Jurnal Teknologi

Full Paper

EFFECT OF *MANIHOT ESCULENTA* AQUEOUS EXTRACT AND THERAPEUTIC ULTRASOUND IN ACCELERATING THE WOUND HEALING PROCESS *IN VITRO*

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Article history
Received
9 September 2018
Received in revised form
18 March 2019
Accepted
14 May 2019
Published online
25 June 2019

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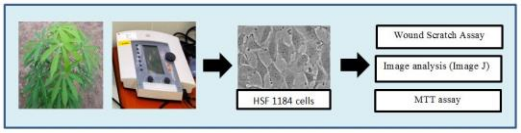
Graphical abstract



Abstract

The aim of this research is to investigate the wound healing process in *in vitro* by combining the *Manihot esculenta* aqueous extract and therapeutic ultrasound. Firstly, the optimization seeding densities of HSF cell 1184 in six-well plate, and then followed by the scratch assay experiment. The scratched that made was treated with the remedial treatments (*Manihot esculenta* aqueous extract only; ascorbic acid+ therapeutic ultrasound; *Manihot esculenta* aqueous extract+ ascorbic acid; *Manihot esculenta* aqueous extract+ therapeutic ultrasound and also the combination of these three materials). The rate of wound closure was observed and analysed at a time interval of 0, 2, 4, 6, 8, 10 and 24 h by using image J software. Then, the cells viability were analysed using the MTT assay. The result showed that *Manihot esculenta* aqueous extract coupled with specific dose therapeutic ultrasound represents a significantly high rate of wound closure at 96.10 % with the cell numbers at 5.44×10^5 cells/mL when compared to the other combination therapy. The finding of this study revealed that *Manihot esculenta* aqueous extract 200 µg/mL and the therapeutic ultrasound specific dose (3 MHz, 300 mWatt/cm^2, 50% in 5 min) have the potential in accelerating wound healing process of cells in *in vitro*.

Keywords: Wound healing, *Manihot esculenta*, therapeutic ultrasound, scratch assay, MTT assay

Abstrak

Tujuan kajian ini adalah untuk menyiasat proses penyembuhan luka *in vitro* dengan menggabungkan ekstrak akueus *Manihot esculenta* dengan terapeutik ultrabunyi. Pertama, pembenihan sel HSF1184 yang dioptimumkan dalam enam plat dan kemudian diikuti oleh percubaan gores. Goresan yang dibuat telah dirawat dengan semua rawatan pemulihan (*Manihot esculenta* ekstrak akueus sahaja; askorbik asid + terapeutik ultrabunyi; *Manihot esculenta* ekstrak akueus + askorbik asid; *Manihot esculenta* ekstrak akueus + terapeutik ultrabunyi dan juga gabungan ketiga bahan). Penutupan luka telah diperhatikan dan dianalisis pada selang waktu 0, 2, 4, 6, 8, 10 dan 24 jam dengan menggunakan perisian imej J. Kemudian, daya maju sel telah dianalisis dengan menggunakan uji MTT. Hasilnya menunjukkan bahawa dengan menggabungkan ekstrak akueus *Manihot esculenta* dengan dos khusus terapi ultrabunyi mempersembahkan kadar penutupan luka paling tinggi pada 96.10% dengan nombor sel pada 5.44×10^5 sel / mL berbanding dengan rawatan pemulihan yang lain dalam kajian ini. Dapatan kajian ini menunjukkan bahawa *Manihot esculenta* ekstrak akueus 200 µg/mL

dengan dos khusus terapeutik ultrabunyi (3 MHz, 300 mWatt / cm^2, 50% dalam 5 minit) untuk mempercepat proses penyembuhan luka sel dalam in vitro.

