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TEST REPORT

EVALUATION OF SKIN IRRITATION ON BERRYCOAT USING *IN VITRO* RECONSTRUCTED HUMAN EPIDERMAL MODEL EPIDERM™ SKIN IRRITATION TEST

Job No. J803/16

Report No. R803/16/B19/43

Sponsor:
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Test Facility:
Industrial Biotechnology Research Centre (IBRC),
Building 19, SIRIM Berhad.

Study Initiation Date:
19 December 2016

Experimental Start Date:
17 January 2017

Experimental End Date:
27 January 2017

Study Completion Date:
3 February 2017

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APPROVAL SIGNATURES

We, the undersigned, declare that the methods, results and data contained in this report faithfully reflect the procedures used and raw data collected throughout the study.

(DR. NURUL IZZA NORDIN)
Deputy Technical Manager
Industrial Biotechnology Research Centre

03 FEB 2017
Date

(NOOR RABIHAH AID)
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SUMMARY

EVALUATION OF SKIN IRRITATION ON BERRYCOAT USING *IN VITRO* EPIDERM™ SKIN IRRITATION TEST (SIT)

In vitro skin irritation test on BerryCoat was performed according to Standard Operating Procedure (SOP) developed at MatTek Corporation. This *in vitro* standard method was validated by European Centre of the Validation of Alternative Methods (ECVAM) as *in vitro* test method based on reconstructed human epidermis (RhE) technology. The test was conducted in line with the requirement of OECD Guidelines for Testing of Chemicals No 439. This test assesses irritability of both cosmetic ingredients and finished products.

The test was conducted to determine whether the test item cause irritation to the *in vitro* skin model EpiDerm™.

In vitro dermal irritation test consists of topical exposure of the BerryCoat to reconstructed human epidermal model EpiDerm™ tissues, followed by a cell viability test. After 60 minutes of exposure, tissues were thoroughly rinsed, blotted to remove the test extract, and transferred to fresh medium. After a 24 hours incubation period, the medium was changed and tissues were incubated for another 18 hours. MTT [(3-4, 5 dimethyl triazole 2-yl) 2, 5-diphenyltetrazoliumbromide] assay was then performed by transferring the tissues to 6-well plates containing MTT medium (1 mg/mL). After 3 hours of incubation, the blue formazan salt formed by cellular mitochondria was extracted with 2.0 mL isopropanol / tissue. The optical density of the extracted formazan was determined using a spectrophotometer at 570 nm. Relative cell viability was calculated for each tissue as percentage (%) of the mean of the negative control tissues. The skin irritation potential was classified according to the remaining cell viability obtained after test item treatment.

The BerryCoat did not reduce viability of the EpiDerm™ tissue to below 50 % of the negative control. Under the condition of this test, BerryCoat is considered as **Non Irritant** to *in vitro* skin model EpiDerm™.

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BACKGROUND

Skin irritation refers to reversible damage to the skin following the application of a test chemical for up to 4 hours as defined by the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS).

For chemical, the reconstructed human epidermal model was developed and designed to predict skin irritation potential of neat test substances in the context of identification and classification of skin irritation hazard according to the European Union (EU) classification system (R 38 or no label). Since the EU and GHS systems were harmonized in 2008, the procedure described in the SOP also allows for hazard identification of irritant substances in accordance to UN GHS.

In vitro skin irritation: Reconstructed Human Epidermis Test allows for assessment of irritation. A sufficient amount of extract was applied on the surface of the three dimensional reconstructed human epidermis (RhE). The RhE model is comprised of non-transformed human-derived epidermal keratinocytes, which have been cultured to form a multilayered, highly differentiated model of the human *epidermis*. It consists of organized basal, spinous and granular layers, and a multilayered *stratum corneum* containing intercellular lamellar lipid layers representing main lipid classes analogous to those found *in vivo*. After a certain incubation period, cell viability is assessed by MTT [(3-4, 5 dimethyl triazole 2-yl) 2, 5-diphenyltetrazoliumbromide] colorimetric test. The reduction of the viability of tissues exposed to chemicals in comparison to negative controls (treated with water) is used to predict the skin irritation potential.

