PHYTOCHEMICAL INVESTIGATION OF THE STEMS OF *TETRACERA INDICA* MERR. AND *IN-VITRO* ANTIDIABETIC EVALUATION OF ISOLATED COMPOUNDS

BY

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ABSTRACT

Tetracera indica Merr. (Family: Dilleniaceae) is a large, woody, rain forest climber species. It is found throughout the Malaysia and is locally and commonly known as Mempelas paya or sand paper plant. Aerial parts of *T. indica* have been traditionally used to cure different disorders including diabetes and its related infirmities in Malaysia. The traditional claims of this plant as an antidiabetic agent have not been properly scientifically evaluated. Hence, the aim of this study was to explore *in vitro* antidiabetic potential of the stems of *T. indica* as well as to isolate the compounds responsible for its *in vitro* antidiabetic property. The *in vitro* antidiabetic activity investigation of the stems ethanol extract, sub-fractions and isolated compounds from the bioactive fraction was carried out on 3T3-L1 pre-adipocytes and adipocytes. Upon thorough investigations, four flavonoids (MHQ1-Wogonin, MHQ-2-Norwogonin, MHQ-3-Quercetin, MHQ-4-Techtochrysin) and two terpenoids (MHQ-5-Stigmasterol, MHQ-6-Betulinic acid) were isolated from the stems ethanol extract of *T. indica* and further subjected to cytotoxicity test against 3T3-L1 adipocytes with regard to evaluate their antidiabetic potential. All the compounds were isolated and purified through silica gel and sephadex LH-20 repeated column chromatography and recrystallization with different solvents. Their structures were elucidated through ^{1}H-and ^{13}C-NMR spectroscopy. Cytotoxicity test was performed through MTT assay on 3T3-L1 pre-adipocytes to determine the safe dose of the extract, sub-fractions and all the isolated compounds for further *in vitro* antidiabetic evaluation on 3T3-L1 pre-adipocytes. The stems ethanol extract, sub-fractions and isolated pure compounds i.e. MHQ1, MHQ2, MHQ3 and MHQ4 were further subjected to adipogenesis to investigate insulin like activity or insulin sensitizing activity for the purpose of evaluating antidiabetic potential. All compounds were introduced to the cells in different safe concentrations as well as in different adipogenic cocktails. The adipogenic cocktails were modified by the addition of compounds to be investigated and rosiglitazone in the presence or absence of insulin. Results vividly showed that MHQ-1, MHQ-2 and MHQ-4 induced adipogenesis like insulin and enhanced adipogenesis like rosiglitazone significantly. Furthermore, MHQ1, MHQ2 and MHQ-4 as well as rosiglitazone as positive control were subjected to fluorescence glucose uptake test by 2-NBDG (fluorescent glucose analogue) on mature adipocytes. Results suggested significant glucose uptake activity by MHQ1, MHQ2 and MHQ-4. It is concluded and suggested that the further in-depth research study on the isolated compounds might help to discover a new safe compound with strong antidiabetic activity. Results further confirm the traditional use of *T. indica* in the management of diabetes in Malaysia.

