CO₂, and 95% relative humidity (RH). The following day, the appropriate tissue inserts were irradiated (UVA) for 60 minutes with 1.7 mW/cm² (=6 J/cm²). 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% CO₂ 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 H₂O
- 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 chlorpromazine, 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/cm² 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% CO₂ 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% CO₂, 95% RH).

C. Tissue Irradiation

Tissue inserts in their 6-well plates are UVA-irradiated for 60 minutes with 6 J/cm² 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% CO₂, 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% CO₂, 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 (OD₅₇₀) 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.

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. Using any test substance at this high of a concentration will have noticeable effects on cellular viability due to the fact that that substance is replacing the cell's nutrients. We can safely say that **PhytoCide Black Currant Powder** is not a photoirritant when used at the suggested use levels of 1 – 3%.

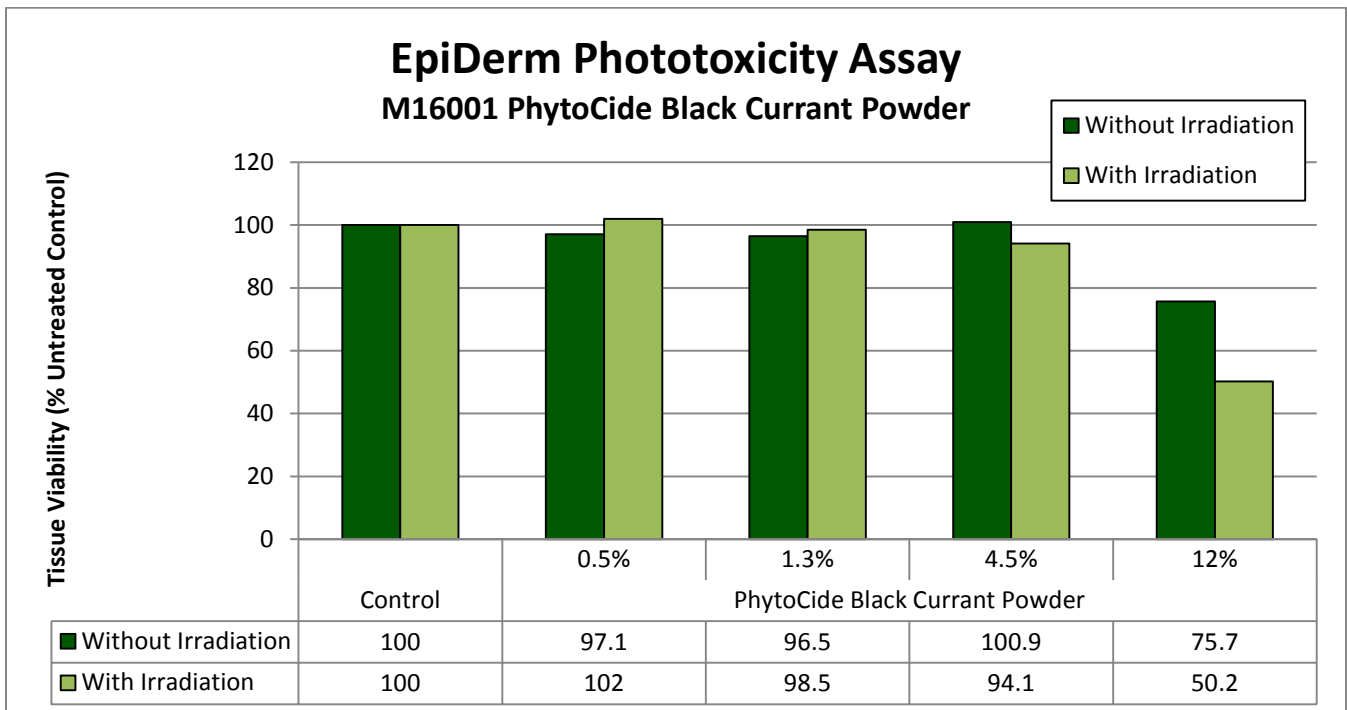


Figure 1: EpiDerm Phototoxicity Graph



OECD 202 Acute *Daphnia* Assay

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

Tradename: PhytoCide Black Currant Powder

Code: M16001

CAS #: 68606-81-5

Test Request Form #: 1047

Lot #: 39836P

Sponsor: Active Concepts, 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 Black Currant 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 EC₅₀ 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 EC₅₀ at 48 hours. EC₅₀ 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 EC₅₀ 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

