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ANTIDIABETIC PROPERTIES OF DELPHINIDIN 3-SAMBUBIOSIDE-ANTHOCYANIN COMPOUNDS ISOLATED FROM *HIBISCUS SABDARIFFA LINN*

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Abstract

Delphinidin 3-sambubioside compound (Dp-3-sam) (patent number MY-193036-A) could be effective against diabetes. Results showed that Dp-3-sam caused BRIN BD11, 3T3F442A, L6 myoblast and HSF 1184 cells to become significantly less viable after three days incubation. By comparison to positive control, Dp-3-sam in 50 µg/ml showed significant inhibitory effects 96.76% (p<0.05). Glucose diffusion was significantly diminished by Dp-3-sam in 50 µg/ml concentration (4.98 mmol/L; p<0.01) compared to control (6.85 mmol/L; p<0.05). With regard to wound healing, migration effect exhibited by Dp-3-sam was more pronounced compared to negative control but weaker than positive control during six hours incubation. Meanwhile, 150 µg/mL Dp-3-sam stimulated insulin production 3.15 times more than positive control (p<0.001) at identical two-fold concentration. Moreover, compared to control, Dp-3-sam could not stimulate glucose assimilation in 3T3F442A adipocytes, and neither could enhance GLUT4 diffusion and regulation on L6 myotubes. Results obtained shed new light on anti-diabetes properties of Dp-3-sam in context of herbal medicine.

Keywords: Delphinidin 3-sambubioside, Anthocyanin, Compound, Antidiabetic, *H. sabdariffa linn*

1.0 INTRODUCTION

A metabolic disorder associated with the endocrine system, diabetes mellitus has an extensive and continuously growing prevalence at global level [1]. It is caused by a high level of glucose in the blood due to inadequate or lack of insulin secretion [2]. The most prevalent type of diabetes is type 2 diabetes, which is characterised by insufficient insulin production or suboptimal use of insulin. Due to the increase in the ageing population, coupled with rising rates of obesity and sedentarism, type 2 diabetes is close to becoming an epidemic [3].

At the moment, diabetes is treated with insulin and different oral anti-diabetes agents, including sulfonylureas, biguanides, a-glucosidase inhibitors, and glinides, which are administered either on their own or mixed so that glycaemia can be regulated more effectively [4]. However, a large proportion of such agents are accompanied by severe side-effects; indeed, diabetes treatment completely free of side-effects remains a problem to be overcome [4], justifying the continued research attention focused on the development of glycaemia-lowering agents of high efficiency and safety. A number of plants have been proven to have potential for diabetes treatment

due to their hypoglycaemic effect, with research being particularly concerned with gaining insight into the manner in which such plants act to lower glycaemia [2].

The extract of *H. sabdariffa* Linn, belonging to the family Malvaceae and more commonly known as roselle, is widely used in beverages, jams, preserves and in traditional medicine [5], being proven to lower blood pressure and aid with inflammation and hepatic diseases [6]. Some studies have even suggested that the plant has effect against tumours, oxidants, and excessive fat levels in the blood [7-10]. Furthermore, evidence derived from recent research on animal models points to the fact that *H. sabdariffa* Linn extract can suppress LDL oxidation and reduce the levels of serum triglycerides, cholesterol and LDL-cholesterol [7, 11].

The purpose of the present study was to investigate the compounds of *H. sabdariffa* Linn anthocyanins, which consist of two major components (i.e. delphinidin-3-sambubioside and cyanidin-3-sambubioside) and two minor components (i.e. delphinidin-3-glucoside and cyanidin-3-glucoside) [12, 13].

The existing knowledge about the anti-diabetes properties of *H. sabdariffa* Linn anthocyanins remains limited. Earlier studies reported that Dp-3-sam caused human leukaemia cells to die [8], targeted oxidants [14], and was effective against inflammation [15]. The present study carried out *in vitro* experiments to explore the effect of Dp-3-sam against diabetes.

2.0 MATERIALS AND METHODS

2.1 Reagents

The Sigma-Aldrich Group (Selangor, Malaysia) was the supplier of the α-glucosidase from *Saccharomyces cerevisiae*, *p*-nitrophenyl *α*-D glucopyranoside, dialysis tubes of cellulose membranes, acarbose, L-ascorbic acid, 3-(4, 5-dimethylthiazol-2, 5-diphenyl tetrazolium bromide (MTT), dimethylsulphoxide (DMSO), D-glucose, diazoxide, glibenclamide, and Dp-3-sam compound. The Spinreact S.A. Company (Sant Esteve de Bas, Girona, Spain) provided the glucose oxidase peroxidase kit, while Mercodia AB (Uppsala, Sweden) supplied the rest of the chemical substances, including the rat insulin ELISA kit. The Invitrogen Corporation (Waltham, USA) was the provider of the Ultima-Gold LLT, sodium pyruvate and Glutamax-I. The Qrec Asia Company (Selangor, Malaysia) provided foetal bovine serum (FBS), Dulbecco Modified Eagle Medium (DMEM), Roswell Park Memorial Institute Medium (RPMI)-1640, trypsin and penicillin/streptomycin.

2.2 Cell Culture

The BRIN BD11 cell culture was maintained with the Roswell Park Memorial Institute (RPMI)-1640 media enriched with 10% foetal bovine serum (FBS) and antibiotic solution under conditions of 5% carbon dioxide and temperature of 37°C. The 3T3F442A fibroblasts, L6 myoblasts and fibroblast cell (HSF 1184) were maintained with Dulbecco's Modified Eagle's Medium (DMEM) enriched with 10% (v/v) FBS and 1% (v/v) antibiotic solution under conditions of 5% carbon dioxide and temperature of 37°C.

Achievement of confluency led to spontaneous differentiation of the 3T3F442A fibroblast-like cells into adipocytes. 5 µg/mL insulin was added to the complete culture medium. Viscous media occurring in every well, with cell production of free fatty acids and subsequent discharge into the media, was indicative of the degree of differentiation. The FBS was decreased from 10% to 2% in the complete culture medium in order to trigger differentiation of L6 myoblasts into myotubes. Cells were kept in this medium for 4-6 days after confluence, with cell multi-nucleation being indicative of the degree of differentiation. Fusion into myotubes was achieved by a proportion of 85-90% of myoblasts in the experiment conducted [16].

