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Evaluation of *in vitro* wound healing efficacy of breadfruit derived starch hydrolysate

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Abstract

To study the wound healing efficacy of breadfruit starch hydrolysate, an in vitro wound scratch assay was conducted, in which the migration rate of wounded NIH 3T3 fibroblasts was determined. Wounds treated with lower dextrose equivalent (DE), (DE 10-14) starch hydrolysate were found capable to improve the wound healing of NIH 3T3 fibroblast cell with the percentage of wound closure improvement of 77%, respectively when compared with higher DE range (DE 15-19 and DE 20-24). The findings obtained in the BrdU uptake and MTT viability assays confirmed the wound healing properties of breadfruit starch hydrolysate as the starch hydrolysate-treated wounded NIH 3T3 fibroblasts were able to proliferate well and no cytotoxicity was observed. Together, these findings indicated that the newly developed breadfruit starch hydrolysate performed better than commercial (COM) starch hydrolysate of the same DE ranges. In conclusion, breadfruit starch hydrolysate had better functional properties than did starch hydrolysates derived from other sources and that they could play a beneficial role in wound healing applications.

Introduction

Breadfruit (*Artocarpus atlitis*) is a starchy staple when mature and rich in nutrients. It is a high-energy food with a moderate glycemic index (Zerega *et al*., 2004). Breadfruit is found throughout the tropics and is cultivated on most Pacific islands (Zerega *et al*., 2004). In a study conducted by Gbadamosi and Oladeji (2013), the suitability of starches from carbohydrate-rich sources, including breadfruit, as a substitute for cassava starch was studied. Based on their results, breadfruit and cassava starch were closely related in terms of chemical composition and physico-functional properties, which made breadfruit starch a potential substitute for cassava starch. This study confirmed that breadfruit is a high-potential source for maltodextrin production because it is a carbohydrate-rich crop.

Starch hydrolysate is a product of starch hydrolysis that is produced via acid or enzymatic treatment. This product contains both simple sugars and polymers of saccharides with lower molecular-weights. Starch hydrolysate is a white hygroscopic powder occurring as different grades that are categorized according to their dextrose equivalent (DE) value, which is a measure of the reducing power of dextrose compared with that of D-glucose on a dry-weight basis (Wang *et al*., 2000). Starch hydrolysate has been widely used as an ingredient in various food products and pharmaceutical delivery systems. Previous finding has reported that starch hydrolysate may possess wound healing potential by promoting the proliferation of fibroblasts cell (Swaim, 1998).

The process of wound healing is complex, and good healing is characterized by the rapid regeneration of the damaged tissue. Wound healing involves the adequate interaction of cells and keratinocytes. Both fibroblasts and endothelial cells that perform cell biological functions are relevant to the process of wound healing. One of the most important aspects of this complex biological process is the epithelial closure, which relies primarily on the concerted action of activated keratinocytes and dermal fibroblasts (Haubner *et al*., 2012). As wounds

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heal, newly regenerated cells fill in the gap of the wound (DeBusk *et al*., 2006). Wound healing is also promoted by wound dressings that are designed to keep the wound clean and free of contamination. Starch hydrolysate is a favorable dressing component because it facilitates the exposure of dermatological agents added to improve healing, assists in contacting all areas of the wound (Silvetti *et al*., 1997) and at the same time, provides topical nutrients to the wound site, creating a natural healing environment that promotes the growth of granulation tissue and epithelial proliferation. Hygroscopic starch hydrolysate creates a dehydrating environment due to the intramolecular hydrogen bonding in amylose, which kills bacteria and hastens wound healing (Moore *et al*., 2005). In fact, starch hydrolysate not only helps to eliminate infections but also contributes to cost saving by reducing the necessity for skin grafting and the overall cost of wound care.

Over the years, corn, potato, sago, tapioca and wheat starch has been widely used in commercial starch hydrolysate production. To date, full spectrum studies on the physico-chemical characteristics of starch hydrolysate produced from breadfruit starchy sources are lacking. Differences in the molecular structure of this breadfruit starch will affect the physico-chemical properties of breadfruit starch hydrolysate that will eventually define its specific functional application in wound healing. The aim of this study is to investigate the effects of breadfruit starch hydrolysate with different DE grade on the migratory behavior and wound healing rate of NIH 3T3 cells, in the latter case using an *in vitro* wound healing scratch assay.

Materials and Methods

Preparation of breadfruit starch hydrolysate

Mature but un-ripened breadfruit was purchased from a local market (Selangor, Malaysia). The starch was prepared at a laboratory scale (Akanbi *et al*., 2009; Koh et al., 2012). The suspension of starch (20%) was prepared with 40 g starch to 200 mL of distilled water. An amount of 0.04 g calcium chloride was added. The pH was adjusted to 6.5 with sodium hydroxide (0.1 N) before hydrolyse using enzyme Ban 480L. Different dextrose equivalent (DE) value of breadfruit starch hydrolysate was prepared according to the specific enzymatic hydrolysis process condition (MARDI in house method). Enzyme inactivation was done by adding hydrochloride acid (0.1 N) to achieve pH value of 3.0 before transferred to centrifuge to separate clear supernatant from crude starch hydrolysate under the condition of 4°C, 10,000 rpm for 10 mins. The liquid starch hydrolysate was spray dried using optimized parameter setting as studied earlier: inlet temperature (170°C); feed flow (9%) and aspirator rate (95%). A total of 10% commercial (COM) starch hydrolysate (cassava base, DE 10-14) was used as the reference material because it was known to be of high quality (Koh *et al*., 2012). A commercial wound dressing agent, Multidex (MUL) containing 99% predominantly sterile NF1 starch hydrolysate with 1% ascorbic acid was used for comparison purpose.