This information is presented in good faith but is not warranted as to accuracy of results. Also, freedom from patent infringement is not implied. This information is offered solely for your investigation, verification, and consideration.

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.

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 EC₅₀ with 95% confidence limits ($p = 0.95$).
- b. Where the standard methods of calculating the EC₅₀ 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 EC₅₀ (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 EC₅₀ 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 EC₅₀

Results

General Information:

Name of new chemical substance	PhytoCide Black Currant Powder		
INCI Nomenclature	<i>Ribes nigrum</i> (Black Currant) Fruit Extract		
CAS number	68606-81-5		
Structural or rational formula (if neither is available, summarize its formulation method)	Botanical: <i>Ribes nigrum</i> fruit		
Molecular weight	242.5 Daltons		
Purity of the new chemical substance used for the test (%)	100%		
Lot number of the new chemical substance used for the test	39836P		
Names and contents of impurities	n/a		
Solubility in water	Soluble up to 3% in solution		
Melting point	n/a		
Boiling point	n/a		
Properties at room temperature	Free flowing light pink 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	<i>Daphnia magna</i>	
	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, 90.9, 41.3, 18.8, 8.5 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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OECD 202 Acute *Daphnia* Assay

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Test Results:

Items		Contents
Toxicity Value	48hr EC50	130.7 mg/L
Exposure Concentrations Used for Calculation	Nominal Values	200, 90.9, 41.3, 18.8, 8.5 mg/L
Remarks		Not harmful to aquatic organisms

Discussion

After 48 hours, the EC50 value for **PhytoCide Black Currant Powder** was determined to be 130.7 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

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

Tradename: PhytoCide Black Currant Powder

Code: M16001

CAS #: 68606-81-5

Test Request Form #: 1048

Lot #: 39441P

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*

Study Director: *Erica Segura*

Principle Investigator: *Meghan Darley*

Test Performed:

OECD 301 B

Ready Biodegradability: CO₂ Evolution (Modified Sturm Test)

Introduction

A study was conducted to assess the ready biodegradability of **PhytoCide Black Currant 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, CO₂ 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 ThCO₂ (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 CO₂ 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 ThCO₂ 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, KH₂PO₄.....8.5g
 - Dipotassium hydrogen orthophosphate, K₂HPO₄.....21.8g
 - Disodium hydrogen orthophosphate dehydrate, Na₂HPO₄·2H₂O.....33.4g
 - Ammonium chloride, NH₄Cl.....0.5g
 - Solution B (Dissolve in water and make up to 1 liter)
 - Calcium chloride, anhydrous, CaCl₂.....27.50g
 - Or
 - Calcium chloride dehydrate, CaCl₂·2H₂O.....36.40g
 - Solution C (Dissolve in water and make up to 1 liter)
 - Magnesium sulphate heptahydrate, MgSO₄·7H₂O..... 22.50g
 - Solution D (Dissolve in water and make up to 1 liter.)
 - Iron (III) chloride hexahydrate, FeCl₃·6H₂O.....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 CO₂-free oxygen and CO₂-free nitrogen from gas cylinders in the correct proportions (20% O₂ : 80% N₂)
 - 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 CO₂-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 CO₂-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 CO₂-free air through the suspensions at a rate of 30-100 mL/minute.
- III. CO₂ Determination
- It is mandatory to follow the CO₂ evolution from the test suspensions and inoculum blanks in parallel and it is advisable to do the same for the other test vessels.
 - During the first ten days it is recommended that analyses of CO₂ 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 CO₂ 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.
 - 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
- Data from the test should be entered onto the attached data sheet.
 - The amount of CO₂ 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.
 - Since 1 mmol of CO₂ is produced for every mol of Ba(OH)₂ reacted to BaCl₂ and 2 mmol of HCl are needed for the titration of the remaining Ba(OH)₂ and given that the molecular weight of CO₂ is 44 g, the weight of CO₂ 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 CO₂ produced is 1.1 in this case. Calculate the weights of CO₂ produced from the inoculum alone and from the inoculum plus test substance using the respective titration values. The difference is the weight of CO₂ produced from the test substance alone.