2.3 Viability of Cells

The cells in the sample that presented feasibility were counted via the MTT assay. A plate with 96 wells was used to seed all cells (BRIN BD11, 3T3F442A, L6 myoblast and HSF 1184), in a concentration 7×10^5 cells per well. The cells were incubated for 24 hours before being treated with Dp-3-sam at concentrations between 7.81 and 1000 µg/mL and subjected to three-day incubation. 5 mg/mL PBS was used for dissolution of the MTT assay and a 0.22 µm Sartorius syringe filter (Sartorius Stedim Malaysia Sdn. Bhd., KL, Malaysia) was subsequently used for filtering the assay. The three days of treatment with Dp-3-sam were followed by addition of 20 µL of MTT solution to every well and four-hour incubation of the plate at 37°C. The next step was elimination of the medium from every well and addition of 225 µL 1N HCl-isopropanol buffer for dissolution of the purple crystals [17]. The Promega GloMax microplate reader (Promega, Wisconsin, USA) was then employed for recording plate absorbance at 560 nm wavelength [18]. The formula for determining the proportion of cells that were viable is indicated below [19]:

$$\% \text{ Cell Viability} = \frac{(\text{Absorbance of the Test} - \text{Absorbance of the Blank})}{(\text{Absorbance of the Control} - \text{Absorbance of the Blank})} \times 100$$

2.4 α-Glucosidase Inhibition Assay

A plate with 96 wells was used to carry out the inhibitory assay of α-glucosidase enzyme, which involved mixing 20 µL Dp-3-sam in different concentrations with 50 µL phosphate buffer (50 mM) and then 10 µL α-glucosidase from *Saccharomyces cerevisiae* (1 U/mL). After five-minute incubation of the resulting mixture at 37°C, 20 µL substrate *p*-

nitrophenylalpha-*D*-glucopyranoside (pNPG) was added. The reaction was permitted to unfold for half an hour at 37°C within the incubator and then it was extinguished through addition of 50 µL sodium carbonate solution. The synthetic drug acarbose available on the market for type 2 diabetes treatment was employed as positive control for suppression of the a-glucosidase enzyme in the small intestine. An Epoch Microplate Spectrophotometer (BioTek instruments Inc., Winooski Vermont, USA) helped with the recording of the absorbance of control and compound at 405 nm wavelength. The formula below was applied to calculate the percentage of suppression [20]:

$$\% \text{ Inhibition} = [\frac{\text{Absorbance of control} - \text{Absorbance of compound}}{\text{Absorbance of control}}] \times 100$$

2.5 Glucose Diffusion Assay

To determine how capable Dp-3-sam was in preventing glucose dispersal within a small portion of intestine [22], a straightforward 15 cm × 25 mm *in vitro* dialysis tube model was employed [21]. A mixture was created of 1 mL of the compound introduced in the dialysis tube and 1 mL of 0.15 M sodium chloride in the presence of 0.22 M D-glucose. After its ends were tied, the dialysis tube was introduced in a glass beaker with 50 mL sodium chloride (0.15 M) before the entire system was placed on an orbital shaker at room temperature [21]. With distilled water serving as control, different concentrations (6.25, 12.5, 25 and 50 mg/mL) and lengths of time (0-24 hours) were used to measure the extent to which glucose dispersal was suppressed by the compound. The glucose oxidase method was employed for the analysis of the concentration of outgoing glucose to the external solution [21, 23].

2.6 Scratch Assay

A plate with six wells was used for seeding the human skin fibroblast cell (HSF 1184) in a concentration of 5 × 10^4 cells per well and left to mature for one day at 37°C and under an atmosphere of 5% carbon dioxide. A 200 µL sterile pipette tip was used to gently scrape the confluent monolayer and the cells were afterwards rinsed with PBS to eliminate the cellular debris [24]. Addition of Dp-3-sam was done in various concentrations. Thanks to its growth factor efficiency in relation to fibroblast expression of collagen gene, 2 µg/mL ascorbic acid served as positive control [25]. Previous studies have concurred that ascorbic acid can help extracellular matrix in chondrocytes, aortic smooth muscle cells and human fibroblasts to be better synthesised [26]. The negative control was the untreated cells [24]. Inverted microscopy (Nikkon Eclipse TS 100, Tokyo) permitted measurement of wound closure and data were analysed via the NIH image J software, with wound closure being estimated based on monitoring the scratch dimension at 0, 6 and 24 hours [27].

2.7 Insulin Secretion Assay

BRIN-BD11 cells were used for assessment of Dp-3-sam in terms of insulin production activity. An earlier study provided an overview of the cell-line with insulin secretion with regard to production and key properties [28]. A somewhat altered method was implemented to perform the insulin secretion assay, with seeding of the cells in density of 7 × 10^5 in a plate with 96 wells and overnight incubation at 37°C before adherence. Krebs-Ringer bicarbonate buffer (KRB) was subsequently used to wash the cells thrice, followed by 40-minute pre-incubation with KRB at 37°C. The cells were subjected to additional half-hour incubation with 1 ml KRB in the case of negative control or with 1 ml KRB comprising different compound concentrations or 1 ml glibenclamide-containing KRB in the case of positive control. Every assessment was carried out at 2 mM glucose. Incubation was followed by the transfer of 1 ml aliquots from every well into 1.5 ml tubes, which were kept at -20°C to measure the concentration of insulin [29].

