Triple-action of the standardized antidiabetic polyherbal extract; Synacinn™ through upregulation of *GLUT4* and inhibition of *DPP(IV)*, α-amylase, and α-glucosidase activity

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ABSTRACT

Introduction: Synacinn™ is a standardized polyherbal supplement for diabetes mellitus which is formulated from *Andrographis paniculata, Curcuma xanthorrhiza, Cinnamomum zeylanicum, Eugenia polyantha,* and *Orthosiphon stamineous*.

Materials and Methods: This study aimed to elucidate the antidiabetic potential of Synacinn™ on three specific actions, including 1) the insulin sensitivity and glucose transport on dexamethasone-induced insulin-resistance 3T3-L1 adipocytes, 2) the inhibitory capacity on postprandial enzyme activity (*α-amylase and α-glucosidase*), and 3) the inhibitory activity of hepatic *DPP(IV)* enzyme.

Results: Results showed that insulin resistance of 3T3-L1 adipocytes may be developed by prolonging the exposure of 1µg/ml of dexamethasone for >48 hours. The insulin-resistance condition was minimized by the treatment of 10 µg/ml of Synacinn™ which significantly improved the insulin-stimulated glucose utilization by 10.6%. Meanwhile, insulin-stimulated glucose utilization in normal adipocytes was also attenuated by 9.2%. At the cellular level, Synacinn™ attenuated glucose utilization mainly by upregulating *GLUT4* protein expression by 1.71 fold. Additionally, Synacinn™ is a potent inhibitor for the activity of *α-amylase* and *α-glucosidase* with IC_{50} of 0.467 mg/mL and 0.245 mg/mL, respectively. Synacinn™ also controlled the glycemic index through inhibition of hepatic *DPP(IV)* enzyme with IC_{50} of 1.11 mg/mL.

Conclusion: Results suggested that Synacinn™ reduced diabetes mellitus through sensitizing the cellular glucose utilization, reducing the postprandial carbohydrate degradation, and inhibiting the hepatic *DPP(IV)* enzyme function.

INTRODUCTION

In recent decades, new drugs and drug classes have become available for type-2 diabetes mellitus (T_2DM) patients that act at different sites of actions including sulfonylureas, meglitinides, biguanides, thiazolidinediones, *DPP(IV)* inhibitors, and *α-amylase* and *α-glucosidase* inhibitor.[1,2] Physicians would prescribe these drugs based on the level of glucose and hemoglobin 1C (HB1C), which may include a single or combination of oral therapy drugs.[1] Despite advanced research on drug development, DM therapies require lifelong drug consumption to control the glycemic condition at a healthy level. Unfortunately, these drugs have limitations and unwanted side effects. For example, metformin increases glucose uptake in body tissues and inhibits gluconeogenesis in the liver, but it causes gastrointestinal problems,[2] hepatotoxicity[3] and is not suitable for patients with kidney problems.[4] Meanwhile, sulphonylureas, an insulin release stimulator, is only ideal for T2DM patients.[5] Potentially natural therapies derived from the plants (single compounds, a group of compounds or whole extract) have become a popular choice to reduce and prevent the DM traditionally.[6,7]

Synacinn™, a traditional polyherbal supplement is recommended for the treatment of DM, and has symptoms including tiredness and high blood glucose level. Synacinn™ is formulated from five herbs, including *Andrographis paniculata, Curcuma xanthorrhiza, Cinnamomum zeylanicum, Eugenia polyantha,* and *Orthosiphon stamineous*. Qualitative and quantitative HPLC fingerprinting of this formulation has been critically developed as reported by Zainol et al.[8] Synacinn™ contains gallic acid, catechin, rosmarinic acid, curcumin, cinnamaldehyde, and andrographolide which are known as therapeutic agents against various diseases. Herb-drug interaction analysis also recommended that Synacinn™ could be consumed separately from a drug known to be metabolized by all tested CYP450 enzymes.[9] It is believed that the synergistic outcomes of this combination involved multiple mechanisms, ultimately in covering all the possible effects of DM in the body. Synacinn™ at 250 (b.i.d.) mg kg^{-1} normalizes the blood glucose level, total glyceride, and cholesterol in STZ-induced rats.[10] It also protects the liver, kidney, and pancreas from the damage caused by STZ administration.[10] However, the fundamental mechanism behind the antihyperglycemic event is still unknown. This study investigated the reversal of insulin-resistance conditions using an *in-vitro* model developed by the acute exposure of dexamethasone (DEX) on 3T3-L1 adipocytes.

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Subsequently, the utilization of glucose and intracellular protein expression was assessed. Furthermore, Synacinn™ was also examined for its ability to inhibit the postprandial enzymes α-amylase and *α-glucosidase* as well as hepatic *DPP(IV)* activity.