- d. The percentage biodegradation is calculated from:

$$\% \text{ Degradation} = \frac{\text{mg CO}_2 \text{ Produced}}{\text{ThCO}_2 \times \text{mg Test Substance Added}} \times 100$$

Or

$$\% \text{ Degradation} = \frac{\text{mg CO}_2 \text{ Produced}}{\text{mg TOC Added in Test} \times 3.67} \times 100$$

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 CO₂ produced after any time interval from the concentration of inorganic carbon and the volume of absorbent used. Calculate the percentage degradation from:

$$\% \text{ ThCO}_2 = \frac{\text{mg IC from Test Flask} - \text{mg IC from Blank}}{\text{mg TOC Added as Test Substances}} \times 100$$

- 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:

$$\% \text{ Abiotic Degradation} = \frac{\text{CO}_2 \text{ Produced by Sterile Flask After 28 Days (mg)}}{\text{ThCO}_2 \text{ (mg)}} \times 100$$

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 CO₂ 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 CO₂/L are obtained, the data and experimental technique should be examined critically.

Data Sheet

Laboratory	Active Concepts Tissue Culture Laboratory		
Test Start Date	12/29/2014		
Test Substance	Name	PhytoCide Black Currant 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
CO₂ Production and Degradability	Method	Ba(OH)₂	0.0125M
		NaOH	N/A
		Other	N/A
Total Contact Time	28 Days		
Total CO₂ Evolved Measurements	Days	2, 4, 11, 17, 23, 28	
Degradation Over Time	92.5%		
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 Black Currant Powder** achieved 92.5% biodegradation after 28 days of testing. The product met method requirements for Readily Biodegradability classification.



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(704) 276-7100 • Fax (704) 276-7101

Date Issued: March 9, 2016

ALLERGEN DECLARATION

RE: *PhytoCide Black Currant Powder (M16001)*

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



Heavy Metals Statement

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

May 10, 2016

To Whom It May Concern,

This letter is to certify that PhytoCide Black Currant Powder (M16001) 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 Antimony and Cadmium do not appear on the Specification for PhytoCide Black Currant 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

www.activeconceptsllc.com



Certificate of Origin

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

PhytoCide Black Currant Powder Code: M16001

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 *Ribes nigrum* and therefore is BSE-Free.

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

Active Micro Technologies, LLC certifies that the above listed ingredient has never been tested on animals.



Safety Data Sheet

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PhytoCide Black Currant Powder

Page: 1/8

Date: 08 / 13 / 2015

Version: 5

Cancels and replaces version: 4

SECTION 1. IDENTIFICATION

Product Name/Identifier	PhytoCide Black Currant Powder
Product Code	M16001
Recommended Use	Topical Cosmetic Use; Antimicrobial
Restrictions on Use	None
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: Not applicable

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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PhytoCide Black Currant Powder

Page: 2/8

Date: 08 / 13 / 2015

Version: 5

Replaces and cancels version: 4

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: Ribes Nigrum (Black Currant) Fruit Extract

Generic name:

Chemical Family: Plant Extract

Description: Substance

<u>Substance</u>	<u>CAS Numbers</u>	<u>EC Numbers</u>	<u>Percentage</u>
Ribes Nigrum (Black Currant) Fruit Extract	68606-81-5	271-749-0	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.