Irritant chemicals are identified by their ability to decrease cell viability below defined threshold levels (*i.e.* ≤ 50 %, for UN GHS Category 2). Depending on the regulatory framework and applicability of the Test Guideline, chemicals that produce cell viability above the defined threshold level may be considered non-irritants (*i.e.* > 50 %, No Category).

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1.0 OBJECTIVE

The objective of this study is to predict skin irritation potential of test item using *in vitro* skin model EpiDerm™.

2.0 STUDY TIMETABLE

2.1 **Receipt of reconstructed human epidermal model EpiDerm™ tissues:**
17 January 2017

2.2 **Tissue Conditioning**
17 January 2017

2.3 **Pre-Incubation**
17 January 2017

2.4 **Treatment**
18 January 2017

2.5 **Change Medium**
19 January 2017

2.6 **MTT Viability Test**
20 January 2017

2.7 **Optical Density Reading**
20 January 2017

2.8 **Data Analysis**
23 January 2017 – 26 January 2017

3.0 MATERIALS

3.1 **Test Item**

3.1.1 Test item: BerryCoat

3.1.2 Lot/Batch No.: Not provided

3.1.3 Sample marking : BerryCoat

3.1.4 Date received: 20 December 2016

3.1.5 Physical appearance: Translucent Liquid

3.1.6 Colour: Translucent

3.1.7 Physical Chemical Properties Data: Not provided

3.1.8 Quantity: 150 mL

3.1.9 pH: Not provided

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3.1.10 Storage condition: Room Temperature

3.1.11 Solubility: Not provided

3.1.12 Stability: Not provided

3.1.13 Expiration date: Not provided

3.2 **Test System**

3.2.1 Test System: Reconstructed human epidermal model EpiDerm™

The reconstructed human epidermal model EpiDerm™ (EPI-200, MatTek, Ashland, USA) consists of normal human-derived epidermal keratinocytes, which have been cultured to form a multilayered highly differentiated model of the human epidermis. It consists of organized basal, spinous and granular layers, and a multilayered stratum corneum containing intercellular lamellar lipid layers arranged in patterns analogous to those found *in vivo*.

The EpiDerm™ tissues are cultured on specially prepared cell culture inserts, containing 24 tissues using serum free medium. Ultrastructurally, the EpiDerm Skin Model closely parallels human skin, thus providing a useful *in vitro* means to assess dermal irritancy and toxicology.

3.2.1.1 Lot No:24959

3.2.1.2 Production Date : 12 January 2017

3.2.1.3 Date of Shipping: 13 January 2017

3.2.1.4 Receipt of EpiDerm™: 17 January 2017, Tuesday,11.45 am

3.2.1.5 Visual quality control of the skin: All tissues in good condition

3.2.1.6 The EpiDerm™ System is manufactured according to defined quality assurance procedures. All biological components of the epidermis and the culture medium are tested by manufacturer for viral, bacterial, fungal and mycoplasma contamination. MatTek determines the ET-50 value following exposure to Triton X-100 (1%) for each EpiDerm™ lot. The ET-50 must fall within a range established based on a historical database of results or acceptability ranges for quality control based on OECD Test Guidelines.

3.3 **Reagent**

3.3.1 Assay Medium: EPI-100-NMM-SIT / Assay Medium
3.3.1.1 Lot No. : 011117CMHB
3.3.1.2 Sterility : Sterile
3.3.1.3 Expiration Date : 8/02/2017
3.3.1.4 Storage : Refrigerator (5 ± 3 ºC)
3.3.1.5 Manufacturer : Mattek Corporation

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3.3.2 Phosphate Buffered Saline without Calcium and Magnesium free, contain the following:
3.3.2.1 Sodium Chloride 8 g/L
3.3.2.1.1 Lot No.: 1409NBXC18A
3.3.2.1.2 Manufacturer : Bio Basic Canada Inc

3.3.2.2 Potassium chloride 0.2 g/L
3.3.2.2.1 Lot No.: 1409BI4J35501
3.3.2.2.2 Manufacturer : Bio Basic Canada Inc

3.3.2.3 Anhydrous potassium dihydrogen orthophosphate 0.2 g/L
3.3.2.3.1 Lot No.: 1211ACN1K12062801
3.3.2.3.2 Manufacturer : Bio Basic Canada Inc