2.8 Glucose Uptake Assay

Dulbecco's Modified Eagle's Medium (DMEM) medium enriched with 10% (v/v) FBS and 1% (v/v) antibiotic solution (50,000IU/L penicillin-streptomycin) was the medium in which the 3T3F442A fibroblasts were kept under conditions of 37°C temperature and 5% carbon dioxide humidification. Achievement of confluency led to spontaneous differentiation of the fibroblast-like cells into adipocytes and 5 µg/mL insulin was added to the complete culture medium. Viscous media occurring in every well, with cell production of free fatty acids and subsequent release into the media, was indicative of the level of differentiation. A plate with 12 wells was used for seeding the confluent cells in a density of 2 × 10^5 cells per well, and in order to achieve adherence before testing, they were kept overnight under conditions of 37°C temperature and 5% carbon dioxide humidification. DMEM without serum was used on the subsequent day to wash the cells three times before two-hour incubation. KRB was used to wash the obtained cells three times following the interval of starvation. Dp-3-sam was used in different concentrations for pre-incubation of adipocyte cells at 37°C for half an hour, with rosiglitazone maleate serving as positive control. The glucose assimilation reaction was induced through the addition of 500 µL 2-deoxy-[^{3}H]-glucose (1 µCi/mL) diluted to 0.1 mM glucose to all wells apart from blank, followed by additional one-hour incubation at 37°C. Subsequently, ice-cold KRB was used to wash the cells three times and 0.1% SDS dissolved in phosphate buffer with pH 7.4 was used for solubilisation of the

cells. The next step was transferring the content of all wells in scintillation vials and addition of 15 mL scintillation cocktail (Ultima Gold™ LLT). The Liquid Scintillation Counter (PerkinElmer, USA) helped to determine how radioactive the cells were.

2.9 Identification of Cell Surface GLUT4

An ELISA kit (Uscn Life Science Inc., United States) available on the market was employed for the measurement of GLUT4 in the content of L6 myoblast cells. The sandwiched enzyme immunoassay Uscn Life Science GLUT4- ELISA kit is intended to quantitatively assess GLUT4 in human tissue homogenates, cell lysates and other biological fluids *in vitro*. A GLUT4-specific antibody was used to pre-coat the microplate in the kit. The standard curve constructed on the basis of GLUT4 concentrations was extrapolated to establish the concentration of GLUT4 in the sample. The addition of 100 µL of each dilution of the standard, blank and samples into the suitable wells was followed by 60-minute incubation at 37°C. The next step was removal of the liquid from all wells without washing, addition of 100 µL of detection (reagent A) and 60-minute incubation at 37°C. After aspiration of the solution, 350 µL washing solution was used for swabbing the solution three times. This was followed by addition of 100 µL of detection (reagent B) and half-hour incubation at 37°C. The procedures of aspiration and washing were performed again and 90 µL substrate solution was added to every well, followed by incubation at 37°C for 10-20 minutes. The last step was addition of 50 µL stop solution to every well and running the microplate reader for measurement performance at 450 nm wavelength.

2.10 Ultra-Performance Liquid Chromatography Tandem Mass Spectrometry (UPLC-MS/MS)

The bioactive compound in *H. sabdariffa* Linn fruit methanolic extract, ethyl acetate and butanol fraction were determined via the UPLC-MS/MS test, extracts optained from different samples depend on solvent polarity which was conducted at the Institute of Bioproduct Development (UTM, Johor Bharu, Malaysia). The requirements for this procedure included the UPLC Waters Acquity (Milford, MA, USA) system interfaced with a triple quadrupole-linear ion trap tandem mass spectrometer (Applied Biosystems 4000 QTRAP, Life Technologies Corporation, Carlsbad, CA, USA), an electro-spray ionisation (ESI) source and a 150 mm × 2.1 mm × 1.7 µm C18 reversed phase Acquity column. 5 µl solvent A, consisting of water and 0.1% formic acid, and 5 µl solvent B, consisting of acetonitrile and 0.1% formic acid, were injected to achieve separation at 0.15 ml/min flow rate. The gradient in solvent B proportions (v/v) that was used was 0 to 20 minutes, 10-90%; 20 to 25 minutes, 90%; 25 to 32 minutes, 90-10%; 32 to 35 minutes; 10%. The data from the mass spectrometry were all in negative ionisation modality [30]. Multiple reaction monitoring (MRM) was applied to examine transitions from parent to product ion (m/z) for Dp-3-sam (595>301) and thus achieve identification [31].

2.11 Statistical Analysis

The expression of the results took the form of mean ± standard deviation (SD) from triplicate values and the data were statistically analysed via the SPSS16.0 software (SPSS 16.0 for Windows Evaluation Version software, SPSS Inc., USA). The Shapiro-Wilk test helped to assess data normality, while the statistical significance of normal and non-normal data was respectively assessed via an independent *t*-test and the Mann-Whitney test. Significance was indicated by *p*-value <0.05 [32].

3.0 RESULTS

3.1 Cytotoxicity

The types of cells listed in Table 1 A-D were subjected to MTT assay to assess how the viability of BRIN BD11, 3T3F442A, L6 myoblast and HSF 1184 cells was affected by Dp-3-sam. A decrease in cell viability by half was established as the IC_{50} value, which was computed by plotting a line graph of percentage viability against the compound logarithmic concentration. The additional experiments that were conducted also made use of the non-toxic concentration derived from the compound. Plant extract toxicity was classified in an earlier study as follows: IC_{50} value < 20 µg/ml indicative of high toxicity, IC_{50} value within the range 21- 40 µg/ml indicative of moderate toxicity, and IC_{50} value > 41 µg/ml indicative of non-toxicity [33, 34]. Dp-3-sam was concluded in the present study to more or less lack toxicity to all cell types as the IC_{50} value exceeded 41 µg/ml. The compound was found to have low cytotoxicity towards BRIN BD11 (IC_{50} = 169 µg/mL) 3T3F442A (IC_{50} = 588 µg/mL), L6 myoblast (IC_{50} = 128 µg/mL) and HSF 1184 cells (IC_{50} = 138 µg/mL). Meanwhile, the Dp-3-sam activities of insulin secretion and glucose assimilation in BRIN BD11 and 3T3F442A adipocytes were assessed against glibenclamide and rosiglitazone maleate. Hence, it was necessary to determine how cytotoxic glibenclamide and rosiglitazone maleate were towards the two types of cells.

3.2 a-Glucosidase Inhibition Assay

The suppressing effect of Dp-3-sam (see Figure 1) against a-glucosidase was confirmed by the *in vitro* a-glucosidase inhibition experiments. Exhibiting close similarity to acarbose, which served as positive control, Dp-3-sam showed a stepwise pattern of a-glucosidase inhibition proportional with concentration increase. However, at identical 50 µg/ml concentration, the suppressing effect of Dp-3-sam, albeit significant (p<0.05), was not as high as that of acarbose (96.76% vs 98.31%).