Determination of dextrose equivalent (DE) value

The Lane and Eynon method was used to analyze the DE value of breadfruit starch hydrolysate samples. Fehling Solutions were standardized against standard dextrose obtained from the Bureau of Standards. To determine the Fehling Factor, 0.5 g anhydrous grams of dextrose was weighed per 200 mL of distilled water and used as the test solution. Fehling Factor was calculated as follow:

$$\text{Fehling Factor} = \frac{(100)\ x\ (mL\ used\ in\ titration)\ x\ (\frac{g\ dextrose}{mL})}{100}$$

A starch hydrolysate solution (10 g/ 200 mL) with the known concentration of an anhydrous starch basis was prepared. The starch hydrolysate solution was transferred to a 50 mL burette. To 50 mL of distilled water in a 500 mL Erlenmeyer flask, 5 mL of each Fehling A and Fehling B were added in. The contents of the flask were brought to boil over a hot plate. When the water starts boiling, add in 2 drops of methylene blue indicator and stirred continuously. The titration was completed by adding the starch hydrolysate solution dropwise until the blue colour disappears. The volume of starch hydrolysate solution used was recorded. The DE was calculated based on below stated formula:

$$DE = \frac{(Fehling\ Factor)}{\left(\frac{g}{mL}\ starch\ hydrolysate\ concentration\right) x\ mL\ starch\ hydrolysate\ solution}\ x\ 100$$

Determination of glucose polymer profiling using liquid chromatography-mass spectrometry (LC-MS)

LCMS experiment was performed on a Shimadzu LCMS 2020 coupled with an electrospray ion (ESI) source. The single stage quadruple mass spectrometer provides an upper mass unit limit of 2000 dalton. Nebulizer gas flow was 1.5 L/min and MS parameter setting was optimized to the following: detector voltage = 1 kV, CDL = 25V, DL temperature = 250°C and block temperature was 200°C.

Polyamine-bonded polymeric gel column (apHeraTM NH_2, 25 cm x 4.6 mm, 5 µm) were used to separate different glucose polymer chain length of

starch hydrolysate with the controlled temperature of 40°C. Mobile phase composition was consisted of acetonitrile (A) and 0.1% formic acid in water (B) and was conducted in the gradient mode: 0-3 min, 65% B; 3-15 min, 10% B; 15-20 min, 10% B; 20-20.1 min, 65% B and 20.1-25 min, 65% B with the flow rate of 0.7 mL/min. The quantification of different glucose polymer chain length was done using calibration curves obtained by injecting known amount of each glucose polymer chain length standard with the known retention times.

Culture of cell lines

NIH 3T3 cell line was obtained from ATCC (USA) and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum (FBS) in a humidified incubator with 5% carbon dioxide. The cell line was detached from culture flasks using a trypsin- Ethylenediaminetetraacetic acid (EDTA) solution (0.25-0.025%) and re-suspended as a single cell suspension in RPMI 1640 culture medium.

In vitro *wound scratch assay and microscopy evaluation*

NIH 3T3 cells were seeded in a tissue culture 6-well plate at an initial density of 2.4 x 10^5 cells/ cm^2 overnight. A micropipette tip was used to create a wound in the monolayer by scraping. A total of 10% (w/v) breadfruit starch hydrolysate was added in each treatment well with or without an addition of 100 ppm of various additives including *aloe vera*, curcumin, hydroxyproline, ascorbic acid, L-arginine, lactic acid and kojic acid, which were added separately into each well. Another two wells were treated with commercial wound dressing agent (MUL) and media only (control), respectively. Wound closure was observed by a phase-contrast microscopy (NIKON, Japan) and digital images were taken at the interval time of 3 h up to 24 h. The % of wound healing was calculated based on the length of wound measured at the specific time as described below:

$$\% \text{ wound healing} = \frac{\text{Length of wound (0 hour)} - \text{Length of wound (Y hours)}}{\text{Length of wound (0 hour)}} \times 100\%$$

whereby '0 hour' indicates the length of which the wound was initially created and 'Y hours' represents the length of the wound measured at the specific time.

Determination of NIH 3T3 cell viability via trypan blue cell count assay

Trypan blue cell count was carried out to identify the amount of viable cells present in each sample. Harvested cell suspension (10 µL) was added with equal volume of 0.4% trypan blue stain. Hemocytometer was used for cell counting under inverted light microscope (NIKON, Japan). Viable cells are those excluded from the stain.

5-bromo-2'-deoxyuridine (BrdU) ELISA cell proliferation assay

Starch hydrolysate treated and untreated NIH 3T3 cell proliferation was measured using the Bromodeoxyuridine (BrdU) Cell Proliferation Kit (Merck, USA). The cells were seeded in a 96 well plate at a concentration of 0.8 x 10^5 cells/ mL overnight. A total of 10% (w/v) breadfruit starch hydrolysate was added separately with or without the addition of 100 ppm of various additives, including aloe vera, curcumin and hydroxyproline and incubated for 24, 48 and 72 h, respectively at 37°C and 5% CO_2. Another two wells were treated with MUL and media only to serve as untreated control. After the corresponding period, BrdU label was added into all wells and incubated for another 24 h. At the respected incubation hours, the cells were fixed and incubated at 4°C for approximately 30 min. After that, the plates were washed twice before added with 100 µL detector antibodies into each well and incubated for 1 h. Then, 100 µL of Goat anti-mouse IgG-HRP conjugated was added and incubated for 30 min. Then, the plates were incubated with 100 µL of 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) substrate for another 30 min. Finally, 100 µL of stop solution (sulfuric acid) was added and the absorbance was measured at 450 nm, using an ELISA microplate reader (Biotech Instruments, USA).