MATERIALS AND METHODS

Materials

Standardized water extract of Synacinn™ was supplied by Naturemedics Laboratories Sdn. Bhd. Terengganu, Malaysia. DEX,3-isobutyl-1-methylxanthine (IBMX), insulin,and rosiglitazone (ROS) were purchased from Sigma Aldrich (St. Louis, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Invitrogen (Carlsbad, CA, USA). Mouse 3T3-L1 preadipocyte was purchased from American Type Culture Collection, Manassas, USA. Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS),and penicillin strep (PS) were purchased from Gibco, Life Technologies (Rockville, MD, USA).

Cells maintenance and differentiation

3T3-L1 preadipocytes were cultured and maintained between 80% and 90% confluency in DMEM supplemented with 10% of FBS and 1% of PS. To initiate differentiation, two-days post confluent cells were incubated with differentiation medium (DMEM supplemented with 10% FBS, 0.5 mM IBMX, 2 mM DEX, and 1.7 mM insulin). After 48–72 h, spent media was replaced by DMEM supplemented with 10% FBS and 1.7 mM insulin. Differentiated adipocytes were maintained in DMEM until day ten.

Cytotoxicity assay

All procedures were referred to Ismail et al.[11] with slight modifications. Preadipocytes were treated with Syancinn™ ranging from 5 to 10000 µg/mL diluted in DMEM for 24 h. MTT solution was added to each well and incubated for 4 hours at 37°C. The developed formazan was dissolved in 200µL of DMSO and analyzed using microplate reader (Biotec, ELx 808, Vermont, USA) at 570 nm via the KC Junior program. Treatment was conducted in six replications. Untreated cells were used as controls.

Induction and validation of insulin resistance inadipocytes

Fully differentiated adipocytes were serum-starved and treated with1 µM of DEX diluted in DMEM for 24, 48, and 72 h. Upon completion of insulin-resistance induction, cells were treated with 50 µM ROS for 48 h. The development of insulin resistance was considered successful if the glucose utilization was significantly lower ($p<0.05$) than that of the control.

Synacinn™ treatment on insulin-sensitive and insulin-resistance adipocytes

Prior to Synacinn™ treatment, cells were starved for 3 h in the basal DMEM. Normaland insulin-resistance adipocytes were treated with Synacinn™ at concentrations of 1, 10, and 100 µg/mL with and without the 1 µg/mL of insulin. After 48 h of incubation, spent media were collected, and glucose utilization was assayed using the Cobas C111. Subsequently, cells were lysed,and the supernatant was collected and stored at –80°C for protein analysis. ROS was used as a positive control. Treatment was conducted in triplicates.

Western blotting

Samples (30µg) were separated by electrophoresis, transferred and blocked by the 5% of skimmed milk. Primary antibody anti-*GLUT4* (1:2000) (PA519621, Thermo Scientific), *IRS-1* (1:2000) (PA11057, Thermo Scientific), *PI3K* (1:2000) (4257P, Cell Signaling Technology), *AKT* (1:2000) (4691P, Cell Signaling Technology), were added for overnight incubation at 4°C with the continuous shaking. Then, the membrane was incubated with a secondary antibody-AP conjugate (1:7500) (0031210, Thermo Scientific) for 1 h at room temperature. The developed band was scanned and quantified using ImageJ.

DPP (IV) inhibitor assay

DPP(IV) inhibition assay was conducted according to the *DPP (IV)* inhibitor screening assay kit (Cayman; 700210). Reaction was initiated by mixing 30µL of assay buffer, 10µL of *DPP(IV)* enzyme, 10µL of samples, and 50µL of substrate solution. The mixture was incubated for 30 min at 37°C. Fluorescence reading was obtained by using an excitation wavelength of 355 nm and an emission wavelength of 458 nm with a multimode reader (Varioskan Flash, Thermo Scientific).

α-Amylase inhibitor assay

The activity of α-amylase was assayed according to the manufacturer protocol (Abcam; ab102523) with modification. A total of 5 µL of 0.5U/µL Aspergillus oryzae α-amylase was preincubated with 45 µL of samples for 30 min at 37°C. Total amount of 100 µL reaction mix was added to each reaction and mixed carefully. The mixtures were allowed to react for 20 min, followed by the measurement of absorbance at 405 nm. Acarbose was used as a positive control. Percent of inhibition was calculated by using Equation(1).

$$\%inhibition = \frac{^{OD}control - ^{OD}sample}{^{OD}control} \times 100 \quad (1)$$

α-Glucosidase inhibitor assay

The inhibitory activity of *α-glucosidase* was conducted according to the procedures provided by the QuantiChromTM *α-Glucosidase* Assay Kit (DAGD-100) with modifications. About 10µL of 1.0U/mL of *α-glucosidase* from baker's yeast was preincubated with 10µL of samples for 15 min at 37°C. A total of 200µL of working reagent was added and allowed to react for 30 min. The reaction was measured at 405nm. Acarbose was used as a positive control. Percent of inhibition was calculated usingthe Equation(1) mentioned above.