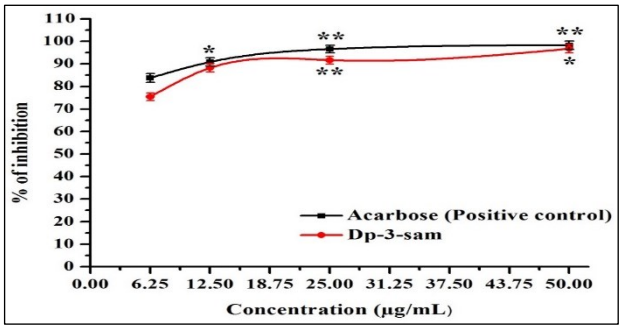

Figure 1 The suppressing effect of Dp-3-sam and acarbose in different concentrations on a-glucosidase enzyme. Expression of values takes the form of mean ± SD from three replicates, with *p<0.05, **p<0.01 by comparison to control

3.3 Glucose Diffusion Assay

The recording of glucose as it moved from closed dialysis tube to exterior solution was performed at regulated time intervals of 0, 4, 8, 12, 16, 20 and 24 hours. The glucose oxidase method was adopted for glucose quantification, in keeping with the procedure suggested by Spinreact S.A. Company. Glucose movement into the exterior solution plateaued after 24 hours. Notably, glucose dispersal was significantly suppressed by Dp-3-sam (P < 0.05) compared to control (Figure 2). On the other hand, compared to the negative control, Dp-3-sam at 50 µg/mL concentration was observed to be a possible inhibitor after 24 hours (6.85 mmol/L vs 4.98 mmol/L).

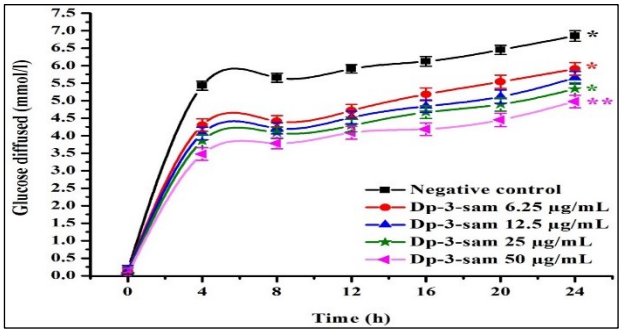

Figure 2 Graph indicating how the time-dependent movement of glucose from sealed dialysis tube to exterior solution was affected by Dp-3-sam. Expression of values takes the form of mean ± SD from three replicates, with *p<0.05, **p<0.01 by comparison to control

Table 1 The impact of Dp-3-sam, glibenclamide and rosiglitazone on the viability of **(A)** BRIN BD11, **(B)** 3T3F442A, **(C)** L6 myoblast and **(D)** HSF 1184 cell. By comparison to control, *p <0.05, **p <0.01, and ***p <0.001 are the mean ± SD values for three replicates

(A)

Concentration (µg/mL)	Control	7.81	15.63	31.25	62.5	125	250	500	1000	IC_{50} µg/ml
		***	*		**			*	*	
Dp-3-sam	100 ±0.011	92.51 ±0.032	91.74 ±0.018	85.89 ±0.033	77.69 ±0.039	59.56 ±0.032	38.24 ±0.084	28.88 ±0.074	14.28 ±0.013	169
		***	*		*	*	***	**	**	
Glibenclamide	100 ± 0.013	98.43± 0.017	97.12± 0.027	92.63± 0.017	83.81± 0.052	75.38± 0.047	57.83± 0.016	49.23± 0.034	27.43± 0.024	464

(B)

Concentration (µg/mL)	Control	7.81	15.63	31.25	62.5	125	250	500	1000	IC_{50} (µg/ml)
		**	*		**	*		**	*	
Dp-3-sam	100 ±0.011	95.35 ±0.032	93.44 ±0.018	88.97 ±0.033	77.82 ±0.039	75.79 ±0.032	61.69 ±0.084	51.78 ±0.074	42.48 ±0.013	588
		***	*	**	**	***	*	***		
Rosiglitazone maleate	100 ±0.023	97.67 ±0.035	90.57 ±0.026	84.15 ±0.031	79.38 ±0.043	75.42 ±0.028	65.52 ±0.019	52.08 ±0.049	44.64 ±0.061	590

(C)

Concentration (µg/mL)	Control	7.81	15.63	31.25	62.5	125	250	500	1000	IC_{50} (µg/ml)
		*	*		***		*	**		
Dp-3-sam	100 ±0.021	87.63 ±0.047	77.21 ±0.037	63.96 ±0.012	56.65 ±0.019	47.82 ±0.062	28.34 ±0.038	21.75 ±0.048	17.57 ±0.016	110

(D)

Concentration (µg/mL)	Control	7.81	15.63	31.25	62.5	125	250	500	1000	IC_{50} (µg/ml)
		*	**		**		**	*		
Dp-3-sam	100 ±0.021	86.47 ±0.047	81.08 ±0.037	75.23 ±0.012	65.62 ±0.019	52.22 ±0.062	41.35 ±0.038	33.98 ±0.048	13.59 ±0.016	150

3.4 Wound Healing Assay

The scratch assay helped to examine how HSF 1184 cells were affected by Dp-3-sam diffusion and migration efficiency. The images produced by this assay of HSF 1184 at 0, 6 and 24 hours after injury whilst treated with Dp-3-sam, untreated (negative control) and treated with ascorbic acid (positive control) are illustrated in Figure 3. It is obvious how would healing progressed on the scratched cells. Compared to lack of treatment, Dp-3-sam treatment improved and sped up migration and wound healing. Thanks to its growth factor efficiency in relation to fibroblast expression of collagen gene, ascorbic acid served as positive control, with previous studies recognising that ascorbic acid could help extracellular matrix in chondrocytes, aortic smooth muscle cells and human fibroblasts to be better synthesised. The extent to which the migration of HSF 1184 was affected by Dp-3-sam in the context of the scratch assay following incubation for 0, 6 and 24 hours at 37°C in DMEM media enriched with 10% FBS is shown in Figure 4. It was observed that, compared to untreated cells, quicker migration was exhibited by the highest concentration of treated cells following six-hour incubation. Furthermore, the cells treated with ascorbic acid exhibited the fasted reproduction and migration for purposes of wound healing. Thus, wound healing was better promoted by treatment with Dp-3-sam in various concentrations than by lack of treatment, but its performance did not reach that of treatment with ascorbic acid.