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) cell viability assay on NIH 3T3 cells

NIH 3T3 cells were seeded on 96 well microtiter plates overnight. An amount of 10% breadfruit starch hydrolysate was added in each treatment well. A total of 100 ppm of various additives including aloe vera, curcumin and hydroxyproline was added separately into each well. Both MUL and media were acted as a control. The fibroblast cells treated with various samples was exposed to the culture medium up to 72 h. At each interval time of 24 h, a total of 20 µL/ well of MTT solution (Calbhiochem, USA) were added, followed by incubation at 37°C for a period of 4 h in an atmosphere of air with 5% CO_2. After that, supernatants were removed from the wells and 100 µL/well of dimethyl sulfoxide (DMSO) (Fisher, USA) were added to solubilize formazan. The absorbance was quantified at 570 nm using ELISA

Table 1. Glucose polymer profiling of breadfruit starch hydrolysate

Sample	DE Group	DP1 (%)	DP2 (%)	DP3 (%)	DP4 (%)	DP5 (%)	DP6 (%)	DP7 (%)	DP1-7 (%)	DP>7 (%)
BB	10 – 14	0.089±0.02^{a}	4.506±0.3^{a}	6.548±0.01^{a}	4.988±0.02^{a}	4.406±0.02^{a}	9.850±0.2^{a}	13.386±0.04^{b}	44.490±0.25^{a}	55.510±0.24^{c}
	15 – 19	1.385±0.01^{b}	7.071±0.08^{b}	8.745±0.18^{b}	6.318±0.1^{b}	5.560±0.15^{b}	12.758±0.2^{b}	14.615±0.3^{c}	56.450±0.7^{b}	43.550±0.07^{b}
	20 – 24	3.766±0.03^{c}	13.898±0.2^{c}	12.386±0.3^{c}	7.818±0.02^{c}	8.550±0.1^{c}	17.992±0.1^{c}	9.432±0.11^{a}	73.840±0.8^{c}	26.160±0.08^{a}

[a]Each data was expressed as mean ± standard deviation of triplicate determinations. Mean values with different superscripts in the same column are significantly different $p<0.01$. (Abbreviations: BB = breadfruit starch hydrolysate; DE=dextrose equivalent; DP1=Glucose; DP2 = Maltose; DP3= Maltotriose; DP4 = Maltotetraose; DP5, Maltopentaose; DP6 = Maltohexaose; DP7 = Maltoheptaose)

microplate reader (Biotech Instruments, USA).

Cell cycle analysis

The cells were seeded in a 6-well plate at a density of 2.4 x 10^5 cells/ mL and were grown overnight. Then, the cells were treated with 5% (w/v) breadfruit starch hydrolysate, with or without the addition of 100 ppm of various additives, including aloe vera, curcumin and hydroxyproline, which were added separately. Another two wells were treated with MUL or culture medium, which served as the controls. After 24 h, the cells were trypsinized and washed using phosphate-buffered saline (PBS). The cell pellets were fixed using 70% ethanol and were stored at -20°C. After a week, the fixed cells were washed with PBS, treated using a solution of RNase-A containing Triton-X and were stained using Propidium Iodide (PI). Finally, the cells were subjected to flow cytometry using a BD FACS Calibur instrument (Becton Dickinson, USA). All of the chemicals used in the cell-cycle analysis assay were purchased from Sigma (USA).

In vitro *red blood cell (RBC) irritation assay*

Approximately 2 mL of RBC obtained from a volunteer (Pusat Kesihatan, UPM) and was washed in PBS in a ratio of 1:10, followed by centrifuged for 10 min at 1500 rpm with a controlled temperature of 10°C and this step was repeated triplicate. The RBC was then diluted with PBS to a 1% concentration. Each 100 µL of breadfruit starch hydrolysate, COM starch hydrolysate and MUL were loaded into the first well in a 96 well-plate separately, followed by 50 µL of PBS loaded from the 2nd to the 12th well. A serial dilution was carried out from the 1st well to the 11th well and the 12th well was treated as the positive control. Then, a total of 50 µL of 1% RBC was added to each well before incubated at room temperature for 30 min. At the end of incubation, the suspension was centrifuged at 500 rpm at 10°C for 10 min.

Statistical analysis

Data was statistically analyzed by one-way analysis of variance (SPSS statistics version 16). Significant differences ($p<0.01$) between means were determined by Duncan's multiple range test.

Results and Discussion

Determination of glucose polymer profiling using liquid chromatography-mass spectrometry (LC-MS)

In this study, liquid chromatography-mass spectrometry with electrospray was used to identify the glucose polymer profile of starch hydrolysate. Oligosaccharides are not easily ionized, therefore, combination of high acetonitrile mobile phase with 0.1% of formic acid in water is required to achieve better mass ion peaks. The retention of oligosaccharides in high acetonitrile-containing mobile phase on polyamine-bonded polymeric gel column (apHeraTM NH_2) is based on the hydrogen bonding interaction between the hydroxyl groups of oligosaccharides and polyamine-bonded stationary phase. The gradient elution consisting of 100% acetonitrile and 0.1% formic acid in water with column temperature set at 40°C had successfully separated all the 7 mixtures of oligosaccharides standard (DP1 = glucose; DP2 = maltose; DP3 = maltotriose; DP4 = maltotetraose; DP5 = maltopentaose; DP6 = maltohexaose; DP7 = maltoheptaose). Table 1 shows the representative glucose polymer profiling of breadfruit starch hydrolysate from three different DE groups: a) DE 10-14; b) DE 15-19 and c) DE 20-24. The DE value is inversely related to molecular weight. It is an indicator of the degree of starch hydrolysis (Sun *et al*., 2010). Based on the data obtained, starch hydrolysate DE 20-24 contained the highest amount of smaller molecular structure of oligosaccharides ranging from DP 1 to DP 7, indicating the least amount of longer glucose chain length (DP > 7). DP

Table 2. The percentage of wound closure by NIH 3T3 cells treated with 10% breadfruit starch hydrolysate (BB) DE 10-14, DE 15-19 or DE 20-24 with or without the addition of 100 ppm of various additives