Kata kunci: Penyembuhan luka, *Manihot esculenta*, Terapeutik ultrabunyi, Uji Gores, Uji MTT

1.0 INTRODUCTION

Chronic wound is a wound which does not heal in an orderly phase of wound healing (haemostasis, inflammatory, proliferative and remodelling phase). While venous leg ulcers, diabetic foot ulcers, and pressure ulcers are some of the examples of chronic wound disease [1, 2]. Currently, compression therapy, antibiotic, antibacterial, irrigation and debridement are commonly used to enhance the healing of these chronic wounds [3, 4]. Compression therapy increased blood flow towards the heart and reduce venous reflux to heal venous leg ulcers [5]. According to Tan *et al*. (2007), therapeutic ultrasound at the lower frequencies had improved in wound healing process for venous ulcers when compared to compression therapy treatment [6]. Therapeutic ultrasound has advantages at the first and second phases (inflammatory and proliferative phase) of wound healing process for diabetic foot ulcers; it prevented patients with diabetes to undergo amputation [7].

Therapeutic ultrasound is secure and a beneficial treatment to combine with the biochemical therapy such as plant extract in order to heal the chronic wounds [8]. Nevertheless, to our best of knowledge the combination of therapeutic ultrasound with the plant extract has not been proven scientifically in accelerating the wound healing in *in vitro*. The medicinal plant that traditionally used for wound healing are such as Aloe vera, Carica papaya, Curcuma longa, Moringa oleifera Lam and Zingiber officinale [9, 10]. Cassava (*Manihot esculenta*) leaves is believed by developing country such as Indonesia to be used for the wound healing process [11, 12]. *Manihot esculenta* have many beneficial from roots, stem to leaves and its leaves consist of protein, vitamin C (ascorbic acid), flavonoid, saponin, tannin and triterpenoid which is higher than other vegetables [11, 12, 13]. Furthermore, ascorbic acid has role in accelerating the wound healing process [14]. Concentration 0.06 mM of ascorbic acid was studied to stimulate proliferation of human skin fibroblast cells, provoke collagen synthesis (as cofactor for prolyl hydroxylase) and its deposition in the extracellular matrix [15]. Thus, it used for positive control in this research.

According to Meilawaty (2013), *Manihot esculenta* leaves extract have a role in accelerating wound healing by reducing the inflammation [16]. It has potential in decreasing the neutrophil cells during the wound healing process in *in vivo* [12]. In related to that, therapeutic ultrasound play a role in first phase of wound healing process (inflammatory phase) in the degranulation of mast cells [2]. In our study, *Manihot esculenta* aqueous extract and therapeutic ultrasound were introduced to identify their capabilities and potential to accelerate the wound healing process rather than single treatment alone.

2.0 METHODOLOGY

2.1 Materials

DMEM (Dulbecco's Modified Eagle's Medium) were purchased from the Interscience Company (Shah Alam, Selangor Darul Ehsan, Malaysia). Trypsin, Trypsin-EDTA and Fetal Bovine Serum (FBS) standard were acquired from Biowest Company (Puchong, Selangor, Malaysia). Penicillin-Streptomycin (Pen/Strep), Ascorbic acid, MTT (Thiazolyl Blue Tetrazolium Bromide) powder and Trypan blue solution were purchased from Sigma-Aldrich Group (Subang Jaya, Selangor, Malaysia).

2.2 Preparation of *Manihot esculenta* Aqueous Extract

The leaves of *Manihot esculenta* were plucked, washed, and cut into pieces before dried under shade condition at room temperature for 6 to 7 days [11]. Direct exposure of sunlight would cause destruction of the bioactive compound contained in the leaves extract [17]. The dried leaves of *Manihot esculenta* were then ground into powder form [18] and soaked into deionized water at room temperature for 24 h in ratio of 1:10 (w/v) [19-22]. After that, it was filtered and sent for evaporation using the EYELA N-1000 rotary evaporator (Tokyo Riakakikai Co., Tokyo) [23]. The gummy extract obtained was freezed dried (Beta 2-4 LD plus LT, Martin Chris, Germany) in order to remove the remaining solvent. *Manihot esculenta* aqueous extract was then kept at 4°C prior to use [24].