Statistical analysis

All data were expressed in mean ± standard error (SEM). Statistical analysis was performed using SPSS program with one-way ANOVA and Tukey test. Significant differences were considered asp< 0.05.

RESULTS

Effect of Synacinn™ on insulin-resistance in-vitro model

Development of insulin-resistance adipocytes by dexamethasone

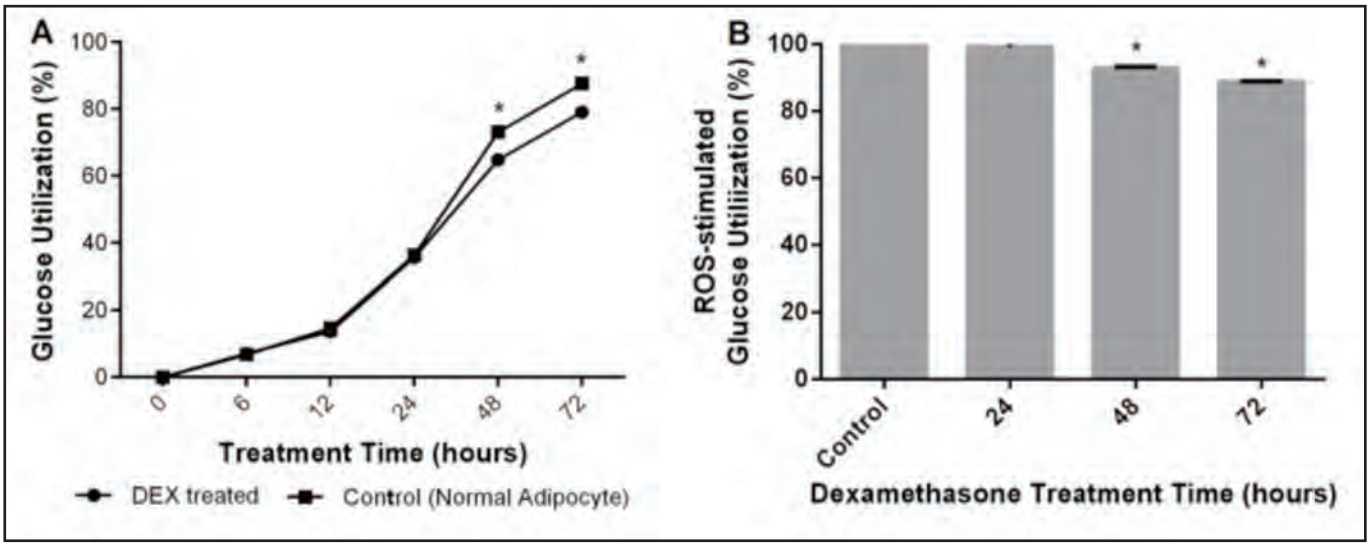

Fig. 1: A)Insulin-stimulated glucose utilization during the induction of insulin resistance by DEX. [*] Significant differences ($p< 0.05$) of 8.3% and 8.5% were quantified at 48 h and 72 h, respectively, as compared to control. Treatment was carried out in triplicates. B)Validation of insulin-resistance model by rosiglitazone (ROS). Significant differences of 7.1% and 11.2% were measured at 48 h and 72 h of treatment, respectively.

