

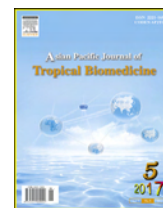
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ABSTRACT

Objective: To provide *in vitro* evidence for antidiabetic activity through potential inhibition of α -glucosidase enzyme, glucose diffusion and enhancement in the wound healing using methanolic extract and fractions from *Hibiscus sabdariffa* Linn. fruit.**Methods:** The inhibitory action of methanolic extract and fractions of such fruit on α -glucosidase enzyme and glucose movement through *in vitro* assay assessment was reported. Their activities on wound healing were tested using the scratch assay.**Results:** Ethyl acetate fraction at 50 mg/mL concentration exhibited significant α -glucosidase inhibition (95.79 mg/mL) with $P < 0.05$. At the same concentration, the methanolic extract as well as other fractions revealed lower α -glucosidase inhibition and higher glucose diffusion retardation across the dialysis tube than the control. Ethyl acetate and butanol fractions displayed notably higher glucose diffusion inhibitory activity of 5.21 mmol/L and 5.2 mmol/L, respectively as compared to methanolic extract and *n*-hexane fraction of 6.58 mmol/L and 6.49 mmol/L, respectively. Conversely, compared to other fractions the methanolic extract and ethyl acetate fraction manifested proliferative effect at the incubation time of 6 h during the wound healing study.**Conclusions:** It is established that methanolic extract and fractions from *H. sabdariffa* Linn. fruit can inhibit the α -glucosidase enzyme and glucose movement as well as influence the wound healing activity positively.

1. Introduction

The presence of high levels of blood glucose, complications in the metabolism of protein, fat and carbohydrate being the most common syndromes of the chronic disease so called diabetes mellitus need proper monitoring and cure [1]. Currently, it is one of foremost health concern to humankind [2]. One of the most popular therapies for plummeting the postprandial

hyperglycemia is the inhibition of α -glycosidase enzyme, which is responsible for the carbohydrates absorption and digestion [3]. Complex polysaccharides that are digested by enteric digestive enzymes (α -glycosidase) are degraded in the form of glucose [4]. Upon absorption, such glucose penetrates the blood stream, causing the postprandial hyperglycemia. Thus, slowing down the digestion of such starch by inhibiting the α -glycosidase enzyme can be regarded as a key therapeutic approach for diabetes cure [5]. It is found that the concentration of postprandial blood glucose can be reduced using natural products that contain multifarious polysaccharides. It can be achieved via the inhibition of glucose diffusion within the intestinal membrane in the course of absorption and digestion [6].

Lately, the use of natural therapeutic drugs in the absence of any side effects appear promising towards chronic wounds healing [7]. Since ancient civilization, plants have been potentially used towards accelerated wound healing process without having any scientific proof of their efficacy. In fact, fundamental understanding regarding the effectiveness of these plants

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extracted compounds and their mode of actions remains unclear [8]. Various types of plants can be used to cure diverse injuries including cut or burning. On top, using simple formulation one can make them suitable candidates for natural remedies [9].

Hibiscus sabdariffa Linn. (*H. sabdariffa*) belongs to the family of Malvaceae, which is also known as karkade or roselle [10]. Traditionally, it has effectively been used against hypertension, inflammation, and liver disorders [10]. Previous studies revealed that an aqueous extract of dried roselle flowers containing high concentration of polyphenol is prospective for effective treatment of leukemia and gastric carcinoma [11], antihypoglycaemic [12], hypolipidaemic [11], antioxidant [13], and estrogenic-like effects [14]. Inspired by these notable attributes of nature gift so called roselle, we scrutinized the potential activity of methanolic extract and fractions of *H. sabdariffa* Linn. fruit on the inhibition of the α -glucosidase enzyme and glucose diffusion as well as speedy wound healing process.