2.3 Optimizing Cells Seeding Density

Human Skin Fibroblast (HSF) 1184 cells were seeded in six-well plate containing DMEM complete medium with

a range 1×10^5 cells/mL to 6×10^5 cells/mL densities in order to ensure the optimum cell seeding density [25]. Their confluency (80-90%) was observed under the inverted microscope (Nikon Eclipse Ti-S microscope with Q-imaging Retiga 2000R camera) for 24 h before it will be further used in this study.

2.4 MTT Cytotoxicity Assay of *Manihot esculenta*

Manihot esculenta aqueous extract with different concentrations ranging from 1.95 µg/mL to 500 µg/mL was prepared. It was tested for *in vitro* cytotoxicity, using HSF 1184 cell lines. 20 µL of MTT solution was added in each well after 72 h [26]. After that, the solution in each well was discarded and MTT dissolving buffer was added into each well. The absorbance was read at 577 nm by using Promega GloMax Multi Detection System (Interscience Company, Shah Alam, Selangor Darul Ehsan, Malaysia).

2.5 Wound Scratch Assay

The scratch assay is known as an appropriate and economical tool to observe cell movement in *in vitro* [27]. By using a fine tip marker, a straight line at the bottom of six well plates was made as the guidance line for scratch assay. Then, fibroblast cells were seeded into a six-well plate and allowed to attach, spread, and grow to confluence in six-well plates [28]. After forming a confluent monolayer in 24 h, a straight line was created by using a sterile yellow pipette tip across each the well of plates [29]. Wound scratch assay was done softly and at a low speed at the centre of well plates in straight line in one way [27].

2.5.1 *Manihot esculenta* Aqueous Extract Treatment

The old medium in each well was sucked out and washed gently with the PBS twice to remove the detached cells [30]. Then, the fresh medium and *Manihot esculenta* aqueous extract 200 µg/mL was added into the treatment groups well while the control group was kept with the same condition as the treatment groups without any *Manihot esculenta* aqueous extract treatment. Both groups were run in triplicates.

2.5.2 Therapeutic Ultrasound Exposure

The Sonopuls 492 (JH Enraf-Nonius, Subang Jaya, Selangor Darul Ehsan, Malaysia) was used in this study. The exposure of therapeutic ultrasound was set up at 3 MHz, intensity 0.3 Watt/cm2 and pulse 50 % for 5 min [31]. Frequency of 3 MHz was used for superficial lesions at depths less than 2 cm wherein fibroblast cells located [32, 33, 34]. The six-well plate with fibroblast cells in DMEM medium was located on top of ultrasound transducer.

2.5.3 Ascorbic Acid Treatment

Treatment with the ascorbic acid (positive control drug) was done. Ascorbic acid with 0.4 mg dissolved in 5 mL sterile water. The deionized water was sterilized through a 0.2 µm nylon filter Whatmann [35]. Subsequently, ascorbic acid solution was diluted to the concentration of 10 µg/mL. Concentration 0.06 mM of ascorbic acid was reported to stimulate proliferation of human skin fibroblast cells [36]. Lastly, ascorbic acid solution was added to each treatment well.

2.6 Image Analysis

The scratch images were taken using the camera (Q-imaging Retiga 2000R camera, Canada) attached to the inverted microscope (Nikon Eclipse Ti-S microscope, USA) after the cells were treated with the remedial treatments (*Manihot esculenta* aqueous extract alone; ascorbic acid+ therapeutic ultrasound; *Manihot esculenta* aqueous extract+ ascorbic acid; *Manihot esculenta* aqueous extract+ therapeutic ultrasound; and also the combination of these three materials) at the specific time interval (0, 2, 4, 6, 8, 10 and 24 h). The images were then analysed by using Image J software [37].

2.7 MTT Assay

The (3-(4,5-dimethylthiazol-2-yl) -2, 5-diphenyl tetrazolium bromide) (MTT) assay is a compatible and low-cost method for determining living cell number by forming dissolved blue formazan as mitochondrial enzyme activities in *in vitro* experiments [38].