3.3.2.4 Anhydrous disodium hydrogen orthophosphate 1.15 g/L
3.3.2.4.1 Lot No.: 1306ACK2NA12020101
3.3.2.4.2 Manufacturer : Bio Basic Canada Inc

3.3.3 MTT – 2mL (5mg/mL)
3.3.3.1 Lot No. : MKBW0025V
3.3.3.2 Manufacturer : SIGMA

3.3.4 MTT Diluent – 8mL
3.3.4.1 As above 3.3.1

3.3.5 Extractant Solution – Isopropanol
3.3.5.1 Lot No. : 632261
3.3.5.2 Storage : Room Temperature
3.3.5.3 Manufacturer : Fisher Scientific

3.3.6 Positive Control: 5 % SDS Solution
3.3.6.1 Part No. : TC-SDS-5 %
3.3.6.2 Lot No. : 010516ACD
3.3.6.3 Expiration Date : 05/01/2017
3.3.6.4 Storage : Room Temperature
3.3.6.5 Manufacturer : Mattek Corporation

3.3.7 Vehicle Control: As above 3.3.2

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4.0 METHOD

Upon receipt of the reconstructed human epidermal model EpiDerm™, the tissue kits and the solutions were stored according to the manufacturer's directions for unpacking and storage.

4.1 Tests for Interference of Test Item

4.1.1 Assessment of Coloured or Staining Materials

Tissue-Binding of Coloured or Staining Materials

30 µL of the test item was applied onto a single EpiDerm™ tissue. In parallel, a tissue was exposed to negative control. After 60 minutes of exposure, tissues were thoroughly rinsed, blotted to remove the test extract, and transferred to fresh medium.

After a 24 hours incubation period, the medium was changed and tissues were incubated for a further 18 hours. The medium was then changed again and incubated for 3 hours. After incubation was completed, the tissues were rinsed and extracted using 2.0 mL of isopropanol per tissue. The optical density of the extracted tissue was determined using spectrophotometer at 570 nm.

4.1.2 Test for Interference of Test Item with MTT

30 µL of the test item was added to 1 mL of the 1 mg/mL MTT and incubated at (37 ± 1)° C, (5 ± 1)% CO_2, 95 % relative humidity for 60 minutes. Untreated MTT medium was used as control. If the MTT solution turns blue/purple, the test item reduces MTT and additional functional check must be performed.

4.1.3 Test for Mesh Compatibility

A mesh was placed on a glass slide and 30 µL of test item was applied. After 60 minutes of exposure, the reaction between the mesh and test item was observed under microscope.

4.2 Tissue Conditioning – Day 0

Under sterile conditions, a sealed 24-well plate containing the EpiDerm™, was opened. Visual inspection of each insert containing the epidermal tissue was done prior to tissue conditioning.

0.9 mL of assay medium was dispensed into each well of six-well plate, the EpiDerm™ tissue cultures were transferred into the wells and the plates was incubated for (60 ± 5) minutes at (37 ± 1)° C, (5 ± 1)% CO_2, 95 % relative humidity. At the end of the first 60 minutes pre-incubation, the assay medium was renewed for further pre-incubation. Each insert was aseptically transferred into well containing 0.9 mL assay medium and pre-incubation was done at (37 ± 1)° C, (5 ± 1)% CO_2, 95 % relative humidity for (18 ± 3) hours.

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4.3 **Dosing protocol – Day 1**

30 µL of test item was applied onto three single EpiDerm™ tissues. Negative and positive controls were conducted in parallel using identical method to the dosed cultures. The cultures were incubated at (37 ± 1) °C, (5 ± 1) % CO_2, 95 % relative humidity for (35 ± 1) minute.

After incubation, each dosed EpiDerm™ tissue was removed from the incubator and placed at room temperature in the biological safety cabinet for 25 minutes. At the end of the exposure period, the EpiDerm™ tissue cultures were removed from the assay plates and gently rinsed with phosphate buffered saline to eliminate any residual test material. The EpiDerm™ tissue cultures were then transferred into wells containing 0.9 mL assay medium and the plates incubated at (37 ± 1) °C,(5 ± 1) % CO_2, 95 % relative humidity for (24 ± 2) hours.

