WOGONIN'S STRUCTURE ACTIVITY RELATIONSHIP STUDY ON ANTIDIABETIC ACTIVITY

BY

MURNI NAZIRA BINTI SARIAN

A thesis submitted in fulfillment of the requirement for the degree of Doctor of Philosophy in Pharmaceutical Sciences (Pharmaceutical Chemistry)

Kulliyyah of Pharmacy
International Islamic University Malaysia

JUNE 2018

ABSTRACT

Wogonin has been reported to exert antihyperglycemic effect and has potential to enhance the current therapy options against type 2 diabetes mellitus. However, the structure-activity relationships (SAR) studies of wogonin against this disease have not been carried out. In this study, thirteen structurally similar compounds to wogonin were taken into account to understand wogonin's SAR on antidiabetic activity. Initially, powdered leaves of *Tetracera indica* Merr. (*T. indica)* was macerated with methanol for 72 h to obtain methanol extract which was subjected to column chromatography (silica gel and sephadex LH 20) to isolate wogonin (MN1). Along with the isolation of wogonin, techtochrysin (MN4), and norwogonin (MN7) were also isolated. Isoscutellarein (MN9), hypolaetin (MN10), kaempferol (MN11) and quercetin (MN12) were also isolated from the *Tetracera scandens (T. scandens)* leaves methanol extract via similar procedure. To understand the SAR of wogonin, methyl ether of wogonin (MN2), acetate of wogonin (MN3) and acetate of norwogonin (MN8) were also synthesized. Their structures were elucidated through the interpretation of spectroscopic data. Some commercial compounds which have related chemical structures were also bought and compared for their biological activities viz., 8-hydroxy-7-methoxy flavone (MN5), chrysin (MN6), (+) catechin (MN13) and (-) epicatechin (MN14). Wogonin (MN1) obtained as the major flavone from *T. indica* was administered through intraperitoneal to STZ-NA induced diabetic rats (25, 40, 80 mg/b.w) to examine its antidiabetic potential. The biochemical assays, insulin release and histological alteration were evaluated and compared to standard hypoglycemic drug, metformin (0.5 mg/kg b.w). Then, all compounds were evaluated for their *in vitro* antioxidant activities using rapid test by dot blot, DPPH, $ABTS^{+}$, xanthine oxidase inhibitory and FRAP assays. Subsequently, *in-vitro* antidiabetic activities through DPP-IV and α-glucosidase inhibitory assays were assessed. Subsequently, cell viability of RIN-5F pancreatic cell and pre-adipocyte were initially tested then insulin secretion of RIN-5F as well as adipogenesis, and glucose uptake measurement of adipocyte were investigated. Next, protein expression studies through adipokines (leptin, adiponectin, TNF-α, RBP-4) as well as western blotting against GLUT4 and C/EBP-α were analyzed. The results showed that wogonin at 40 mg/b.w and 80 mg/b.w exhibited significant antihyperglycemic activity without showing any toxicity effect of the liver ($p<0.05$). *In vitro* antioxidant and antidiabetic activities (DPP-IV, α-glucosidase) clearly highlighted the importance of the total number and configuration of hydroxyl group, as well as disadvantage of the absence of ketonic group at C-4 and C-2-C-3 double bond. Nevertheless, the results of animal study, cell culture (insulin secretion, adipogenesis, glucose uptake), and protein expression showed that the methyl ether group at position C-8 might be responsible for wogonin's antidiabetic capacity via β-cells of islets of Langerhans' recovery as well as through glucose uptake mechanism which was indicated by up regulation of GLUT4 and C/EBP-α. The mechanism could be enhanced by the addition of acetate group at C-5 and C-7 positions. These finding have facilitated us to understand the key pharmacophore of wogonin via SAR and should be encouraged for further future studies, which could lead to the development of nutritional product and semi synthetic analogs that retain substantial antidiabetic capacity with minimal adverse effects.