2. Materials and methods

2.1. Materials

The study was performed using the raw materials such as α -glucosidase from *saccharomyces cerevisiae*, p-nitrophenyl α -D-glucopyranoside, dialysis tubes of cellulose membranes, acarbose and 3-(4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide (MTT) (Sigma–Aldrich Group, Malaysia). Glucose oxidase peroxidase kit was procured (Spinreact S.A. Company, Spain). Other chemicals including fetal bovine serum (FBS), Dulbecco Modified Eagle Medium (DMEM), trypsin and penicillin/streptomycin were employed (Qrec, Malaysia).

2.2. Plant material

The whole fresh fruit of *H. sabdariffa* Linn. were purchased from a local herbal shop (Johor Bahru, Malaysia) which were then washed using tap water and air dried for 1 week before being grounded (via a grinding machine) to get fine powder. It is important to note that the existence of this plant was already documented by the forest research institute Malaysia (FRIM) with the catalog number 280615-14.

2.3. Preparation of the extract and fractions

The powdered form of processed *H. sabdariffa* Linn. fruits was soaked in the methanol and water with the ratio of 4:1 for 96 h to obtain the first batch of crude methanolic extract [15]. Then, the collected extract was evaporated using rotary evaporator (EYELA N-1000, Japan). The gummy extract was further dried completely using a freeze dryer (Beta 2-4 LD plus LT, Martin Christ, Germany). Following the same protocol four batches of crude extracts were obtained and pooled [16]. Later, the extract was subjected to successive solvent–solvent partitioning from least polar (*n*-hexane) to high polarities (ethyl acetate and butanol). Finally, the *in vitro* potency of the methanolic extract and fractions in inhibiting α -glucosidase enzyme, glucose diffusion and wound healing were evaluated [16].

2.4. α -Glucosidase inhibition assay

The inhibitory assay of α -glucosidase was performed in a 96 well plate. In this process, 20 μ L of various concentrations of *H. sabdariffa* Linn. fruit methanolic extract and fractions were mixed with 50 μ L of 50 mM phosphate buffer followed by 10 μ L of α -glucosidase from *saccharomyces cerevisiae* (1 U/mL). The obtained mixture was then incubated for 5 min at 37 °C before adding 20 μ L substrate of p-nitrophenyl- α -D-glucopyranoside (pNPG). Next, the reaction was allowed to proceed for 30 min at 37 °C inside the incubator before being terminated by adding sodium carbonate solution (50 μ L). To inhibit the α -glucosidase enzyme within the small intestine, the commercially available synthetic drug acarbose (used for Type II diabetes patient) was used as a positive control. Absorbance of both control and extract was recorded at 405 nm wavelength using an Epoch Microplate Spectrophotometer (BioTek instruments Inc., USA). The inhibition percentage was computed via [17]:

$$\text{Inhibition} = \frac{A_{co} - A_{ex}}{A_{co}} \times 100$$

where A_{co} is the absorbance of control and A_{ex} is the absorbance of extract.

2.5. Glucose diffusion assay

A simple *in vitro* dialysis tube model was used to assess the potential of *H. sabdariffa* Linn. fruit methanolic extract and fractions in retarding the glucose diffusion inside a small intestine tract [18]. This *in vitro* model used a dialysis tube of dimension 15 cm \times 25 mm [19]. Plant extract of 1 mL was incorporated in this dialysis tube and thoroughly mixed with 0.15 M NaCl of 1 mL in the presence of 0.22 M D-glucose. Then, both end of the dialysis tube was closed before being positioned in a glass beaker that contained 50 mL of 0.15 M NaCl. Subsequently, the whole system was positioned on an orbital shaker at room temperature [19]. The effect of methanolic extract and fractions on the inhibition of glucose diffusion was determined at varying concentrations (6.25, 12.5, 25 and 50 mg/mL) under controlled time periods in the range of 0–24 h, where distilled water was used as a control. The concentration of glucose that has moved out to the external solution was analyzed by glucose oxidase method [19,20].

2.6. Cell culture

The fibroblast cell (HSF 1184) of human skin was obtained from European Collection of Cell Cultures (ECACC, England). This HSF 1184 cell was cultured in DMEM added with 10% FBS and 1% penicillin/streptomycin in a humidified incubator containing 5% CO₂ and 95% air at 37 °C. The cells were passaged in every 3–5 days.