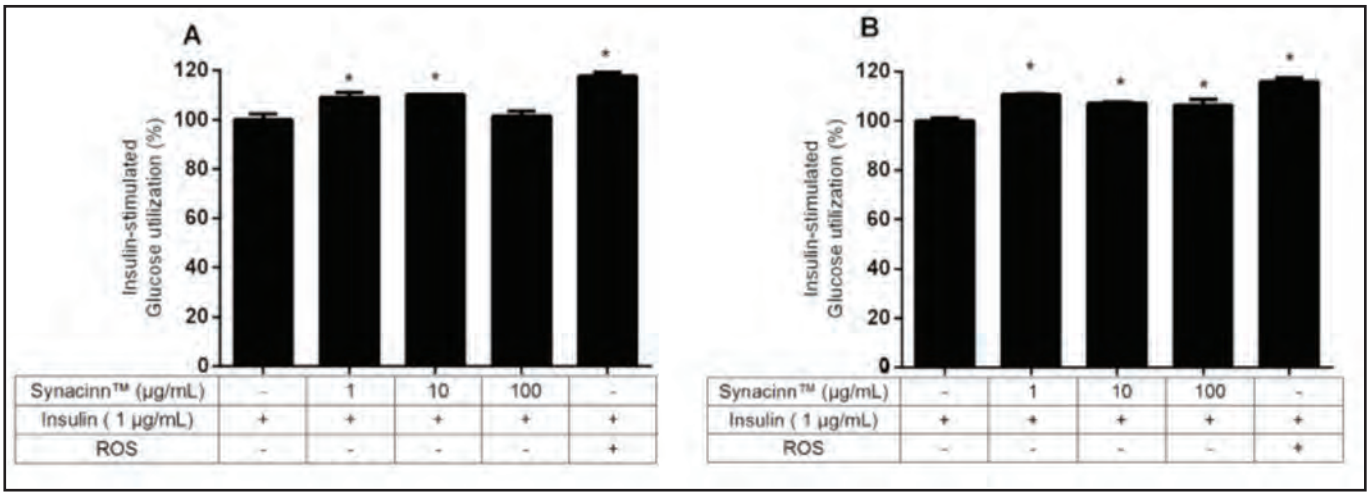

Fig. 2: Effect of Synacinn™ on insulin-stimulated glucose utilization. A) Effect of Synacinn™ on glucose utilization in normal adipocytes. B) Effect of Synacinn™ on the glucose utilization in insulin resistant adipocytes. Rosiglitazone (ROS) was used as a positive control. [*] Significance was considered as $p< 0.05$.

Induction of insulin resistance by DEX was carried out in the presence of insulin, which manifests the condition inthe human body. As illustrated in Fig 1 (A), treatment of 1µM DEXfor 72 h showed a time-dependent inhibition on glucose utilization. The presence of DEX partially disturbed the glucose utilization starting at 48 h of treatment, and continuously inhibited until the end of the experiment. Significant differences ($p < 0.05$) of 8.3% and 8.5% were quantified at 48 h and 72 h of DEX treatment, respectively, as compared to the control (normal adipocytes).

In addition, following the induction of DEX, the insulin-resistance condition was validated in the presence of 50µM ROS as presented in Fig 1 (B).The ROS-stimulated glucose utilization was consistent with the results in Fig 1. Significant differences were observed starting at 48 h (7.1%) and 72 h (11.2%).

Synacinn™ sensitized the insulin-stimulated glucose utilization in normal adipocytes and insulin-resistance adipocytes

Glucose utilization stimulated by insulin is predominant in insulin-sensitive tissues like muscles and adipose. In this study, normal adipocytes and insulin-resistance adipocytes were treated with Synacinn™ for 48 h, and the glucose concentration in spent media was measured to estimate the utilization of glucose by cells. As in Fig. 2 (A), insulin-stimulated glucose utilization in normal adipocytes was