ABSTRACT

التيتراسيرا انديكا مير(العائلة: الديلنية) أحد الأنواع الخشبية الكبيرة المتسلقة في الغابات المطيرة. تتواجد في كافة الغابات الماليزية، وتعرف محلياً باسم "ميمبيلاس بايا" أو نبات ورقة الرمل. الأجزاء الهوائية للتي.إنديكا استعملت قديماً في علاج العديد من الأمراض ومن ضمنها السكري والمشاكل المصاحبة له في ماليزيا. الإدعاءات القديمة على فعالية هذا النبات كعلاج للسكري لم تثبت علمياً بعد، لذلك هدفت هذه الدراسة لإستكشاف الخصائص المضادة للسكري لساق التي. إنديكا في الخلايا مخبرياً، بالإضافة لعزل المركبات المسؤولة عن الفعالية ضد السكري. دراسة الفعالية ضد السكري للمستخلص الايثانولي للساق والمستخلصات الفرعية والمركبات المعزولة من المستخلصات الفعالة أجريت على الخلايا الدهنية وقبل الدهنية نوع 3T3-L1 . بعد البحث؛ أربعة فلافونويدات (MHQ-3-Norwogonin, MHQ-2-Wogonin, MHQ1-Quercetin, MHQ-4-Techtochrysin) واثنين من التيربينودات (MHQ-6-Stigmasterol, MHQ-5-Betulinic acid) تم عزلها من المستخلص الايثانولي للساق للتي. إنديكا، وتم فحص سميتها الخلوية عبر اختبارها في خلايا 3T3-L1 الدهنية فيما يتعلق بفعاليتها ضد السكري. جميع المركبات عزلت ونُقّيت من خلال جل السيلكا والفصل باستعمال عمود sephadex LH-20 لعدة مرات، وتمت إعادة بلورتها يعدة مذيبات. تم الكشف عن تركيبها بواسطة أطياف 1H-and 13C-NMR. فحص سميتها على الخلايا تم بواسطة فحص MTTعلى خلايا 3T3-L1 قبل الدهنية للتحقق من الجرعة الآمنة للمستخلص والمستخلصات الفرعية والمركبات النقية مثل: MHQ1, MHQ2, MHQ3 MHQ4 والتي بدورها عُرّضت لعمليات بناء الدهون لدراسة النشاط المشابه للإنسولين أو النشاط المحفز لحساسية الإنسولين بغاية تقييم الفعالية المحتملة ضد السكري. جميع المركبات أدخلت للخلايا بعدة جرعات آمنة وأخرى محفزة لإنتاج الدهون.الجرعات المحفزة لإنتاج الدهون عُدّلت بإضافة المركبات المراد دراستها إلى روزيغليتازون بوجود او غياب الإنسولين. أظهرت النتائج بشكل واضح أن MHQ-1, MHQ-2 and MHQ-4 تحفز إنتاج الدهون بشكل مماثل للإنسولين وكذلك روزيغليتازون. في سياق آخر، MHQ-1, MHQ-2 and MHQ-4 بالإضافة للروزيغليتازون كمجموعة تحكم عُرّضت لفحص أخذ الجلوكوز بالإشعاع بواسطة 2-NBDG على خلايا دهنية ناضجة. أظهرت النتائج إرتفاعاً ملحوظاً لأخذ الجلوكوز بواسطة MHQ1, MHQ2 وMHQ-4 . نخلص مما سبق إلى ضرورة إستكمال البحث والدراسة على المركبات المعزولة والذي بدوره قد يساعد على اكتشاف مركبات جديدة وآمنة مع فعالية قوية ضد السكري. وكما أكدت الدراسة على الاستعمال التقليدي للتي. إنديكا في السيطرة على مرض السكري في ماليزيا.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Pharmaceutical Chemistry.

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DECLARATION

I hereby declare that this dissertation 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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This thesis is dedicated to my parents for making me be who I am and my siblings for supporting me all the way!

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LIST OF SYMBOLS

d	Doublet
Hz	Hertz
J	Coupling Constant
Kg	Kilogram
m	Multiplet
mg	Milligram
s	Singlet
µg	Microgram
%	Percentage
*P	P value <0.05
**p	P value <0.005

LIST OF ABBREVIATIONS

ACETONE-D_6	Deuterated Acetone
APT	Attached Proton Test
$CDCl_3$	Deuterated chloroform
DCM	Dicholoro Methane
DM	Diabetes Mellitus
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DPP	Dipeptidyl Peptidase
EtOH	Ethanol
FTIR	Fourier transform infrared spectroscopy
GLUT	Glucose Transporter
IC_{50}	Inhibitory Concentration where the response reduced by half
MOA	Mechanism of Action
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
NHMS	National Health and Morbidity Survey
ORO	Oil Red O Staining
PBS	Phosphate Buffered Saline
PPM	Parts Per Million
SPSS	Statistical Package for the Social Sciences
TEF	Toluene: Benzene: Formic Acid
TLC	Thin Layer Chromatography
^{1}H NMR	Proton Nuclear Magnetic Resonance
^{13}C NMR	^{13}Carbon Nuclear Magnetic Resonance
2-NBDG	2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose

CHAPTER ONE

INTRODUCTION

1.1 INTRODUCTION

Diabetes mellitus is a metabolic disorder which gradually leads a person to death. In Malaysia, the rate of DM is dramatically increasing and therefor it has become a major threat to all Malaysians of different age groups. The prevalence of diabetes in Malaysia was recorded 0.65% in 1960. However, according to the First National Health and Morbidity Survey, the DM prevalence was found to be 6.3% in 1986 which was further increased to 8.2% in 1996 (NHMS II). More interestingly, the recent survey revealed very appalling information as the prevalence of DM was found to be about 20.8% which is astonishingly an increase of 80% in a decade at 8% rising rate every year (NHMS I & NHMS II). This indicates that, the prevalence of DM in Malaysia is alarming and has doubled in the last decade (NHMS III). Another major concern about DM is diabetes comorbidity (Meyers et al., 2014). DM leads to many other complications like cardiovascular diseases, obesity, hypoglycemic shock etc. which collectively responsible to decrease the quality of life and eventually lead to death (Abdelmalik et al., 2007).

