**Effects of *Spirulina platensis* on insulin secretion, DPP-IV activity and both carbohydrate digestion and absorption indicate potential as an adjunctive therapy for diabetes**

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**Shortened version of title**: **Antidiabetic actions of *Spirulina platensis***

**Keywords:** Diabetes; plant therapies; glucose; insulin

**Abstract**

*Spirulina platensis* (*S. platensis*) has previously been shown in both animals and humans to be useful in the treatment of type 2 diabetes. The present study aims to elucidate the effects of ethanol extract and butanol fraction of *S. platensis* on insulin release and glucose homeostasis in type 2 diabetic rats, together with their mechanism of actions. Both *in vitro* and *in vivo* methods were used including cellular studies to determine potential role of ion channels and cAMP in the insulinotropic actions of the extracts. The ethanol extract and butanol fraction stimulated insulin release from mouse islets and pancreatic β-cells in a concentration-dependent manner. The butanol fraction also similarly stimulated insulin release from perfused rat pancreas. The insulin releasing action was augmented by glucose, isobutyl methylxanthine, tolbutamide and a depolarizing concentration of KCl. The insulin secretory effect was attenuated with diazoxide and verapamil and by omission of extracellular Ca2+. Butanol fraction was found to significantly inhibit DPP-IV enzyme activity. Moreover, butanol fraction improved glucose tolerance following oral glucose administration (2.5 g/kg b.w.). The butanol fraction was tested on 24 h starved rats given an oral sucrose load (2.5 g/kg b.w.) to examine possible effects on carbohydrate digestion and absorption. *S. platensis* substantially decreased post-prandial hyperglycemia after oral sucrose load and increased unabsorbed sucrose content throughout the gut. During *in situ* intestinal perfusion with glucose, the butanol fraction reduced glucose absorption and promoted gut motility. Finally, chronic oral administration of butanol fraction for 28 days significantly decreased blood glucose, increased plasma insulin, pancreatic insulin stores, liver glycogen and improved lipid profile. The characterization of active compounds from butanol fraction revealed the presence of p-coumaric acid, β-carotene, catechine and other antioxidant polyphenols. These findings indicate that *S. platensis* could be a novel adjunctive therapy for the management of type 2 diabetes.

**Introduction**

Diabetes mellitus (DM) is a metabolic syndrome where pancreatic β-cells fail to meet the body’s need for insulin with resultant hyperglycemia and increased risk of diabetic complications. The World Health Organization (WHO) recognized DM as the world’s fastest growing metabolic disorder. Type 1 diabetes is the result of total or near total β-cell destruction, whereas Type 2 diabetes mellitus (T2DM) is the result of β-cells dysfunction and insulin resistance. About 90% of diabetic patients suffer from T2DM. This condition is associated with the alterations in the metabolism of lipids, carbohydrates and proteins (1), which causes multiple complications including cardiovascular disease (2), retinopathy (3), neuropathy, cognitive decline (4), nephropathy and end-stage renal disease (3).

T2DM treatments include diet together with either a single or combination of oral anti-hyperglycemic agents, to manage dysglycemia (5, 6). Although, advances have been made recently in the treatment options to achieve better glycemic control, they are often expensive and associated with notable adverse effects (7, 8). This focusses attention particularly in poorer countries, towards herbal therapy and dietary supplements as alternative approaches to the mainstream medical treatment of T2DM. Unlike contemporary treatment, herb-based medicines are entirely natural, they possess very few adverse effects and are generally affordable (9). It has been reported that nearly 30–76% of T2DM patients from different countries are using herbal medicines (10, 11), and that this approach is managing T2DM with safety (12).

Over the last decades, functional foods received attention as a potential source of useful bioactive protein hydrolysates and peptides that have health benefits and reduce the risk of disease (13). In this way, many aquatic species have been studied with generation of new and useful bioactive peptides sources. For instance, the eukaryotic microalgae and prokaryotic cyanobacteria (often called blue‐green algae) utilization in the food industry has become globally popular (14, 15). Edible microalgal and cyanobacterial proteins have been established as suitable precursors for the production of biologically active peptides (14). Thus, a series of peptides isolated from microalgal or cyanobacterial hydrolysates has been demonstrated to exhibit desirable bioactivities, such as antioxidative, anticancer, anti‐inflammatory and antihypertensive properties (13, 16-18).

*S. platensis* is a unicellular Cyanobacterium belonging to the Cyanophyceae class, Oscillatoriaceae family (19). This organism (cyanobacterium) is characterised by spiral chains of the cells enclosed in a thin sheath. It contains very potent naturally occurring antioxidants, and free radical scavenging agents (20). *S. platensis* is nontoxic, bioavailable, and is believe to provide significant multiorgan protection against many drugs and chemically induced toxic assaults (21). Active constituents exhibit anti-inflammatory, neuroprotective, hepatoprotective, immunomodulatory, and anticancer activities (22). A recent animal study reported potential anti-diabetic effects of *S. platensis* but the mechanisms underlying such effects are unknown (23-25). In this paper we have made a detailed study of effects of *S. platensis* on pancreatic insulin release, DPP-IV inhibition, and various gastrointestinal tract actions to elucidate the mechanism and therapeutic potential of *S. platensis* for improvement of diabetes control.

**Materials and methods**

**1. Plant material and preparation of ethanol extract**

*S. platensis* was purchased from Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka, Bangladesh and was authenticated by a botanical taxonomist. A voucher specimen was deposited in the Bangladesh National Herbarium (Mirpur, Dhaka). The whole plants were dried at 40 °C (oven) and proceed into fine powder by a cyclotec-grinding machine. The powder (2 kg) was extracted with 80% ethanol (10 L) in a conical flask and put in to orbital shaker (550 rpm, 48 - 72 hr). The extract was filtered using a Whatman filter paper and ethanol was removed using a rotary evaporator (Figure 1). A Varian 801 LY-3-TT freeze dryer (Varian, Lexington, MA, USA) was used to freeze-dry the extract which was stored at 4°C until used.

**2. Preparation of Butanol fraction of *S. platensis***

According to the previously described method (26), the ethanol extract (50g) was partitioned into hexane (0.5L×3) and water (0.5L). Hexane fraction was separated and soluble material (15g) isolated after evaporation to dryness. The water layer was additionally partitioned using 1-butanol (0.5L×3), and soluble materials of 1-butanol (10.5g) were obtained after evaporation to dryness. The leftover watery portion was further concentrated using a rotary evaporator and the end product was dried (20g) using a freeze dryer, and stored in refrigerator at 40C until used (Figure 1). The ethanol extract and butanol fraction were analysed for bioactivity in the present studies.

**3. Insulin secretion from isolated islets and beta cell lines**

The effects of *S. platensis* on insulin release from BRIN-BD11 cells and isolated mouse islets were assessed as described previously (27). A range of concentrations of plant extract or butanol fraction or known modulators of insulin secretion were incubated with BRIN-BD11 cells in the presence or absence of glucose (1.1, 5.6 or 16.7 mM) during 40- or 20-min incubation at 37 oC. Islets were isolated from the pancreas of NIH Swiss mice (27). Groups of 10 islets were cultured for 24 - 48 hr in RPMI media prior to pre-incubation in KRB buffer at 1.4 mM glucose for 60 min. Test incubations were performed in the presence of 16.7 mM glucose for 60 min. After incubation, the supernatants were collected and stored at -20 oC until analysed by insulin radioimmunoassay (28).