2.7. MTT assay

The number of feasible cells in the sample was determined using MTT assay. Human skin fibroblast cell (HSF 1184) were

trypsinized and seeded into 96-well plate with the seeding density ranging from 1×10^4 to 8×10^4 cells/well [21]. The cells were then incubated in CO₂ incubator until it reaches about 70%–80% confluency [22]. After 72 h, 80% of the confluent seeding density (5×10^4 cells/well) was chosen and captured using a camera interfaced with ISCapture software (Xintu Photonics Co., Ltd.). Then, it was treated with *H. sabdariffa* Linn. fruits methanolic extract and fractions for 72 h. The MTT (3-(4, 5-dimethylthiazol-2, 5-diphenyl tetrazolium bromide)) were dissolved in phosphate buffer saline (PBS) at 5 mg/mL and filtered using 0.22 μ m syringe for sterilization. After 72 h of incubation, MTT solution of 20 μ L was mixed to every well and the plate was subjected to incubation at 37 °C for 4 h. Later, each well medium was isolated carefully and 225 μ L of 1 NHCl/isopropanol solution was supplemented to all wells to solubilize the purple crystals [23]. Finally, the absorbance was measured at 570 nm wavelength using PromegaGloMax microplate reader (Promega, Wisconsin, USA) [24].

2.8. Scratch assay

Human skin fibroblast cell (HSF 1184) was seeded in 6-well plate with seeding density in the range of 9×10^4 to 7×10^5 cells/well. Then, the optimum seeding density (4×10^5 cells/well) was permitted to grow at 37 °C for 24 h in the 5% of CO₂ atmosphere. The confluent monolayer was scratched (linear and tiny) by gently scraping it using a sterile pipette tips (200 μ L). Subsequently, the cellular debris was removed by thoroughly rinsing these cells using PBS. Ascorbic acid was used as a positive control due to its growth factor effectiveness regarding the expression of collagen gene by fibroblasts [25]. Literature revealed that ascorbic acid can enhance the synthesis of extracellular matrix in chondrocytes, aortic smooth muscle cells and fibroblasts of human [26]. The cells without treatment was used as negative control [9]. Wound closure was measured using inverted microscopy (Nikon Eclipse TS 100, Tokyo). NIH image J software was used for data analyses, where the scratch dimension at different duration (0, 6 and 24 h) was monitored to estimate the wound closure [27].

2.9. Statistical analysis

Each measurement was performed three times to calculate the average and standard deviation (SD) using SPSS 21 (Windows Evaluation Version, SPSS Inc., USA). The Shapiro–Wilk test was carried out to determine the data normality. Independence t-test was used to analyze the normal data. Meanwhile, the Mann–Whitney test was carried out for data which are not normal. If the probability (*P*) is less than 0.05 the data was regarded as significant and vice versa [28].

3. Results

3.1. In vitro α -glucosidase inhibition

The *in vitro* α -glucosidase inhibition studies demonstrated that all samples of *H. sabdariffa* Linn. fruit had α -glucosidase inhibitory activity. Figure 1 illustrates the inhibitory activity of methanolic extract and fractions. The α -glucosidase inhibition

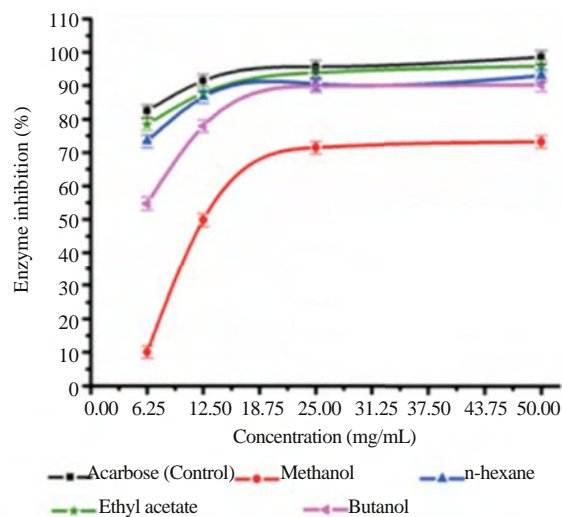


Figure 1. Inhibitory effect of methanolic extract and fractions of *H. sabdariffa* Linn. fruit and acarbose against α -glucosidase enzyme activation at varying concentrations.