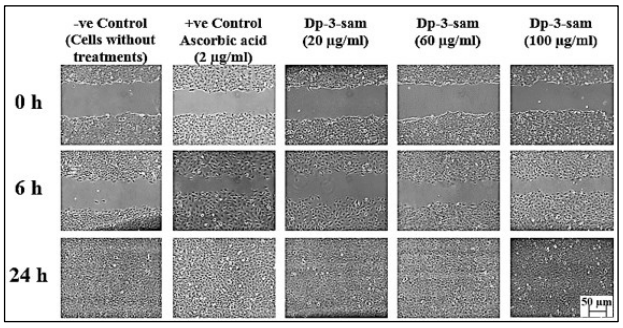

Figure 3 The migration of concentration-dependent, Dp-3-sam-treated and scratched HSF 1184 cells in the context of *in vitro* scratch assay following incubation for 0, 6 and 24 hours. Images were taken at 10× magnification and 50 µm scale bar.

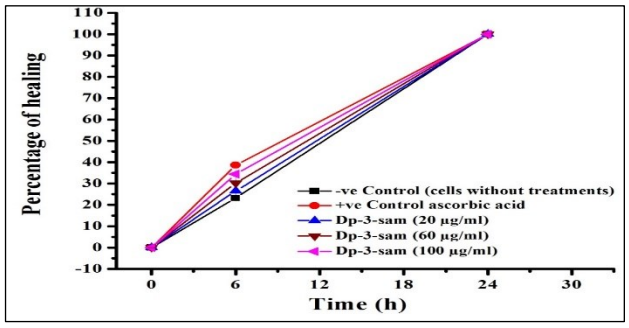

Figure 4 The proportion of migratory activities of Dp-3-sam-treated HSF 1184 cells at various durations of incubation

3.5 Insulin Secretion Assay

The extent to which the capability of BRIN BD11 cells to secrete insulin was affected by Dp-3-sam and glibenclamide is shown in Figures 5 and 6. At a concentration of 150 µg/mL, Dp-3-sam increased potential activity by 3.15-fold compared to the negative control (p<0.001). However, insulin production was suppressed to some degree by Dp-3-sam and glibenclamide at all concentrations that exceeded IC_{50}. This was labelled as insulin suppression mediated by cytotoxicity. By contrast, at maximum concentration of 400 µg/mL, insulin production was significantly promoted by glibenclamide by 3.23-fold (p<0.001). Thus, based on the results obtained, insulin production appears to have been more effectively stimulated by Dp-3-sam than by negative control. Furthermore, despite seeming to be less efficient than glibenclamide (positive control) at maximum concentration, Dp-3-sam displayed greater potency at 150 µg/mL concentration than glibenclamide at 400 µg/mL concentration.

Unlike glibenclamide at 150 µg/mL concentration, which increased insulin production by two-fold, Dp-3-sam at the same concentration increased insulin production by 3.15-fold, indicating that the latter possessed greater potency than the former compound. Hence, Dp-3-sam shows more promise for development as a novel agent for glycaemia reduction.

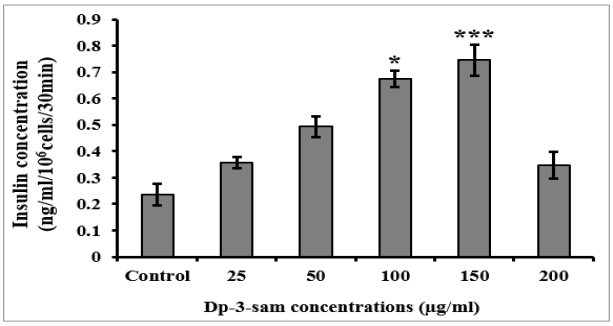

Figure 5 The impact of Dp-3-sam on BRIN BD11 cells in terms of insulin production activity. Expression of values takes the form of mean ± SD from three replicates, with *p<0.05, **p<0.001 by comparison to control

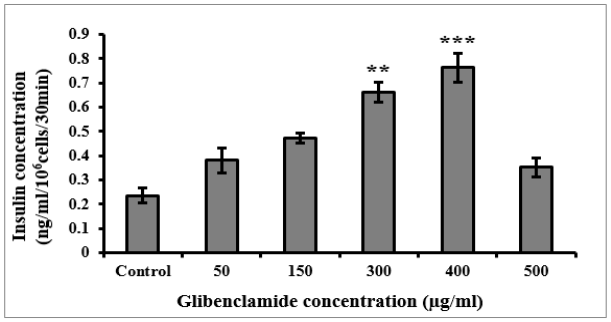

Figure 6 The impact of glibenclamide on BRIN BD11 cells in terms of insulin production activity. Expression of values takes the form of mean ± SD from three replicates, with *p<0.001 and **p<0.001 by comparison to control

3.6 Glucose Uptake Assay

The impact of Dp-3-sam and rosiglitazone maleate on the activity of glucose assimilation of 3T3442A adipocytes is illustrated in Figure 7 and Figure 8. Results showed that this activity was not as significantly impacted by Dp-3-sam as insulin (100 nM), which increased glucose assimilation by 1.51-gold (p<0.001) compared to control.

Under basal and insulin-mediated condition, glucose assimilation activity in 3T3F442A adipocytes was increased by Dp-3-sam by 0.38-fold at 10 µg/mL concentration, by 0.75-fold at 50 µg/mL concentration, by 1.02-fold at 150 µg/mL concentration, by 0.71-fold at 300 µg/mL concentration, and by 0.87-fold at 500 µg/mL concentration, compared to control (Figure 7). None of these values exceeded 100 nM of insulin, suggesting an assimilation of 1.51-fold (p<0.001) compared to control. Basically, by comparison to the negative control and 100 nM insulin, every concentration examined exhibited effects that lacked significance.

The impact of rosiglitazone maleate on basal- and insulin-mediated glucose assimilation by 3T3F442A adipocytes is shown in Figure 8. More specifically, by comparison to negative control and 100 nM insulin, glucose assimilation under basal- and insulin-mediated condition was increased by 1.98-fold (p <0.001), 1.61-fold (p <0.01), 1.59-fold (p <0.001), 2.08-fold (p <0.001), 2.14-fold (p <0.001) and 3.10-fold (p <0.001).