**4. Insulin secretion from perfused pancreas**

Long-Evans male rats (180 - 250 g b.w.) were anaesthetized with Na-pentobarbital (50 mg/kg, i.p.) and the pancreas was isolated and perfused at 37 oC according to the method of Giroux *et al.* (29). KRB buﬀer supplemented with 1.25 g/L BSA and 40 g/L dextran T70 and 2.8- or 11.2-mM glucose. A mixture of O2: CO2 (95: 5) was continuously used to gas the perfusate. The composition of the perfusate was changed after the first 20 min of equilibration as indicated in Figure 4. Samples were stored at -20 oC prior to measurement of insulin using ELISA kits supplied by Crystal Chem (Downers Grove, IL, USA).

**5. Membrane potential** **and intracellular calcium ([Ca2+]i)**

The effects of *S. platensis* on membrane potential and intracellular calcium were measured using BRIN-BD11 cells monolayers as previously described (30,31). Cells were seeded onto 96-well plates (black-walled, clear-bottomed microplates, Greiner) and washed with Krebs-Ringer bicarbonate (KRB) buffer prior to addition of FLEX assay reagents (Molecular Devices, Sunnyvale, CA, USA) addition. Effects of introducing extract or butanol fraction and other reagents were monitored fluorometrically using Flex Station 3 (Molecular Devices) at a wavelength of 450nm and 525 nm respectively.

**6. DPP-IV activity**

Effects of *S. platensis* on DPP-IV enzyme activity were determined using a fluorometric method. Enzyme activity were determined using 96-well black-walled, clear-bottomed microplates (Greiner) containing 8 mU/ml of DPP-IV enzyme and 200 µM of substrate (Gly-Pro-AMC) as described previously (32). Flex Station 3 (Molecular Devices, CA, USA) was used to measure the changes in fluorescence with an excitation and emission at 370 nm and 440 nm with 2.5 nm slit width.

**7. Induction of experimental diabetes**

Long-Evans male rats (150 - 200 gm) were purchased from the International Center for Diarrheal Disease Research, Bangladesh (ICDDRB). Standard environmental conditions with temperature of 22 ± 5 oC, relative humidity of 55% - 65% and 12 hr light and dark cycle were maintained. Food and fresh water were supplied *ad libitum*. The composition of the pelleted diet (metabolisable energy of 11.8 MJ/kg/2820 kcal/kg) was described previously (33). T2DM was induced in neonatal rats at 2 days of age by a single intraperitoneal injection of streptozotocin (90 mg/kg b.w.). T2DM rats were selected for the experiments at 12 weeks of age after an oral glucose tolerance test (OGTT). Animals exhibiting blood glucose levels of 8 - 12 mmol/L were chosen as type-2 diabetes rats. The ‘Principles of Laboratory Animals Care’ (National Institutes of Health Publication no. 86-23, revised 1985) and the UK Animals Scientific Procedures Act 1986 were followed.

**8. Residual gut sucrose content**

The effects of *S. platensis* on sucrose digestion and absorption from the gastrointestinal tract were assessed after the oral administration of sucrose solution (2.5 g/kg b.w.) to 24h fasted T2DM rats with or without butanol fraction of *S. platensis* (250 mg/kg, body wt.). Blood samples were collected from the tip of the tail prior to and after 30, 60, 120 and 240 min for glucose analysis. To measure the unabsorbed sucrose content of the gastrointestinal tract, rats were sacrificed at the same time points and the tract was excised and divided in to six parts: the stomach, the upper (20 cm), middle and lower (20 cm) of the small intestine, the caecum, and the large intestine. Each segment was washed with acidified ice-cold saline and centrifuged for 10 min at 3000 rpm (1000 g). The resulting supernatant was boiled for 2h to hydrolyse the sucrose and pH was adjusted (7.0 - 7.4) by adding NaOH. The concentration of serum glucose and the total amount of glucose liberated from GI tract were determined (34).

**9. Intestinal glucose absorption**

The effects of *S. platensis* on intestinal glucose absorption were determined using *in situ* intestinal perfusion technique (35). Non-diabetic rats were fasted for 36 hr followed by induction of anaesthesia with sodium pentobarbital (50 g/kg body weight). Butanol fraction of *S. platensis* (5 mg/ml equal to 0.25 g/kg) dissolved in KRB buffer containing glucose (54 g/l) was infused via the rat pylori and perfusate was collected at the end of ileum. The control group was treated with KRB buffer only in the presence of glucose. Perfusion of intestine was carried out at a constant rate of 0.5 ml/min for 30 min at 37oC. The results were expressed as the percentage of glucose absorbed, measured from the amount of glucose in solution before and after the perfusion of intestine.

**10. Intestinal disaccharidase activity and gastrointestinal motility**

Intestinal disaccharidase enzyme activity was determined as described previously (33). Nondiabetic rats were fasted for 20 hr followed by the oral administration of *S. platensis* (250 mg/kg) or water alone. The rats were sacrificed after 1 hr and the small intestine was collected, cut longitudinally and rinsed with ice-cold saline. Volume was made up to 10 mL by adding saline (0.9% NaCl) and the tissue was homogenized. Aliquots of the homogenate were incubated at 37 oC in a 40 mM sucrose solution for 60 min. Disaccharidase enzyme activity was measured as µmol/mg protein/h. Acarbose (200 mg/kg), an established disaccharidase enzyme inhibitor was used as control. GI motility was measured according to the method of Chatterjee (36). The butanol fraction of *S. platensis* (250 mg/kg b.w.) or water (10 ml/kg) were administered orally to nondiabetic rats. One hour later, a suspension of BaSO4 milk (10% BaSO4 & 0.5% carboxymethyl cellulose; w/v) was administered orally. Rats of both groups were sacrificed after 15 min of administration of BaSO4. The length travelled by BaSO4 milk was calculated and expressed as the % of the total distance of the small intestine (pylorus to the ileo-caecal junction). The established drugs: Loperamide (LPM) (5 mg/kg b.w.) and Sennoside (10 mg/kg b.w.) were used as positive controls.

**11. Glucose tolerance and chronic effects in type 2 diabetic rats**

The effects of butanol fraction of *S. platensis* on oral glucose tolerance were measured after fasting the T2DM rats for 12 hr. Blood samples were obtained from the cut tip of the tail at given time points (0, 30, 60, 120 and 180 min) before and after oral administration of glucose (2.5 g/kg body weight) with (Treated group) or without (Control group) *S. platensis* (250 mg/kg). The long-term effects of *S. platensis* on glucose homeostasis in T2DM rats were measured by twice daily administration (oral gavage) of butanol fraction (250 mg/kg) for 28 days. Control rats received oral gavage of water alone. Blood samples were collected and serum was separated by centrifugation and stored at -20 oC until measurement of glucose, insulin and lipid profiles. Glucose and insulin were measured via GOD-PAP method (glucose kit, Randox™, UK) and rat insulin ELISA Kit (Crystal Chem™, USA)*.*

**12. Effects of *S. platensis* on liver glycogen content**

The effects of *S*. *platensis* treatment for 28 days on liver glycogen content were determined as previously described (37). Briefly, the weight of liver was measured and finely homogenized with 10 ml of 5% trichloroacetic acid (TCA). The precipitated proteins were filtered, and glycogen content was analyzed from the clear filtrate. 1 mL of the filtrate was mixed with 2 mL of 10 N KOH and boiled for 1 hr at 100o C. After cooling, 1 mL of glacial acetic acid was added, and the solution was made up to 10 mL by adding deionized water. Then 1 mL of this solution was mixed on ice with 2 mL of anthrone solution (100 mg anthrone dissolved in 50 mL of conc. H2SO4), this mixture was boiled for additional 10 minutes at 100o C and then cooled. Aliquots were taken in a microplate reader and the absorbance was measured at 490 nm.

