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TEST REPORT

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Applicant : TEVO Creations Sdn Bhd

Manufacturer / Company : No 2, Lorong Beringin 1,
Taman Industri Beringin,
14100 Simpang Ampat,
Pulau Pinang.

Sample : Berry C

Reference standard / Method of Test : *In – Vitro* Scratch Wound Healing Assay

Description of Sample : Received one sample with the following identification for the analysis:

1) Berry C : Translucent

Date Received : 1st December 2023

Job No. : J1404/23

Issue Date : 5th February 2024

Approved signatories,

(HARMAYUMI WAHID)
Analyst
Industrial Biotechnology Research Centre
SIRIM Berhad

(Dr Theanmalar A/P Masilamani)
Reviewer
Industrial Biotechnology Research Centre
SIRIM Berhad

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In –Vitro Scratch Wound Healing Assay

1.0 OBJECTIVE

To determine the cell migration and wound closure rate in the wound healing process.

2.0 PRINCIPLE

There are four main phases in wound healing: coagulation, inflammation, migration-proliferation (including matrix deposition), and remodelling. These phases do not represent distinct events, but rather overlap and are continuous. After tissue injury and under the influence of various growth factors and cytokines, keratinocytes at the rear of the wound margins may display a high proliferative activity. These cells then migrate forward onto the wound bed and help restore the epidermal barrier structure and function. This overall process involves cell migration, proliferation, and differentiation. In smaller wounds, the critical event is keratinocyte migration rather than proliferation. Cell migration begins several hours after injury. Epidermal cells adjacent to the wound margin become polarized (driven by the actin cytoskeleton) and develop pseudopodium-like projections preferentially oriented outward, into the free space, and within 24 hours, the cells detach from the basal lamina and are ready for migration. Lamellipodial crawling refers to the pattern of motion that epidermal cells exhibit during migration.

In–Vitro Scratch Wound Healing Assay is a method based on the observation that, upon creation of a new artificial gap, so-called “scratch”, on a confluent cell monolayer, the cells on the edge of the newly created gap will move toward the opening to close the “scratch” until new cell-cell contacts are established again. The basic steps involve the creation of a “scratch” on monolayer cells, capture of images at the beginning and regular intervals during cell migration to close the scratch, and comparison of the images to determine the rate of cell migration.

3.0 ACCEPTANCE CRITERIA

Allantoin was used as a positive control. The value of allantoin (1mg/ml) shall indicate at least 50% of wound closure with more than 80% cell viability. For this experiment, 1 mg/ml of allantoin promotes 80.8 ± 3.0 % of wound closure with 100.8 ± 6.1 % cell viability. This indicates the integrity of the assay, and the test system is acceptable.



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4.0 METHOD

4.1 Sample Preparation

Sample (Berry C) was first dissolved in aqueous and finally tested in assay at final concentration of 0.31 mg/ml to a maximum of 10 mg/ml (weight/volume). The concentration is ten-fold diluted from the actual prepared concentration.

4.2 Measurement of cell wound closure and cell viability

Specifically, Human keratinocytes cells line (HaCaT) was seeded at a density of 2×10^5 cells into 24 well plates and incubated with complete medium at 37°C and 5% CO₂ until they reached 90% confluence. After 24h of incubation time, the monolayer confluent cells were scrapped horizontally with a sterile P200 pipette tip. The debris was removed by washing with PBS. The cells were treated with samples for 48 hours. The non-treated and treated cells with Allantoin were used as the control and positive control, respectively. The scratch induced by the represented wound was photographed at 0 h using phase-contrast microscopy at 4X magnification at 0 h, before incubation with the sample and positive control. After 48 hrs of incubation, the second set of images was photographed. To determine the migration rate, the images were analysed using "ImageJ" software, and the percentage of the closed area (μm^2) unit was measured and compared with the value of control at 0 hr. An increase in the percentage of the closed area indicated the migration of cells. Experiments were performed in a triplicate manner and the data were recorded and analysed.