reveals a stepwise pattern with increasing concentrations of methanolic extract and fractions, which is greatly similar to those characterized for Acarbose (positive control drug). Furthermore, ethyl acetate fraction displayed highest inhibitory activity of 95.79% at the concentration of 50 mg/ml as compared to acarbose (98.51%) at the same concentration.

3.2. Glucose diffusion

The glucose movement from a closed dialysis tube into exterior solution was recorded at controlled time duration of 0, 4, 8, 12, 16, 20 and 24 h. Following the recommended protocols provided by Spinreact S.A. Company, the amount of glucose was measured using the glucose oxidase method. It is observed that after 24 h the movement of glucose (for the control in the absence of extract from fruit) into the exterior solution attained a plateau. Interestingly, all the *H. sabdariffa* Linn. fruit samples were found to be significant inhibitors of glucose diffusion ($P < 0.05$) than that of the control as depicted in Figure 2a–d. Conversely, the ethyl acetate and butanol fractions at the concentration of 50 mg/mL after 24 h were found to be potential inhibitors with 5.21 mmol/L and 5.2 mmol/L, respectively than the control (6.975 mmol/L).

3.3. Cytotoxicity effects

The cytotoxicity of the *H. sabdariffa* Linn. fruit methanolic extract and fractions were tested against human skin fibroblast cell (HSF 1184) for eight different concentrations as enlisted in Table 1. It is clear that the both the methanolic extract and ethyl acetate fraction reveal high cytotoxic effect against HSF 1184 cell with IC₅₀ value of 79 μ g/mL. However, hexane and butanol fractions displays low toxicity effect against HSF 1184 cell with IC₅₀ value of 125 μ g/mL and 112 μ g/mL, respectively. The IC₅₀ value is the 50% reduction of the viable cells, which was determined by plotting a line graph of percentage of viability against logarithmic concentration of extract. Thus, such non-toxic methanolic extract and fractions of *H. sabdariffa* Linn. fruit were used for further experimentations.