**13. Insulin content in pancreases**

Rats were sacrificed after 28 days treatment with *S*. *platensis* (250 mg/kg) and the pancreatic tissues were dissected, weighed, homogenized and extracted in (10 ml) acid alcohol solution (23.5% distilled H2O, 75% ethanol and 1.5% HCl 12 mM). After centrifugation, the supernatant samples were stored at -20 oC. Pancreatic insulin level was determined using rat insulin ELISA Kit (Crystal ChemTM, USA).

**14. Chemical characterization by RP-HPLC**

Crude extract was re-dissolved in solvent A (0.12% (v/v) TFA/Water) and purified by reversed-phase high-performance liquid chromatography (RP-HPLC). The prepared crude extract solution was injected in to a Vydac 218TP1022 (C-18) reversed-phase HPLC column (Grace, Deerfield, IL, USA) equilibrated with 0.12% (v/v) TFA/water at a flow rate of 1.0ml/min. The concentration of acetonitrile within the eluting solvent was expanded using linear gradients 0 to 20% over 10min, and to 70% over a period of 25min. The wavelength of 254nm and 360nm were used to measure the absorbance.

**14. Statistical analysis**

Statistical analyses were performed by using SPSS for windows (V.20). The results are represented as mean ± SEM. Data was analyzed using repeated measures ANOVA followed by Dunnett adjustment and unpaired t-test where applicable. P<0.05 was considered as the level of significance.

**Results**

**1. Effects of *S. platensis* on insulin secretion from clonal pancreatic β-cells (BRIN BD11)**

Figure 2 (A & B) shows the effects of ethanol extract and butanol fraction of *S. platensis* on insulin secretion from BRIN-BD11 cells. Alanine (10 mM) was used as a positive control. Both extract and fraction (1.6 – 5000 µg/ml) stimulated insulin release concentrations-dependently compared with control (5.6 mM glucose). Higher concentrations (1000 – 5000 µg/ml) also induced insulin release but were associated with decreased cell viability. Further tests revealed that the insulinotropic effects of a non -toxic dose of *S. platensis* (40 µg/ml) were significantly enhanced in the presence of 16.7 mM glucose (p<0.001), isobutyl-methyl xanthine (IBMX, (p<0.001) and tolbutamide (p<0.001, Figure 2 E - F). The phosphodiesterase inhibitor IBMX and tolbutamide were used to modulate the glucose-induced cAMP production and insulin secretion. In contrast, the effects of both ethanol extract and butanol fraction of *S. platensis* were inhibited by 40% -50% by diazoxide (p<0.05) and verapamil (P<0.01, Figure 2 E - F). Both diazoxide, a KATP channel opener and verapamil, voltage-gated calcium channel blocker inhibited pancreatic insulin secretion by reducing intracellular Ca2+influx. The ethanol extract and butanol fraction also maintained an ability to increase insulin secretion in cells depolarised with 30 mM KCl (p<0.001, Figure 2 E - F). Absence of Ca2+ (Figure 2 G - H) significantly inhibited but did not totally abolish insulin secretion induced by *S. platensis.*

**2. Effects of *S. platensis* on insulin secretion from isolated islets**

The ethanol extract and butanol fraction significantly increased insulin secretion from isolated mouse islets compared with 16.7 mM glucose alone (P<0.05 to P<0.001; Figure 2 C & D). Increasing the concentrations from 25 to 200 µg/ml resulted in a 2 and 2.5-fold increase in insulin release compared with control (16.7 mM glucose); GLP-1 (10-6 & 10-8 M) and alanine (10 mM) were used in this experiment as positive control (Figure 2 C & D).

**3.** **Eﬀects of *S. platensis* on insulin secretion from perfused pancreas**

In a pilot study, the pancreas retained insulin secretory capacity during 70 min with exposure to glucose and arginine (Figure 4E). Butanol fraction of *S. platensis* produced a significant (P<0.001) biphasic increase in insulin release with a 20-fold elevation above the basal level (2.8 mM) (Figure 4 A). Subsequent exposure for 10 min to 11 mM glucose caused a sharp rise of insulin release from the basal level of 0.05 - 0.01 ng/ml to a peak of 2.4 - 1.5 ng/ml (P<0.001). After adding butanol fraction to 11 mM glucose, a further enhancement in insulin release was noted (Figure 4 A), which opposed the decline in insulin release under the continuous exposure to 11 mM glucose alone. Theophylline (10mM) is a methylxanthine derivative that inhibits cyclic nucleotide phosphodiesterase and increases the intracellular cyclic 3′,5′-AMP level. Addition of theophylline (10 mM) to butanol fraction increased insulin secretion further (Figure 4B). As shown in Figure 4C - D, perfusion in the presence of verapamil and diazoxide at 11.1 mM glucose decreased insulin releasing activity of the butanol fraction by 20 to 30%.

**4. Eﬀects of *S. platensis* on membrane potential and intracellular calcium ([Ca2+]i) in the clonal BRIN-BD11 cell line**

The ethanol extract and butanol fraction of *S. platensis* increased membrane potential and intracellular calcium ([Ca2+]i) at the presence of 5.6 mM glucose (p<0.05; Figure 3 A-D and E-H) as compared to control (5.6mM glucose alone). KCl (30mM) and alanine (10mM) were used as positive controls in this experiment.

**5. Effects of *S. platensis* on DPP-IV activity *in vitro***

The butanol fraction significantly (p<0.05, p< 0.01 and p<0.001) inhibited the liberation of AMC (7-amino-4-methylcoumarin), by 7 - 70% at concentrations of 200 - 5000 µg/ml (Figure 5 F). Sitagliptin, as an established DPP-IV inhibitor, reduced DPP-IV activity by 10% to 97% (p<0.05, p < 0.01 and p<0.001; Figure 5 E) at concentrations between 16x10-4 µM to 5 µM.

**6. Effects of *S. platensis* on unabsorbed sucrose content in the gut**

One hour following sucrose load (2.5 g/kg b.w.), a significant (p<0.05-0.01) amount of sucrose was measured in the stomach and the upper, middle, lower, and large intestine (Figure 6A-F). An extensive (p<0.01) amount of sucrose found in the stomach, upper and middle intestine at 30 min, while at 2 hr (p<0.05) increased amounts of unabsorbed sucrose were measured in caecum and lower intestine. The administration of butanol fraction (250 mg/kg b.w.) with sucrose reduced sucrose absorption significantly (p<0.01) after 30 min and up to 2 hr (Figure 5). At 4 hr, sucrose content was almost nil throughout the GI tract, although a small fraction remained in the ceacum and lower intestine, indicating rapid hydrolysis and absorption of sucrose in the upper part of the intestine (Figure 6).