A standard curve experiment was done to define the total cell number for MTT scratch assay by using seeding density of 1×10^5 to 6×10^5 cells/mL. According to Suzuki *et al*. (2011), standard curve is a tool used as quantitative research purposes by plotting data to build a graph [39]. The absorbance obtained from the MTT reading is the value for cell contents in well so that standard curve was prepared to determine the total cell number of HSF 1184 cells from the MTT reading [40]. Firstly, the fibroblast cells were seeded onto six-well plates and cultured for 24 h. Afterwards, the absorbance of the MTT assay experiment was plotted to obtain a standard curve for each seeding density of fibroblast cells.

The six-well plates with the remedial treatments were incubated for 24 h. Later, MTT assay was done by adding 300 µL of MTT reagent into each control and treatment wells. The plate was wrapped well with aluminium foil and stayed in CO2 incubator (NuAire IR Autoflow CO2 Water-Jacketed Incubator, USA) at 37°C for 4 h. The old medium was sucked out from each well and 3 mL of MTT dissolving buffer was added into each well. The absorbance of the purple colour solution was read at 577 nm using Promega GloMax Multi Detection System.

2.8 Statistical Analysis

The normality of the data was determined using the Shapiro-Wilk test. The independent t-test was used to analyse the normal data while non-normal data was analysed by the Mann-Whitney test. Analysis was used to determine the significant differences between groups. The number of asterisks represented the significance level of correlation among both groups which were *p< 0.05, ** p< 0.01, *** p< 0.001 [41].

3.0 RESULTS AND DISCUSSION

3.1 Optimization of Seeding Cells Density on Six-Well Plate

HSF 1184 cells were seeded in six-well plate with a specific range of seeding number (1×10^5 to 6×10^5 cells/mL). The result showed that the cell seeding number of 4×10^5 cells/mL in six-well plate for HSF cell lines was the most suitable for the experiments due to 80 % of cell confluency in 24 h.

3.2 MTT Cytotoxicity Assay of *Manihot esculenta* Aqueous Extract

Based on Figure 1, it can be calculated that IC50 value was 320 µg/mL. According to U.S National Cancer Institute plant screening program, in the preliminary experiment, a crude extract is considered to have high cytotoxic activity if the IC50 is 20 µg/mL or less [42]. Hence, the *Manihot esculenta* aqueous extract was found to have a weak cytotoxic effect on HSF 1184 cells tested which IC50 was 201-500 µg/ml [43].

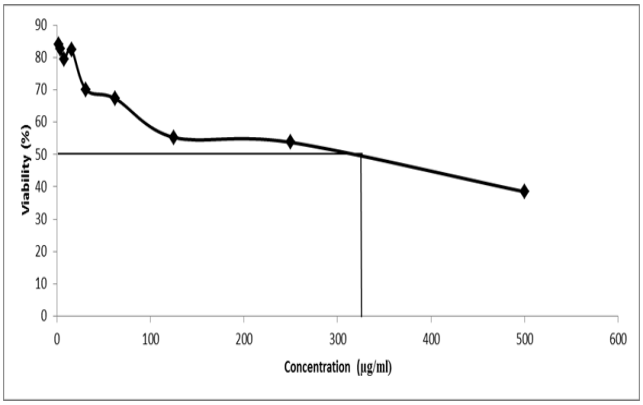


Figure 1 Graph of cell viability from different concentration of *Manihot esculenta* aqueous extract on the HSF cells

Based on the IC50 (320 µg/mL) value from the MTT assay, the experiment was continued with the scratch assay. The scratch assay was done with three different concentrations; 100µg/mL, 200µg/mL, and 300 µg/mL. These concentrations were less than IC50 value which was not toxic to the cells [44].

