CHARACTERISATION OF α-GLUCOSIDASE INHIBITORS FROM *Clinacanthus nutans* (Burm.f.) Lindau LEAVES EXTRACT USING METABOLOMICS APPROACH AND MOLECULAR DOCKING SIMULATION

BY

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ABSTRACT

The use of medicinal plants to combat various diseases has grown intensively due to the side effects from commercial synthetic drugs. *Clinacanthus nutans* (Burm.f.) Lindau is a traditional medicinal plant native to Malaysia, yet to be explored for its antidiabetic effect. Therefore, this study aimed to investigate the antidiabetic potential of the plant using metabolomics approach and molecular docking simulation. The 80% methanolic crude extract of this plant leaves was partitioned using different polarity solvents viz., *n*-hexane, *n*-hexane: ethyl acetate (1:1, v/v), ethyl acetate, ethyl acetate: methanol (1:1, v/v) and methanol. All fractions were screened for antioxidant and antidiabetic activity via bioassays techniques. The α-glucosidase inhibitors of the plant were identified using gas and liquid chromatography fitted with mass spectrometry (GCMS and LCMS, respectively) based metabolomics. All the inhibitors identified were then docked to α-glucosidase enzyme crystal structure to predict its ligand-protein interaction. The study also developed a validated regression model using Fourier transform infrared (FTIR) spectroscopy. Lastly, the most active fraction was investigated for its toxicity level using zebrafish (*Danio rerio*) embryos. Despite a moderate antioxidant capacity, *n*-hexane fraction exhibited a good α-glucosidase and dipeptidyl peptidase-IV inhibiting activities. The *n*-hexane fraction also improved glucose uptake in a dose-dependent manner. Chemical profiling utilising GCMS based metabolomics derived 11 bioactive compounds namely; palmitic acid, phytol, hexadecanoic acid (methyl ester), 1-monopalmitin, stigmast-5-ene, pentadecanoic acid, heptadecanoic acid, 1-linolenoylglycerol, glycerol monostearate, α-tocospiro B and stigmasterol corresponding to the distinct biological activity. Meanwhile, LCMS revealed 4 compounds tentatively identified as; 4,6,8-megastigmatrien-3-one; N-isobutyl-2-nonen-6,8-diynamide; 1',2'-bis(acetyloxy)-3',4'-didehydro-2'-hydro-β, ψ-carotene and 22-acetate-3-hydroxy-21-(6-methyl-2,4-octadienoate)-olean-12-en-28-oic acid. The docking results predicted the interaction of all the identified compounds to be in non-competitive mode with majorly involving hydrophobic interactions with the protein residues. Some of the residues involved include LYS156, THR310, PRO312, LEU313, GLU411 and ASN415, etc with hydrogen bond while TRP15, VAL232, HIE280, ALA292, PHE314, ARG315, etc in hydrophobic contact. The partial least square model generated using FTIR analysis was validated using external sample and could potentially predict the α-glucosidase inhibitory activity of *C. nutans* leaves extracts thus can be applied for quality control purposes. The toxicity assessment showed the morphological defects caused by *n*-hexane fraction such as hyperactivity, delayed hatching, crooked backbone, reduced pigmentation, awkward position and oedema at 125 µg/mL. The LC_{50} value was calculated to be 75.49 µg/mL. However, the plant extract can be developed as an antidiabetic agent after the removal of the toxicants. Conclusively, the metabolomics approach in this study has revealed the antidiabetic potential of *C. nutans* leaves through identification of the active α-glucosidase inhibitors from this plant.