4.4 **Change Medium – Day 2**

After 24 hour's incubation, the medium was changed and EpiDerm™ tissues were incubated for a further 18 hours.

4.5 **MTT Viability Test**

4.5.1 Day 3

The MTT assay was performed by transferring each EpiDerm™ tissues to 6-well plates containing 300 µL MTT medium (1 mg/mL) in each well. After 3 hours of MTT incubation, the blue formazan salt formed by cellular mitochondria was extracted with 2.0 mL isopropanol / tissue (extractant solution). The extraction plates were sealed with parafilm and agitated for at least 2 hours at room temperature. As alternative, overnight extraction at room temperature in the dark also possible.

4.5.2 At the end of the extraction period, EpiDerm™ tissue was pierced with an injection needle and the extract was decanted into the well from which insert was taken. The insert was then discarded. The extraction solution was pipetted up and down to ensure complete mixing. Finally, 200 µL was transferred into a 96 well microtiter plate for absorbance measurement (OD=optical density) at 570 nm without using a reference filter. 200 µL of isopropanol was used as blank.

Relative cell viability for each tissue was calculated as percentage (%) relative to mean of the negative control tissues viability.

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5.0 DATA ANALYSIS

5.1 Tests for Interference of Test Item

5.1.1 Assessment of Coloured or Staining Materials

The percentage difference of the optical density between a coloured test item (CTI) and the negative control (NC) was calculated according to the following formula:

$$[(\text{Mean OD}_{CTI} - \text{Mean OD}_{NC}) / \text{Mean of OD}_{NC}] \times 100$$

OD reading of treated tissue by coloured test item	Action
Below 5 % (<5 %) of the negative control ; Tissue viability determined in MTT Assay not close to classification cut-off (50%)	Correction of the results is not necessary
Between 5 % and 30 % of the negative control	Further test on more tissue
Above 30 % (>30 %) of the negative control	Additional step and expert judgment ; Incompatible with the test system

The real MTT OD (unaffected by interference with the color or staining materials) was calculated using the following formula:

OD = OD Coloured tissue (MTT assay) – OD Coloured tissue (no MTT assay)

5.1.2 Test for Interference of Test Item with MTT

The test item is presumed to have reduced the MTT if the MTT solution colour turns blue/ purple.

5.2 Workbook EpiDerm™-SIT

A blank, password protected MS EXCEL workbook EpiDerm™-SIT-SPREAD.XLS was provided by MatTek Corporation. The workbook consists of two single spreadsheets named: Import and Spread.

5.3 Raw Data of Optical Densities (ODs)

Raw data of optical densities generated by the microplate reader (without blank subtraction) were copied from the reader software and then pasted into the Import spreadsheet of the Excel workbook. The blank corrections, calculation of results and statistical parameters are automatically calculated in the Spread spreadsheet of the workbook.

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5.4 **Calculation**

5.4.1 After data entry, the spreadsheet performs the following calculations:

5.4.2 Blank correction

5.4.3 For each individual tissue treated with a test item (TI), the positive control (PC) and the negative control (NC) the individual relative tissue viability was calculated according to the following formulas;

Relative viability TI (%) = [OD_{TI} / Mean of OD_{NC}] x 100
Relative viability NC (%) = [OD_{NC} / mean of OD_{NC}] x 100
Relative viability PC (%) = [OD_{PC} / mean of OD_{NC}] x 100

5.4.4 For each test item, negative control and positive control, the mean relative viability of the three individual tissues was calculated and used for classification according to the Prediction Model (Refer to 8.0).

6.0 ACCEPTABILITY RANGES FOR QUALITY CONTROL

	Lower acceptance limit	Upper acceptance limit
EpiDerm™ SIT (EPI-200) (1% Triton X-100)	ET_{50} = 4.0 hour	ET_{50} = 8.7 hour

7.0 ACCEPTABILITY RANGES FOR NEGATIVE CONTROL OD VALUES OF THE TEST METHODS

	Lower acceptance limit	Upper acceptance limit
EpiDerm™ SIT (EPI -200)	≥ 1.0	≤ 2.5

8.0 ACCEPTANCE CRITERIA FOR POSITIVE CONTROL

The assay meets the acceptance criterion if the mean viability of positive control tissues expressed as percent of the negative control tissues is ≤ 20%. The standard deviation shall be below 18 for all substances and controls.