3.3 Scratch Assay Analysis of *Manihot esculenta* Aqueous Extract Treatment

Based on the scratch assay analysis, all of the concentration of *Manihot esculenta* aqueous extract showed a wound closure in this study. However, at the concentration of 200 µg/mL it shows the highest wound closure than concentration of 100 µg/mL and 300 µg/mL (Figure 2)

3.4 Scratch Assay Analysis of the Combining of *Manihot esculenta* Aqueous Extract, Therapeutic Ultrasound and Ascorbic Acid

Based on the graph (Figure 3), the treated HSF 1184 cells with *Manihot esculenta* (ME) aqueous extract 200 µg/mL shows a significantly higher wound closure at time interval 4, 6, 8, and 10 h (*p< 0.05, **p< 0.01). On the contrary, the treated HSF 1184 cells with *Manihot esculenta* aqueous extract 200 µg/mL and ascorbic acid (AA) 10 µg/mL shows a low progress of cell migrations with a significant value at intervals of 2, 4 and 10 h (*p< 0.05, **p< 0.01).

The treated HSF 1184 cells with *Manihot esculenta* aqueous extract 200 µg/mL and therapeutic ultrasound (3 MHz, 300 mWatt/cm2, 50 % in 5 min) shows a high progress of cell migrations (Figure 3). From the graph, it shows that the combining of *Manihot esculenta* aqueous extract and therapeutic ultrasound (US) gives positive effects to cell migrations and displays a uniform pattern of cell migration.

The combining therapy among *Manihot esculenta* aqueous extract, ascorbic acid and therapeutic ultrasound gives positive effects, but still lower than *Manihot esculenta* aqueous extract coupled with therapeutic ultrasound. The result had revealed that the combination of these three materials had the rate of wound closure at 89.79 % after 10 h while the combination of *Manihot esculenta* aqueous extract coupled with therapeutic ultrasound had a significantly higher rate of wound closure at 96.10 % after 10 h (**p< 0.01).

The role of ascorbic acid in this experiment is as positive control. Concentration 0.06 mM of ascorbic acid was studied to stimulate proliferation of human skin fibroblast cells, provoke collagen synthesis (as cofactor for prolyl hydroxylase) and its deposition in the extracellular matrix [15]. However, it is also reported that ascorbic acid at markedly higher concentrations used in medium are lethal, which can inhibit cell proliferation as well as cell apoptosis [45]. This study shows that ascorbic acid alone gives positive effects, but still lower than *Manihot esculenta* aqueous extract coupled with therapeutic ultrasound.