خلاصة البحث

أثبتت التقارير قدرة مركب الوقونين (wogonin) كمخفض لمستويات السكر في الدم وبذلك وجود احتمالية استعمالها في تحسين خيارات العلاج الحالية ضد داء السكري من النمط الثاني، ومع ذلك لم يكن هنالك أي درسات على علاقات النشاط بالهيكل لمركب الوقونين ضد هذا المرض. تم في هذه الدراسة نقع مسحوق أوراق نبتة التيتراسيرا إنديكا مير (*Tetracera indica* Merr.) في الميثانول لمدة 72 ساعة للحصول على مستخلص الميثانول، والذي تم تحليله بالكروماتوجرافيا العمودية (هلام السيليكا وسيفادكس LH 20) لعزل الوقونين (MN1). بالإضافة إلى عزل الوقونين، تم أيضا عزل التيكتوكريسين (MN4)، والنوروقونين (MN7). تم عزل الإيسوسكوتلارين (MN9)، والهبوليتين (MN10)، والكامبفيرول (MN11)، والكيرسيتين (MN12) من مستخلصات التيتراسيرا سكاندنز (*Tetracera scandens*) الإيثانولية بنفس الطريقة. لفهم علاقات النشاط بالهيكل، استحدثت المركبات الآتية جزئيا: ميثيل الأثير من الوقونين (MN2)، وخلات الوقونين (MN3)، وخلات النوروقونين (MN8)، ومن ثم تم توضيح هياكلها من خلال تفسير بياناتها الطيفية. تم شراء بعض المركبات التجارية ذي هياكل كيميائية مماثلة ومقارنة أنشطتها البيولوجية والتي تضمنت: 8-هيدروكسي-7-ميثوكسيفلافون (MN5)، و الكريسين (MN6)، و (+) كاتشين (MN13، و (-) إبيكاتشين (MN14). تم إعطاء الوقونين التي تم الحصول عليها كالفلافون الرئيسي من نبتة التيتراسيرا إنديكا للجرذان المصابة بالسكري المستحدث بمركب الإس تي زيد على جرع 25، و40، و80 ملغ/كج من وزن الجسم لدراسة فعاليتها المضادة للسكري. تم تقييم القياسات البيوكيميائية، وإفرازات الأنسولين، والتغيير النسيجي ومقارنتها مع نتائج العقار النموذجي لتخفيض سكر الدم، الميتفورمين. ثم تم تقييم الأنشطة المضادة للأكسدة لجميع المركبات مخبريا باستخدام اختبار التخطيط النقطي السريع، و DPPH، و $ABTS^{+}$، وفحص تثبيط الزانثين أوكسيديز، وتحاليل FRAP. تم لاحقا تقييم الأنشطة المضادة للسكري في المختبر لتحاليل DPP-IV وتحاليل تثبيط ألفا-غلوكوزيديز. تم بعدها اختبار حيوية خلايا RIN-5F البنكرياسية، والخلايا القبل شحمية، ومن ثم تم التحقيق في إفراز الأنسولين من RIN-5F وكذلك تكون الشحم، وقياس امتصاص الجلوكوز للخلايا الشحمية. بعد ذلك، تم تحليل دراسة التعبير البروتيني من خلال قياس هرمونات الأديبوكين (اللبتين، أديبونيكتين، RBP-4, TNF-α) بواسطة ELISA وكذلك من خلال لطخة ويسترن ضد GLUT4 و C/EBP-α. أظهرت النتائج أن لدى الوقونين نشاطا كبيرا مضاد لارتفاع سكر الدم بدون أي تأثير سمي ($p < 0.05$) على جرعات 40 و 80 مغ/كج من وزن الجسم. سلطت الأنشطة المختبرية المضادة للأكسدة والفحوصات المخبرية المضادة للسكري (DPP-IV، ألفا-جلوكوزيديس) الضوء على أهمية العدد الإجمالي وتكوين مجموعة الهيدروكسيل، فضلا عن سلبية غياب مجموعة الكيتونيك في الروابط المزدوجة في C-4 و C-3- C-2. ومع ذلك، أظهرت نتائج الدراسة الحيوانية، والزراعة الخلوية المضادة للسكري (إفراز الأنسولين، تشكل الشحوم، امتصاص الجلوكوز) والتعبير البروتيني أن مجموعة الأثير الميثيلي في الموقع C-8 هي المسؤولة عن قدرة المضادة للسكري لمركب الوقونين عبر استرجاع خلايا بيتا في جزر لانجرهانز وكذلك من خلال آلية امتصاص الجلوكوز التي أشار إليها التنظيم الرفعي للبروتينات GLUT 4 و C/EBP-α . بالإمكان تعزيز هذه الآلية بإضافة مجموعة خلات في C-5 و C-7. سهلت هذه النتائج فهم الفارماكوفور الرئيسي للوقونين عن طريق علاقات النشاط بالهيكل، والتي ينبغي تشجيعها لإجراء المزيد من الدراسات المستقبلية، والتي يمكن أن تؤدي إلى تطوير منتجات صحية ونظائر شبه اصطناعية محتفظة بقدرتها المضادة للسكري بآثار جانبية قليلة.

APPROVAL PAGE

The thesis of Murni Nazira Binti Sarian has been approved by the following:

_____
Qamar Uddin Ahmed
Supervisor

_____
Siti Zaiton Mat So'ad
Co-Supervisor

_____
Solachuddin Jauhari Arief Ichwan
Co-Supervisor

_____
Muhammad Taher Bakhtiar
Co-Supervisor

_____
Ridhwan Abdul Wahab
Internal Examiner

_____
Azran Azhim Noor Azmi
Chairman

DECLARATION

I hereby declare that this thesis is the result of my own investigation, 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.

Murni Nazira Binti Sarian

Signature……..…. Date …..**.....**

INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

DECLARATION OF COPYRIGHT AND AFFIRMATION OF FAIR USE OF UNPUBLISHED RESEARCH

WOGONIN'S STRUCTURE ACTIVITY RELATIONSHIP STUDY ON ANTIDIABETIC ACTIVITY

I declare that the copyright holders of this thesis are jointly owned by the student and IIUM.

Affirmed by Murni Nazira Binti Sarian

……..…….. ……..
Signature Date

I humbly present this little gift to my beloved father, Sarian Bin Samingan, who has suffered from diabetes mellitus type 2, my loving mother, Mahiran Binti Hashim, as well as to my dearly-departed aunty, Allahyarhamah Jaemah Binti Samingan (1940-2017). Not to forget, to my husband and dearly-loved son, billion of thanks to both of you for your sweat, blood and tear, accompanying me throughout this tough journey.

ACKNOWLEDGEMENTS

In the name of Allah, The Most Gracious, The Most Merciful. Salawat and remembrance for the Holy Prophet, Muhammad S.A.W.

Firstly, I would like to express my highest gratitude towards Allah the Almighty, due to His blessing, I managed to complete my PhD project, Alhamdulillah. The pleasure that He gives us will never be reimbursed by our quality of piousness.