In Malaysia, the similar scenario has been observed as there is a gradual increase of diabetes rate among the Malaysian adults aged 30 years or above from 6.3 to 8.3 in the year 1986 and 1996 which reached to 14.9% in 2006 (Nazaimoon et al., 2013). Since DM is a chronic disorder, it creates many health complications which lead to financial crisis to the patients. Apart from using insulin, many oral medications are utilized in the management of DM. Most of the medications used in DM treatment have been reported

to exert deleterious side effects and they are costly too. At some point of the prolonged period of DM treatment, people either stop taking prescribed synthetic medicines or diverted to herbal medicines (Ettaro et al., 2004).

The search for a new class of safe antidiabetic agents is regarded as an important scientific endeavor to overcome chronic diabetes and its related infirmities. Therefore, there is always a continuous search for alternative drugs. Medicinal plants are considered the best source to obtain a variety of drugs according to the World Health Organization (Farnsworth et al., 1985). With respect to treat hypoglycaemic as well as hyperglycaemic conditions, many medicinal plants are used and referred to considerable interest to ethno-botanical community as they are recognized to contain valuable and important medicinal properties in different parts of the plant and also a number of plants have shown varying degrees of hypoglycaemic and anti-hyperglycaemic effects as well (Ponnusamy et al., 2011). Many medicinal plants have been reported to afford active principles with desired pharmacological properties to cure ailments such as type-1 and type-2 DM (Fabricant & Farnsworth, 2001).

At present, researchers have become more interested in plant source and a numerous biological studies are currently undergoing in regard to find out safe and effective antidiabetic agents from plants source. In this regard, *Tetracera indica* (Houtt. Ex Christm. & Panz.) Merr. (Dilleniaceae) is one of the Malaysian plants to address this issue effectively. It is a large, woody, rain forest climber of Malaysia which is commonly known as mempelas paya or sand paper plant. It has white colored flower and leaves are simple and medium shaped. It has berry-like fruits which are sour in taste (Christophe, 2002). Different parts of the *T. indica* have been used for healing of fever, flue, sinus symptoms, skin rashes, itching, piles, mouth ulcer, diarrhea, insects bites and diabetes. *T. indica* is also used as one of the active ingredients in a local herbal drug viz,

Plantisol, which is widely prescribed and recommended to effectively manage diabetes in Malaysia by the local herbalist practitioners. *Barringtonia racemosa*, *Pithecellobium jiringa*, *Tinospora crispa* and *Andrographis paniculata* are other active ingredients of Plantisol (http://www.klik4sihat.com/kencingmanis). In this research, our aim is to evaluate an *in-vitro* antidiabetic potential of the stems of *T. indica* and isolated compounds from the stems extract with respect to find out safe and efficacious antidiabetic agents.

1.2 PROBLEM STATEMENT

Currently available therapeutic options for non-insulin-dependent diabetes mellitus (NIDDM) such as dietary modification, oral hypoglycemic, and insulin are not only costly to manage but have limitations of their own. Many of these antidiabetic agents have a number of serious toxic side effects on health; thus management of diabetes without any side effects is still considered as a great challenge to tackle. Hence, the search for more effective and safer antidiabetic agents (i.e. antihyperglycemic agents) has continued to be an important area of investigation for scientists throughout the world. There has been a growing interest in antidiabetic agents from natural products, particularly those derived from traditional medicinal plants. Currently available therapies for diabetes include insulin and various oral antidiabetic agents such as sulfonylureas, biguanides and α-glucosidase inhibitors which are used as monotherapy or in combination to achieve better glycemic regulation. Many of these oral anti-diabetic agents suffer from various deleterious effects, thus, managing diabetes without any side effects is still a great challenge to the scientists (Ahmed et al., 2012), and hence the search for more effective and safer therapeutic agents in managing diabetes has continued to be an important area of investigation. In this regard, *T. indica* could be one

of the candidates to tackle aforementioned problems associated with DM. Aerial parts of *T. indica* are used in the management of diabetes in different parts of Malaysia. However, antidiabtic potential of the compounds present in the aerial parts of *T indica* is yet to be scientifically evaluated appropriately. Therefore, this research work will highlight the development of the compounds present in the stems of *T. indica* into a widely used safe antidiabetic drug to control or prevent diabetes efficaciously. This study will provide preclinical evidence and elucidate the extent of pharmacological activities of the compounds present in the stems of *T. indica* which could prove to be a potential clinical drug in the management of diabetes.