**7. Effects of *S. platensis* on intestinal glucose absorption**

During perfusion, the extent of glucose absorption in the intestine was almost consistent over 30 min. However, supplementation with butanol fraction significantly decreased intestinal glucose absorption (p<0.05 to p<0.01) as illustrated in the Figure 7 A and B.

8. **Effects of *S. platensis* on intestinal disaccharidase activity and gastrointestinal motility**

Butanol fraction of *S. platensis* (250 mg/kg b.w.) did not affect disaccharidase enzyme activity (Figure 7C). However, this fraction significantly promoted gastrointestinal motility (p<0.05, Figure 7D).

**9.** **Acute and chronic effects of *S. platensis* on glucose homeostasis in type 2 diabetic rats**

Oral administration of butanol fraction of *S. platensis* (250 mg/kg b.w.) together with glucose (2.5 g/kg body weight) improved glucose tolerance at 30 and 60 min (p<0.01 to p<0.05) in T2DM rats (Figure 5 A & B). In addition, *S. platensis* (250 mg/kg b.w.) treatment lowered serum glucose significantly after sucrose load (p<0.05; Figure 5 C & D) at 60 and 120 min in T2DM rats.

Furthermore, twice daily oral administration of the butanol fraction of *S. platensis* (250 mg/kg b.w.) for 28 days significantly lowered serum glucose levels (p<0.05) and increased serum insulin level (p<0.05) compared to controls (Table 1). Pancreatic insulin and liver glycogen were increased (p<0.05) as shown in Table 1. Measurement of lipid profile showed that the butanol fraction of *S. platensis* (250 mg/kg b.w.) increased HDL, while the LDL and total cholesterol (TC) were significantly decreased (p<0.05-0.01; Table 1).

**10. Chemical characterization by RP-HPLC**

The phytochemical screening of butanol fraction by HPLC (Figure 8) revealed presence of β-carotene, catechine and some other previously identified compounds in *S. platensis* such as zeaxanthin, astaxanthin, p-coumaric, and apigenin (38, 39). The concentrations of nutritional chemical characterization of *S. platensis* are listed in the Table 2.

**Discussion**

*S. platensis* has been reported recently to exhibit antihyperglycaemic effects but the mechanism of action was not elucidated (23-25). This study has examined the insulinotropic effects of *S. platensis* using the perfused rat pancreas, isolated mouse islets and the clonal BRIN-BD11 beta cell line. The results suggest that the anti-hyperglycemic effects are partly mediated through stimulating the insulin secretion from pancreatic β-cells (27), which is further supported by observation of incremental increases in serum and pancreatic insulin after 28 days of chronic treatment.

Both the ethanol extract and butanol fraction dose-dependently enhanced insulin release from isolated islets and clonal BRIN-BD11 cells. The butanol fraction was more insulinotropic than the ethanol extract. Likewise, the butanol fraction exerted substantial insulin release from perfused rat pancreas. Non-toxic concentrations of *S. platensis* were used to examine mechanisms underlying stimulation of insulin secretion in the absence and presence of known modulators of β-cell function. Sulfonylureas are known to act by closing KATP channels, depolarizing the plasma membrane, as induced by 30 mM KCl, and stimulating Ca2+ entry by activation of voltage-dependent calcium channels (26, 40). *S. platensis* stimulated insulin release enhanced by tolbutamide and KCl (30mM), suggesting its ability to potentiate insulin secretion via other pathways such as the adenylate cyclase/cAMP or the phosphatidylinositol pathway, or as a direct effect on exocytosis (26). *S. platensis* also clearly stimulated β-cells via its effects on Ca2+ ion channels. Thus, diazoxide, a KATP-channel opener (41) inhibited the insulin-releasing effects of both the extract and fraction. This suggests that *S. platensis* closes KATP channels to induce insulinotropic action. Furthermore, the L-type voltage-dependent Ca2+ channel blocker, verapamil, (42) also reduced the insulin-releasing effects of *S. platensis*. This suggests a dependency on Ca2+ channel to induce insulin release. Similar intracellular Ca2+-dependency was found in BRIN-BD11 cells, where, *S. platensis* increased insulin release, which was inhibited by the L-channel blocker verapamil. Furthermore, studies with BRIN BD11 cells showed the *S. platensis* induced membrane depolarization and increased [Ca2+]i.

The insulin releasing effects of *S. platensis* were also markedly increased by the phosphodiesterase inhibitors IBMX and theophylline implicating involvement of the cAMP pathway (43). Recent studies have shown that use of *S. platensis* in asthma as adjunct therapy has improved the condition significantly (44). The anti-asthmatic actions have been attributed to elevation of cAMP in bronchial smooth muscle cells, promoting airway relaxation and blocking replication of smooth muscle cells (45). Overall, these results indicate that the polar solvents ethanol and butanol contain active molecules of *S. platensis* that exert multiple effects on the β-cells mediated most importantly via ion channels.

Dipeptidyl peptidase IV (DPP-IV) is an enzyme that metabolises incretin hormones and as a result terminate the insulin releasing and glucose lowering actions of both GLP-1 and GIP (46). DPP-IV inhibitors have been developed to enhance endogenous incretin action and treat T2DM (47). The two incretin hormones have been shown to have multiple action by increasing pancreatic insulin secretion and reducing glucagon secretion (46). In present study, *S. platensis* significantly (P<0.05, P<0.01 and P<0.001) inhibited DPP-IV enzyme activity, indicating the butanol fraction can enhance endogenous GLP-1 and GIP activity. A recent study has shown that flavonol glycosides from the seeds of *Lens culinaris* Medikus (Fabaceae) inhibited DPP-IV enzyme activity in the dose-dependent manner (48). Therefore, it is anticipated that the same phytochemicalconstituents of *S. platensis* may be responsible for the observed DPP-IV inhibitory activity effect.

Administration of *S. platensis* significantly lowered blood glucose and improved glucose tolerance in T2DM rats. The butanol fraction also significantly inhibited glucose absorption during gut perfusion. As anticipated, significant amounts of unabsorbed sucrose were found in postprandial state throughout the gut. This postprandial effect may be related to the interference of intestinal glucose absorption (33). Butanol fraction of *S. platensis* did not inhibit intestinal disaccharidase enzyme activity and thus inhibition of digestion of carbohydrate is not involved in its mechanism of antihyperglycaemic action. However, increased GI motility, examined using BaSO4 milk may inhibit carbohydrate absorption in the gut. Dietary fibers reduce postprandial food transit time in GI tract (49), thus, shorter time is available for the carbohydrates absorption (50) and therefore postprandial hyperglycemia is reduced. High sucrose content in the GI tract indicates reduced sucrose digestion. As a result, a significantly higher concentration of sucrose reaches to the large intestine and caecum and is excreted. *S. platensis* reduced postprandial sucrose absorption and enhanced GI motility, possibly by forming glucose-fiber complexes that reduce post prandial transit time or gastric emptying time.

A recent study using *S. platensis* reported potential protective activity against fat induced apoptosis and decreasing intestinal cholesterol absorption (51, 52). However, in the present chronic study, administration of butanol fraction of *S. platensis* for 28 days in type 2 diabetic rats significantly increased liver glycogen content and HDL, while it reduced LDL substantially. Interestingly, chronic treatment also enhanced plasma insulin and pancreatic insulin content in type 2 diabetic rats. Therefore, the stimulation of insulin release from β-cells as well as insulin action is possibly thereby co-related with the improvement of hepatic glucose uptake.