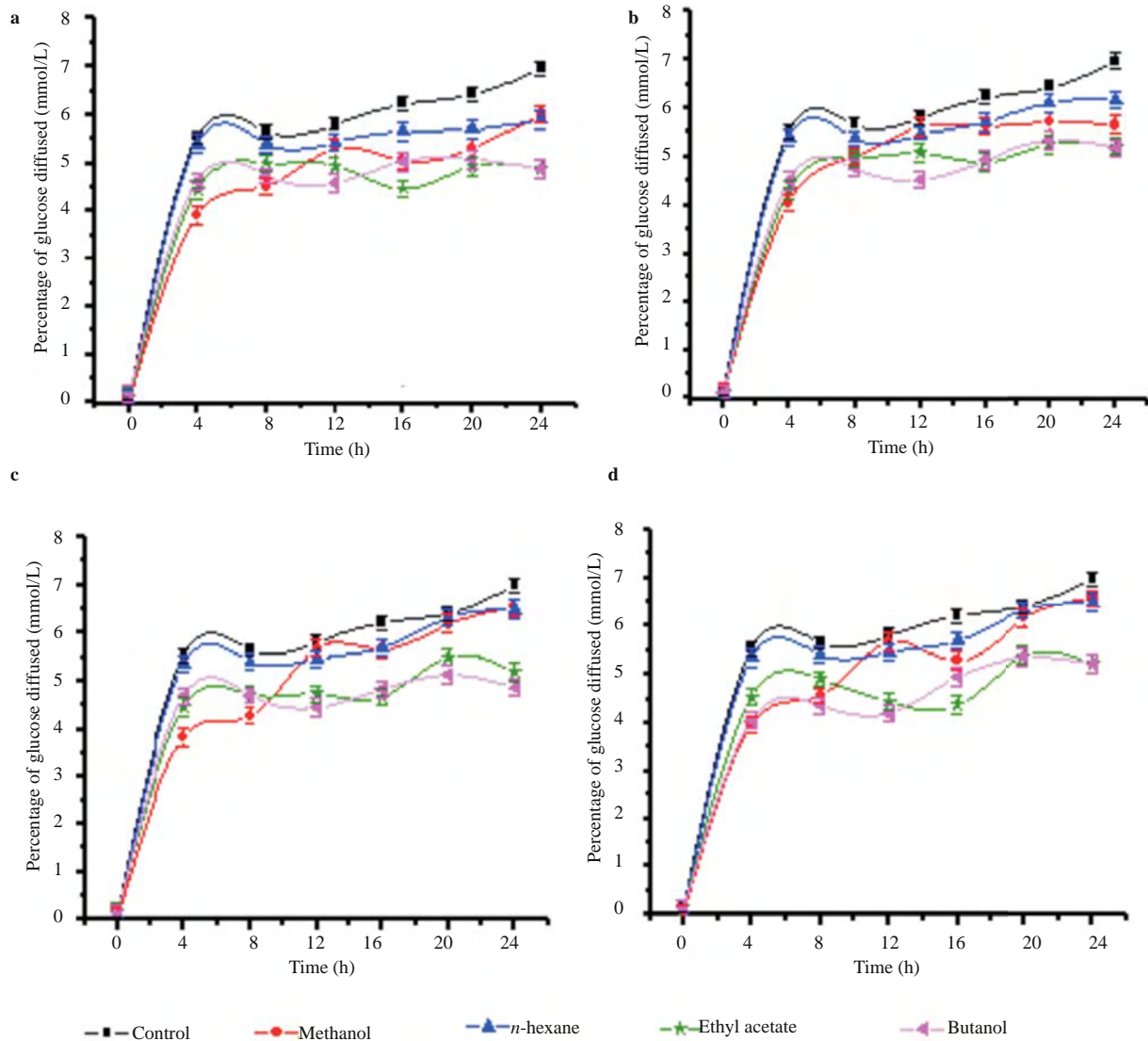


Figure 2. Influence of *H. sabdariffa* Linn fruit methanolic extract and fractions on the time dependent glucose movement from a sealed dialysis tube into exterior solution at varying concentrations of a) 6.25 mg/mL; b) 12.5 mg/mg; c) 25 mg/mL; and d) 50 mg/mL.

Table 1

Percentage of cell viability of HSF 1184 cells treated with different concentrations of *H. sabdariffa* Linn. fruits methanolic extract and fractions.

Concentration (µg/mL)	Control	7.81	15.63	31.25	62.5	125	250	500	1000	IC ₅₀ (µg/mL)
Methanol	100 ± 0.006	81.4 ± 0.003 ^{***}	77.4 ± 0.009 [*]	65.8 ± 0.007 ^{***}	51.4 ± 0.004	41.4 ± 0.006 [*]	31.2 ± 0.019 ^{***}	22.2 ± 0.218 ^{**}	11.2 ± 0.022 [*]	79