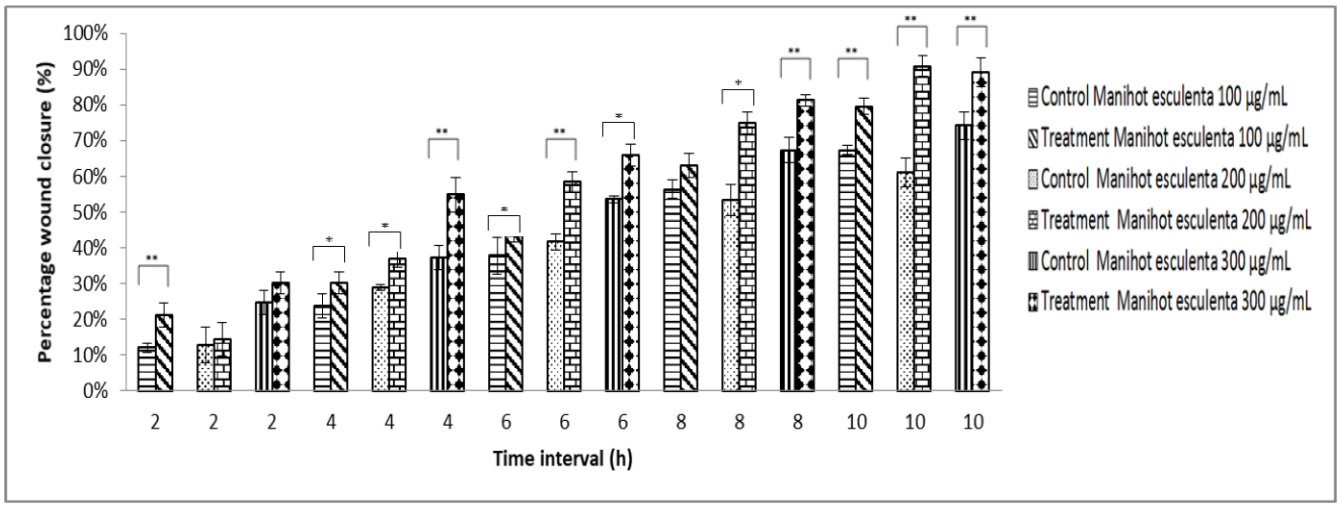


Figure 2 Percentage of wound closure after treated with the Manihot esculenta aqueous extract with a specific time interval (0, 2, 4, 6, 8 and 10 h). Significant (*p< 0.05, **p< 0.01, ***p< 0.001) differences between the groups are indicated by asterisks

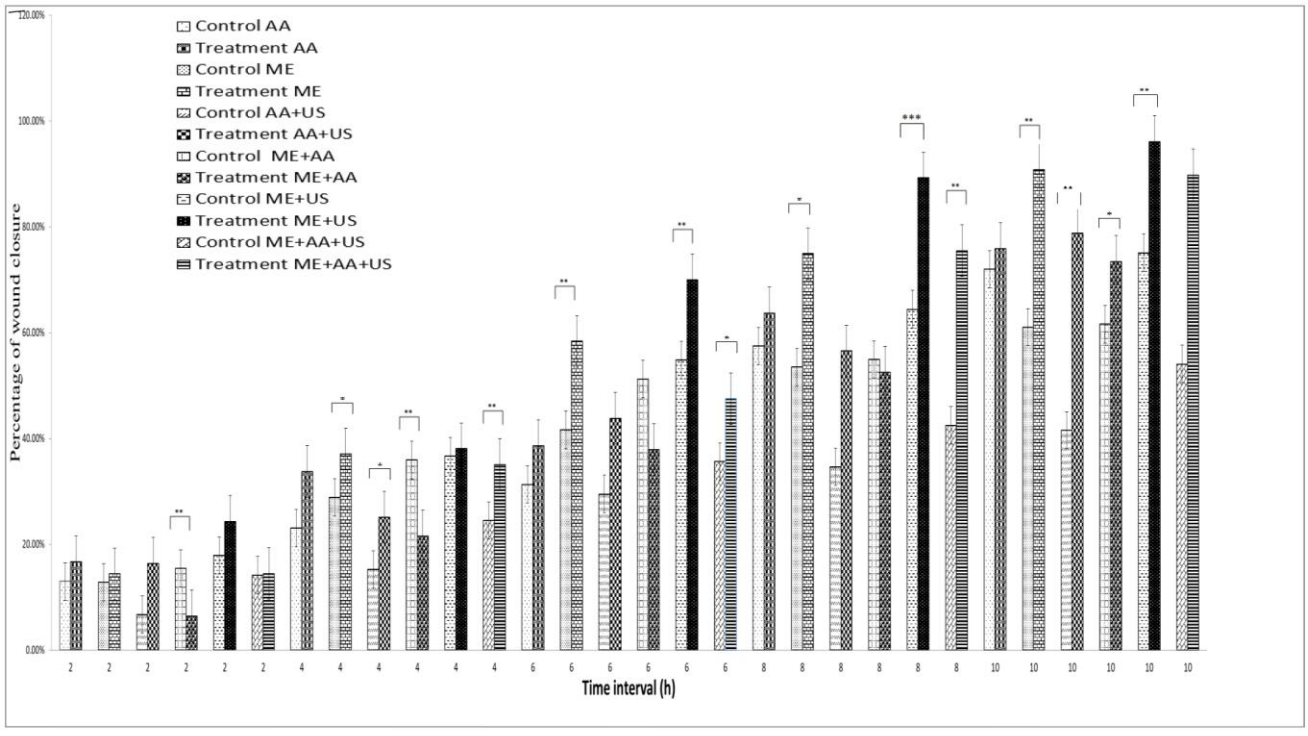


Figure 3 Percentage of wound closure after treated with five remedial treatments with a specific time interval (0, 4, 6, 8 and 10 h). Significant (*p< 0.05, **p< 0.01, ***p< 0.001) differences between the groups are indicated by asterisks