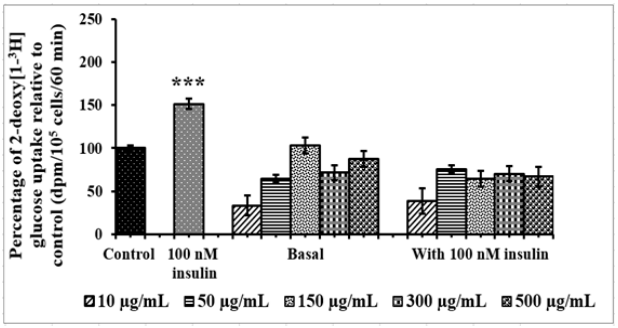

Figure 7 The impact of Dp-3-sam on 3T3F442A adipocytes in terms of basal- and insulin-mediated glucose assimilation. Expression of values takes the form of mean ± SD from three replicates, with ***p<0.001 by comparison to control

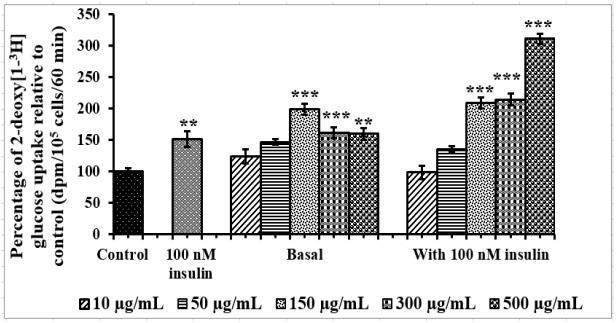

Figure 8 The impact of rosiglitazone maleate on 3T3F442A adipocytes in terms of basal- and insulin-mediated glucose assimilation. Expression of values takes the form of mean ± SD from three replicates, with **p<0.01 and ***p<0.001 by comparison to control

3.7 GLUT4 Detection Assay

The ELISA kit was employed to quantitatively measure GLUT4 *in vitro* to investigate the extent to which the regulatory activity of GLUT4 in L6 myotubes was affected by Dp-3-sam (Figure 9). Compared to negative control, this sample achieved a dose-dependent relative enhancement in the GLUT4 surface distribution, with Dp-3-sam concentration of 100 µg/mL being associated with 3.68% increase in activity. On the other hand, GLUT4 surface distribution was more significantly enhanced (p<0.01) by 5.41% by the positive control represented by 100 nM insulin. The inhibition mediated by cytotoxicity was identified as the main reason for the effect of Dp-3-sam on GLUT4 regulation at concentration higher than IC_{50}.

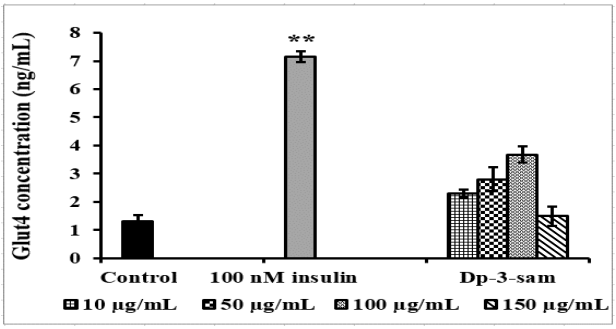

Figure 9 The impact of Dp-3-sam on GLUT4 regulation in L6 myotubes. Expression of values takes the form of mean ± SD from three replicates, with **p<0.01 by comparison to control

3.8 Compound Analysis Based on UPLC-MS/MS

UPLC-MS/MS was employed under condition of negative ionisation to determine the main compound in *H. sabdariffa* Linn fruit methanolic extract, ethyl acetate and butanol fraction. The pattern of fragmentation and comparative analysis against data from previous studies underpinned this process of compound determination [35, 36]. Figures 10-12 show the chromatogram of *H. sabdariffa* Linn fruit methanolic extract, ethyl acetate and butanol fraction, respectively. The examination of the fragmentation pattern indicated that the main compound was associated with [M - H]$^-$ at mass-to charge ratios (m/z) 595.5, fragmentation pattern m/z 301 at 4.4 minutes compared to the data from the literature [36], and this compound was recognised as Dp-3-sam (see Figure 13 for chemical configuration). Thus, Dp-3-sam was suggested to be the main compound in the *H. sabdariffa* Linn fruit methanolic extract, ethyl acetate and butanol fractions.