1.3 SIGNIFICANCE OF THE STUDY

In United States, 56.9% people with diagnosed diabetes take only oral medications (Diabetes statistics report, 2010-12; CDC). As diabetes is considered a chronic disease with comorbidity, it is a major financial threat for the patients and their families as well. The oral medications available in the market are also not free of side effects and they are costly too. So patients are becoming more interested in the traditional medicines which are mainly prepared from the plants extracts of traditional medicinal plants widely used to cure various diseases throughout the world (Christensen et al., 2009).

Different parts of *T. indica* have been traditionally used to manage DM in Malaysia. However, no attempt has been made to discover biologically active compounds from this plant. Hence, our aim in this research study is to explore *in vitro* antidiabetic potential of the stems of *T. indica* and isolate active principles responsible for the *in-vitro* antidiabetic activity of the stems of *T. indica*. Through meticulous investigation on the isolated compounds and *in-vitro* antidiabetic activity testing, we anticipate to find out the phytoconstituents which could be responsible for the

antidiabetic activity of the stems of *T. indica* and the resultant compounds showing promising *in vitro* antidiabetics effect might provide lead for the discovery of safe antidiabetic agents in the management of diabetes.

1.4 RESEARCH OBJECTIVES

The objective of the research was as follows:

1. To investigate an *in vitro* antidiabetic activity of the stems ethanol extract of *T.*

   indica on 3T3-L1 adipocytes.

2. To isolate phytoconstituents of the stems ethanol extract of *T. indica*.

3. To check the cytotoxicity of the fractions and isolated compounds on 3T3-L1 pre-adipocytes.

4. To investigate *in-vitro* antidiabetic activity of all isolated compounds on 3T3-L1 adipocytes.

1.5 THEORETICAL FRAMEWORK

For type II diabetes mellitus several types of therapeutic approaches are taken into account viz. increase insulin secretion by pancreas, increase insulin sensitivity to target organ or increase glucose uptake to adipocyte cells. In this study, insulin-like and insulin-sensitizing activity of the isolated compounds has been checked in both pre-adipocytes and adipocytes. MTT viability assay has been followed to determine the concentration to which the compound is toxic to the cells. Later on, activity of the compounds at safe concentration has been checked on 3T3-L1 adipocytes. Adipogenesis is a complex process where pre-adipocytes become mature with hundreds

of genes alteration that lead to reduction of glucose level in blood (Rosen & Spiegelman, 2000).

Again, glucose uptake of mature adipocytes is stimulated by insulin through re-localization of glucose transporter type 4 (GLUT 4) from intracellular stores to the plasma membrane (Kuppusamy et al., 2014). Glucose uptake was measured by a newly developed fluorometric method whereby a fluorescence analog of glucose 2-NBDG was used (Manaharan et al., 2013). All the mechanisms mentioned were investigated on the isolated compounds from *T. indica*. The structure of the isolated compound has been elucidated through extensive NMR studies which shaded some light on further structural modification of the biologically active compounds.

The main objective of the study was to investigate the antidiabetic effect of the stems of *T. indica* and isolated compounds from the stems ethanol extract and finding out the mechanism of action of ethanol extract as well as all the isolated compounds from the same extract on 3T3-L1 adipocytes.

1.6 RESEARCH HYPOTHESIS

The primary hypothesis of the study was as follows:

H1: *T. indica* is traditionally used in the management of diabetes in Malaysia and contains antidiabetic agents.

The specific hypothesis of the study is given below:

H2: *T. indica* and isolated compounds induce adipogenesis like insulin.

H3: *T. indica* and isolated compounds enhance adipogenesis and have insulin sensitizing activity like rosiglitazone.

H4: *T. indica* and isolated compounds can uptake fluorescent glucose analog (2-NBDG) like insulin.