To my admirable supervisor, Assoc. Prof. Dr. Qamar Uddin Ahmed, may Allah bless him and his family in this world and akhirah for guided me, allowed me to design my project accordingly, taught me on how to become a better scientist, motivated me when my spirit was at the par as well as gave his full support throughout this study. I sincerely pray for his sheer success here and hereafter.

I would like to thank my co-supervisors; Assoc. Prof. Dr. Siti Zaiton Mat So'ad, Assoc. Prof. Dr. Solachuddin Jauhari Arief Ichwan and Assoc. Prof. Dr. Muhammad Taher, for their trustworthy and opportunity were given to me, to complete my study. Heartfelt thanks to the colleagues and staff of Kulliyyah of Pharmacy; Ms. Hanisuhana Hamidon, Mrs. Nurlailinajmi, Ibu Wastuti, Ms. Suganya Murugesu, Ms. Vikneswari Perumal, Ms. Syarifah Akilah Binti Syed Muhammad, Mr. Alhassan, Mr. Mahmudul Hasan, Mr. Razif, Mrs. Fatimah, Mrs.Noridayu, and etc, for their valuable support.

To my family; I thank so much to my husband, Muhammad Farhan Ishak and my adorable baby, Muhammad Fahri Arjuna for your sacrifices and support. To my father, Sarian Samingan and my mother, Mahiran Hashim, your love and dua will never be paid by any means in the entire universe. To my little twin sisters, Syahdu and Syahda, I hope my achievement will enlighten you to pursue your ambitions. Not to forget, my parents in-law, and all in-laws that always provide support, care and love at any circumstances.

Finally, I would like to express my gratitude to Kulliyyah of Pharmacy, International Islamic University Kuantan Campus, and to Ministry of Higher Education Malaysia that has provided me MyBRAIN15 scholarship and research grant; Fundamental Research Grant Scheme, FRGS13-089-0330 which has sponsored my entire study.

TABLE OF CONTENTS

Abstract …… ii
Abstract in Arabic…… iii
Approval Page…… iv
Declaration…… v
Copyright…… vi
Dedication…… vii
Acknowledgements…… viii
List of Tables…… xiv
List of Figures…… xv
List of Equations…… xix
List of Abbreviations …… xx

CHAPTER ONE: INTRODUCTION…… 1
1.1 General Overview…… 1
1.2 Problem Statement…… 4
1.3 Objectives…… 6
1.3.1 General Objective…… 6
1.3.2 Specific Objectives…… 6
1.4 Significant of the Study…… 7
1.5 Hypothesis…… 7
1.6 Experimental Design…… 9

CHAPTER TWO: LITERATURE REVIEW…… 10
2.1 General Overview…… 10
2.1.1 Drug Discovery and Natural Products…… 11
2.1.2 Medicinal Plants Research in Malaysia…… 11
2.1.3 Structure Activity Relationship Based Study…… 13
2.2 Diabetes Mellitus…… 14
2.2.1 Prevalence of Diabetes in Malaysia…… 14
2.2.2 Classification of Diabetes Mellitus…… 15
2.2.3 Interaction between Insulin Resistance and Insulin Secretion in the Development of Glucose Intolerance…… 17
2.2.4 Mechanisms Associating Obesity to Insulin Resistance and Type 2 Diabetes Mellitus…… 19
2.2.5 Hyperglycemia Induced Oxidative Stress and Role of Antioxidants…… 21
2.2.6 Complication of Diabetes Mellitus Related to Insulin Resistance and its Metabolic Justification…… 23
2.3 Pharmacotherapy For Diabetes Mellitus…… 25
2.3.1 Exercise and Weight Loss…… 25
2.3.2 α-Glucosidase Inhibition…… 25
2.3.3 Delay Gastric Emptying…… 27
2.3.4 Increase Pancreatic Insulin Secretion…… 27
2.3.5 Increase Insulin Sensitivity…… 28
2.3.6 Reduce Hepatic Glucose Output (HGO) …… 29
2.3.7 Insulin Intervention…… 29
2.3.8 Insulin Mimitec Agents…… 30
2.3.9 Oral Hypoglycemic Agents…… 30
2.3.10 Herbal Medicines for Diabetes Mellitus Management..... 32

2.4 Medicinal Plants with Antidiabetic Activity…….….. 35
2.4.1 *Tetracera indica* Merr……..…. 35
2.4.1.1 Traditional Uses, Phytochemical & Pharmacological Assessment of *T. indica*……..…… 37
2.4.1.2 Wogonin……..…… 38
2.4.2 *Tetracera scanden* Linn.……... 40
2.4.2.1 Traditional Uses, Phytochemical & Pharmacological Assessment of *T. scanden* …….……..… 40
2.5 Flavonoids as Secondary Metabolites with Antidiabetic Activity…….……. 42
2.5.1 Flavonoids……...….. 42
2.6 Animal Model For Study of Antihyperglycaemic Effect……..... 46
2.6.1 Animal Model of Hyperglycemia…… 46
2.6.2 Alloxan and Streptozotocin-induced Diabetes…….…. 46
2.6.3 Streptozocin (STZ) and Nicotimide (NA) Induction…… 49
2.7 Cellular And Molecular Model For Antidiabetic Effect…….. 52
2.7.1 Pancreatic β cell Line as A Model Study on Insulin Secretion…….…… 52
2.7.2 3T3-L1 Preadipocytes and Adipocyte As A Model Study on Glucose and Lipid Metabolism……..... 53
2.7.3 Adipocyte Differentiation…… 55
2.7.4 Role of Adipokiness…….. 56
2.7.4.1 Leptin…….. 56
2.7.4.2 Adiponectin……..….. 57
2.7.4.3 Tumor necrosis factor α (TNF-α)……..…. 57
2.7.4.4 Retinol Binding Factor –IV (RBP-IV)……..….. 58
2.7.5 Transcriptional Regulation of Adipogenesis……..….. 58
2.7.6 GLUT4 Protein: Mechanism of Action and Their Role..... 59