TIME, h	BB	BBCUR	BBHYD	BBAA	BBARG	BBLA	BBKA	MUL	CTRL
i). The percentage wound closure of NIH 3T3 cell treated with 10% BB (DE 10-14) with 100 ppm of various additives									
0	←				$0.00\pm0.00^{A}{}_{a}$				→
6	$37.34\pm2.80^{D}{}_{c}$	$30.27\pm0.00^{B}{}_{c}$	$27.78\pm0.00^{A}{}_{b}$	$32.26\pm0.00^{C}{}_{c}$	$32.65\pm3.12^{C}{}_{c}$	$30.16\pm0.00^{B}{}_{c}$	$22.34\pm3.47^{AB}{}_{bc}$		$27.19\pm7.59^{A}{}_{c}$
12	$65.19\pm4.74^{D}{}_{e}$	$55.26\pm4.49^{B}{}_{e}$	$62.96\pm0.00^{BC}{}_{d}$	$54.84\pm0.00^{CD}{}_{e}$	$53.06\pm1.84^{B}{}_{d}$	$52.38\pm3.72^{AB}{}_{e}$	$46.50\pm6.45^{A}{}_{d}$	$0.00\pm0.00^{A}{}_{a}$	$42.42\pm3.03^{AB}{}_{d}$
24	$100.00\pm0.00^{A}{}_{f}$	$100.00\pm0.00^{A}{}_{f}$	$100.00\pm0.00^{A}{}_{e}$	$100.00\pm0.00^{A}{}_{f}$	$100.00\pm0.00^{A}{}_{e}$	$100.00\pm0.00^{A}{}_{f}$	$100.00\pm0.00^{A}{}_{e}$		$100.00\pm0.00^{A}{}_{d}$
ii).The percentage wound closure of NIH 3T3 cell treated with 10% BB (DE 15-19) with 100 ppm of various additives									
6	$32.69\pm0.00^{A}{}_{c}$	$29.82\pm1.51^{A}{}_{c}$	$28.95\pm5.50^{A}{}_{c}$	$23.33\pm0.00^{A}{}_{c}$	$31.78\pm0.00^{A}{}_{c}$	$28.09\pm0.00^{A}{}_{c}$	$21.66\pm0.00^{A}{}_{c}$		$27.19\pm7.59^{A}{}_{c}$
12	$61.13\pm0.00^{BC}{}_{e}$	$52.97\pm0.00^{C}{}_{e}$	$51.52\pm5.49^{BC}{}_{d}$	$47.78\pm0.00^{D}{}_{e}$	$47.66\pm0.00^{BC}{}_{e}$	$44.94\pm0.00^{C}{}_{e}$	$32.98\pm0.00^{B}{}_{e}$	$0.00\pm0.00^{A}{}_{a}$	$42.42\pm3.03^{AB}{}_{d}$
24	$100.00\pm0.00^{A}{}_{f}$	$100.00\pm0.00^{A}{}_{f}$	$100.00\pm0.00^{A}{}_{e}$	$100.00\pm0.00^{A}{}_{f}$	$100.00\pm0.00^{A}{}_{f}$	$100.00\pm0.00^{A}{}_{f}$	$100.00\pm0.00^{A}{}_{f}$		$100.00\pm0.00^{A}{}_{d}$
iii).The percentage wound closure of NIH 3T3 cell treated with 10% BB (DE 20-24) with 100 ppm of various additives									
6	$37.34\pm4.75^{F}{}_{c}$	$30.27\pm2.98^{EF}{}_{c}$	$28.95\pm0.00^{CD}{}_{c}$	$23.33\pm0.00^{B}{}_{b}$	$18.42\pm6.18^{B}{}_{ab}$	$14.00\pm0.00^{A}{}_{ab}$	$18.42\pm0.00^{B}{}_{b}$		$27.19\pm7.59^{A}{}_{c}$
12	$65.19\pm5.98^{E}{}_{e}$	$52.97\pm2.01^{DE}{}_{e}$	$44.74\pm0.00^{CD}{}_{d}$	$47.78\pm4.12^{CD}{}_{d}$	$36.84\pm13.20^{BC}{}_{b}$	$28.00\pm8.24^{AB}{}_{c}$	$18.42\pm0.00^{A}{}_{b}$	$0.00\pm0.00^{A}{}_{a}$	$42.42\pm3.03^{AB}{}_{d}$
24	$86.08\pm3.11^{D}{}_{f}$	$70.27\pm4.91^{C}{}_{f}$	$64.47\pm0.00^{B}{}_{d}$	$56.67\pm0.00^{B}{}_{d}$	$55.26\pm3.64^{C}{}_{c}$	$33.00\pm8.25^{AB}{}_{d}$	$39.47\pm0.00^{A}{}_{c}$		$100.00\pm0.00^{A}{}_{d}$

[a]Each data was expressed as mean ± standard deviation of triplicate determinations. Mean values with different superscripts in the same column are significantly different $p<0.01$. Mean values with different superscripts in the same row are significantly different $p<0.01$

(Abbreviations: BB: Breadfruit starch hydrolysate; CUR: Curcumin; HYD: Hydroxyproline; AA: Ascorbic acid; ARG: L-Arginine; LA: Lactic acid; KA: Kojic acid; MUL; commercial wound dressing agent; CTRL: Control)

1-7 was the total oligosaccharides with smaller chain length of sugar molecules (from glucose DP1 up to maltoheptoase DP7), while DP > 7 was the glucose molecule chain length longer than maltoheptaose. The characteristics of breafruit starch hydrolysate DE 20-24 resulted in a more viscous solution as it contained higher sugar composition compared to starch hydrolysate DE 10-14 and 15-19. Based on Dokic *et al*. (2004) study, the viscosity increases with the increasing DE value of the starch hydrolysate, because the percentage of saccharides with lower degree of polymerization in the mixture is higher. The difference in sugar composition between starch hydrolysate with different DE ranges (DE 10-14, DE 15-19 and DE 20-24) is believed to have caused an effect on their role in the wound healing of NIH 3T3 cells process. Because of that, a wound healing study was conducted to analyze the NIH 3T3 wounded cell migratory rate in *in vitro* wound healing study using different grades of breadfruit starch hydrolysate DE to proof this statement.