	Mean of Viability (%)	Standard Deviation of Viability (SD %)
EpiDerm™ SIT (EPI-200)	≤ 20	<18

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9.0 DATA INTERPRETATION PROCEDURE (PREDICTION MODEL)

An irritant is predicted if the mean relative tissue viability of three individual tissues exposed to the test substance is reduced below 50% of the mean viability of the negative controls.

***In vitro* result**	***In vivo* prediction**
mean tissue viability ≤ 50%	Irritant (I), (R38 or GHS category 2)
mean tissue viability > 50%	non-irritant (NI)

10.0 RESULT AND DISCUSSION

10.1 Tests for Interference of Test Item

10.1.1 Assessment of Coloured or Staining Materials

Tissue-Binding of a Coloured Test Item

The relative OD reading of treated tissue by coloured test item is below 5% of the negative control. Correction of the results is not necessary.

10.1.2 Test for Interference of Test Item with MTT

There was no change in MTT colour therefore the test item did not interfere with MTT.

10.2 Viability Measurement

10.2.1 Raw data of optical densities (ODs) of Blank

Refer To Table 1

10.2.2 Blank Correction

Refer to Table 2

10.3 The Quality Control value meets the acceptance range criteria

EpiDerm™ SIT (EPI-200)	**Hour**
ET_{50}	7.80

10.4 The negative control OD value meets the acceptance range criteria

EpiDerm™ SIT (EPI-200)	**Lower OD**	**Upper OD**
Negative Control	1.928	2.039

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10.5 **The positive control OD value meets the acceptance range criteria**

EpiDerm™ SIT (EPI-200)	**Mean of Viability (%)**	**Standard Deviation of Viability (SD %)**
Positive Control	3.2	0.15

10.6 **Classification**

Test item, negative control and positive control are qualified according to prediction model. The mean relative viability of the three individual tissues was calculated and used for classification

Refer to Table 3

10.7 **Graph**

10.7.1 The spreadsheet shows a graph of the results (% of relative viability ± standard deviation)

Refer to Figure 1

10.8 **Prediction Model**

	Mean of Viability (%)	**Standard Deviation of Viability (SD %)**	***In vitro* result**	***In vivo* prediction**
BerryCoat	101.1	6.26	mean tissue viability > 50 %	**Non-Irritant (NI)**
Negative Control	100.0	2.91	mean tissue viability > 50 %	**Non-Irritant (NI)**
Positive Control	3.2	0.15	mean tissue viability ≤ 50 %	**Irritant (I), (R38 or GHS category 2)**

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11.0 CONCLUSION

Under the condition of this test, BerryCoat is considered as **Non Irritant** to *in vitro* skin model EpiDerm™.

12.0 RETENTION OF RECORDS AND TEST ITEM

One report will be forwarded to the Sponsor. The other report, together with all generated raw data is maintained at the Industrial Biotechnology Research Centre Archives.

13.0 REFERENCES

13.1 OECD (2013), In *Vitro* Skin Irritation: Reconstructed Human *Epidermis* Test Method – OECD Guidelines for Testing of Chemicals No. 439

13.2 Protocol for: In *Vitro* EpiDerm™ Skin Irritation Test (EPI-200-SIT) – Reconstructed Human Epidermal Model EpiDerm (EPI-200-SIT). For use with MatTek Corporation

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Table 1 Optical Densities (ODs) of Blank

Optical Densities (ODs) of Blank	Mean Optical Densities (ODs) of Blank
0.0444	0.0424
0.0415	
0.0421	
0.0411	
0.0423	
0.0430	

Table 2 Blank Corrected Data

	Tissue	Raw data		Blank corrected data		Mean	% of Viability
		1	2	1	2		
BerryCoat	1	2.1842	2.18	2.142	2.138	2.140	108.3
	2	1.9968	1.9427	1.954	1.900	1.927	97.6
	3	1.9445	1.9878	1.902	1.945	1.924	97.4
Negative Control	1	1.9927	2.013	1.950	1.971	1.960	99.2
	2	1.9768	1.9631	1.934	1.921	1.928	97.6
	3	2.0237	2.1396	1.981	2.097	2.039	103.2
Positive Control	1	0.1074	0.1085	0.065	0.066	0.066	3.3
	2	0.1061	0.1075	0.064	0.065	0.064	3.3
	3	0.1006	0.1044	0.058	0.062	0.060	3.0