CHAPTER THREE: METHODOLOGY……...…… 62
3.1 Experimental Materials ……..... 62
3.1.1 Collection and Preparation of Plant Material……..... 62
3.1.2 Chemicals…….. 62
3.2 Plant Extraction And Isolation……. 64
3.2.1 Preparation of Methanol Extract of the Leaves of *T. indica*…… 64
3.2.2 Isolation and Purification of Wogonin From The Methanol Extract of *T.indica*…….…… 65
3.2.3 Fractionation of *T. indica* and *T. scandens* Leaves Methanol Extracts and Flavonoids Isolation……..…… 66
3.2.4 Semi-synthetic analog…….…..... 68
3.2.4.1 Methylation of Wogonin…… 68
3.2.4.2 Acetylation of Wogonin and Norwogonin……..…… 68
3.3 *In vivo* Anti-Diabetic Evaluation of Wogonin (MN1)……..…… 69
3.3.1 Experimental Design for Diabetic Rats……...….. 69
3.3.1.1 Streptozotocin (STZ) and Nicotinamide (NA) Induction……. 70
3.3.1.2 Collection of Blood…… 72
3.3.1.3 Serum Biochemical Analysis……..…. 72
3.3.2 Histopathological Assessment……..…… 72
3.3.2.1 Tissue Processing…….…..... 72
3.3.2.2 Haematoxylin & Eosin (H&E) Staining……. 73
3.4 *In Vitro* Anti-Diabetic Evaluation of Wogonin and its Chemical Anologs……..... 74

3.4.1 Determination of Antioxidant Activities……..... 74
3.4.1.1 Rapid Screening of Radical Scavenging Activity by Dot Blot Assay……..... 74
3.4.1.2 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity……..... 75
3.4.1.3 $ABTS^{+}$ Cation Radical Scavenging Activity…….. 76
3.4.1.4 Ferric Reducing Power Capacity (FRAP)…….. 77
3.4.1.5 Xanthine oxidase inhibitory activity (XO)……. 77
3.4.2 α-Glucosidase Inhibitory Assay……..... 78
3.4.3 Dipeptidyl Peptidase IV (DPP-IV) Inhibitory Assay……...……. 79
3.5 Cell Culture *In Vitro* Anti-Diabetic Evaluation of Wogonin and its Chemical Anologs……..…… 80
3.5.1 RIN-5F Pancreatic Cell Lines …….. 80
3.5.2 3T3-L1 Preadipocyte Cell Lines……...…… 81
3.5.3 Cell Viability MTT Assay……. 81
3.5.3.1 RIN-5F Pancreatic Cell ……..…… 81
3.5.3.2 3T3-L1 Preadipocyte Cells ……... 81
3.5.3.3 Insulin Secretion Assay…….. 82
3.5.4 Adipogenesis……. 84
3.5.4.1 Induction of Differentiation (3T3-L1 preadipocyte) …… 84
3.5.4.2 Oil Red O Staining……..…… 86
3.5.5 Fluorescence Glucose [2NDBG] Uptake Measurement……..…. 86
3.6 Proteomic Studies…… 87
3.6.1 Adipokines Measurement via Enzyme-linked Immunosorbent Assay (ELISA) ……..... 87
3.6.1.1 Leptin…… 87
3.6.1.2 Adiponectin…… 88
3.6.1.3 Tumor Necrosis Factor-α (TNF- α) ……... 89
3.6.1.4 Retinol Binding Protein-IV (RBP-IV) ……... 90
3.6.2 Harvesting Cells Lysate…… 91
3.6.3 Quantification of Protein Concentration of Cell Lysates…… 92
3.6.4 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) ……..……..... 92
3.6.5 Western Blotting……... 93
3.7 *In Silico* Molecular Docking…… 95
3.7.1 Receptor and Ligand Preparation…….. 95
3.7.1.1 α-Glucosidase……...…….. 95
3.7.1.2 Glucose Transporter 1 (GLUT1)…….... 96
3.8 Statistical Analysis…….…….. 97

CHAPTER FOUR: RESULTS …… 98
4.1 Preparation of Methanol Extract…….……. 98
4.2 Spectral Data of Tested Compounds…… 98
4.3 *In Vivo* Anti-Diabetic Evaluation of Wogonin (MN1)…… 107
4.3.1 Effect of Wogonin (MN1) on Body Weight……. 107
4.3.2 Effect of Wogonin on Blood Glucose Measurement…… 107
4.3.3 Effect of Wogonin on Insulin Release…….….. 110
4.3.4 Effect of Wogonin on Biochemical Serum Index……. 110
4.3.5 Effect of Wogonin on Histolopathological Morphology…….. 112
4.4 *In Vitro* Anti-Diabetic Evaluation of Wogonin and its Chemical Analogs……. 116
4.4.1 Determination of antioxidant activities…….…… 116