The phytochemical screening of *S. platensis* using RP-HPLC revealed the presence of several phenolic acids like p-coumaric and other bioactive molecules such as pheophytin A, catechine, zeaxanthin, astaxanthin, apigenin and carotenoid pigments including β-carotene. This composition is in general agreement with previous research (38, 39). Recent studies also claim that these compounds have antioxidant properties, trigger insulin secretion and have antihyperglycaemic activity (53-55).

In conclusion, this study has shown that ethanol extractof *S. platensis* and its butanol fraction exert prominent stimulatory effects on insulin secretion from β-cells via physiological pathways. *In vivo* studies in T2DM rats indicate that the butanol fraction decreased blood glucose, increased gut motility, reduced glucose absorption in GIT and improved both plasma and pancreatic insulin levels. *S. platensis* contains important phytochemicals and valuable micro/macronutrients, consistent with the use of this microalgae as a prophylactic or dietary supplement in the treatment of diabetes.

**Acknowledgments**

The authors would like to thank the School of Biomedical Sciences and members of the Diabetes Research Group for providing access to their laboratory and the use of facilities to carry out this research.

**Conflict of Interest**

The authors declare that there is no conflict of interest.

**Financial support**

The present study was supported by the Ulster University Strategic Research Funding and award of Vice Chancellor’s research studentship to P.A.

**Statement of authorship**

Y. H. A. A.W. and P. R. F. were responsible for the conception and design of research and also contributed equally to the supervision of the study. P. A., J. M. A. H. and S. A. performed the experiments. P. A and S. A. analysed the data; P. A. and J. M. A. H. interpreted the results of experiments; P. A. prepared the figures; P. A. and S. A. drafted the manuscript; Y. H. A. A. W., P. R. F. and P.A. edited the revised manuscript; all authors approved the final version of the manuscript.

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**Figure Legends:**

**Figure 1:** **Schematic diagram of preparation of ethanol extract and butanol partition fraction of *S. platensis*.**

**Figure 2: Effects of ethanol extract and butanol fraction of *S. platensis* on insulin release from (A & B) BRIN-BD11 cells, (C & D) Islets of Langerhans and, (E-H) BRIN BD11 cells in the presence of established stimulators or inhibitors of insulin secretion.** Values are Mean ± SEM, n = 8 and 4 for insulin release. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 compared to 5.6 mM and 16.7 mM glucose alone. ϕP<0.05, ϕϕP< 0.01 and ϕϕϕP<0.001 compared to 5.6 mM glucose in the presence of the extract or fraction. ΔP<0.05, ΔΔP<0.001, ΔΔΔP<0.001 compared to respective incubation in the absence of the extract or fraction.

**Figure 3: Effects of ethanol extract and butanol fraction of *S. platensis* on (A, B & C, D) membrane potential and (E, F & G, H) intracellular calcium in BRIN-BD11 cells expressed as RFU and respective area under the curve.** Values are Mean ± SEM, n = 6 for membrane potential and intracellular calcium. \*\*\*P<0.001 compared to 5.6 mM glucose alone.

**Figure 4: Effects of butanol fraction of *S. platensis* on insulin release from perfused rat pancreas in the (A) absence or (B) presence of theophylline (10mM), (C) Verapamil (50µM) and (D) Diazoxide (8mM) at 11mM glucose and (E) control group: Arginine (19mM) alone.** Values are Mean ± SEM with n = 4. Pancreas was perfused (1 ml/min) with butanol fraction of *S. platensis* at a dose of 5mg/ml in the presence or absence of theophylline (10 mM), verapamil (50 µM) and diazoxide (8 mM) at 11mM glucose and control group: Arginine (19mM) alone. The glucose concentration was raised from the basal level of 2.8mM (basal) to 11mM. G: Glucose, THEO: Theophylline and BSP: Butanol fraction of *S. platensis.*

**Figure 5: Effects of butanol fraction of *S. platensis* on (A & B) glucose tolerance (GTT), (C & D) serum glucose after sucrose load (SGASL) in type 2 diabetic rats and (E & F) DPP-IV activity *in vitro*.** Rats were fasted for 12 and 24 hr and administered glucose or sucrose solution (2.5 g/kg b.w.) by oral gavage in presence or absence of butanol fraction of *S. platensis* (250 mg/kg b.w.). Sitagliptin was used as established DPP-IV inhibitor. Values are Mean ± SEM represented by vertical bars (n = 6, for GTT and SGASL and n = 3 for DPP-IV). \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 compared to control.

Figure 6: Effects of butanol fraction of *S. platensis* on (A-F) gastrointestinal sucrose content after oral sucrose loading in type 2 diabetic rats. Type 2 diabetic rats were fasted for 24 hours prior to the oral administration of sucrose solution (2.5 g/kg b.w.) in the presence (treated group) or absence of (control group) butanol fraction of *S. platensis* (250 mg/kg b.w.). Values are Mean ± SEM represented by vertical bars (n = 6). \*P<0.05 and \*\*P<0.01 compared with type 2 diabetic control rats.

Figure 7: Effects of butanol fraction of *S. platensis* on (A & B) intestinal glucose absorption, (C) disaccharidase enzyme activity and (D) gastrointestinal motility (by BaSO4 traversed) in non-diabetic rats. Rats were fasted for 36 hrs and intestine was perfused with glucose (54 g/l) in the presence (treated group) or absence of (control group) butanol fraction of *S. platensis* (10 mg/ml). BaSO4 was administered at 60 min following oral feeding of *S. platensis*. Acarbose (ACB) (200 mg/kg); and Loperamide (LPM) (5 mg/kg) and Sennoside (10 mg/kg) were used as positive controls for determinations of disaccharidase activity and gastrointestinal motility respectively. Values are Mean ± SEM represented by vertical bars (n = 8). (\*P<0.05, \*\*P<0.01 and \*\*\*P<0.001) compared with controls.

**Figure 8:** **Representative HPLC profile of butanol fraction of *S. platensis* using analytical C-18 column over the period of acetonitrile.** The column was equilibrated with 0.1% (v/v) TFA/water at flow rate of 1.0ml/min. The concentration of the eluting solution was raised using linear gradients from 0 to 20% acetonitrile over 10 min, to 70% over 25 min. Details of peaks corresponding to butanol fraction are presented in the chromatogram. UV detection was set at 254 and 360 nm and 1mg/ml sample was injected each run. Peaks 1-9 of unknown compounds were detected at different retention time (RT).