The cell viability (Figure 4) –shows that *Manihot esculenta* aqueous extract coupled with therapeutic ultrasounds (3 MHz, 0.3 Watt/cm^2 with 50 % pulse mode in 5 min) significantly increased the cell numbers (*p< 0.05) when compared to the other combination therapy (*Manihot esculenta* aqueous extract alone; ascorbic acid+ therapeutic ultrasound; *Manihot esculenta* aqueous extract+ ascorbic acid; and the combination of these three materials). The combination of *Manihot esculenta* aqueous extract and therapeutic ultrasound enhance the cell numbers at 5.44×10^5 cells/mL, proved that the potential effect of *Manihot esculenta* aqueous extract combined with therapeutic ultrasounds with a specific dose.

The result presented here suggest that *Manihot esculenta* aqueous extract coupled with therapeutic ultrasound treatment had a good effect on scratch assay analysis and cell viability. Based on the graph (Figure 3), the treated HSF 1184 cells with *Manihot esculenta* aqueous extract 200 µg/mL and therapeutic ultrasound (3 MHz, 300 mWatt/cm^2, 50 % in 5 min) had shown a significantly high progress of cell migrations at intervals of 4, 6 and 10 h (**p< 0.01, ***p< 0.001). This finding was supported by previous study that reported that *Manihot esculenta* extract had a role in inflammatory phase of wound healing [16].

In related to that, therapeutic ultrasound activates inflammatory cells that play a role in the production of chemical mediators, resulting in the activation of fibroblasts proliferation [46, 47].

Due to the potential of phytochemical content in *Manihot esculenta* leaves, *Manihot esculenta* extract has capability to accelerate wound healing process [7]. Saponin and flavonoid has anti-inflammation activity and tannin and triterpenoid are known to have antioxidant activity [48, 49]. Vitamin C (ascorbic acid) in *Manihot esculenta* leaf helps synthesizes collagen of the proliferation process and flavonoid helps to protect the oxidation of ascorbic acid, thus improving the process of collagen synthesis [50, 51].

The cell viability of the *Manihot esculenta* aqueous extract 200 µg/mL (Figure 4) had appreciable effect to stimulates cell significantly (*p< 0.05) with the cell numbers at 5.01×10^5 cells/mL. According to Nisa *et al*. (2013), the extract of *Manihot esculenta* had potential to improve the quality of connective and epithelial tissue in the wound healing process in *in vivo* [11]. Furthermore, the fibroblast is one of the connective tissue cells which has role in wound healing process and had been used in this study [51].

Therapeutic ultrasound is secure and beneficial treatment to combine with biochemical therapy in order to heal the wounds [8]. Furthermore, therapeutic ultrasounds with a dose of 3 MHz, 0.3 Watt/cm2 with 50 % pulse mode had demonstrated to have positive effect on HSF 1184 fibroblast cell lineage after 5 minutes of exposure [31]. Based on Figure 4, the combination of *Manihot esculenta* aqueous extract and therapeutic ultrasound enhance the cell numbers at 5.44×10^5 cells/mL, proved that the potential effect of *Manihot esculenta* aqueous extract combined with therapeutic ultrasounds with a specific dose. In comparison with Mahdzir's work, these combining therapies have better effect than the therapeutic ultrasound alone.

