

ANTI-DIABETES MECHANISM OF ACTION BY SYNACINN™ IN
ADIPOCYTES

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“Verily after every difficulty, there’s a relief” (Surah Al-Inshirah)

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ABSTRACT

Type 2 diabetes mellitus is described as a defective action of insulin to properly regulate glucose transport within cells and clinically known as insulin resistance. Several factors such as elevated free fatty acid, glucocorticoids as well as abnormal levels of cytokines have been reported to cause insulin resistance. Potentially, phytochemical agents with insulin resistance reverting effect could serve as an alternative treatment for type 2 diabetes mellitus. Synacinn™ is a polyherbal supplement formulated from *Andrographis paniculata*, *Curcuma xanthorrhiza*, *Cinnamomum zeylanicum*, *Eugenia polyantha* and *Orthosiphon stamineous*. It is claimed as a hypoglycemic agent for diabetes mellitus treatment. Even though the anti-diabetes potential of each singular herbs has been well examined, scientific evidence has never been reported for polyherbal combination. In this study, the potential anti-diabetes mechanism of standardized Syancinn™ was investigated using two types of *in vitro* models; normal and insulin-resistant 3T3-L1 adipocytes. It was found that Synacinn™ improved the insulin-mediated glucose utilization in both models. For normal adipocytes, the increase of glucose utilization resulted in overexpression of glucose transporter 4, insulin receptor 1 and protein kinase B. Meanwhile in insulin resistant adipocytes, Synacinn™ increased the glucose transporter 4 expression without affecting the insulin receptor 1 and protein kinase B expression, indicating that Synacinn™ mechanism of action is dominant on the glucose transport rather than repairing the insulin signal transduction. This study also shows that Synacinn™ is a mild peroxisome proliferator-activated receptor gamma ligand and pro-adipogenic agent. During adipogenesis, Synacinn™ stimulated the peroxisome proliferator-activated receptor gamma nuclear transcriptional activity, as well as expression of (cytosine-cytosine-adenosine-adenosine-thymidine)-enhancer-binding protein alpha, adiponectin, glucose transporter 4 and protein kinase B. In terms of safety, standardized Syancinn™ was free from heavy metals and microbial contaminations and the concentrations used in the experiment do not affect the normal embryogenesis of zebrafish. Collectively, results suggest that Synacinn™ is a peroxisome proliferator-activated receptor gamma ligand agent which acted through two mechanisms: 1) restores the insulin-mediated glucose utilization by activating the glucose transporter 4 expression in insulin resistant adipocytes and 2) enhances the adipogenesis into insulin-sensitive adipocytes containing abundance of glucose transporter 4 resulted from activation of (cytosine-cytosine-adenosine-adenosine-thymidine)-enhancer-binding protein alpha and proliferator-activated receptor gamma.