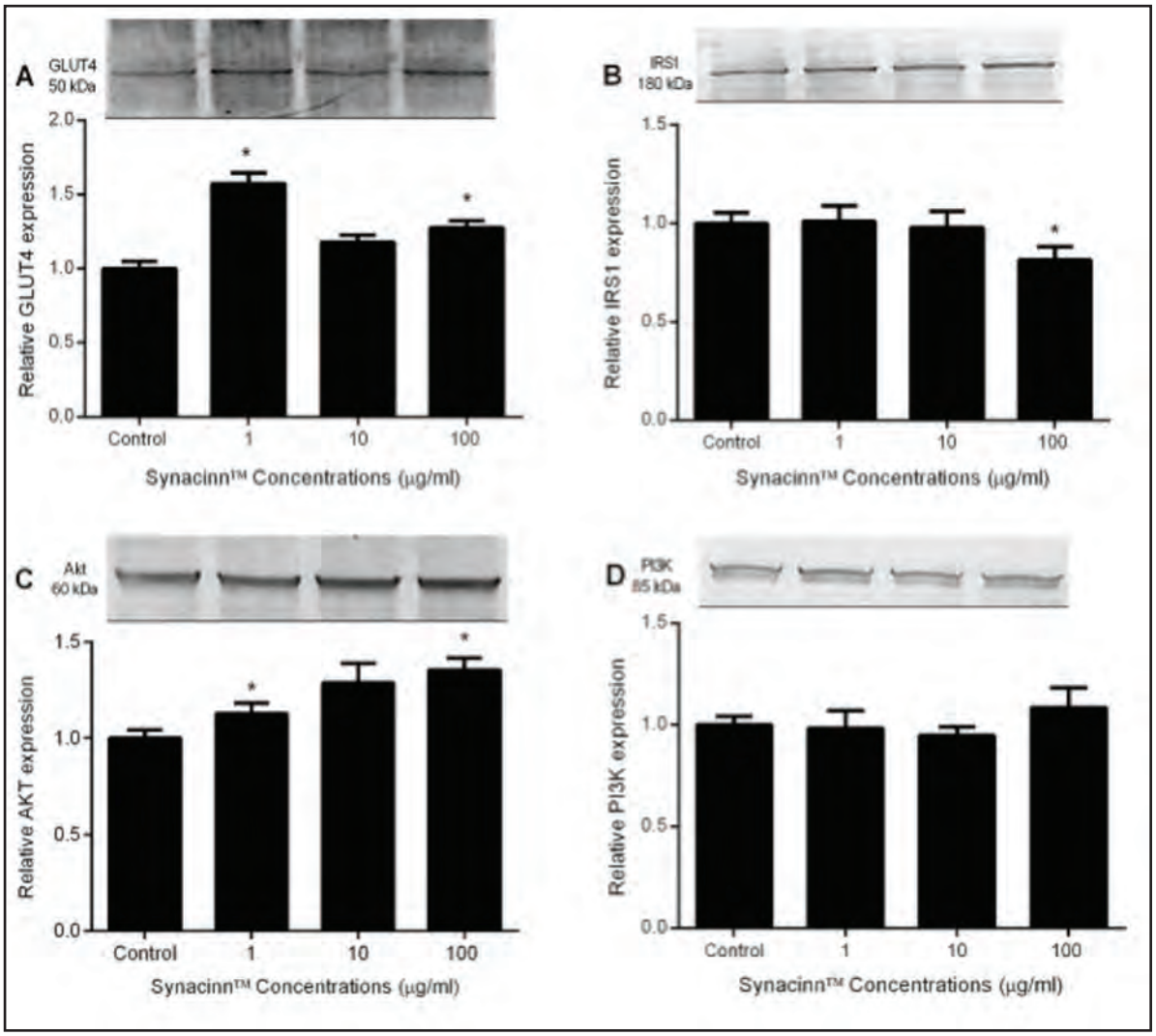

Fig. 3: Effect of Syancinn™ on the expression of insulin signaling pathway proteins in normal adipocytes. A) Relative expression of *GLUT4*, B) relative expression of *IRS-1*, C) relative expression of *AKT*, and D) Relative expression of *PI3K*. [*]Significance was considered as $p< 0.05$.

significantly increased during the treatment of Synacinn™. At a concentration of 1 and 10 µg/mL, Synacinn™ significantly ($p< 0.05$) attenuated glucose utilization by 9.2% and 10.2%, respectively. However, total of 100 µg/mL failed to increase the glucose utilization. As expected, ROS as positive control enhanced the glucose utilization by 17.6%.

The effect of Synacinn™ on glucose utilization was further investigated using DEX-induced insulin-resistance adipocytes. Fig.2 (B) demonstrates that Synacinn™ in the presence of insulin restored the impaired glucose utilization process. In comparison with the control (insulin only), significant improvement ($p< 0.05$) on glucose utilization was observed with 10.6%, 7.2%, and 6.3% increment for 1, 10, and 100 µg/mL of Synacinn™ treatment, respectively.

Synacinn™ increased glucose utilization through upregulation of GLUT4

Previously, it was discovered that Synacinn™ enhanced glucose utilization in normal and insulin resistant adipocytes. Further analysis of the expression of proteins related to the insulin signaling pathway has shown that Synacinn™ treatment on normal adipocytes enhances the expression of *GLUT4* and *AKT*. The total *GLUT4* expression was markedly increased during the treatment of all concentrations with 1.55-, 1.17-,and 1.28-fold for 1, 10, and 100 µg/mL,respectively (Fig.3A). Meanwhile, the expressions of AKT were increased by 1.13-, 1.29-, and 1.22-fold during treatment of similar doses of Synacinn™ (Fig.3C). No changes in the expression of IRS-1 and *PI3K* were detected upon Synacinn™ treatment (Fig.3B and Fig.3D).

The treatment of Synacinn™ on insulin resistant adipocytes showed that Synacinn™ treatment in the presence of insulin specifically improved the expression of total *GLUT4* (Fig.4). Treatment of 1, 10, and 100 µg/mL Syancinn™ significantly increased the total *GLUT4* expression by 1.39-, 1.71-, and 1.59-fold, respectively (Fig.4A). However, the changes of expression of *IRS-1*, *AKT*, and *PI3K* were not significant during Syancinn™ treatment.