**Table 1:** **Long-term effects of butanol fraction of *S. platensis* on blood glucose, plasma insulin, pancreatic insulin content and other parameters in type 2 diabetic rats after 28-day study**

Table 1

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Parameters | 0 days | | 28 days | |
| Control | Butanol fraction | Control | Butanol fraction |
| Glucose (mmol/l) | 9.71 ± 1.41 | 9.53 ± 1.27 | 9.67 ± 1.45 | 7.31 ± 1.17 \* |
| Plasma Insulin (ng/ml) | 0.47 ± 0.19 | 0.48 ± 0.29 | 0.46 ± 0.18 | 0.67 ± 0.15\* |
| Pancreatic insulin (nmol/g) | - | - | 0.81 ± 0.15 | 1.1 ± 0.11\* |
| Liver glycogen (g/100g) | - | - | 1.45 ± 0.22 | 2.26 ± 0.30\* |
| Total cholesterol (mg/dL) | - | - | 63.59 ± 2.59 | 46.83 ± 2.03\* |
| HDL (mg/dL) | - | - | 43.49 ± 2.55 | 56.83 ± 2.02\* |
| LDL (mg/dL) | - | - | 36.97 ± 6.14 | 13.55 ± 4.30 \*\* |

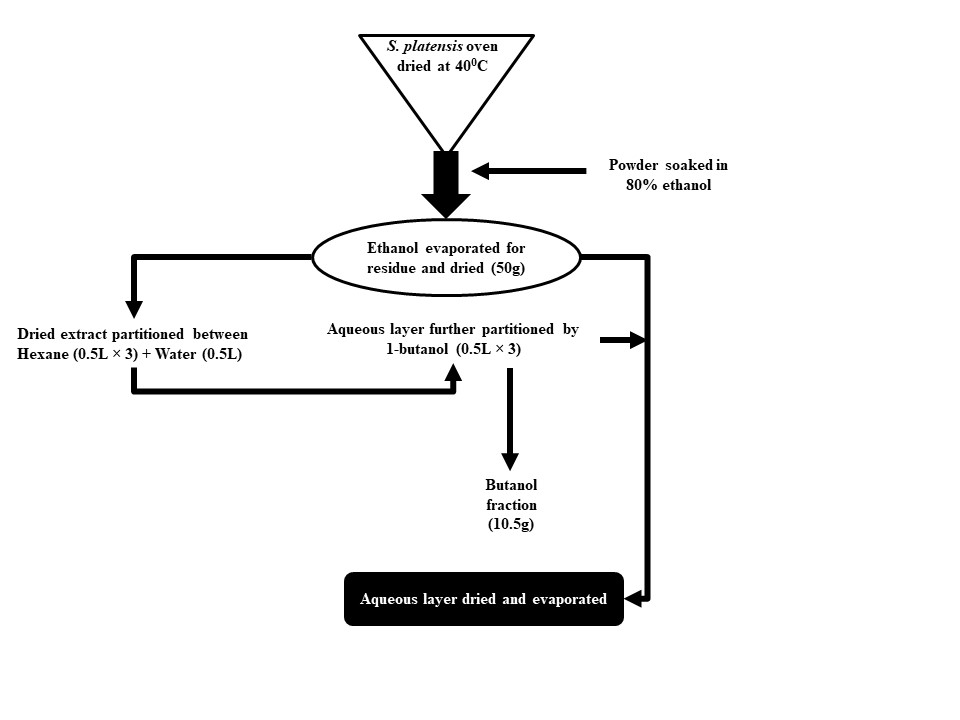
Butanol fractionof *S. platensis* (250 mg/kg b.w.)) or only saline (control) were administered orally to rats for 28 days. Values are Mean ± SEM (n = 8). \*P<0.05 and \*\*P<0.01 compared with type 2 diabetic control rats.

**Table 2:** Major compounds identified by RP-HPLC of butanol fraction *of S. platensis*.

|  |  |  |  |
| --- | --- | --- | --- |
| **Peaks** | **Compounds** | **RT (min)** | **Concentration**  **(µg/ml)** |
| 1 | Siphonein | 10.3 | 20.6 |
| 2 | Zeaxanthin | 12.2 | 30.5 |
| 3 | Astaxanthin | 12.7 | 17.8 |
| 4 | Unidentified carotenoids | 14.9 | 122.4 |
| 5 | Catechine | 23.5 | 15.6 |
| 6 | Pheophytin A | 26.2 | 15.6 |
| 7 | P-coumaric | 30.5 | 51.3 |
| 8 | β-carotene | 30.8 | 22.1 |
| 9 | Apigenin | 33.5 | 29.7 |