خلاصة البحث

استخدام النباتات الطبية لمكافحة العديد من الأمراض نمت بشكل مكثف بسبب الآثار الجانبية الناتجة عن الأدوية المصنعة تجاري كليناكانتس نيوتنس هو نبات ماليزي أصلي و معروف عادة باستخدامه ضد امراض النقرس و فرط حمض يوريك الدم و التهابات و الحمى و الطفح جلدي و غير ذلك ؛ ومع ذلك لا يزال يتعين استكشاف تأثيرها علي مرض السكر. ولذلك ، تهدفت الدراسة إلى التحقق من فاعلية النبات على مرض السكر باستخدام نهج الاستقلاب ومحاكاة الالتصاق الجزيئي. خام مستخلص الميثانول بنسبة 80٪ من أوراق النبات تم استخلاصه باستخدام مذيبات قطبية مختلفة. جميع المستخلصات تم التحقق من تأثيره مرض السكر و مضادات الاكسدة. تم تحديد مثبطات الالفا كليكوزيدز للنبات باستخدام الغاز والكروماتوغرافيا السائلة المزودة بمقياس الطيف الكتلي (LCMS وGCMS) مع نهج الاستقلاب . تم ربط جميع الموانع المحددة إلى بنية بلورة إنزيم الالفا كليكوزيدز للتنبؤ بتفاعل المركب . كما طورت الدراسة نموذج انحدار معتمد باستخدام التحليل الطيفي للأشعة تحت الحمراء(FTIR) . وأخيرا تم التحقيق الجزء الأكثر نشاطا لمستوى سميته باستخدام الأجنة (دانيو ريريو) الزرد. على الرغم من قدرة مضادات الأكسدة المعتدلة ، عرض جزء ن – هكسان جيد الالفا كليكوزيدز وأنشطة تثبيط-DPP IV.تحسّن أيضًا جزء ن – هكسان من امتصاص الغلوكوز بطريقة تعتمد على الجرعة. التنميط الكيميائي باستخدام نهج الاستقلاب مع GCMS مستمدة من 11 مركبات المقابلة للنشاط البيولوجي المميز. وفي الوقت نفسه ، كشف LCMS عن 4 مركبات تم تحديدها مبدئياً .-تنبأت نتائج الالتحام بتفاعل جميع المركبات التي تم تحديدها في وضع غير تنافسي مع وجود تفاعلات كارهة للماء مع مخلّفات البروتين. تتضمن بعض الوحدات البنائية المتضمنة LYS156 وTHR31 وPRO31 وLEU313 و GLU411وASN415 ، إلخ مع رابطة هيدروجينية بينما TRP15 وVAL232 و HIE280 وALA292 وPHE314 وARG315 وما إلى ذلك في اتصال مسعور. تم التحقق من صحة النموذج الجزئي الأقل مربع الذي تم إنشاؤه باستخدام تحليل FTIR باستخدام عينة خارجية ويمكن أن يتنبأ على الأرجح بالأنشطة المثبطة الالفا كليكوزيدز لمستخلصات أوراق كليناكانتس وبالتالي يمكن تطبيقها لأغراض مراقبة الجودة. أظهر تقييم التشوهات المورفولوجية التي تسببها أجزاء ن – هكسان مثل فرط النشاط ، الفقس المتأخر ، العمود الفقري المتعرج ، التصبغ المخفف ، الموضع الغريب والوذمة عند 125 ميكروغرام / مل. تم حساب قيمة LC_{50} لتكون 75.49 ميكروغرام / مل. و لذلك يمكن تطوير المستخلص النباتي كعامل مضاد لمرض السكر.

APPROVAL PAGE

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I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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To Appa and Amma,

Mr. Murugesu and Mrs. Anjalai

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LIST OF ABBREVIATIONS

ACD	Advanced Chemistry Database
AGI	α-Glucosidase inhibitory
ALA	Alanine
ASH	Protonated Aspartic acid
ASN	Asparagine
ASP	Aspartic acid
ARG	Arginine
DEX	Dexamethasone
DM	Diabetes Mellitus
DMEM	Dulbecco's Modified Eagle Media
DPM	Differentiation Progression Medium
DPPH	2,2- Diphenyl-1-picrylhydrazyl
FBG	Fasting Blood Glucose
FBS	Fetal Bovine Serum
FRAP	Ferric Reducing Antioxidant Potential
GLH	Protonated Glutamic acid
GAD	Glutamic Acid Decarboxylase
GCMS	Gas Chromatography Mass Spectrometry
GIP	Glucose-Dependent Insulinotropic Polypeptide
GLN	Glutamine
GLP	Glucagon-like Peptide
GLU	Glutamic acid
HID	Histidine neutral δ-protonated
HIE	Histidine neutral ε-protonated
HSV	Herpes Simplex Virus
IBMX	3-isobutyl-1-methylxanthine
ILE	Isoleucine
IS	Internal Standard
JOD	Juvenile Onset Diabetes
LDL	Low Density Lipid
LEU	Leusine
LYS	Lysine
MSD	Mass Selective Detector
NAC	N-acetylcysteine
NCBI	National Center for Biotechnology Information
NHMS	National Health and Morbidity Surveys
NIST14	National Institute of Standards and Technology
NMR	Nuclear Magnetic Resonance