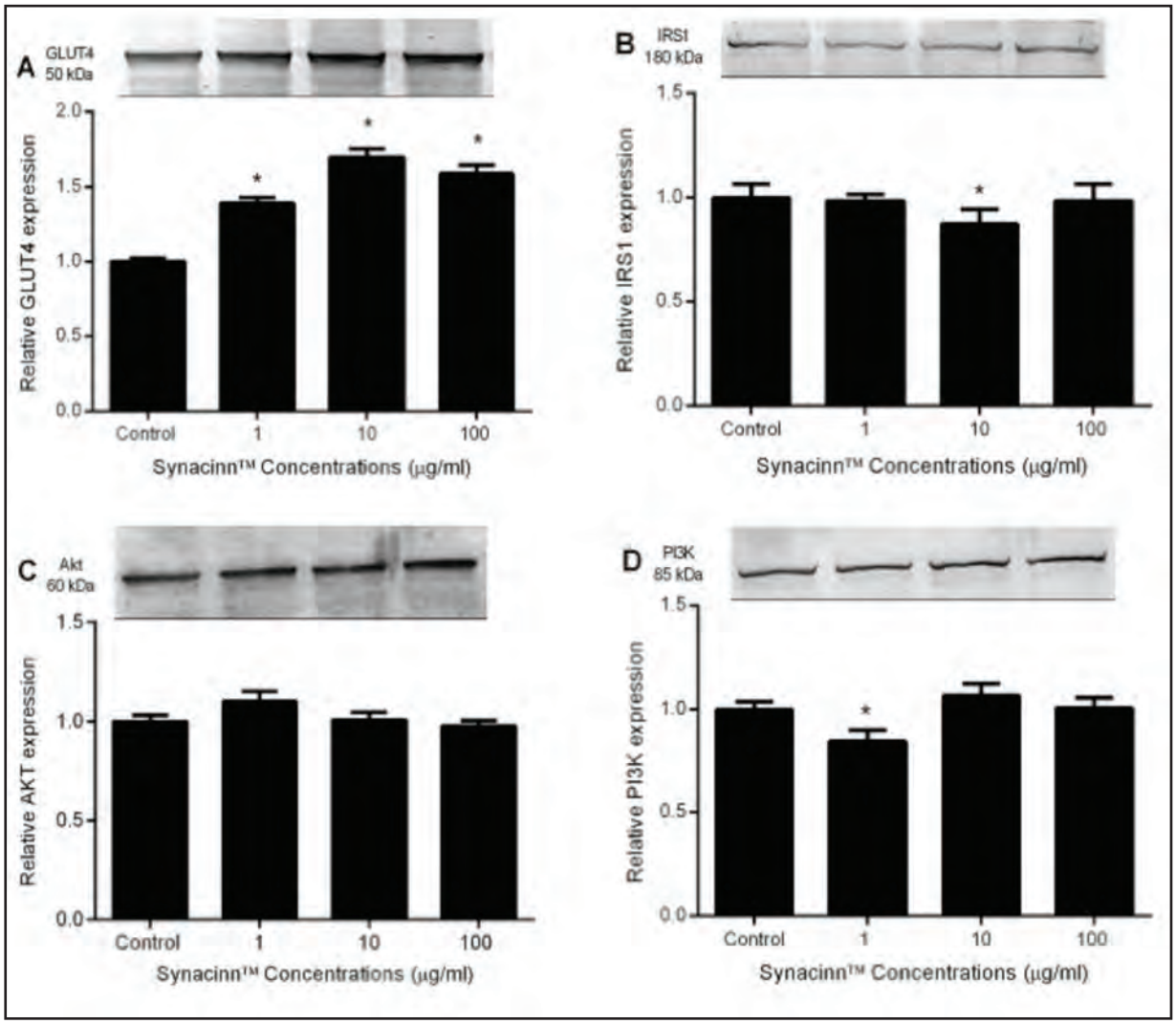

Fig. 4: Effect of Syancinn™ on the expression of insulin signaling pathway proteins for insulin-resistance adipocytes. A) Relative expression of *GLUT4*, B) relative expression of *IRS-1*, C) relative expression of *AKT*, and D) relative expression of *PI3K*. [*] Significance was considered as $p< 0.05$

Effect of Synacinn™ on the inhibition of DPP(IV), α-amylase, and α-glucosidase activities

Another route of antidiabetic therapies is by inhibiting DPP(IV) enzyme activity from converting glycogen in the liver to glucose, which in turn will increase the glycemic index in the blood. In this study, adose-dependent inhibition trend was observed during the treatment of Synacinn™ on *DPP(IV)* activity (Fig 5). At the highest tested concentration, 4 mg/mL, Synacinn™ inhibited 94.3% of its activity with IC_{50} of 1.11 mg/mL.