ABSTRAK

Diabetes mellitus jenis 2 digambarkan sebagai kecacatan terhadap tindak balas insulin untuk mengawal pengangkutan glukosa di antara sel dan secara klinikal dikenali sebagai rintangan terhadap insulin. Beberapa faktor seperti peningkatan asid lemak bebas, glukokortikoid serta tahap sitokin yang tidak normal telah dilaporkan menyebabkan rintangan terhadap insulin. Agen fitokimia dengan potensi berbalik terhadap kesan rintangan ke atas insulin mampu dijadikan rawatan alternatif terutamanya untuk diabetes mellitus jenis 2. Synacinn™ adalah suplemen pelbagai herba yang diformulasikan dari *Andrographis paniculata*, *Curcuma xanthorrhiza*, *Cinnamomum zeylanicum*, *Eugenia polyantha* dan *Orthosiphon stamineous*. Ia dikatakan sebagai agen hipoglisemik untuk rawatan diabetes mellitus. Walaupun potensi anti-diabetes untuk setiap herba telah pun dikaji dengan terperinci, akan tetapi, tiada laporan saintifik yang telah diterbitkan dalam bentuk gabungan pelbagai herba. Dalam kajian ini, potensi mekanisme anti-diabetes bagi Synacinn™ yang telah dipiawaikan, diselidik menggunakan dua jenis model *in vitro*; sel lemak normal dan sel lemak yang rintang terhadap insulin (3T3-L1). Kajian mendapati bahawa, Synacinn™ menambah baik penggunaan glukosa yang didorong oleh insulin di dalam kedua-dua model. Bagi sel lemak normal, peningkatan penggunaan glukosa menyebabkan ekspresi lebih pada pengangkut glukosa 4, reseptor insulin 1 dan kinase protein B. Manakala bagi sel lemak yang rintang terhadap insulin, Synacinn™ meningkatkan ekspresi pengangkut glukosa 4 tanpa mempengaruhi reseptor insulin 1 dan kinase protein B dan ini menunjukkan bahawa mekanisme tindakan bagi Synacinn™ adalah dominan pada pengangkutan glukosa dan bukannya pada memperbaiki transduksi isyarat insulin. Kajian juga menunjukkan bahawa Synacinn™ adalah ligan reseptor pengaktifan proliferasi peroksisom gamma yang sederhana dan agen pro-adipogenik. Semasa *adipogenesis*, Synacinn™ merangsang aktiviti transkripsi reseptor pengaktifan proliferasi peroksisom gamma nukleus, serta ekspresi protein penggalak ikatan alfa (sitosina-sitosina-adenosina-adenosina-timidina), adiponektin, pengangkut glukosa 4 dan kinase protein B. Dari segi keselamatan, Synacinn™ yang telah dipiawaikan ini adalah bebas daripada pencemaran logam berat dan mikroba dan kepekatan yang digunakan dalam eksperimen tidak mempengaruhi embriogenesis normal zebrafish. Secara keseluruhannya, keputusan menunjukkan bahawa Synacinn™ adalah agen ligan reseptor pengaktifan proliferasi peroksisom gamma yang bertindak melalui dua mekanisme: 1) mengembalikan penggunaan glukosa yang didorong oleh insulin dengan mengaktifkan ekspresi pengangkut glukosa 4 didalam sel lemak yang rintang terhadap insulin dan 2) meningkatkan *adipogenesis* kepada sel lemak yang sensitif terhadap insulin yang mana mengandungi limpahan pengangkut glukosa 4 hasil daripada pengaktifan protein penggalak ikatan alfa (sitosina-sitosina-adenosina-adenosina-timidina) dan reseptor pengaktifan proliferasi peroksisom gamma.

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LIST OF ABBREVIATIONS

AKT/ PKB	Protein kinase B
AP	<i>Andrographis paniculata</i>
AS160	Akt substrate of 160 kDa
aPKC	Atypical protein kinase C
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
C/EBP α	CCAAT-enhancer-binding protein alpha
C/EBP β	CCAAT-enhancer-binding protein beta
C/EBP δ	CCAAT-enhancer-binding protein delta
CCAAT	Cytosine-cytosine-adenosine-adenosine-thymidine
CD1	Complete DMEM 1
CD2	Complete DMEM 2
CX	<i>Curcuma xanthorrhiza</i>
CZ	<i>Cinnamomum zeylanicum</i>
DEX	Dexamethasone
DM	Diabetes mellitus
DM1	Differentiation media 1
DM2	Differentiation media 2
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
EP	<i>Eugenia polyantha</i>
FFA	Free fatty acid
FBS	Fetal bovine serum
FCS	Fetal calf serum
FTIR	Fourier Transmission Infra-Red spectroscopy
GCs	Glucocorticoids
GSV	Glucose transporter storage vesicle

GC-MS	Gas chromatography-mass spectrometry
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
G6P	Glucose-6-phosphatase
HBA1C	Hemoglobin A1C
hpf	Hour post fertilization
IBMX	Methylisobutylxanthine
INS	Insulin
IL-6	Interleukin -6
IRs	Insulin receptors
IRS1	Insulin receptor 1
IRS2	Insulin receptor 2
HPLC	High performances liquid chromatograhpy
KLFs	Kruppel-like Factors
LDL	Low density lipid
MTT	3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MAPKinases	Mitogen-activated protein kinase
NAFLD	Nonalcoholic fatty liver disease
NF- κ B	Nuclear factor-kappaB
NIDDM	Non-insulin-dependent diabetes mellitus
ORO	Oil red O
OS	<i>Orthosiphon stamineous</i>
p38MAPK	P38 mitogen-activated protein kinases
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate-Buffered Saline
PDK1	Pyruvate dehydrogenase kinase 1
PDK2	mTOR-RICTOR
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-kinase
PIP2	3'-phosphatidylinositol 3,4,5-bisphopsphate
PIP3	3'-phosphatidylinositol 3,4,5-trisphopsphate
PS	Penicillin strep
PPAR- γ	Peroxisome proliferator-activated receptor gamma

PTP1B	Protein tyrosine phosphatase 1B
ROS	Rosiglitazone
SGK	Glucocorticoid-inducible kinase
SREBP-1	Sterol regulatory element-binding protein 1
TBS	Tris Buffer Saline
TBST	Tris Buffer Saline – Tween 20
TCM	Traditional Chinese Medicine
TNF- α	Tumor necrosis factor α
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TZDs	Thiazolidinediones
WI	With insulin
WO	Without insulin