RT: retention time; min: minutes

**Figure: 1**



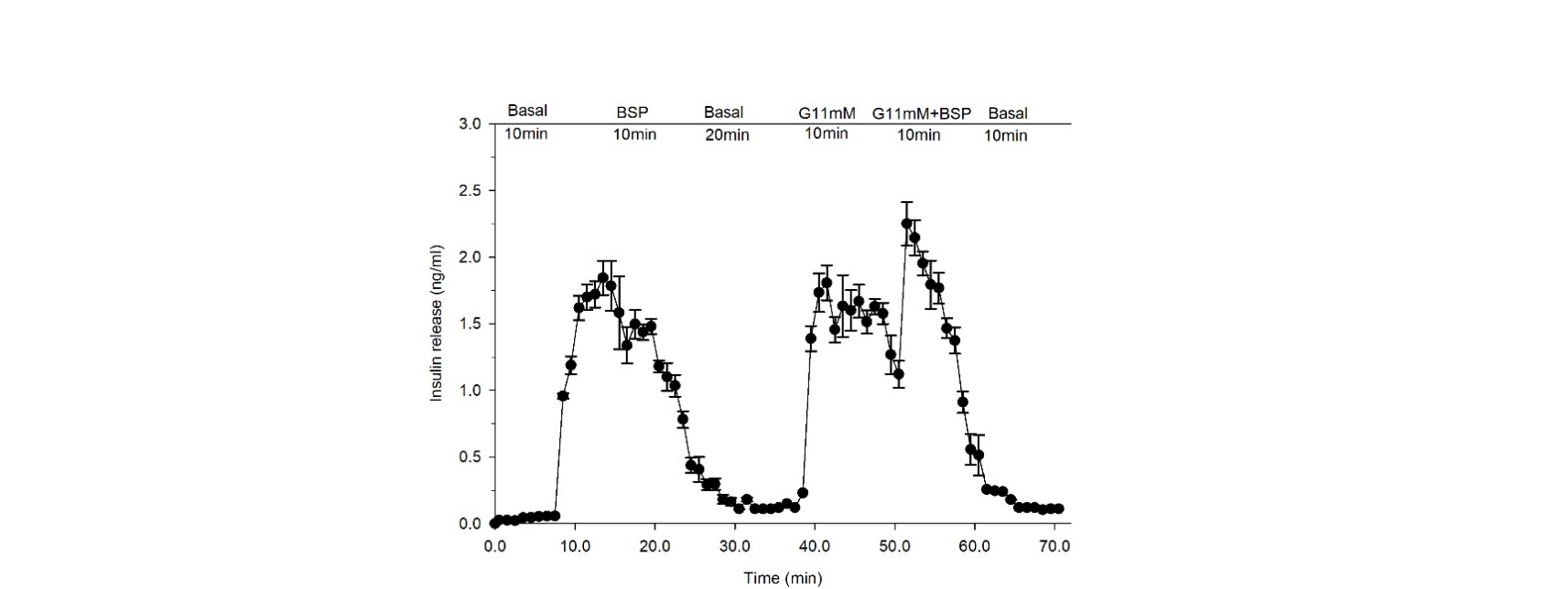
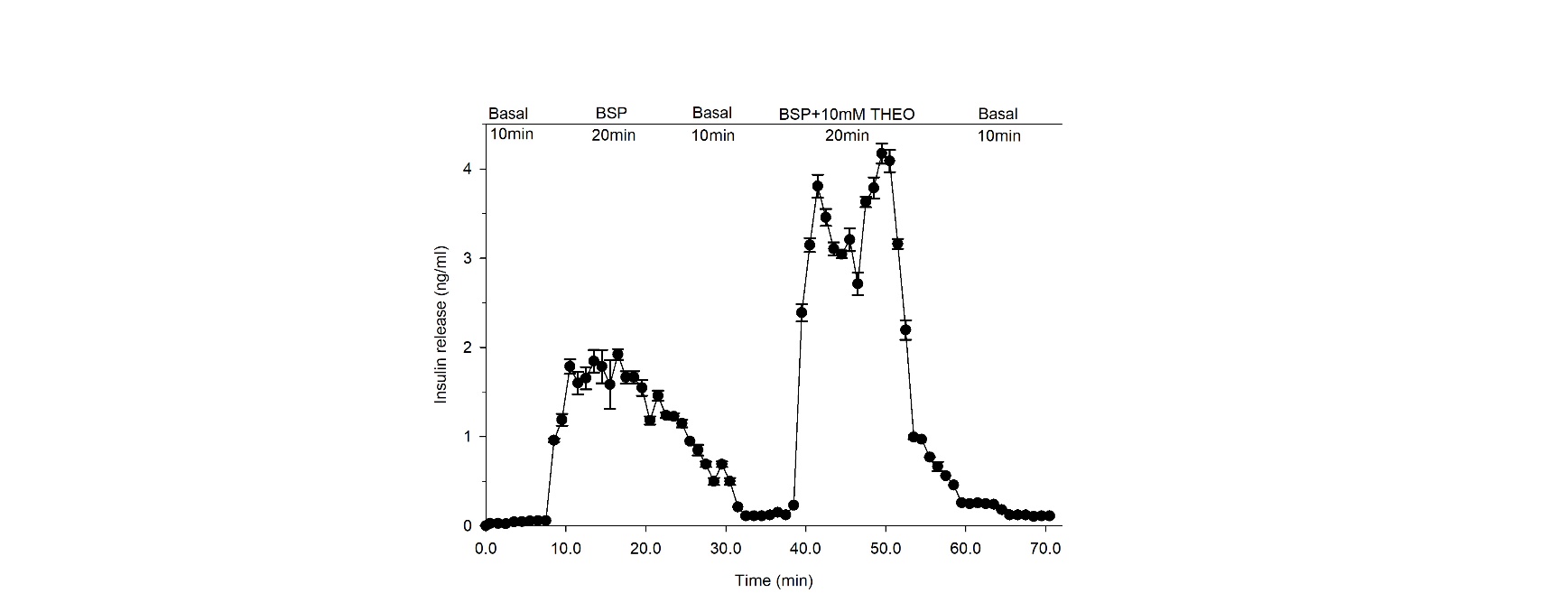
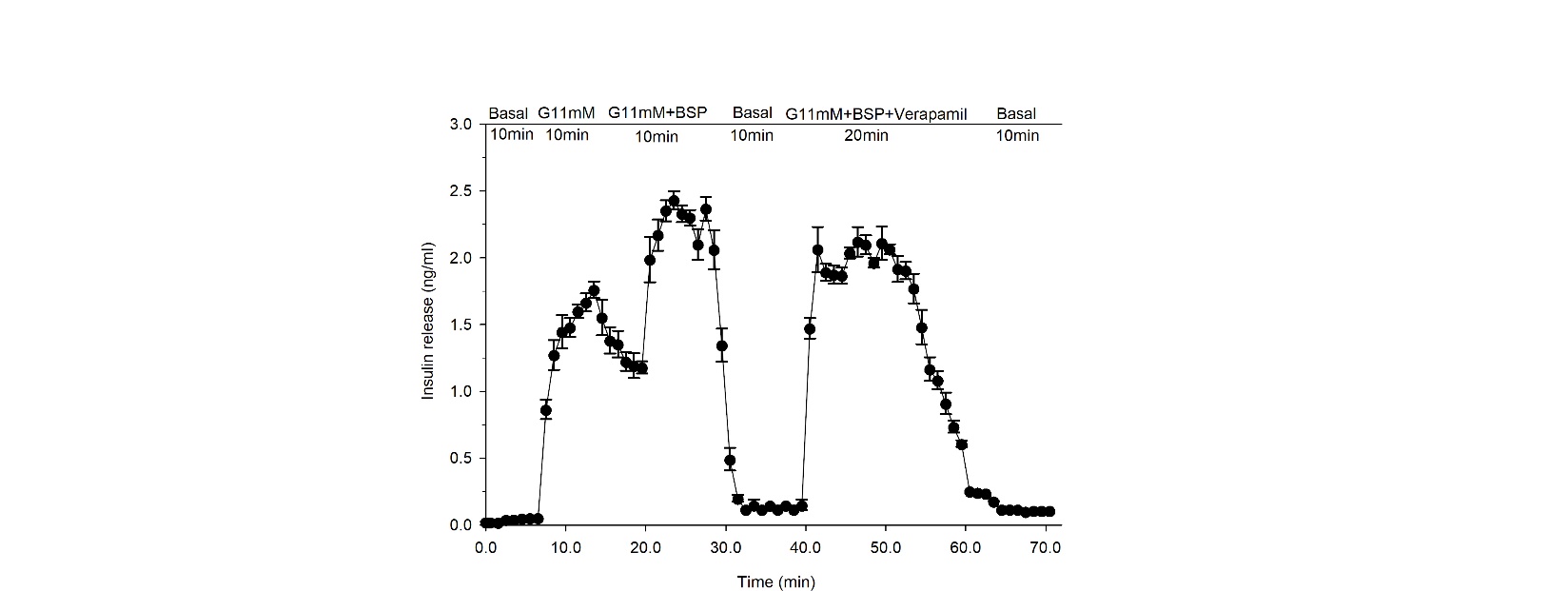
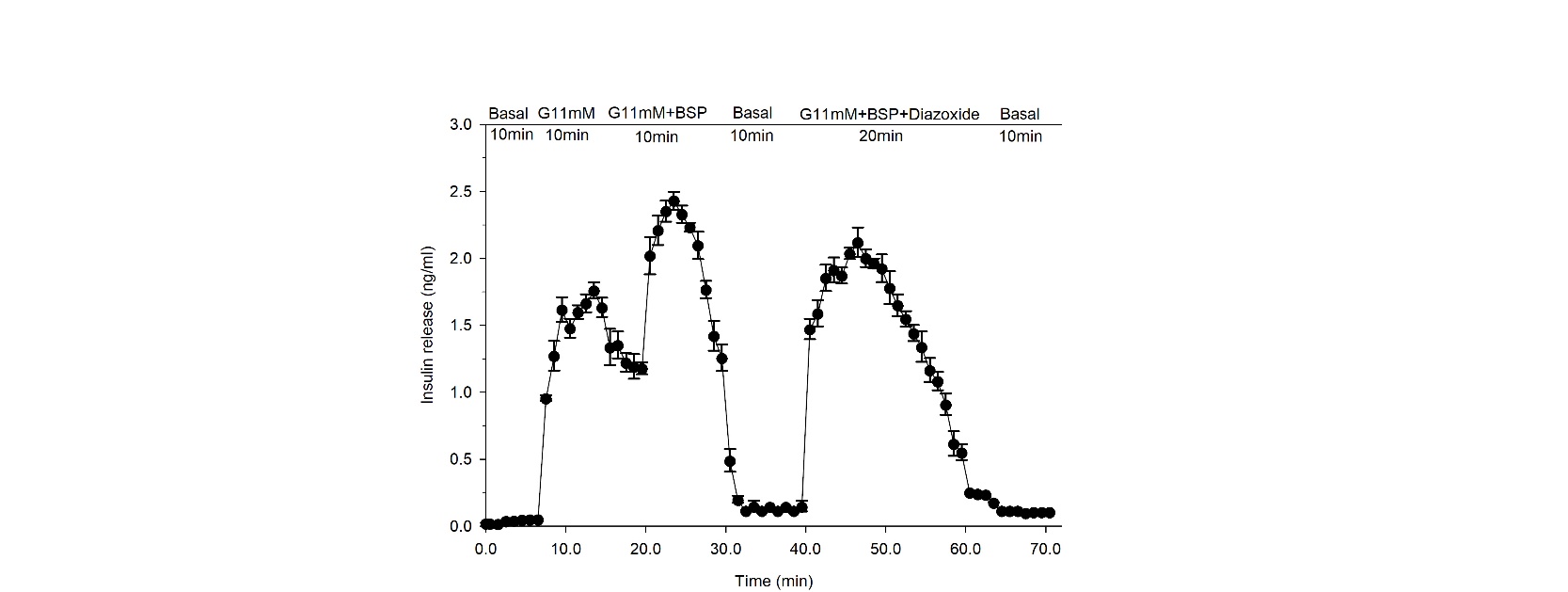
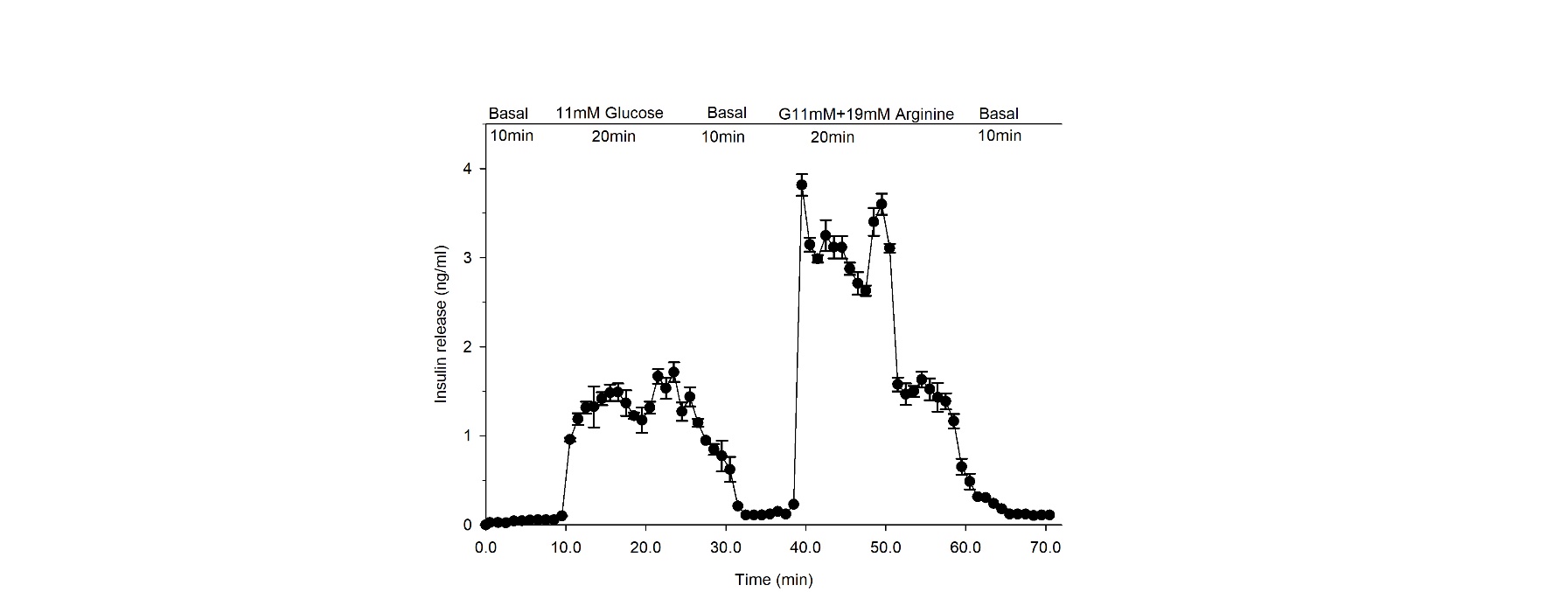