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Table 3 Classification of Test Item

	Mean of OD	SD of OD	Mean of Viability [%]	SD of Viability	CV [%]	*In vitro* result	Classification
BerryCoat	1.997	0.124	101.1	6.26	6.19	Non-Irritant	Qualified
Negative Control	1.976	0.057	100.0	2.91	2.91	Non-Irritant	Qualified
Positive Control	0.063	0.003	3.2	0.15	4.53	Irritant	Qualified

OD- Optical Density, SD- Standard Deviation, CV- Coefficient of Variation

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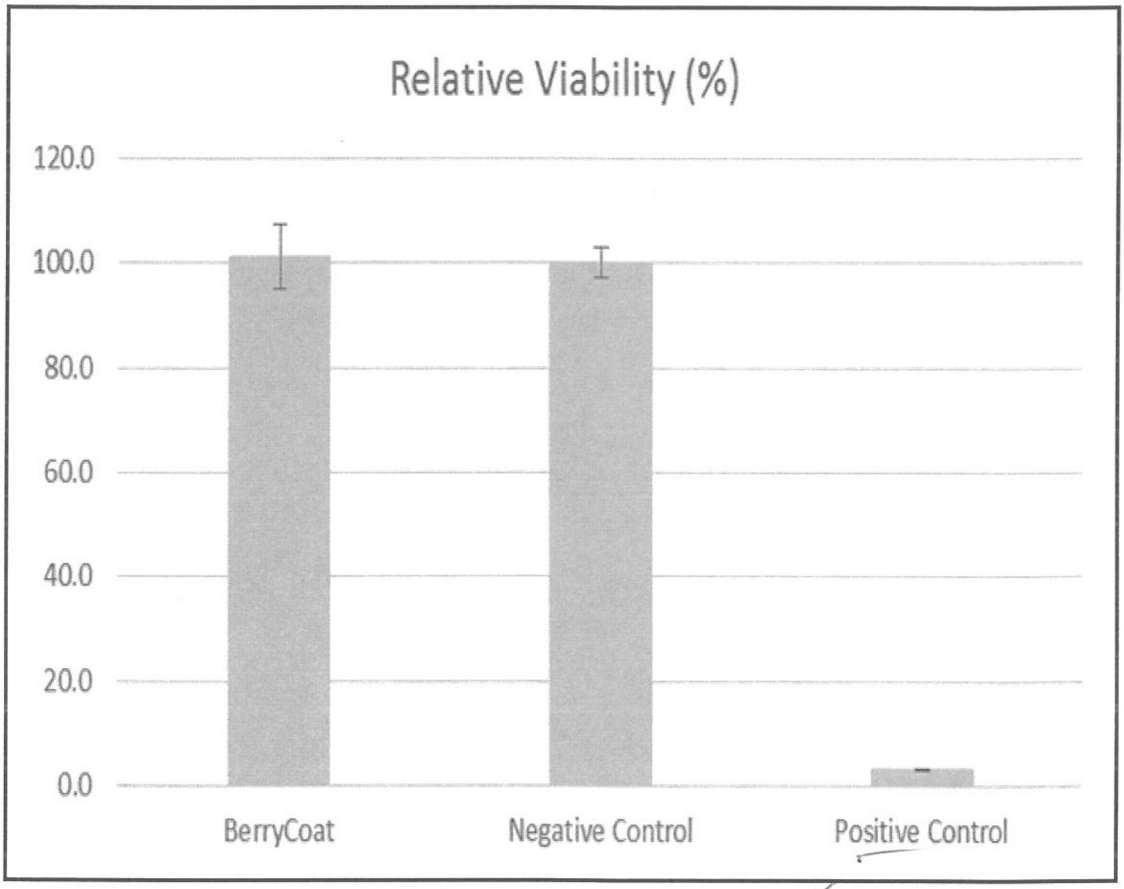

Figure 1 Relative Viability

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