Evaluation of different DE grades of breadfruit starch hydrolysate effect on wound closure rate via in vitro *wound healing of NIH 3T3 cells assay*

In vitro wounding of a confluent monolayer initiates cells to start migrating and proliferating at the edge of the wounds (Schreier *et al*., 1993). Table 2 shows the results of *in vitro* wound healing of NIH 3T3 cells treated with breadfruit starch hydrolysate DE 10-14, 15-19 and 20-24 with or without the addition of 100 ppm selected additives. The selected additives including curcumin, hydroxyproline, ascorbic acid, L-arginine, lactic acid and kojic acid were chosen based on previous reports of their significant wound healing properties (Choi *et al*., 2006; Panchatcharam *et al*., 2006; Debats *et al*., 2009; Hansen *et al*., 2009; Wu *et al*., 2011; Hussein *et al*., 2012). The use of *aloe vera* in wound healing is being considered in many studies. *Aloe vera* is known to contain anthraquinones, saccharides, vitamin E and C, zinc, enzymes, acetyl salicyclic and others (Reddy *et al*., 2011). A major carbohydrate fraction obtained from the *aloe vera* leaf is called, acemannan, which is the key promoter of wound healing (Reddy *et al*., 2011). A study conducted by Maenthaisong *et al*. (2007) reported that burn healings were 8.79 days faster than those in the control group when treated with topical *aloe vera*. Therefore, the proliferation effect of breadfruit starch hydrolysate with addition of 100 ppm aloe vera on wounded NIH 3T3 cells was examined in the proceeding analysis. According to Schreier *et al*. (1993), compared with that of cells in the absence of the additive, the addition of an additive (platelet-derived growth factor) notably increased the rate of cell migration to the 'wounded' area. MUL, one of the commonly utilized commercial wound dressings was used as a negative control.

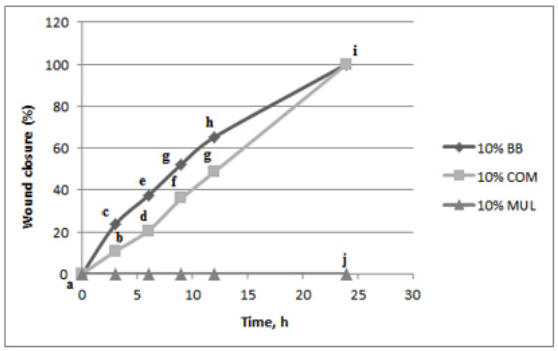
Based on the results obtained in Table 2, breadfruit starch hydrolysate with a DE range of 10-14 possessed significantly ($p<0.01$) the highest rate of wound healing in relative to breadfruit starch hydrolysate DE 15-19 and DE 20-24 ranges. As starch hydrolysate is a product of low hydrolysis degree, it

still contains high percentage of highly polymerized saccharides (Dokic *et al*., 2004). The DE value of starch hydrolysate will affect the viscosity, sugar composition and its characteristics (Moore *et al*., 2005). According to a study conducted by Dokic *et al*. (2004) to determine the characteristics of different DE of starch hydrolysate, it was shown that the viscosity of starch hydrolysis increases with the increment of DE value, because the percentage of saccharides with low degree of polymerization in the mixture is higher. The latter statement explains the poor performance of breadfruit starch hydrolysate in both DE 15-19 and DE 20-24 treatments. The high DE values (DE 15-19 and 20-24) of breadfruit starch hydrolysate exhibited viscous and contain more concentrated of smaller molecular structure sugar which reduced the ability of cell migration and thus decreased the ability of the starch hydrolysate to diffuse into the cell monolayer (Swaim *et al*., 1998). Breadfruit starch hydrolysate with a lower DE value is preferable to facilitate the exposure of dermatological agents added to improve the healing and facilitates the contact to all areas of the wound (Silvetti *et al*., 1993).

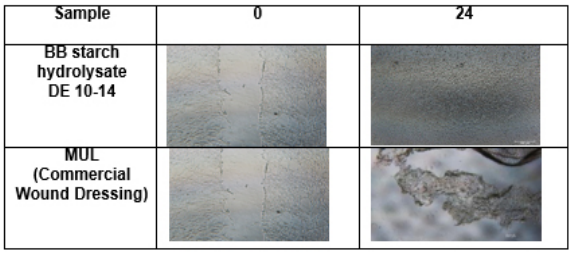
The addition of 100 ppm additives were seen to improve the percentage of wound closure compared to media alone. Nevertheless, breadfruit starch hydrolysate DE 10-14 alone had significantly shown the best recovery rate ($p<0.01$) in wound healing compared to other treatments with addition of various additives. The purpose of adding various types of additives is only meant to compare their healing effects with our breadfruit starch hydrolysate. Based on our findings, breadfruit starch hydrolysate DE 10-14 possessed the best criteria as a wound healing agent and confirmed its effectiveness to facilitate the wound healing process of NIH 3T3 cells.

To further investigate the wound healing activity of breadfruit starch hydrolysate on NIH 3T3 cell wounds, a commercial (COM) starch hydrolysate obtained from cassava was studied under the same conditions. Cassava base was selected because it is a high quality starch hydrolysate that is widely used in food and pharmaceutical applications. A comparative study of breadfruit and cassava starches by Koh *et al*. (2012) demonstrated differences in their physico-chemical properties that would affect the functional properties of the starch hydrolysates produced from the hydrolysis of different starchy sources.