The antidiabetic effect of Synacinn™ was further analyzed on the inhibition activity of the postprandial enzymes *α-amylase* and *α-glucosidase* as presented in Fig. 6. A dose-dependent inhibition was achieved by Synacinn™ toward the activity of both enzymes. At the highest concentration of 4 mg/mL, Synacinn™ inhibited 98.5% of *α-amylase* activity and 96.6% for *α-glucosidase* with IC_{50} of 0.467 mg/mL and 0.245 mg/mL, respectively.

DISCUSSION

Synacinn™, a polyherbal supplement for DM, is formulated from five different types of Malaysian herbs, including *A. paniculata, C. zeylanicum, C. xanthorrhiza, E. polyantha,* and *O. stamineus*. It is believed that this combination triggers a synergistic mechanism ultimately to overcome all the possible effects of DM. In a recent study, *in-vitro* pharmacodynamics tests were conducted to identify the antidiabetic potential of the standardized extract of Synacinn™. We discovered that this novel polyherbal formulation is a multifunctional mediator for DM with the following abilities: 1) increases the glucose utilization in normal and insulin-resistance adipocytes through upregulation of *GLUT4*, 2) inhibits the activities of the postprandial enzymes, and 3) inhibits *DPP(IV)* enzyme activities.

To investigate the mechanism of action of Synacinn™, the in-vitro insulin-resistance model that mimics T2DM was

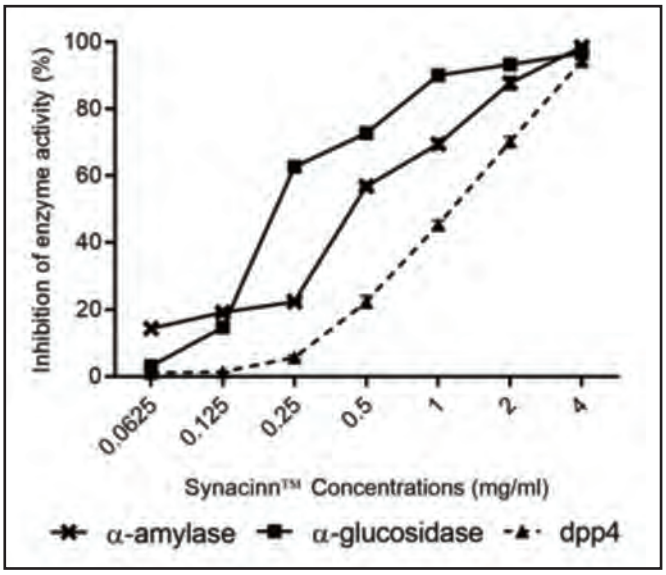

Fig. 5: Effect of Syancinn™ on the activity of hepatic *DPP(IV)*, *α-amylase*, and *α-glucosidase*. Dose-dependent inhibition was observed for the *DPP(IV)* enzyme activity with a maximum inhibition at 4 mg/mL (94.3%) (IC_{50} = 1.11 mg/mL) For *α-amylase* and *α-glucosidase*, the maximum inhibition were 98.5% (IC_{50} = 0.467 mg/mL) and 96.6% (IC_{50} = 0.245 mg/mL), respectively.

developed by treating the *3T3-L1* adipocytes with 1 µM DEX with the presence of insulin for 72 h. During the development of insulin resistance, we discovered that the disturbance of insulin-stimulated glucose utilization was initiated at 48 hand worsened at 72 h of DEX treatment. This condition was further validated by 50µM ROS, which produced similar results. In contrast with Sangeetha et al.[12] in the absence of insulin, 50% reduction in glucose uptake during the insulin-resistance state was achieved as early as 24 hours. The differences suggested that insulin might have a protective mechanism toward the early stage of DEX-induced insulin resistance. However, the protective effect was later diminished ata longer treatment period. In addition, no phenotypic changes such as cell death, changes in size and shape (data not shown) were observed during this period, suggesting that the inhibitory effect of glucose utilization was due to the resistance imposed on the cells. ROS has been reported to improve insulin sensitivity in in-vitro insulin-resistance models, such as *3T3-L1* adipocytes, human embryonic kidney 293 *(HEK 293)*, and C_2C_{12} skeletal muscle cells.[12-14] As a *PPARγ* ligand, ROS binds specifically and activates the *PPARγ* nuclear receptor. The activated *PPARγ* binds to the retinoid X receptor and forms a complex. This complex will assist the transcription of another gene especially in insulin signaling and adipogenesis pathway.[15] In isolated fetal rat primary brown adipocytes, ROS treatment has correspondingly increased the expression of *IRS-1* and *IRS-2Tyr* phosphorylation, which subsequently activates the *PI3K* and *AKT* proteins. The mRNA level of *GLUT4* was not changed, but ROS increases the translocation of *GLUT4* to the plasma membrane resulting in a significant increase of basal and insulin-stimulated glucose uptake.[16] ROS was also shown to improve glucose transport in vastus lateralis muscles and adipocytes of Goto–Kakizaki diabetic rats and independently increase the expression of PKC-ζ/λ without the improvement in the activation of *PI3K* and *PKB*.[17]