The percentage of Wound closure (%) calculation:

$$\frac{\text{Measurement at 0 hr } (\mu\text{m}^2) - \text{Measurement at 48 hr } (\mu\text{m}^2)}{\text{Measurement at 0 hr } (\mu\text{m}^2)} \times 100\%$$

MTT reagent was added into each well and cells incubated for 3 hours at 37°C. Cells were then centrifuged and dissolved with dimethyl sulfoxide (DMSO). Absorbance was measured using spectrophotometer at 570 nm. Cell viability was determined as:

$$(\% \text{ Cell Viability}) = \frac{B}{A} \times 100\%$$

A: Absorbance of non-treated cells

B: Absorbance of treated cells



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5.0 RESULTS

5.1 Cell Viability

Remark	Tested Concentration (mg/ml)	Cell Viability (%)				
		R1	R2	R3	Average	StDev
Positive control:						
Allantoin	1.00	105.08	103.52	93.75	100.8	6.1
Sample:						
Berry C	0.31	88.67	93.36	95.31	92.4	3.4
	0.63	73.05	73.83	68.75	71.9	2.7
	1.25	57.03	57.42	56.64	57.0	0.4
	2.50	43.36	34.38	40.63	39.5	4.6
	5.00	35.16	27.34	26.56	29.7	4.8
	10.0	21.88	18.75	20.70	20.4	1.6

5.2 Wound Closure

Remark	Tested Concentration (mg/ml)	Wound Closure (%)				
		R1	R2	R3	Average	StDev
Positive control:						
Allantoin	1.00	78.0	80.4	83.9	80.8	3.0
Sample:						
Berry C	0.31	35.1	42.3	38.0	38.5	3.6

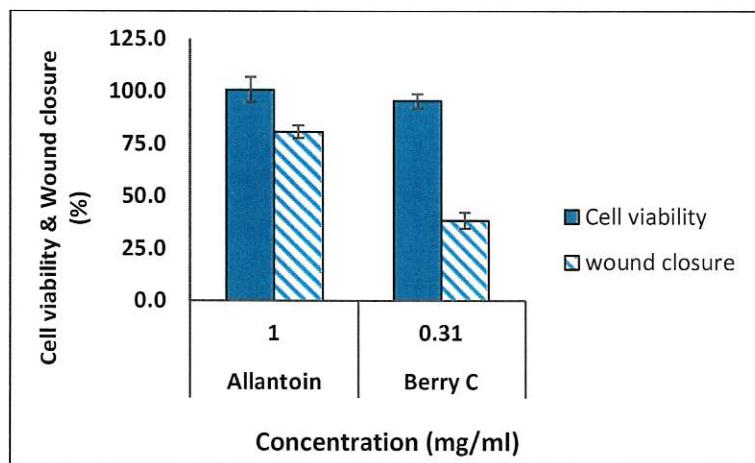


Figure 1: Effects of positive control and sample (Berry C) on the rate of wound closure and cell viability.



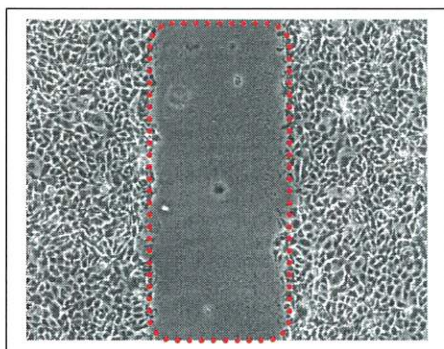
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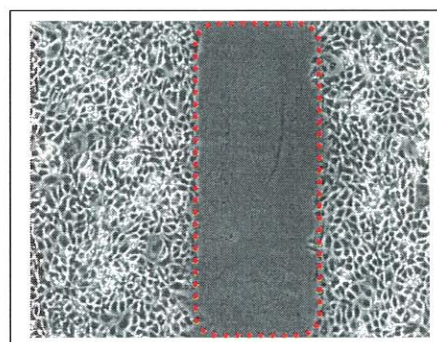
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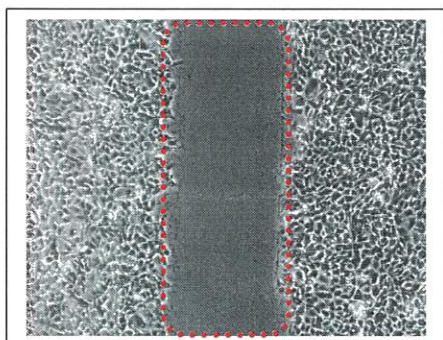
5.3 Microscopy Image of Wound Healing Activity