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CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Diabetes mellitus (DM) is a metabolic disorder disease. The condition of DM is clinically diagnosed by the high level of glucose in circulating blood and known as hyperglycemia. It is triggered by insufficient and ineffective insulin actions in regulating blood glucose properly. The high glucose level affects several important body metabolisms including glucose, proteins and triglyceride metabolism which cause kidney and renal failure, heart and cardiovascular disease, blindness and nerves degeneration (Katiyar *et al.*, 2015). Clinical investigations have identified several factors linked to DM such as autoimmune reactions, hereditary factors, unhealthy diet, lack of exercises and overweight. Approximately, 17.5% of Malaysian adults above 18 years old were affected by DM in 2015 and the highest among Asian countries (Hanum, 2017). In fact, 9.2% of DM patients are unaware of their hidden disease (Hanum, 2017). This large-scale epidemic has cost millions of lives worldwide and billion dollars for treatments. In Malaysia, our government has to spend RM 2684.24 per diabetic patient, while each patient with kidney failure needs RM 1400 to RM 3200 monthly for hemodialysis (Hanum, 2017).

DM is categorized into two main groups as clinically manifested. Type 1 diabetes mellitus (T1DM) or juvenile diabetes is an autoimmune destruction and dysfunction of pancreatic beta cells, a specialized cell for insulin production. Patients with this type of DM require external insulin injection due to insulin insufficiency. Meanwhile for Type 2 diabetes mellitus (T2DM), which comprises 9/10 of total DM

patients, usually occurs in adults and clinically diagnosed by a high level of blood glucose and hemoglobin A1C (HBA1C). However, a recent report had found an increasing trend of T2DM among young ages in Japan and USA (Alberti *et al.*, 2004).

High level of blood glucose in T2DM is caused by the ineffective cellular response towards the insulin action. This phenomenon is known as insulin resistance. It occurs mainly in insulin-sensitive tissues such as liver, muscles and adipose. In insulin resistance cells, it was reported that the defective insulin signaling pathway blocked the phosphorylation signals at various steps which subsequently reduced the translocation of glucose transporters (Saini, 2010). One of the factors that induced the insulin resistance occurrence is glucocorticoids (GCs). GCs such as dexamethasone, cortisol and cortisone are clinically used to treat a range of human diseases including autoimmune diseases, cancer as well as prevention agent in organ transplantation from rejection (Ferris and Kahn, 2012). However, circulated GCs in body system also increases the adiposity and enhance the lipolysis, leading to elevate of free fatty acid (FFA) and insulin resistance (Lee *et al.*, 2014; Rhee *et al.*, 2004). In *in vitro* models, DEX-induced insulin resistance in adipocytes and muscles cells. It was observed that DEX inhibited the activity of downstream cytokines in the insulin signaling pathway including phosphatidylinositol 3-kinase (PI3K), protein kinase B (AKT), Akt substrate of 160 kDa (AS160) and glucose transporter 4 (GLUT4) (Tappy *et al.*, 1994).

Thiazolidinediones (TZDs), an oral anti-diabetes drug class has shown to reduce the insulin resistance effect by sensitizing the insulin activity during glucose uptake (Arner, 2003). Rosiglitazone and pioglitazone are the examples of TZDs drug available in the market. In adipocytes, TZDs acted as peroxisome proliferator-activated receptor gamma (PPAR γ) ligand agent which stimulates the transcription of various genes including IRS1, GLUT4, CCAAT-enhancer-binding protein (C/EBPs), AKT, PI3K and adiponectin (Cuzzocrea *et al.*, 2004). On other aspects, TZDs also enhances the adipogenesis process (Hausman *et al.*, 2008). Adipogenesis is a differentiation process of preadipocytes to mature adipocytes which involves changes in cells phenotypes and genotypes regulation (Lowe *et al.*, 2011). Replacement of

enlarging and inflamed adipocytes with small adipocytes has been proposed as an alternative method for obesity and anti-diabetes treatment (Müller, 2011).

In search of DM therapeutic drugs, plant derived products have shown exciting potential as hypoglycemic agents. In Malaysia, herbal products have become the popular alternative for health-promoting supplement with annual income approximately RM 17 billion in 2013 and increasing 8-15% each year (Embong, 2007; Zakaria, 2015). Traditional medicine such as Traditional Chinese Medicine (TCM), Ayurvedic and Campa are prepared in a polyherbal mixture containing several herbs, spices and plants to enhance the bioactive effectiveness as well as eliminate undesired adverse events (Katiyar *et al.*, 2015; Yeo *et al.*, 2011). There are various polyherbal formulations have been developed to treat DM including Diabet, Diabecon, Diabeta, Diakyur, Diasulin, Dihar Dihar, DRF/AY/5001, ESF/AY/500, 5EPHF, Karmin Plus, Okudibet, PM021 (Katiyar *et al.*, 2015).