4.4.1.1 Rapid Screening of Radical Scavenging Activity…….. 116
4.4.1.2 DPPH Radical Scavenging Assay…….. 118
4.4.1.3 $ABTS^{+}$ Radical Scavenging Assay……. 118
4.4.1.4 Xanthine Oxidase Inhibition (XOI) Assay……. 119
4.4.1.5 Ferric Reducing Antioxidant Power (FRAP) Assay …….. 121
4.4.2 α-Glucosidase Inhibitory Assay…… 123
4.4.3 Dipeptidyl peptidase IV (DPP-IV) Inhibitory Assay…….. 123
4.5 Cell Culture *In Vitro* Anti-Diabetic Evaluation of Wogonin and its Chemical Analogs…… 125
4.5.1 Cell Viability Study of RIN-5F Pancreatic Cell Lines…….. 125
4.5.2 Effect of Wogonin and its Chemical Analogs on Insulin Secretion (Cell RIN-5F) ……. 127
4.5.3 Cell Viability Study of 3T3-Li Preadipocyte Cell Lines…….. 129
4.5.4 Effect of Wogonin and its Chemical Analogs Adipogenesis (Oil Red O Staining) …….. 131
4.5.5 Effect of Wogonin and its Chemical Analogs Flourescence Glucose [2-NDBG] Uptake Measurement……. 134
4.6 Proteomic Anti-Diabetic Evaluation of 3T3-L1…….. 136
4.6.1 Adipokines Measurement via Enzyme Linked Immunosorbent Assay (ELISA) ……. 136
4.6.2 Western Blotting……. 139
4.7 *In silico* Molecular Docking…….. 141
4.7.1 α-Glucosidase Receptor…….. 141
4.7.2 Glucose Transporter -1 (GLUT1) Receptor…….. 148

CHAPTER FIVE: DISCUSSION…….. 154
5.1 General Discussion…….. 154
5.2 Plant Extraction, Isolation and Semi-Synthesis…….. 155
5.3 *In Vivo* Antidiabetic Evaluation of Wogonin (MN1)……... 157
5.4 *In Vitro* Antidiabetic Evaluation of Wogonin and its Chemical Analogs…… 158
5.4.1 Determination of Antioxidant and Radical Scavenging Activities…… 159
5.4.2 α-Glucosidase Inhibitory Assay……. 161
5.4.3 Dipeptidyl Peptidase IV (DPP-IV) Inhibitory Assay……. 163
5.5 Cell Culture: *In-vitro* Antidiabetic Evaluation of Wogonin and its Chemical Analogs…… 164
5.5.1 Effect of Wogonin and its Chemical Analogs on Insulin Secretion of RIN-5F Pancreatic Cells…… 164
5.5.2 Effect of Wogonin and its Chemical Analogs on Adipogenesis (Oil Red O Staining)……... 165
5.5.3 Effect of Wogonin and its Chemical Analogs on Flourescent Glucose [2-NDBG] Uptake Measurement…….. 166
5.6 Proteomic Anti-Diabetic Evaluation Of 3T3-L1……. 167
5.6.1 Effect of Wogonin and its Chemical Analogs on Adipokines Measurement…….. 167
5.6.2 Western Blotting…….. 170
5.7 *In Silico* Molecular Docking…….. 171
5.7.1 α-Glucosidase ……..…… 171
5.7.2 GLUT1 ……..…… 173
5.8 Summary……..…… 177

CHAPTER SIX: CONCLUSION…… 182
6.1 General……..… 182
6.2 Significant of the Study……..…… 183

185
REFERENCES……...……

APPENDIX…….…… 208
HNMR of Wogonin (MN1)……..…… 208
CNMR of Wogonin (MN1)…….. 209
HNMR of Methyl ether of Wogonin (MN2)…… 210
CNMR of Methyl ether of Wogonin (MN2)…….…… 211
HNMR of Acetate of Wogonin (MN3)…….…… 212
CNMR of Acetate of Wogonin (MN3)…….…… 213
HNMR of Techtochrysin (MN4)……...…… 214
CNMR of Techtochrysin (MN4)……...…… 215
HNMR of Chrysin (MN6)…….…… 216
CNMR of Chrysin (MN6)…….…… 217
HNMR of Norwogonin (MN7)…….…… 218
CNMR of Norwogonin (MN7)…….… 219
HNMR of Acetate of Norwogonin (MN8)…… 220
CNMR of Acetate of Norwogonin (MN8)…… 221
HNMR of Quercetin (MN12)…… 222
CNMR of Quercetin (MN12)…… 223
HNMR of (+) Catechin (MN13)……...…… 224
CNMR of (+) Catechin (MN13)…….…… 225
HNMR of (-) Epicatechin (MN14)…….…… 226
CNMR of (-) Epicatechin (MN14)…….…… 227
IACUC-IIUM Approval…….…… 228
List of Conferences…… 229
List of Awards…… 230
List of Publications…… 230
Certificate of Award…….……..…… 231
Publication……..…… 234

LIST OF TABLES

Table 2.1	Oral antidiabetic agents with their mechanism of action and adverse effects	31
Table 2.2	Plants used as herbal medicine in diabetic management	34
Table 2.3	Classification of *Tetracera indica* Merr .	36
Table 2.4	Differences between widely used cytotoxic glucose analogues	48
Table 2.5	Comparison of adipogenic cell models in proliferation and differentiation capacity	54
Table 3.1	Animal groups	71
Table 3.2	Overview of differentiation of 3T3-L1	85
Table 3.3	Antibody used in this study	94
Table 4.1	Physical properties of flavonoids' compounds	102
Table 4.2	IC_{50} values for DPPH, $ABTS^{+}$ and xanthine oxidase inhibition assays	120
Table 4.3	IC_{50} values of flavonoids for α-glucosidase and DPP-IV inhibition assays	124
Table 4.4	Docking Scores against α-Glucosidase receptor	142
Table 4.5	Docking Score against GLUT1 receptor	150
Table 5.1	Chemical structures and substituents of selected flavonoids	179