Figure 1a shows the wound healing performance of breadfruit starch hydrolysate, commercial starch hydrolysate (COM) and commercial dressing agent (MUL). The graph shows that the percentage of wound closure by NIH 3T3 cells treated with breadfruit starch hydrolysate was the highest,



(a)

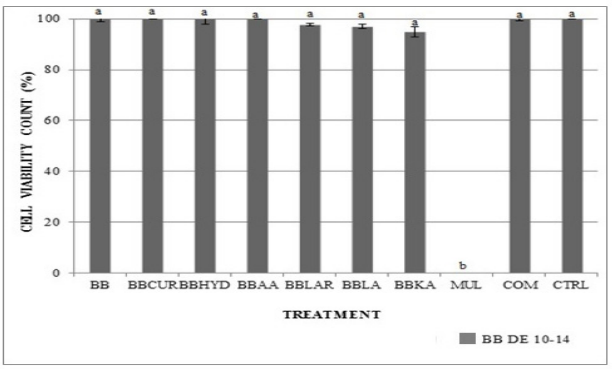


(b)

[a]The values were expressed as the mean values ± standard deviation of triplicate determinations. The mean values indicated using different superscript letters were significantly different at $p<0.01$
(Abbreviations: BB: breadfruit starch hydrolysate; COM: commercial starch hydrolysate; MUL: commercial wound dressing)

Figure 1. Results of the *in vitro* scratch assays of NIH 3T3 fibroblasts treated using starch hydrolysates obtained from different sources: a) % wound closure; b) image of wound closure between BB starch hydrolysate DE 10-14 and MUL sample at 0 hour and 24 hours

followed by COM starch hydrolysate. Whereas, the commercial dressing agent (MUL) had caused death to the NIH 3T3 cell (Figure 1b). Although breadfruit and COM starch hydrolysate shared similar range of DE values (10-14), the time necessary for wound closure significantly varied ($p<0.01$) depending on the source of the starch hydrolysate. Our finding is consistent with the result described earlier showing that starch hydrolysate with the same range of DE values obtained from different starchy sources have different functional and physico-chemical properties that are highly dependent on the starch molecular structure (Moore *et al*., 2005; Sun *et al*., 2010). As reported herein, breadfruit starch hydrolysate stimulated the proliferation of NIH 3T3 cells significantly better ($p<0.01$) than did COM starch hydrolysate. One of the possible explanations for the less efficient performance of COM starch hydrolysate in wound healing relative to breadfruit starch hydrolysate is its size. According to Koh *et al*. (2012), as shown using microscopy, cassava starch exhibited the largest molecule size compared



[a]The values were expressed as the mean values ± standard deviation of triplicate determinations. The mean values indicated using different superscript letters were significantly different at $p<0.01$

(Abbreviations: BB: breadfruit starch hydrolysate; CUR: curcumin; HYD: hydroxyproline; AA: ascorbic acid; LAR: L-arginine; LA: lactic acid; KA: kojic acid; MUL: commercial wound dressing; COM: commercial starch hydrolysate; CTRL: control)

Figure 2. The percentage of viable cells treated breadfruit starch hydrolysate (DE 10-14) with 100 ppm of various additives

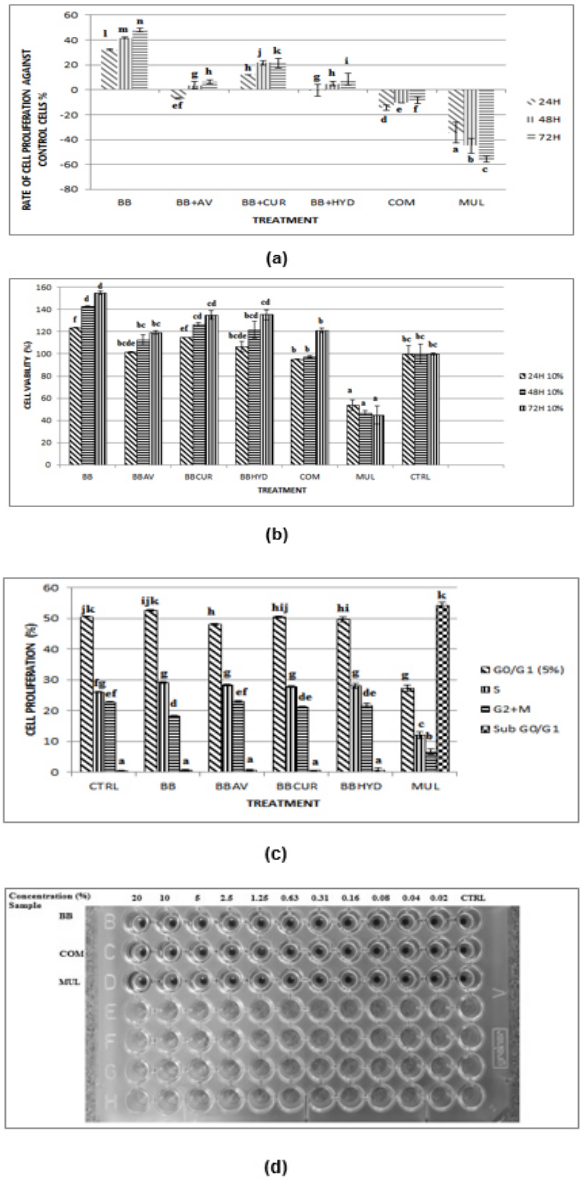
with that of breadfruit starches. The larger size of COM starch hydrolysate molecules may have led to a poor nutrient distribution to the NIH 3T3 cells, thus restricting the rate of migration. Overall, breadfruit starch hydrolysates were clearly shown to positively affect wound healing, substantiating their utility as wound healing agents. In this study, we demonstrated that breadfruit starch hydrolysate performed significantly better ($p<0.01$) than COM starch hydrolysate as a wound healing agent.