Ingestion: Consult with a physician.

Protection of first-aiders: No special protection required.

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Safety Data Sheet

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PhytoCide Black Currant Powder

Page: 3/8

Date: 08 / 13 / 2015

Version: 5

Cancels and replaces version: 4

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.

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.

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Safety Data Sheet

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

PhytoCide Black Currant Powder

Page: 4/8

Date: 08 / 13 / 2015

Version: 5

Cancels and replaces version: 4

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
Color: Light pink with darker specks

Odor: Characteristic

pH (1% Solution in Water): 3.0 – 6.0

Loss on Drying (1g-1hr-105°C): 8.0% Maximum

Solubility (in Water): Partially water soluble

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PhytoCide Black Currant Powder

Page: 5/8

Date: 08 / 13 / 2015

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Citric Acid:	1.5 – 4.0%
Heavy Metals (Total):	< 20 ppm
Lead:	< 10 ppm
Arsenic:	< 3 ppm
Mercury:	< 1 ppm
Specific Gravity:	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:	Partially 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 Irritation Model)
Ocular:	Non-Irritant (Ocular Irritation Model)
Inhalation:	Not Determined
Acute toxicity data:	EC50 (Acute Daphnia): 130.7 mg/L - Not harmful to aquatic organisms
Sensitization:	Non-Primary Sensitizer (In-Vitro Skin Sensitization Report & Direct Peptide Reactivity Assay)

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PhytoCide Black Currant Powder

Page: 6/8

Date: 08 / 13 / 2015

Version: 5

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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: Not Determined

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

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

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

PhytoCide Black Currant Powder

Page: 7/8

Date: 08 / 13 / 2015

Version: 5

Cancels and replaces version: 4

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:
EC regulations: This product does not need to be labeled in accordance with EC Directives or respective national laws

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

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(704) 276-7100 • Fax (704) 276-7101

PhytoCide Black Currant Powder

Page: 8/8

Date: 08 / 13 / 2015

Version: 5

Cancels and replaces version: 4

Other regulations:

EINECS inventory status:	Ribes Nigrum Fruit Extract:	271-749-0
TSCA inventory status:	Exempt	
AICS inventory status:	Listed: 68606-81-5	
Canadian (CEPA DSL) inventory status:	Exempt: Ribes Nigrum (Black Currant) Fruit Extract (68606-81-5)	
Japan (MITI list):	Ribes Nigrum (Black Currant) Fruit Extract	
Korea:	Ribes Nigrum (Black Currant) Fruit Extract	
China inventory status:	Ribes Nigrum (Black Currant) Fruit Extract	
Philippines inventory status:	Listed: Currant, Ribes nigrum, ext.	