LIST OF FIGURES

Figure 1	Flow chart of the study	9
Figure 2.1	Mechanism of β-cell adaptation to insulin resistance	18
Figure 2.2	Impaired insulin released in linking obesity with insulin resistance and T2DM	20
Figure 2.3	Mechanism of hyperglycemia-induced cellular damage mediated by oxidative stress	22
Figure 2.4	Hyperglycemia-induced pathways of oxidative stress in obesity, including the polyol pathway, the AGE pathway and glucose auto-oxidation	22
Figure 2.5	*T. indica* (a) Flower and fruit); (b) Leaves	36
Figure 2.6	Wogonin (5, 7-Dihydroxy-8-methoxyflavone) Phenylbenzopyrans (C6-C3-C6 backbone)	38
Figure 2.7	*T. scandens* (L.) (a) Leaves; (b) Flower	42
Figure 2.8	Basic structure of flavonoids consists of a fused A and C rings, with phenyl B ring attached through its 1′ position to the 2-position of the C ring	43
Figure 2.9	Phenylbenzopyrans (C6-C3-C6 backbone)	44
Figure 2.10	The schematic presentation of the toxicicty action of streptozotocin (STZ) and the protective effect of nicotinamide (NA) on β-cell. PARP 1-Poly Adenosine triphosphate ribose polymerase 1; PPRP-5phosphoribosylpyrophosphate; NMN- Nicotinamide mononucletide; Nampt-Nicotinamide phosporibosyltransferase; Nmnat-Nicotinamide mononucleotide adanylyltransferase. Red arrow- decrease activity, grey arrow- increase activity	51
Figure 2.11	Uptake of glucose to intracellular compartments following insulin binding initiating signaling cascade. Fatty acids (FA) impairs this pathway by producing defects on insulin receptors (IR), insulin receptor substrate (IRS-1), Phosphatidylinositol-3-Kinase(PI3K), Phosphoinositide-dependent kinase 1(PKD-1), Akt/Protein Kinase B (PKB) and Glucose Transport 4	61
Figure 4.1	Structure of tested flavonoids (MN1-MN4)	103
Figure 4.2	Structure of tested flavonoids (MN5-MN8)	104
Figure 4.3	Structure of tested flavonoids (MN9-MN12)	105
Figure 4.4	Structure of tested flavonoids (MN13-MN14)	106

Figure 4.5	The effect of wogonin (MN1) on body weight of rats. Diabetic control showed statistically significant ($p<0.05$) lower as compared to all groups	108
Figure 4.6	The effect of wogonin on blood glucose serum of rats	109
Figure 4.7	The effect of wogonin on rat's insulin release	111
Figure 4.8	Concentration of serum lipid profiles after 30 days of wogonin treatment	111
Figure 4.9	Pancreas histology	113
Figure 4.10	Liver histology	114
Figure 4.11	Kidney histology	115
Figure 4.12	Rapid screening of radical scavenging activity by dot blot assay on silica sheets stained with DPPH solution at 16 different concentrations	117
Figure 4.13	Result of FRAP in ascorbic acid equivalent (AAE)	122
Figure 4.14	The percentage of cell viability of RIN-5F ranging from 0.39 – 25 μg/well in 1 h	126
Figure 4.15	The effect of the flavonoid on insulin secretion activity of RIN-5F cell	128
Figure 4.16	The percentage of cell viability of 3T3-L1 preadipocyte cell measured by MTT assay, ranging from 0.78 to 100 μg/ well for 48 h	130
Figure 4.17	Quantification of lipid droplet after treatment	132
Figure 4.18	Lipid droplets formations of 3T3-L1 adipocyte stained with Oil Red O	133
Figure 4.19	The effect of flavonoids on glucose uptake measurement of adipocyte cells.	135
Figure 4.20	Adipokines measurement of adipocyte after flavonoids treatment at 12.5 μg/mL; a) Leptin, b) Adiponectin, c) TNF-α, d) RBP-IV.	138
Figure 4.21	Image of protein bands of GLUT4, C/EBP-α and β-actin (internal control) on PVDF membrane	140
Figure 4.22	The density of protein bands of GLUT4 and C/EBP-α per β-actin	140
Figure 4.23	Active site of yeast α-glucosidase (PDB ID: 3A4A) showing amino acid residues (green) interaction with maltose (black).	142
Figure 4.24	Binding interactions of isoscutellarein (MN9) with active site residues of yeast α-glucosidase (PDB ID: 3a4a). The compound is colured green while amino acids are brown. Green, purple and dashes depict hydrogen bond, hydrophobic bond and electrostatic interactions respectively.	144