OGTT	Oral Glucose Tolerance Test
OPLS	Orthogonal Partial Least Square
PCA	Principal Component Analysis
PHE	Phenylalanine
PLS	Partial Least Square
PRO	Proline
Q-ToF LCMS	Quadrupole Time-of-Flight Liquid Chromatography Mass Spectrometry
RBG	Random Blood Glucose
$RMSE_E$	Root Mean Square Error of Estimation
$RMSE_{CV}$	Root Mean Square Error of Cross Validation
SER	Serine
STZ	Streptozotocin
THR	Threonine
TRP	Tryptophan
TYR	Tyrosine
TZD	Thiazolidinediones
VAL	Valine
XO	Xanthine Oxidase
µg	microgram
µL	microliter
mL	mililiter
g	gram
nM	nanomolar
nm	nanometer
w/v	weight per volume
m/z	mass per charge

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

A natural product derived from natural sources especially from herbs have been observed in a wide range of pharmacological studies and commercialized by established pharmaceutical industries. Herbal plants have been discovered and utilized for various purposes to cure and combat diseases since ancient times (Babiaka et al., 2014). At recent times, researchers are investigating and producing more scientific findings on the use and utilizations of plant sources as alternative medicines to cure various ailments. Likewise, pharmaceutical industries have been widely producing natural derived products to be used as an alternative treatment in multiple cases (Katz and Baltz, 2016). However, precise scientific findings are needing to be acquired before commercialisation of any natural product could take place. The current study highlights on the use of the medicinal plant as an alternative cure for one of the major disorders suffered by most Malaysians; diabetes mellitus. It will aid in identifying the specific bioactive compound(s) responsible to manage the disorder.

Diabetes mellitus (DM) is indeed known as a chronic metabolic disorder caused by genetic defects (most commonly inherited from both parents) or due to the deficiency in insulin secretion and irresponsiveness of the organs to secreted insulin. This deficiency in turn will lead to hyperglycemia which is characterised by increased blood glucose level which causes interruption in some of the body's systems and organ damages including blood vessels and nerves (Inzucchi et al., 2015). DM is a global metabolic epidemic and increasing in the proportions throughout the world. It is a major leading cause of mortality, morbidity and disability across the world, thus affecting

some of the essential biochemical activities in almost every age group being affected (Choudhary et al., 2014). DM affects major global population and the number of people suffering of diabetes is rising rapidly in most parts of the world, especially in developing countries like Malaysia, Thailand, Indonesia and India (Mohamed et al., 2012). The two most recent National Health and Morbidity Surveys (NHMS) demonstrated a drastic increment in the prevalence of diabetes from 8.3% out of 1996 to 14.9% out of 2006 for Malaysian adults aged 30 years or above. This is an expansion of 80% over 10 years' time. Moreover, about 36% of the diabetic populace are left undiagnosed. The same survey also demonstrated that the predominance of obesity had increased tragically from 4.4% to 14.0% from 1996 to 2006 among adults in Malaysia aged 18 years and above. Moreover, the Second National Health and Morbidity survey indicated that 3.4 million Malaysians were diabetic in 2010. It is an alarming situation where, Malaysia has the greatest number of overweight and obese individuals in Asia compared with only 24.1%, 10 years ago. Obesity is a primary cause of diabetes as 54% of the grown-up populace are corpulence. This causes 7 out of 10 Malaysian adults to suffer chronic illnesses which are associated with diabetes (Tee and Yap, 2017).

Digested carbohydrates from our diet increases the postprandial plasma glucose levels. A sudden raise of the blood glucose level leads to hyperglycemia in type 2 DM patients which is typically facilitated by enteric enzymes which includes pancreatic α-amylase and α-glucosidase that are attached to the brush border of intestinal cells. Monosaccharides (e.g. glucose and fructose) can be transported out of the intestinal lumen into the bloodstream while complex starches like disaccharides and oligosaccharides must be broken down into monosaccharides before they can be absorbed into the bloodstream via duodenum and upper jejunum (Ching et al., 2013; Wong et al., 2014).

Hyperglycemia is critical in the early treatment of DM and for reducing chronic vascular complications. One of the effective ways for the management of type 2 DM is via strong inhibition of intestinal α-glucosidases and the mild inhibition of pancreatic α-amylase (Marín-Peñalver et al., 2016), which will eventually retard the absorption of glucose in the digestive tract, thus reducing postprandial hyperglycemia in patients with DM, achieved by preventing the absorption of carbohydrates after food uptake. Inhibitors of these enzymes delay carbohydrate digestion as well prolong the overall carbohydrate digestion time, thus reduce the rate of glucose absorption and consequently blunting the postprandial blood glucose rise. Currently, various α-glucosidase inhibitors including acarbose, miglitol and voglibose that are used as oral anti-hyperglycaemic drugs, with metformin as the most commonly prescribed oral antidiabetic drug in Malaysia for type 2 DM patients. However, these therapeutic drugs have biggest drawbacks that cause side effects to human health in the long run and with limited efficacy is still a challenge to the medical system (Inzucchi et al., 2015; Ismail and Deshmukh; 2012).