*Listed on 2010 INCI Standard Chinese Name Directory

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

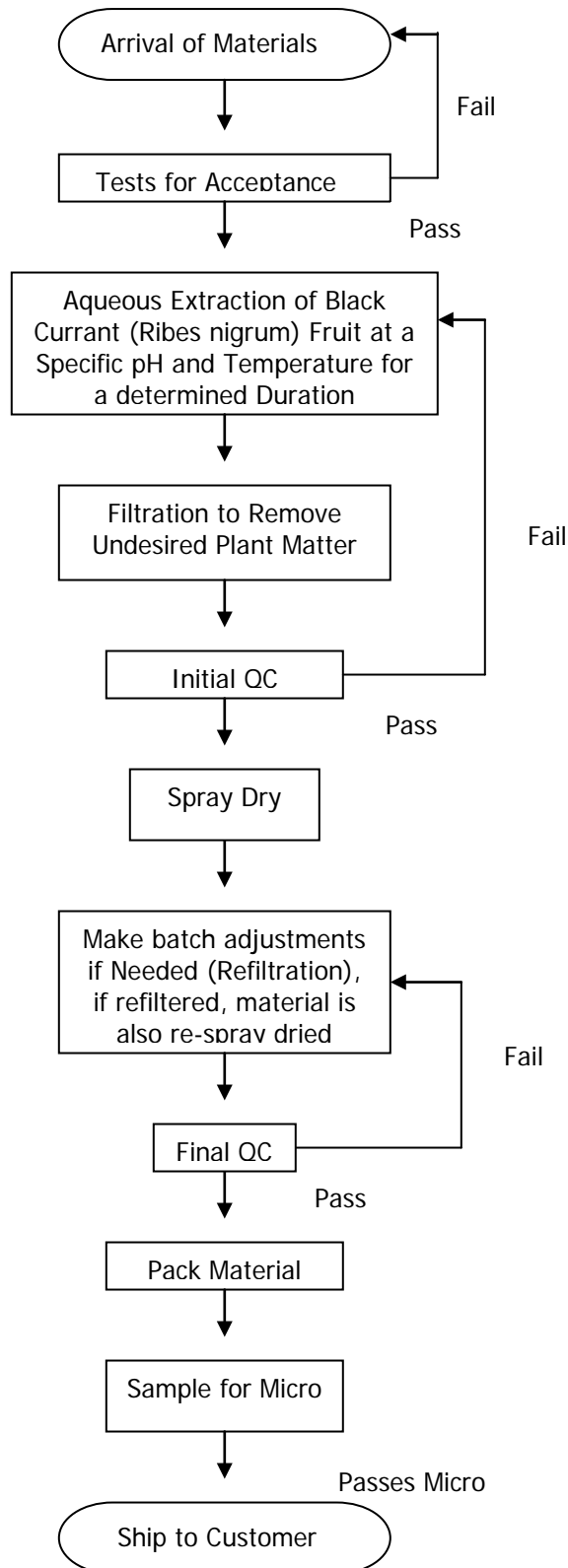
- Added Precautionary Statements - Section 2 (Hazards Identification)
- Added Mutagenicity Data – Section 11 (Toxicological Information) & Updated Transport Information – Section 14 (Transport Information)
- Added Acute Toxicity Data – Section 11 (Toxicological Information)
- Added Sensitization Data – Section 11 (Toxicological Information)

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.



PhytoCide Black Currant Powder Manufacturing Flow Chart

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

PhytoCide Black Currant Powder Certificate of Compliance

Code: M16001
INCI Name: Ribes Nigrum (Black Currant) Fruit Extract
INCI Status: Approved
CAS#: 68606-81-5
EINECS # 271-749-0

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
USA (TSCA)	Exempt
Australia (AICS)	Compliant
Japan (METI)	Compliant
Canada (DSL)	Compliant
China (IECSC)	Compliant
Brazil (ANVISA)	Compliant
Korea (KECI)	Compliant
Philippines (PICCS)	Compliant

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(704) 276-7100 • Fax (704) 276-7101

PhytoCide Black Currant Powder Code: M16001

Attention must be paid to the use of PhytoCide Black Currant 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 Black Currant Powder and its components and impurities are in compliance with the rules governing cosmetic products in the European Union (Directive 76/768/ECC & Regulation No. 1223/2009). The recommended use levels for PhytoCide Black Currant Powder is 1.00 – 3.00%.

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

PhytoCide Black Currant 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 Black Currant 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 Black Currant Powder does not contain any materials prohibited by Halal laws.

PhytoCide Black Currant Powder is REACH Compliant and free of the following:

- Formaldehyde or formaldehyde donors
- Glycol ethers
- Gluten
- 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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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.

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:

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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CREDIT MUTUEL 2200 20246201 29 - SIREN 509 534 095 RCS AUCH - APE 7120B

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:

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 until 31/12/2016

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