Figure 4.25	Binding interactions of hypoletin (MN10) with active site residues of yeast α-glucosidase (PDB ID: 3a4a). The compound is colured green while amino acids are brown. Green, purple and dashes depict hydrogen bond, hydrophobic bond and electrostatic interactions, respectively.	145
Figure 4.26	Binding interactions of kampferol (MN11) with active site residues of yeast α-glucosidase (PDB ID: 3a4a). The compound is colured green while amino acids are brown. Green, purple and dashes depict hydrogen bond, hydrophobic bond and electrostatic interactions respectively.	146
Figure 4.27	Binding interactions of quercetin (MN12) with active site residues of yeast α-glucosidase (PDB ID: 3a4a). The compound is colured green while amino acids are brown. Green and purple dashes depict hydrogen bond and hydrophobic bond, respectively.	147
Figure 4.28	Active site residues of GLUT1 (PDB ID: 5EQI)	151
Figure 4.29	Binding interactions of glucose (a) with active site residues of GLUT1 (PDB ID: 5EQI) .The compounds are colured black while amino acids are green. Green and purple dashes depict hydrogen bond and hydrophobic bond respectively. (b) Surface structure of glucose in active site of GLUT1. Blue and brown colours indicate polar and hydrophobic regions, respectively.	151
Figure 4.30	Binding interactions of wogonin(MN1) (a), methylether of wogonin (MN2) (b), acetate of wogonin (MN3)(c), and tectochrysin (MN4) (d) with active site residues of GLUT1 (PDB ID: 5EQI).The compounds are colured black while amino acids are green.Green and purple dashes depict hydrogen bond and hydrophobic bond respectively.	152
Figure 4.31	Binding interactions of norwogonin (MN7) (a) and acetate of norwogonin (b) with active site residues of Glut1 (PDB ID: 5EQI). The compounds are colured black while amino acids are green. Green and purple dashes depict hydrogen bond and hydrophobic bond, respectively.	153
Figure 4.32	Binding interactions of kaempferol (MN11) (a), (-) epicatechin (MN14)(b) with active site residues of Glut1 (PDB ID: 5EQI). The compounds are colured black while amino acids are green. Green and purple dashes depict hydrogen bond and hydrophobic bond, respectively.	153
Figure 5.1	Structure of the different classes of flavonoids	158
Figure 5.2	3D surface structure of GLUT1 with wogonin in the active site. Blue and brown colours indicate polar and hydrophobic regions	176
Figure 5.3	3D surface structure of GLUT1 with cytochalasin B (an inhibitor) in the active site. Blue and brown colours indicate polar and hydrophobic regions.	176

Figure 5.4 Summary of SAR study of antidiabetic evaluations of flavonoids (MN1-MN14) 178

LIST OF EQUATIONS

Equation		Page No.
1.1	$DPPH\ Scavenging\ Activity = \left[\frac{Abs\ (control) - Abs\ (sample)}{Abs\ (control)}\right] \times 100$	75
1.2	$\%\ inhibition = \left[\frac{Abs\ (control) - Abs\ (sample)}{Abs\ (control)}\right] \times 100$	76
1.3	$\%\ of\ inhibition = \left[\frac{Abs\ control - Abs\ sample}{Abs\ control}\right] \times 100$	78
1.4	$Inhibitory\ activity\ (\%) = \left[\frac{(\text{Abs control} - \text{Abs sample})}{(\text{Abs control})}\right] \times 100\ \%$	79
1.5	$Inhibitory\ activity\ (\%) = \left[\frac{(\text{Initial Activity} - \text{Sample})}{(\text{Initial Activity})}\right] \times 100\ \%$	80
1.6	$Viability\ (\%) = \left[\frac{(Abs\ sample - Abs\ blank)}{(Abs\ control - Abs\ blank)}\right] \times 100$	82
1.7	$100\%\ Specific\ Absorbance = Abs\ of\ insulin\ induced\ 2NDBG - Abs\ of\ non\ insulin\ induced\ 2NDBG$	87

LIST OF ABBREVIATION

Abs	Absorbance
ABTS+	2,2'azino-bis(3-ethylbenzothiazoline-6-sulphonic acid cation
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
C/EBPα	CCAAT/enhancer-binding protein α
DEX	Dexamethasone
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
DPP-IV/4	Dipeptidyl peptidase-IV/4
DPPH	2,2-diphenyl-1-picrylhydrazyl
EtOH	Ethanol
FBS	Fetal bovine serum
FRAP	Ferric reducing antioxidant power
GLUT1	Glucose transporter-1
GLUT2	Glucose transporter-2
GLUT4	Glucose transporter-4
IBMX	3-isobutyl-1-methylxanthine
IC50	Inhibition concentration at 50%
IR	Insulin receptor
mA	Milliampere
Mg/b.w	Milligram per body weight
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5,diphenyltetrazolium bromide
MS	Mass spectrometry
NA	Nicotinamide
NMR	Nuclear magnetic resonance
ORO	Oil-Red-O
PBS	Phosphate Buffer Saline
PI3K/IRS-1	Phosphatidylinositol-3-kinase/Insulin receptor substrate-1
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PKB	Protein kinase B
PPARα	Perixosome proliferator-activated receptor α
PPARγ	Perixosome proliferator-activated receptor γ
RBP-4	Retinol binding protein-4
Rf	Retention factor
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RPMI 1640	Roswell Park Memorial Institute medium 1640
SAR	Structure activity relationship
STZ	Streptozocin
TZD	Thiazolidinediones
T2D	Type 2 diabetes
UV	Ultraviolet
V	Voltage
v/v	Volume per volume
XO	Xanthine oxidase

CHAPTER ONE

INTRODUCTION

1.1 GENERAL OVERVIEW

Medicinal plants are known as the best resource to obtain variety of drugs. Since decades, research studies focused on natural products have increased all over the world. Special insight on medicinal plants in tropical countries has inspired researchers to discover new lead compounds and pharmacologically viable derivatives for drug design and therapeutic purposes (Pan et al., 2013).