Based on the positive results obtained from breadfruit starch hydrolysate with low DE range (DE 10-14) compared to the high DE starch hydrolysates (DE 15-19 and 20-24), several assays were conducted on breadfruit starch hydrolysate DE 10-14 to further analyze its efficacy on NIH 3T3 cell proliferation and viability.

Determination of NIH 3T3 cell viability via trypan blue cell count assay

Determination of cell viability is a well-recognized requirement of most biological investigations using cellular preparations (Medzihradsky *et al*., 1975). Trypan blue is one of the most widely applied methods used to demonstrate cell viability in the exclusion of dyes (Medzihradsky *et al*., 1975). Trypan blue is a vital dye containing a negatively charged chromopore that enter cells when the plasma membrane is damaged (Freshney *et al*., 1987). Therefore, cells that exclude this dye are considered viable.

Figure 2 shows the results of the trypan blue viability assay of NIH 3T3 cells treated with breadfruit starch hydrolysate (DE 10-14) that was



[a]The values were expressed as the mean values ± standard deviation of triplicate determinations. The mean values indicated using different superscript letters were significantly different at $p<0.01$

(Abbreviations: BB: breadfruit starch hydrolysate; AV: *Aloe vera* extract; CUR: curcumin; HYD: hydroxyproline; COM: commercial maltodextrin; MUL: commercial wound dressing)

Figure 3. Results of: a) BrdU-based cell proliferation assay; b) MTT-based cell viability assay; c) cell-cycle analysis of various treatment group on NIH 3T3 fibroblast cell; d) *In vitro* red blood cell hemolysis assay conducted using various starch hydrolysates

conducted to determine the percentage of viable cells present during the *in vitro* wound healing study. The results of the trypan blue viability assay were consistent with the percentage of wound closure obtained as discussed earlier. It was found that a higher percentage of viable cells supported the treatments that showed better improvement in the wound closure rate.

Based on the counts of viable cells, 100% of all starch hydrolysate-treated populations were viable, except for those treated with additives such as lactic acid, L-arginine and kojic acid (BBLA, BBLAR and BBKA) respectively which only exhibited 93-95% cell viability count. These findings indicated that the presence of lactic acid, L-arginine and kojic acid affected the viability of NIH 3T3 fibroblasts. Although breadfruit starch hydrolysate alone were found to improve wound healing more than did these substances in the presence of additives, most of the cells treated with additives maintained 100% cell viability. It was found that cells treated with MUL, a commercial wound dressing were killed (Figure 1b & 2). This finding indicated that the presence of MUL most likely had caused substantial cellular damage over time, leading to the death of the cells (Guenoun *et al*., 2005). Generally, the results of the trypan blue viability assay had supported the wound healing capability of breadfruit starch hydrolysate with a DE value of 10-14, as shown by the percentage of wound closure in NIH 3T3 cells.

BrdU ELISA cell proliferation assay

A BrdU-based cell proliferation ELISA was used to evaluate the effect of starch hydrolysate on the proliferation of NIH 3T3 fibroblast cells *in vitro*. Cultured cells that entered the log growth phase were pulsed labeled using non-radioactive BrdU (Klein *et al*., 2000). The results of the BrdU-based cell proliferation assay of NIH 3T3 cells treated with breadfruit starch hydrolysate with or without additives are shown in Figure 3(a). Generally, the proliferation rate of NIH 3T3 cells treated with breadfruit starch hydrolysate was noted increased gradually across incubation time compared to the controlled cells, except for COM and MUL treatment, whereby the percentage of cell proliferation of both COM and MUL treatment decreased significantly ($p<0.01$) from 24 to 72 h. The drastic decrease in cells treated with MUL when compared to the controlled cells is supported by the study of Guenoun *et al*. (2005); the preservatives presents in MUL may have contributed to its cytotoxic effect on NIH 3T3 cells.

Overall, all of the NIH 3T3 fibroblast cells (except those treated with COM and MUL) achieved the highest rate of cell proliferation after 72 h of incubation. The increase in the percentage of proliferating fibroblasts may be attributed to the stimulatory effect of starch hydrolysate with DE value of 10-14. This conclusion was reached by Coolen *et al*. (2010) in their comparative study using a tissue model to determine the best wound healing performance by observing the most effective tissue model through increase in the rate of fibroblast proliferation via BrdU incorporation.

Cells treated with breadfruit starch hydrolysate with or without additives showed a higher rate of proliferation than the control group. In general, the highest rate of cell proliferation at 72 h was achieved by treatment with breadfruit starch hydrolysate, which was 48.21% higher in proliferation compared to the control group, followed by breadfruit starch hydrolysate with 100 ppm curcumin (21.26%), breadfruit starch hydrolysate with 100 ppm hydroxyproline (8.49%) and breadfruit starch hydrolysate with 100 ppm *aloe vera* extract (6.47%). Compared with the effect of COM starch hydrolysate obtained from cassava, our newly developed breadfruit-derived starch hydrolysate was found capable to promote higher cell proliferation rate, confirming the other findings that indicated their superior functional properties for wound healing applications. In brief, the percentage of cells proliferating treated with breadfruit starch hydrolysate alone was significantly ($p<0.01$) higher than that observed using others treatments. The higher proliferation rate of NIH 3T3 cells treated with the starch hydrolysates may be attributed to their glucose content, which supplied energy for the metabolism and proliferation of cells (Swaim *et al*., 1998). These findings indicated that wounded NIH 3T3 cells treated with starch hydrolysate received enough nutrient supply, effectively improved their cell proliferation rate compared with that of the untreated cells.