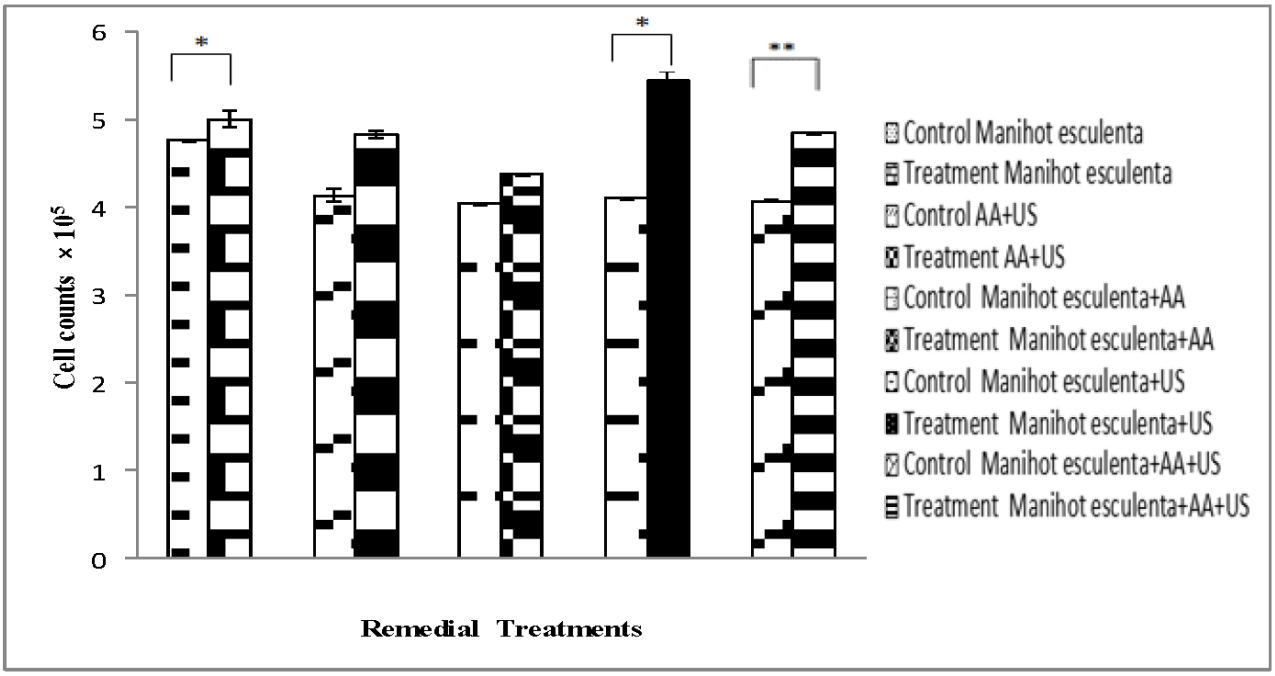


Figure 4 Total cell numbers of HSF 1184 cells from the scratch assay using the MTT assay of five remedial treatments. Significant (*p< 0.05, **p< 0.01, ***p< 0.001) differences between the groups are indicated by asterisks

4.0 CONCLUSION

This study shown that *Manihot esculenta* aqueous extract 200 µg/mL and the therapeutic ultrasound specific dose (3 MHz, 300 mWatt/cm^2, 50% in 5 min) have the potential in accelerating wound healing process of cells in *in vitro*. Furthermore, this research has limitation on the method of wound healing; the scratch assay method can be replaced by gap closure method (using a biocompatible hydrogel chamber), so it will create a consistent circle shape. Hence, the result of image J analysis will not leave a residue that could interfere with cell migration in 6-well plates. On top of that, more *in vivo* studies of the *Manihot esculenta* aqueous extract and therapeutic ultrasound on intact tissue should be conducted to investigate the physiological effects of this combining therapy. Thus, it is recommended that *Manihot esculenta* aqueous extract and the therapeutic ultrasound specific dose can be tested in *in vivo* model.

Acknowledgement

The authors fully acknowledged Universiti Teknologi Malaysia for the facility which makes this important research viable and effective.

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