1.2 Problem Statement

Despite the advanced research in drugs development, DM therapies require lifelong drugs consumption to control the glycemic condition at a normal level. Various oral therapeutic drugs for T2DM are available in market such as metformin, sulphonylureas, and alpha-glucosidase inhibitors with a specific mechanism of action and site of action (Moller, 2001). Unfortunately, these drugs have limitations and unwanted side effects (Chaudhury *et al.*, 2017; Kumar *et al.*, 2016). For example, metformin increases glucose uptake in body tissues and inhibits gluconeogenesis in liver but it is an appetite suppressant and not suitable for patients with liver and kidney problems (The Diabetes Prevention Program Research, 2012). Meanwhile for sulphonylureas, an insulin release stimulator, only suitable for T2DM patients (Kalra *et al.*, 2015). Potentially, natural therapies derived from plants (single compounds, a group of compounds or whole extract) have become an alternative choice to reduce and prevent DM traditionally (Katiyar *et al.*, 2015; Prabhakar and Doble, 2011).

Synacinn™, a traditional supplement has been recommended for treatment of DM. Its testimonials claimed to reduce DM related signs including tiredness and high blood glucose level. Synacinn™ is formulated from a combination of five Malaysian herbs including *Andrographis paniculata*, *Curcuma xanthorrhiza*, *Cinnamomum zeylanicum*, *Eugenia polyantha* and *Orthosiphon stamineous*. Even though there are various reports regarding the anti-diabetes potential of each single herbs, but there is no scientific reports have been published for polyherbal combination. It is believed that the synergistic effect of this combination involved multiple mechanisms ultimately covering all possible effects of DM in our body. Hence, the recent challenge is to identify and understand the mechanism of action of Synacinn™ at cellular levels in treating DM. Insulin-sensitive 3T3-L1 adipocytes were employed as DM *in vitro* model due to the important roles on glucose metabolism and insulin signaling pathway.

1.3 Research Questions

To accomplish the proposed study, several questions need to be answered:

- i. What are the concentrations of phytochemicals in Synacinn™?
- ii. How Synacinn™ reduces the insulin resistance effect?
- iii. Does Synacinn™ produce similar effect as PPAR γ ligand on the development of adipocytes?

1.4 Objectives of the Study

- i. To establish the quality control and safety endpoints of Synacinn™.
- ii. To evaluate the possible anti-diabetes mechanism of Synacinn™ on insulin-resistance 3T3-L1 adipocytes specifically for the glucose transport and insulin signaling pathway.
- iii. To investigate PPAR γ ligand activity on the development of adipocytes.

1.5 Scopes of the Study

Herbal product is composed of multiple bioactive compounds which act synergistically to improve human health. Poor quality control (QC) especially on the raw materials and the manufacturing process will result in variation between batches and possible contamination from hazardous compounds, microbes and heavy metals. Hence, strict QC is imperative to control and standardize the herbal-based production. 3T3-L1 preadipocytes can be differentiated to adipocytes by suitable hormones. Once differentiated, it is sensitive to insulin and play important role in insulin pathway and glucose metabolism (Gregoire *et al.*, 1998; Medina-Gomez *et al.*, 2007; Ntambi and Young-Cheul, 2000). Several scopes were identified based on the Synacinn™ QC aspects as well as adipocytes capability in order to achieve the proposed objectives.

- i. Quality control of phytochemicals in the water extract of Synacinn™ using Fourier Transmission Infra-Red Spectroscopy (FTIR), Gas Chromatography – Mass Spectroscopy (GC-MS) and High Performance Liquid Chromatography (HPLC).
- ii. Adipocytes were treated with dexamethasone to induce insulin resistance condition. Synacinn™ effect on this model is assessed based on the glucose utilization capacity and the restoration of impaired insulin signaling pathway.
- iii. Adipogenesis of 3T3-L1 preadipocytes can be induced by chemical cocktail. The modulation effect of Synacinn™ on the adipogenesis was measured by quantifying the lipid droplet formation, intracellular triglyceride and adipokines expression using immunoblotting and transcription assay.

1.6 Significance of the Study

1. Provides the first possible mechanism of action of Synacinn™ for a better understanding on how it works as anti-diabetes supplement.
2. Leads to further preclinical and clinical tests on higher models.

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