Synacinn™, a standardized polyherbal supplement, is designed to reduce DM and its complications. In a recent study, it was confirmed that Synacinn™ increases glucose utilization in normal adipocytes and reverses DEX-induced insulin resistance in *3T3-L1* adipocytes. Interestingly, lower concentrations of Synacinn™ were more potent in sensitizing insulin-stimulated glucose utilization. Even though the improvement was not as good as ROS within the tested period, it was postulated that for a longer time, Syancinn™ exhibited a promising effect as a glucose-lowering agent. Among the phytochemicals in Synacinn™ that have such an effect on adipocytes are gallic acid and andrographolide.18-20 Meanwhile, 5µM rosmarinic acid increases glucose uptake by 86% in L6 rat myotubes.[21]

In insulin-responsive tissue like muscles and adipose, glucose transportation is regulated by a cascade of intracellular phosphorylation event insulin signaling pathway. Cellular analysis on the insight of DEX-induced insulin resistance identified dephosphorylating several downstream proteins in the insulin signaling pathways including *IRS-1, PI3K,* and *AKT,* which subsequently inhibit the translocation of *GLUT4* from the cytosolic compartment to the cellular membrane.[22,23] In this study, we discovered that the effect of Synacinn™ was dominant in restoring glucose transportation rather than repairing the insulin signal transduction. The restoration of glucose utilization in adipocytes was in fact, mainly stimulated by upregulation of *GLUT4* level, and not by other downstream proteins in the insulin signaling pathway, such as *IRS-1, PI3K*, and *AKT*. The expression of *GLUT4* was hyped up to 1.5-fold suggesting that more glucose will be transported into the cells. Even though Synacinn™ does not improve *IRS-1, PI3K*, and *AKT* expression in insulin-resistance adipocytes, it is postulated that these proteins' activity is sufficient to enhance *GLUT4* activity. Similar results were demonstrated by 10µM gallic acid showed the enhancement of *GLUT4* translocation without any stimulation on the *AKT* and *AMPK* phosphorylation.

In this study, we also discovered that Synacinn™ controls the glycemic index through inhibition of *DPP(IV)* enzyme. Dose-dependent inhibition of the activity of this enzyme was observed (Fig. 6), where the optimum concentration was at 4 mg/mL.Curcumin, gallic acid, and rosmarinic acid have shown potential as natural sources of *DPP(IV)* inhibitor.[24] The inhibition of *DPP(IV)* enzyme activity influences the blood glucose level by prolonging the half-life of active glucagon-like *peptide-1 (GLP1)* to stimulate the insulin secretion, increase beta-cell mass, inhibit glucagon secretion, reduce the rate of gastric emptying,and induce satiety.[25]

The control of postprandial hyperglycemia may be achieved by slowing the absorption of glucose through the inhibition of the carbohydrate hydrolyzing enzymes (*α-amylase* and *α-glucosidase*).Inhibitors of these enzymes delay carbohydrate digestion and prolong the overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently blunting the postprandial blood glucose rise.While most individual herbs in the formulation have

been reported to inhibit carbohydrate hydrolyzing enzymes, Synacinn™as in polyherbal combination exertsgreater potential as an inhibitor for *α-amylase* and *α-glucosidase* with IC_{50} of 0.467 mg/ml and 0.245 mg/ml. In comparison, the binary water-ethanolic extract of Andrographis paniculataexhibited higher IC_{50} for *α-amylase* (35.7 mg/mL) and *α-glucosidase* (4.63 mg/mL).[26]

CONCLUSION

To summarize, the multifunctional standardized polyherbal formulation, Synacinn™, modulates hyperglycemic control through three specific mechanisms of action including:1) enhancing cellular glucose transportation through upregulation of $GLUT_4$, 2) inhibiting postprandial enzymes activities,which delay the degradation and absorption of polysaccharide, and 3) altering the gluconeogenesis process in the liver by inhibiting hepatic *DPP(IV)* enzyme activities.

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