n-Hexane	100 ± 0.020	82.7 ± 0.007 ^{**}	75.3 ± 0.022 ^{**}	68.3 ± 0.037 [*]	61.3 ± 0.068 ^{**}	52.3 ± 0.011 [*]	47.7 ± 0.013 ^{***}	44.7 ± 0.003 ^{**}	24.6 ± 0.005 ^{**}	125
Ethyl acetate	100 ± 0.017	71.1 ± 0.012 [*]	67.5 ± 0.002 ^{**}	60.1 ± 0.004 ^{**}	51.1 ± 0.006 ^{**}	32.7 ± 0.012 [*]	28.4 ± 0.007 ^{***}	24.3 ± 0.021 ^{***}	15.4 ± 0.022 ^{**}	79
Butanol	100 ± 0.017	85.97 ± 0.012 [*]	82.08 ± 0.002 ^{**}	76.23 ± 0.004 [*]	63.62 ± 0.006 ^{***}	51.82 ± 0.012 [*]	44.15 ± 0.007 ^{***}	37.58 ± 0.021 ^{**}	10.89 ± 0.022 ^{***}	112

Values are mean ± SD for three replicates; ^{*}*P* < 0.05, ^{**}*P* < 0.01, ^{***}*P* < 0.001 as compared to the control.