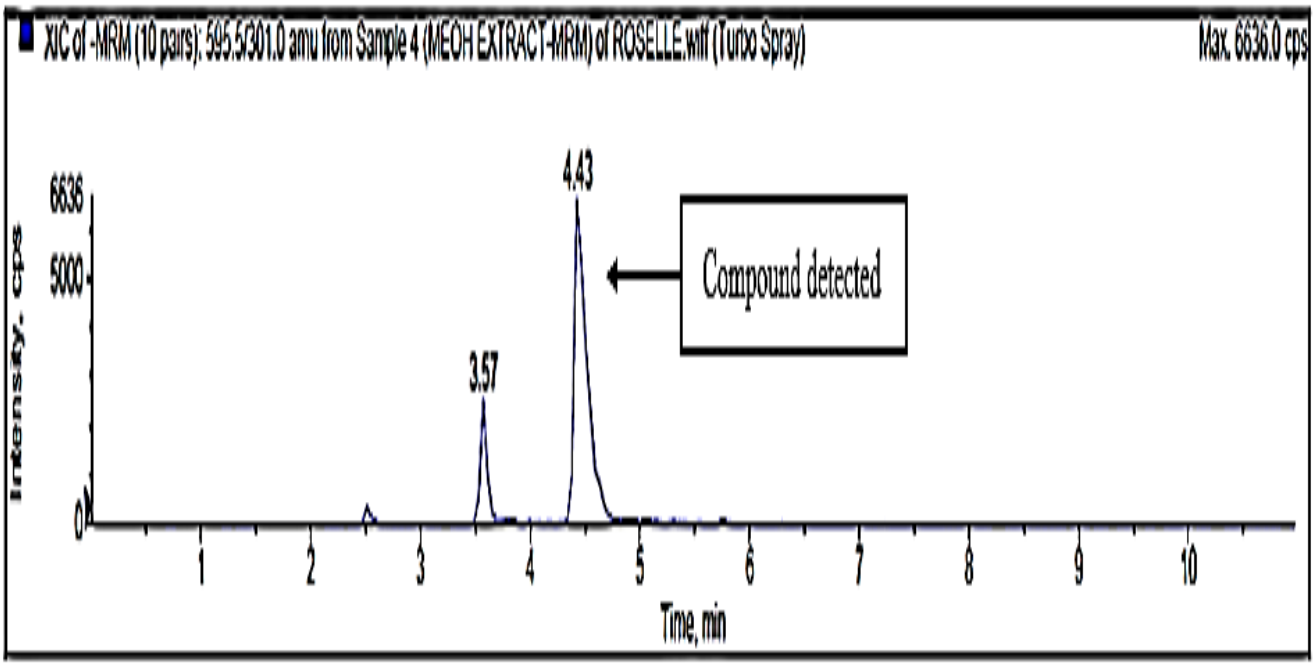

Figure 10 Chromatogram associated with the *H. sabdariffa* Linn fruit methanolic extract

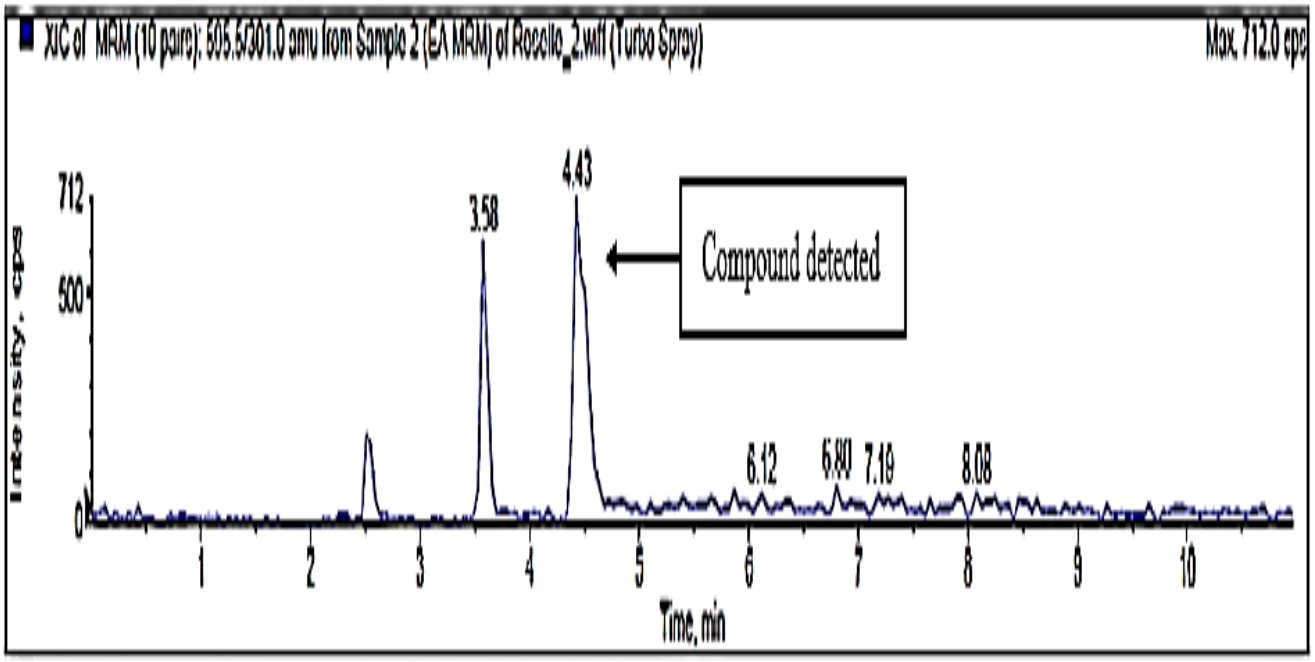

Figure 11 Chromatogram associated with the *H. sabdariffa* Linn fruit ethyl acetate fraction

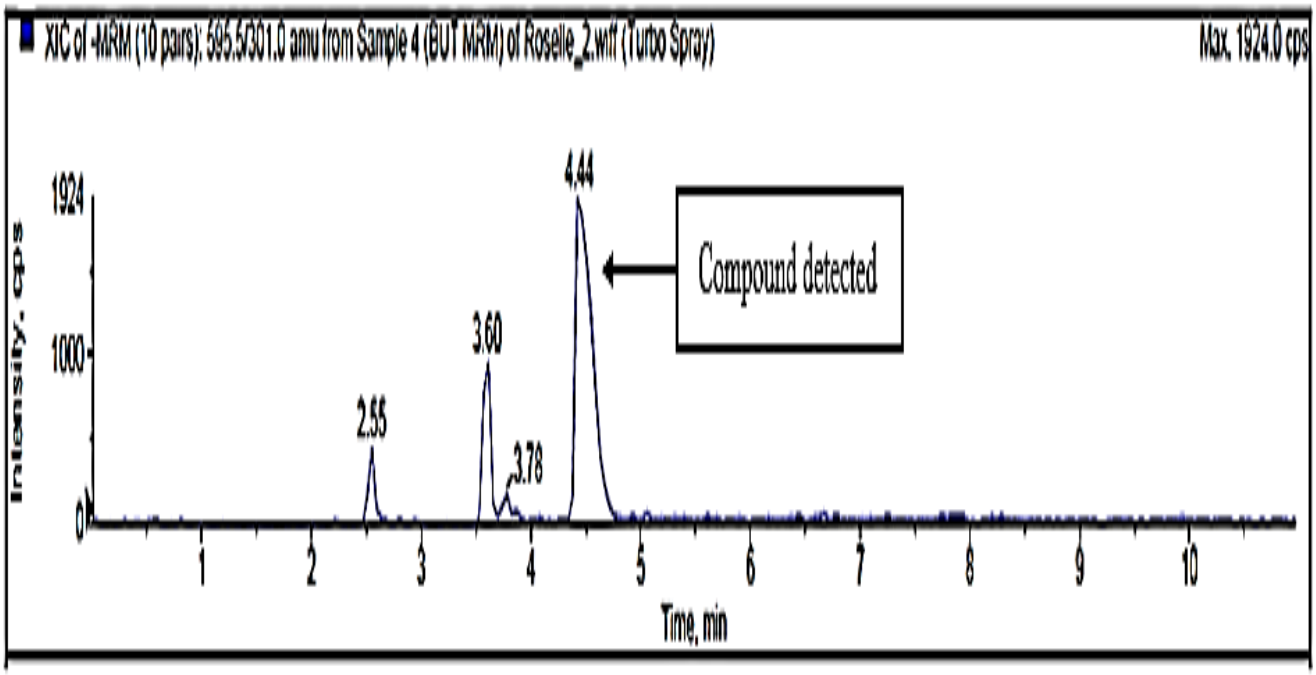

Figure 12 Chromatogram associated with the *H. sabdariffa Linn* fruit butanol fraction