**Figure: 2**

**Figure: 3**

****

**Figure: 4**



**(E)**

**(D)**

**(B)**

**(C)**

**(A)**

**Figure: 5**

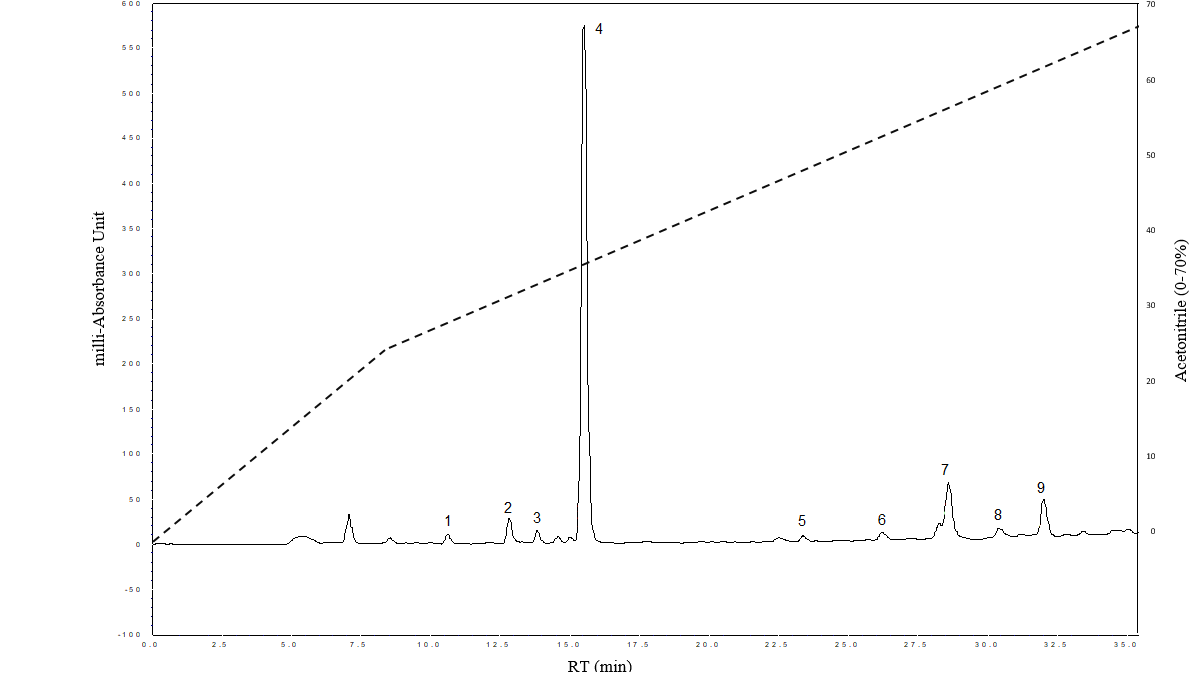


**Figure: 6**



**Figure: 7**





**Figure: 8**