3.4. Spreading and migratory activity

The influences of spreading and migrating effectiveness of the methanolic extract and fractions of *H. sabdariffa* Linn. fruit on the human skin fibroblast cells (HSF 1184) were determined using scratch assay. Figure 3 shows the images of scratched assay on HSF 1184 cells at 0, 6 and 24 h of post injury time without (control) and with cure. These images are captured at 10× magnifications using the inverted microscopy (Nikon Eclipse TS 100, Tokyo). The progression of wound healing on

the scratched cells is clearly evidenced. Furthermore, cells treated with such methanolic extract and fractions revealed enhanced migration and wound healing with rapid proliferation of cells than the untreated one. These effects are comparable with ascorbic acid as a positive control. It is affirmed that the methanolic extract and fractions of *H. sabdariffa* Linn. fruit significantly influenced the untreated cells in terms of wound healing and than those treated with ascorbic acid.

Figure 4 displays the influence of various extracts and fractions on the migration and proliferation activities of HSF

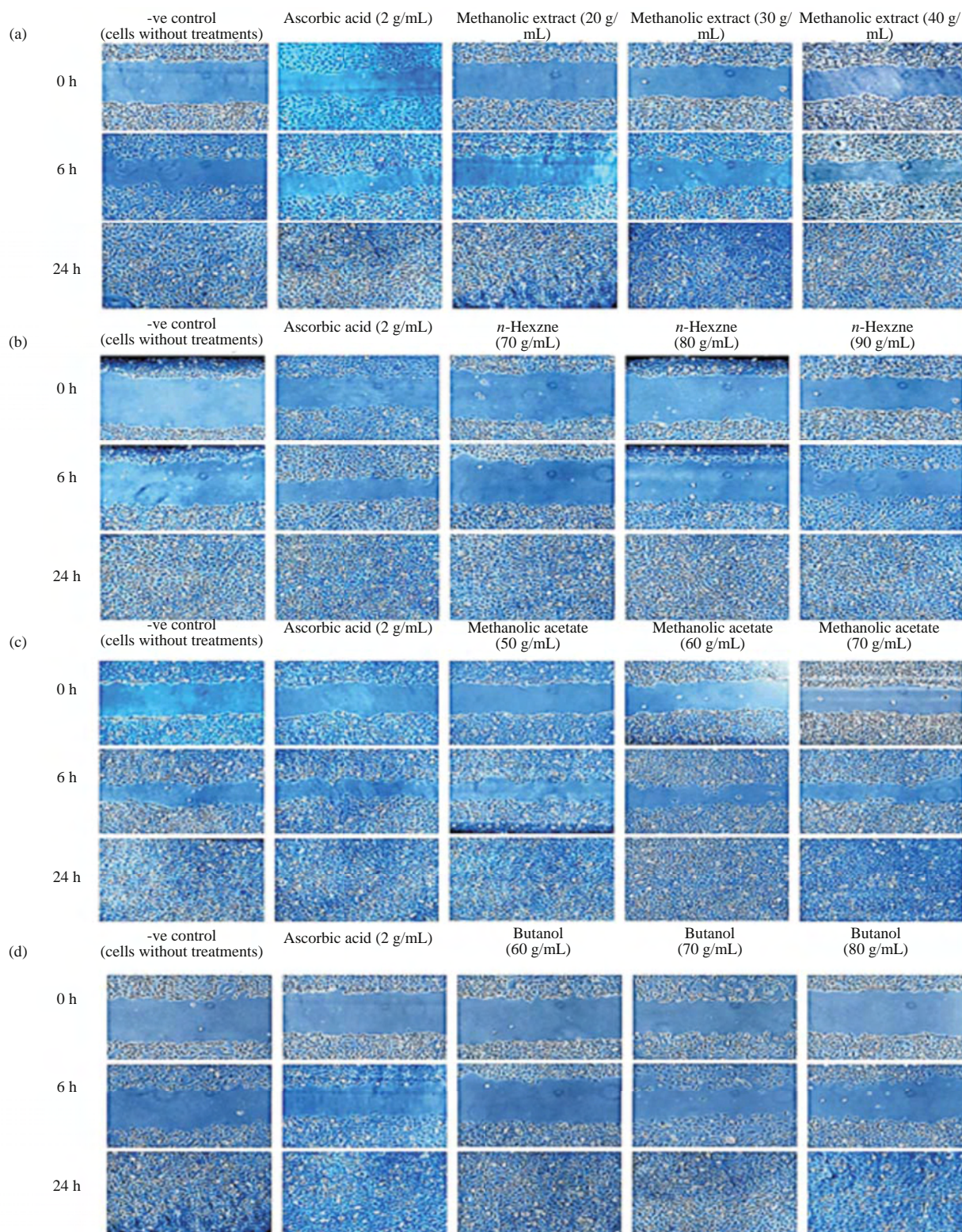


Figure 3. Concentration dependent HSF 1184 cells migration in the *in vitro* scratch assay subjected to scratch and treated with a) methanolic extract; b) *n*-hexane fraction; c) ethyl acetate fraction; and d) butanol fraction after an incubation period of 0, 6 and 24 h.

1184 cells in the scratch assay after an incubation period of 0, 6 and 24 h at 37 °C within DMEM media added with 10% FBS. The methanolic extract and ethyl acetate fraction treated HSF 1184 cells (after 6 h incubation) are migrated more rapidly than the one treated with *n*-hexane and butanol fractions. Meanwhile, the ascorbic acid treated cells are reproduced and moved faster

to heal the injured part than all other samples. In short, *H. sabdariffa* Linn. fruit methanolic extract and fractions at different concentrations significantly ($P < 0.01$) enhanced the wound healing process as compared to negative control (cells without treatments), which is slightly lower for ascorbic acid treated cells.