Many natural resources have been investigated with respect to suppress glucose production from carbohydrates in the gut or glucose absorption through the intestine (Bahmani et al., 2014). In recent times, great attention is given to the management of diabetes using medicinal plants along with dietary restriction (Choudhary et al., 2014). In most regions of the developing countries including Malaysia, traditional medicine using herbs are more an affordable source for healthcare. This leads to increasing interest in the use of medicinal plants as an alternative management of type 2 DM. Herbs play an important role in the development of complementary and alternative medicine. Many herbs have been developed as natural medicine and being utilized as an alternative for modern treatment in many common diseases. The mixture of compounds

in plants which work synergistically on the complex system of human body has made it a choice for alternative and complementary medicine. Besides that, certain compounds in the herbs could help to reduce the toxicity in human body unlike the modern medicine that could be metabolised into dangerous toxin that may lead to chronic diseases in prolong use (Abdul et al., 2011; Ifeoma and Oluwakanyinsol, 2013).

Clinacanthus nutans (Burm.f.) Lindau, (*C. nutans*) or locally known as Sabah snake grass is a native Malaysian plant. *C. nutans* leaves extract has been used traditionally for the treatment of skin rashes, snake bites, allergic reaction, diuretics and diabetes. Experimentally, numerous studies have shown the therapeutic potential of *C. nutans* which include antiviral, anti-inflammatory, antioxidant, neuromodulator and anti-cancer (Alam et al., 2016; Arullappan et al., 2014; Kamarudin et al., 2017; Li et al., 2013; Yuann et al., 2012). Wanikiat et al. (2008) evaluated the anti-inflammatory property of *C. nutans* and a compound (4,5-dinonyl-1,3-dioxolane) possessing the anti-inflammatory and anti-arthritic activities were later isolated and identified by Sreena et al. (2012). Lee et al. (2014) conducted antidiabetic analysis via *in-vitro* bioassay using α-glucosidase inhibition activity for the plant's methanolic extract which exert mild inhibition for its leaves and stem extracts. However, no other evidence on the activity of other solvent extracts or compound(s) responsible for the activity have been reported up until now. Whilst, this study provides the evidence of *C. nutans* extract effectiveness against hyperglycaemia condition. In order, to explore the efficacy of *C. nutans* against hyperglycaemia, the *in vitro* evaluation against α-glucosidase was carried out (Javadi et al., 2014). The chromatography and spectroscopy analytical techniques were utilised in this study that further facilitated the identification of the bioactive compound(s) involved in anti-hyperglycaemia action and the determination of the efficacy of the *C. nutans* leaves extract against diabetes.

Metabolomics approach coupled with chromatography and spectroscopy associated with multivariate data analysis has been exploited to determine the metabolites responsible for the bioactivity observed. Metabolites profiling helps us to understand the metabolic pathways in cells, tissues, organs or organisms' mechanisms (Kuhlisch and Pohnert, 2015). In recent times, metabolomics approach has been practiced in fulfilling the lacking in the identification of bioactive compounds in medicinal herbs. Besides that, it also rationalises the therapeutic superiority of many plant extracts over single isolated constituent. Metabolomics can help in to identify and quantify multiple targets to obtain an overview of all compounds classes and brings an important insight into the natural product by linking putative bioactivity with some compounds in a targeted plant (Wolfender et al., 2015).

1.2 PROBLEM STATEMENT

The available synthetic drugs that are prescribed for diabetes such as voglibose and acarbose have led to many side effects such as diarrhea, nausea, vomiting, bloating and flatulence, to sensitive patients or in prolonged use. Besides that, the use of synthetic drugs in long term will eventually lead to complications involving major organs especially kidney and liver (Alhadramy, 2016; Azmi et al., 2012). Therefore, drug discovery leads to zero side effects and less toxicity were recently implemented. One of the strategies is the use of natural medicinal herbs. Although herbs are considerably safer than commercial synthetic drugs and at the same time can treat the diseases, the scientific proof to use herbs for medicinal purpose is still lacking.

C. nutans leaves extract has been reported to possess various pharmacological actions (Alam et al., 2016; Arullappan et al., 2014; Lau et al., 2014; Li et al., 2013). Arullappan et al. (2014) have mentioned that it has been used traditionally to treat