MTT cell viability assay on NIH 3T3 cells

A MTT assay was performed to evaluate the viability of NIH 3T3 cells treated with breadfruit starch hydrolysate. This test involves the conversion of a tetrazolium salt, 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide (MTT) to an insoluble formazan product, which is quantitated using spectrophotometry (Klein *et al*., 2000). Figure 3(b) presents the result of NIH 3T3 cell viability assay treated with breadfruit starch hydrolysate at 10% of concentration, with or without the addition of 100 ppm of additives. Generally, the viability rates of cells treated with breadfruit starch hydrolysate at concentration of 10% was observed significantly increased ($p<0.01$) with the treatment period. The increase in the cell viability rate indicated that the cells remained viable even after treatments of up to 72 h. The same phenomenon observed in the BrdU-based proliferation assay, the results of the MTT-based viability assay showed a significant decreased ($p<0.01$) in the percentage of viable cells in the

MUL-treated group at 24, 48 and 72 h of exposure. The toxicity effect of 72 h treatment with 10% MUL decreased the percentage of viable cells from 53.85% to 44.83%.

Briefly, NIH 3T3 cell treated with 10% breadfruit starch hydrolysate were found to have the highest percentage of viable cells at 72 h of exposure (154.79%), followed by breadfruit starch hydrolysate with 100 ppm hydroxyproline (135.21%), breadfruit starch hydrolysate with 100 ppm curcumin (135.11%), COM starch hydrolysate (120.96%) and breadfruit starch hydrolysate with 100 ppm *aloe vera* extract (119.36%). Similar to the findings obtained using the BrdU-based cell proliferation assays, treatment with breadfruit starch hydrolysate alone resulted in the higher cell viability compared with that observed using the other treatments.

Cell cycle analysis using flow cytometry

Based on the above described results of the wound healing and cell proliferation studies, treatment with commercial wound dressing (MUL) was found failed to promote the healing of wounded NIH 3T3 cells. It was important to identify whether the concentration of MUL used was the main factor causing the death of these cells. Therefore, flow cytometric cell cycle analysis was conducted to evaluate the effect of 5% breadfruit starch hydrolysate with or without 100 ppm of additives relative to that of same concentration of MUL on the cell cycle progression of NIH 3T3 cells. Figures 3(c) shows the cell cycle distribution of NIH 3T3 cells treated with 5% of both MUL and breadfruit starch hydrolysate respectively. The results showed that NIH 3T3 cells treated with MUL, even at a very low concentration, exhibited a drastic increased in the sub G0/G1 population, indicating that MUL induced DNA fragmentation (Guenoun *et al*., 2005). The sub G0/G1 population was 54.40%, while the population was reduced in the S and G2/M phases, at 5% concentration of MUL. In contrast, 5% breadfruit starch hydrolysate with or without additives increased the cell population which is undergoing DNA synthesis. This finding confirmed that prolonged exposure to MUL was toxic to the cells, consistent with the earlier findings in MTT cell viability assay that confirmed that MUL was not a good *in vitro* wound healing agent, as demonstrated using the *in vitro* wound healing model.

In vitro *red blood cells (RBCs) irritation assay*

The development of a number of *in vitro* methods to reduce the use of animals were reviewed recently due to the criticism regarding the use of classical *in vivo* methods to determine whether a substance causes skin or ocular irritation (Benavides *et al*., 2004). An *in vitro* irritation assay employing RBCs is a good alternative to the in vivo eye-irritation test because it is an inexpensive, rapid method that provides reliable results with good reproducibility and reduces or avoids the use of experimental animals (Lagarto *et al*., 2006). The tested sample is assumed not to irritate cells and thus confirm to be safe for human use on skin if the RBCs remain intact and maintain their normal shape. Lysis of the RBCs indicates that the sample at the tested concentration had an irritation effects.

In this study, a RBCs hemolysis assay was conducted using various concentrations of BB starch hydrolysate, COM starch hydrolysate and MUL. Wells containing either PBS or RBCs served as positive controls. When the starch hydrolysate was added to erythrocytes suspended in an aqueous medium, the molecules were distributed between the erythrocyte membranes and the solution by absorption until an equilibrium was reached (Mitjans *et al*., 2003). Hemolysis most likely began when the erythrocyte membranes were saturated with the treatment molecules (Mitjans *et al*., 2003). Based on the results in figure 3(d), none of treatments had hemolytic activity. This finding demonstrated that the starch hydrolysate were safe for topical use and would not irritate the human skin. MUL also did not cause RBC lysis although it caused cell death in the *in vitro* wound healing assay. The presence of the preservatives in MUL reduced the viability of NIH 3T3 cells. This finding showed that the preservatives in MUL are safe to use on human skin but exhibit not safe for oral consumption or injection onto the skin. In summary, BB starch hydrolysate is confirmed to be safe and have shown no hemolytic effect on RBCs even at a high concentration.

Conclusion

Breadfruit starch hydrolysate with low DE values (DE 10-14) did improve the rate of wound closure better than their high DE counterparts (DE 15-19 & DE 20-24), as demonstrated in an *in vitro* wound healing model. Interestingly, treatments with breadfruit starch hydrolysate of low DE values in the absence of additives resulted in the best rate of wound healing, as shown in the wound scratch assay, the trypan blue assay, the MTT cell viability assay, BrdU-based cell proliferation assay and the cell cycle analysis when compared with those treatments in the presence of additives. The breadfruit starch hydrolysate exhibited higher healing efficacy compared with that of COM starch hydrolysate, as

confirmed in the wound healing, MTT-based cell viability BrdU-based cell proliferation assays and cell cycle analysis. Most importantly, breadfruit starch hydrolysate did not induce any irritation effect as demonstrated in the RBCs assay. In conclusion, breadfruit starch hydrolysate is shown to have a positive effect on the rate of *in vitro* wound closure and could play a beneficial role in wound healing application.

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