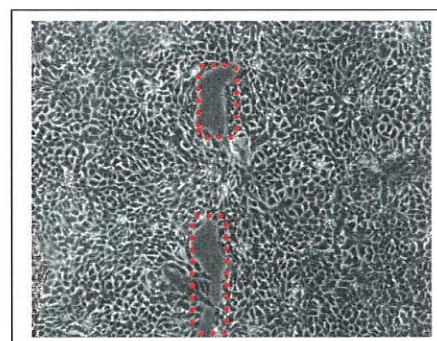
Control at 0 hr



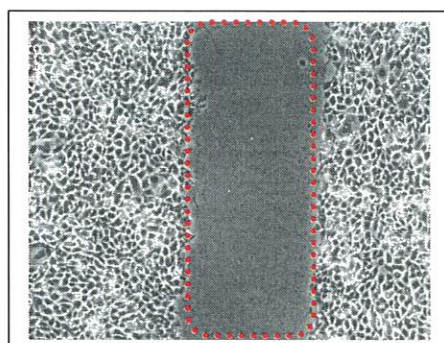
Control at 48 hr



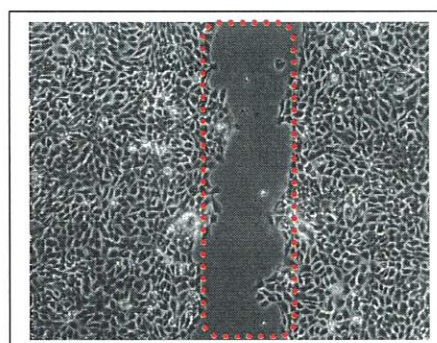
Allantoin (1.0 mg/ml) at 0 hr



Allantoin (1.0 mg/ml) at 48 hr



Berry C (0.31 mg/ml) at 0 hr



Berry C (0.31 mg/ml) at 48 hr

Fig. 2: The figure indicates cells were scratched at (0 hr), and treated with Allantoin (positive control) and Berry C (0.31 mg/ml) at 48 hr, showed cell proliferation and cell migration as compared with control (without treatment). The area was measure in (μm^2) unit using "imageJ" software.



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6.0 DISCUSSION

Based on data in 5.1, sample (Berry C) shows cytotoxicity effect against human keratinocyte cells at concentration range from 0.63 mg/ml to a maximum of 10 mg/ml, which is less than 80% cell viability. Therefore, wound closure at the concentration of less than 0.63 mg/ml shall be considered. Based on data in 5.2, the cells treated with sample (Berry C) promotes cell migration activity and closed the gap of wound healing. Sample (Berry C) at the concentration of 0.31 mg/ml (The concentration is ten-fold diluted from the actual prepared concentration-refer 4.1), promotes 38.5 ± 3.6 % of cell migration activity.

7.0 CONCLUSION

Based on the result, sample (Berry C) has the potential in accelerating wound healing activity.

***This finding partially supported that Berry C has potential in wound healing activity, where further studies are recommended.

8.0 REFERENCES

1. Muniandy, K., Gothai, S., Tan, W. S., Kumar, S. S., Esa, N. M., Chandramohan, G., Arulselvan, P. (2018). *In Vitro Wound Healing Potential of Stem Extract of Alternanthera sessilis*. 2018(2011).
2. Cappiello, F., Casciaro, B., & Mangoni, M. L. (2018). *A Novel In Vitro Wound Healing Assay to Evaluate Cell Migration*. (March), 10–15. <https://doi.org/10.3791/56825>