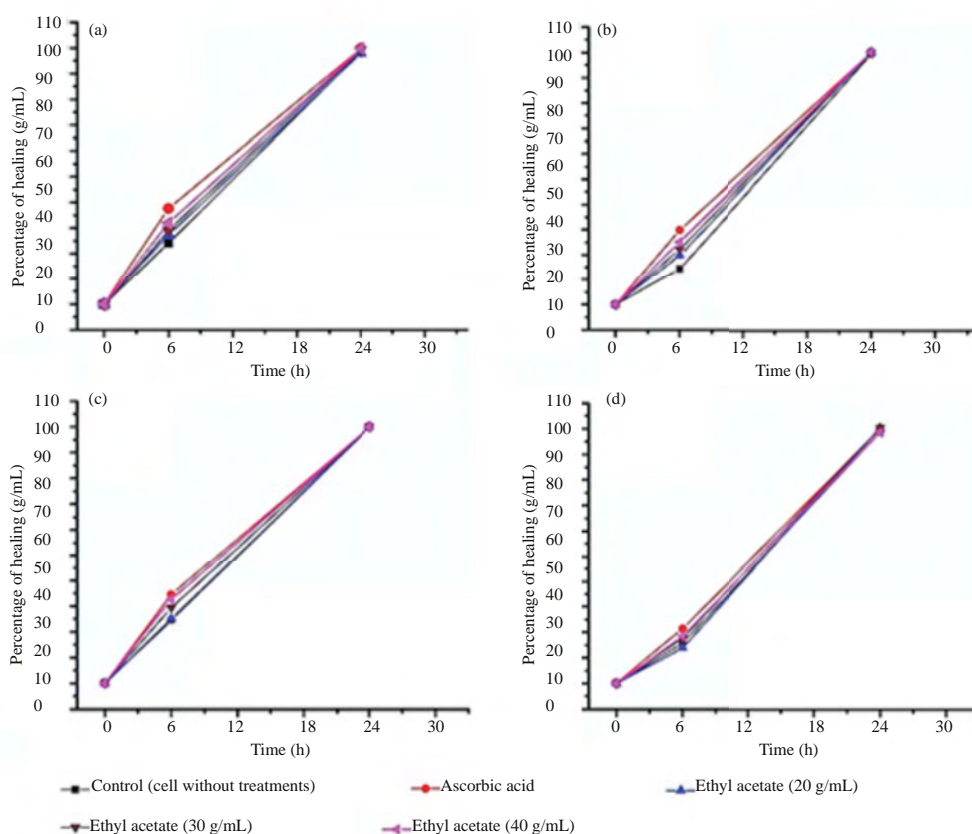


Figure 4. The migration and proliferation activities of HSF 1184 cells in the scratch assay after different incubation period subjected to a) methanolic extract; b) *n*-hexane fraction; c) ethyl acetate fraction; and d) butanol fraction of *H. sabdariffa* Linn fruit.

4. Discussion

Being a dreadful illness, diabetes mellitus leads to various other metabolic disorders. An estimate revealed that the future annual incidence rate will continue to increase worldwide. Diabetes involves the development of micro- and macrovascular complications [29]. In human, glucose tolerance impairs prior to maturity-onset of hyperglycemia [30,31] and is widely used as a clinical index to predict the potentiality of developing diabetes [29]. The objective of this study was to investigate the feasibility of hypoglycemic effect of the methanolic extract and fractions of *H. sabdariffa* Linn. fruit.

Present study demonstrated the *in vitro* α -glucosidase inhibitory potency of all samples of *H. sabdariffa* Linn. fruit. Loh *et al.* [32] examined the inhibitory activity of some Malaysian plants targeted to type II diabetes and hypertension treatment. They acknowledged the great inhibitory efficiency of hexane extract from pucukubi (95.01%) and dichloromethane extract from kacangbotol (38.94%) against α -glucosidase enzyme. However, our ethyl acetate fraction from *H. sabdariffa* Linn. fruit revealed significant ($P < 0.05$) inhibition (95.79 mg/mL) against α -glucosidase enzyme at the highest concentration of 50 mg/mL.

The inhibition potential of ethanolic and aqueous extracts of *Teucrium polium* for retarding the glucose movement from a sealed dialysis tube into exterior solution was reported [33]. However, the entrapment capacity of *T. polium* extracts to retarding the movement of glucose into the exterior solution was found to be insignificant [33]. Thus, the present study established that the *H. sabdariffa* Linn. fruit extract and fractions are more potent than *T. polium* for slowing down the glucose movement from a sealed dialysis tube into exterior

solution. Furthermore, the *H. sabdariffa* Linn. fruit methanolic extract and fractions at different concentrations significantly ($P < 0.01$) enhanced the wound healing process as compared to negative control (cells without treatments), which is slightly lower for ascorbic acid treated cells. The wound healing activity of human dermal fibroblast cells using the methanolic and ethanolic extracts from *Spirulina platensis* was examined. It was acknowledged that the treatment with those extracts through scratch assay was not effective because the lesion area after 24 h of incubation remained un-healed.

We report the inhibition of α -glucosidase enzyme and glucose diffusion together with wound healing activities of *H. sabdariffa* Linn. fruit extracts and fractions. The future prospects of developing antidiabetic drugs for preventing the hyperglycemia using such methanolic extract and fractions from *H. sabdariffa* Linn. fruit are demonstrated. Enhanced wound healing potential of such compounds is attributed to the fibroblast stimulation, the regulation, and coordination of vascularization. It is worth to fractionate and identify the active compounds for unraveling the exact molecular mechanism involves in the antidiabetic action.

Conflict of interest statement

We declare that we have no conflict of interest.

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