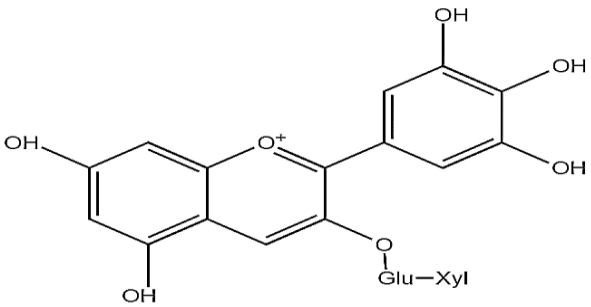

Figure 13 The chemical configuration of Dp-3-sam from *H. sabdariffa* Linn

4.0 DISCUSSION

A chronic disorder affecting the endocrine system, diabetes impairs the metabolic activity of carbohydrates, proteins, fats, electrolytes and water. It is distinguished particularly by the high levels of sugar in the blood, which are due to the fact that the insulin production of the pancreas is insufficient or cell unresponsiveness to insulin [37].

By highlighting the properties of Dp-3-sam, such as suppression of α-glucosidase enzyme and glucose movement via *in vitro* assay, wound healing via scratch assay, insulin production capability via BRIN-BD11 cell lines, increase of glucose assimilation via 3T3 F442A adipocytes and GLUT4 regulation at the surface of L6 myotubes, the present study demonstrated that this compound has potential as an anti-diabetes agent. As a result of this discovery, our finding successfully granted a patent No (MY-193036-A).

The therapeutic potential of certain plants from Malaysia for type 2 diabetes and high blood pressure was investigated in earlier studies, revealing that *Kacangbotol dichloromethane* extract could inhibit the α-glucosidase enzyme in a proportion of 38.94%. The present study, however, showed that Dp-3-sam at maximum concentration of 50 µg/ml could inhibit the α-glucosidase enzyme in a much higher proportion of 96.76%.

Furthermore, this study demonstrated that, compared to *T. polium*, Dp-3-sam had greater potency in retarding the glucose migration from a sealed dialysis tube to external solution, since *polyoleous* extracts were observed to lack significant trapping capability in slowing down such glucose migration.

Moreover, by comparison to negative control, Dp-3-sam in various concentrations enhanced wound healing, but not to the same extent as the positive control (i.e. ascorbic acid). *Spirulina platensis* methanol and ethanol extracts were investigated in terms of treatment potential in relation to human dermal fibroblasts. However, lesion healing failed to occur following incubation for 24 hours, indicating the inefficiency of processing such extracts via a scratch test.

The BRIN BD11 cells indicated that Dp-3-sam stimulated insulin production. Thus, by comparison to numerous other proposed compounds, Dp-3-sam could serve as an anti-diabetes agent of higher efficiency. It has been shown that insulin production in BRIN BD11 cells was triggered by nutrient regulation [29]. Meanwhile, compared to control, insulin production was increased by treatments with nymphayol [38] by 2.1-fold (p<0.05), *C. colocynthis* [39] by 1.8-fold (p<0.001) and *Achillea millefolium* L. (Asteraceae) [40] by 2.3-fold (p<0.001), thanks to *in vitro* glucose stimulation. Moreover, insulin production was also effectively triggered by adding a dose of Hank's balanced salt solution (0.1 mg/mL). Additionally, insulin production was moderately influenced by three novel flavonoids derived from *Pseudarthria hookeri* upon glucose stimulation [41]. However, Dp-3-sam had a more pronounced impact on insulin production than the compounds indicated above.

According to the findings of the present study, Dp-3-sam cannot increase basal- and insulin-mediated glucose assimilation in 3T3F442A adipocytes possibly due to reduced GLUT4 translocation and regulation to the plasma membrane or lack of insulin-mimetic and insulin-sensitising properties associated with the employed compound concentrations. Furthermore, Dp-3-sam could not regulate GLUT4 at the L6 myotube surface either. However, other medicinal plants were found to have a favourable effect on glucose assimilation and regulation of GLUT4 in L6 myotubes. For instance, anti-diabetes effects have been reported for *Cecropia obtusifolia Bertol*, which stimulated glucose assimilation in adipocytes with and without insulin sensitivity, with no significant pro-adipogenic effects [42].

5.0 CONCLUSION

This study found that Dp-3-sam suppressed the α-glucosidase enzyme and glucose diffusion and aided wound healing, thus constituting a promising candidate as an anti-diabetes agent targeting high blood glucose levels. Furthermore, insulin production in BRIN BD11 cells was revealed to be effectively promoted by Dp-3-sam at controlled concentrations. Stimulation of pancreatic β-cells to produce insulin was identified as the basis of the Dp-3-sam property of lowering glycaemia. All in all, Dp-3-sam has great potential for development of an oral hypoglycaemic drug based on the findings of experimental work and detailed analyses.

The main purpose of the present study was to investigate the effect that Dp-3-sam had on regulation of the mechanism of glucose assimilation in

3T3F442A adipocytes GLUT4 regulation at the surface of L6 myotubes. Results indicated that, by comparison to control, Dp-3-sam lacked sensitivity with regard to stimulation of glucose assimilation in 3T3F442A adipocytes. However, GLUT4 did not become significantly more widely distributed on the surface of L6 myotubes thanks to these derivatives. To conclude, it can be said that Dp-3-sam is a promising compound for the development of novel drugs against diabetes.

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Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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