Approximately 420,000 plant species exist on earth, but for most of these only very limited knowledge is available (Pan et al., 2013). According to the National Policy on Biological Diversity (2016-2025), Malaysia is one of the most mega diverse countries in the world. It ranks 12th globally. It is estimated to own more than 15,000 species of vascular plants, with about 8,300 species found in Peninsular Malaysia and approximately 12,000 plant species in Sabah and Sarawak. A policy was made by the Malaysian government in 2014 to provide the direction and framework to conserve our biodiversity and use it sustainably in the face of the increasingly complex challenges. The reserved rainforest of Malaysia offers great chances for research activity due to wide range of available species (Ministry of Natural Resources and Environment, 2014). Most of the plants have been collected for medicinal purposes or applied in herbal preparations. Tropical countries enormously retained their unexplored medicinal plants and active compounds which might contain novel biological activities.

Secondary metabolites produced by plants may exert various biochemical and pharmacological functions in humans and animal kingdom. Certain secondary metabolites may hold vital functions in the living plants. For example, flavonoids are able to eliminate free radicals produced during photosynthesis (Falcone et al., 2012). Terpenoids may engage pollinators, as seed disperse, or inhibit competing plants. Alkaloids in the form of phytoalexins provide protection to plants against herbivores or insect attacks (Falcone et al., 2012). Other secondary metabolites function as cellular signaling molecules or may be responsible for some other functions in the plants (War et al., 2012). Hence, further investigation should be conducted to elucidate the potential bioactive compounds.

The most challenging component while conducting research involving natural products would be the unknown effects, interaction and complexity of each compound that has been isolated. It is worth noting that the tools of scientific approaches in modern society today have provided fundamental skeletons for constructing molecule structures and predicting the bio-interaction of isolated plant-derived compounds. These tools have driven meticulous researches to unravel underlying biological importance, mechanisms and the structure activity relationship.

The prevalence and severity of diabetes mellitus (DM) and the resultant metabolic syndrome is rapidly increasing. As the successful preventive and therapeutic strategies for these life threatening health ailments often come with adverse side effects, nutritional elements are widely used in many countries as preventive therapies to prevent/manage metabolic syndrome. With respect to treat hyperglycemic as well as hypoglycemic conditions, several secondary metabolites especially flavonoids have been investigated as they are well known to contain valuable features and offer significant result in the studies. The search for a new class

of safe antidiabetic agents is considered as an important scientific endeavor to overcome chronic diabetes mellitus and its related infirmities. Therefore, there is always a continuous research for alternative drugs related to plant active compounds.

Tetracera indica (Christm. & Pantz.) Merr. (Dilleniaceae) is one of the Malaysian plants that can be used to tackle this issue effectively. It is a woody, Malaysian rain forest climber which is commonly known as "Mempelas paya" or sand paper plant. It has white-pinkish colored flowers and the leaves are simple and medium shaped. It has berry-like fruits which have been described as sour in taste (Hasan et al., 2017; Christophe, 2002). Traditionally, different parts of *T. indica* have been claimed for healing flu, sinuses symptoms, fever, skin rashes, itching, piles, ulcer, diarrhea, insect bites as well as diabetes mellitus. In addition to that, *T. indica* is used as one of the ingredients in a local herbal drug i.e. Plantisol®, which is commonly prescribed and recommended to effectively manage diabetes in Malaysia by the local herbalist practitioners.

Wogonin (5,7-dihydroxy-8-methoxy flavone), a flavone isolated (Harrison et al., 1994) from the leaves of *T. indica* has been reported for its antidiabetic potential (Hasan et al., 2017; Zhang et al., 2015; Bak et al., 2014). However, structure-activity relationship (SAR) study on the wognin with regard to understand its true antidiabetic potential is yet to be carried out meticulously. Hence, the aim of this study was to evaluate the antidiabetic effects of wogonin and its chemical analogs, mechanism of action and their SAR. Initially, wogonin from the leaves of *T. indica* was isolated using silica gel and sephadex LH_{20} column chromatographies. Besides that, some chemical anologues of wogonin were also isolated from the leaves of *T. indica* and *T. scandens* for SAR study. These compounds structures were elucidated and characterized by spectroscopic analyses (NMR, IR, UV, Mass spectrometry). The

major compound of *T. indica*, wogonin, was used for *in-vivo* test against streptozocin-nicotinamide induced diabetic rats. Then, all compounds (chemical analogues of wogonin) were subjected to *in-vitro* antioxidant assays, *in-vitro* antidiabetic assays, protein expression via enzyme linked immonosorbent assay (ELISA), western blotting and molecular docking to investigate the mechanism and their SAR with the convergence of T2DM pathways.

1.2 PROBLEM STATEMENT

Diabetes mellitus is one of the major metabolic disorders that continues to present as significant health problem worldwide and mostly associated with chronic and disturbances in protein, carbohydrate and lipid metabolism (Hameed et al., 2015). The overall prevalence of DM has increased by more than twofold from 1996 to 2015 (NHMS, 2015). In 2013, a total of 381.8 million adults worldwide were affected with diabetes mellitus and the number is estimated to reach 591.9 million by 2035 (Guariguata et al., 2014). Hence, the search for alternative medicinal plant based drugs is crucial to ameliorate this condition. In Malaysia, the most comprehensive and nationally representative available health data (National Health and Morbidity Surveys (NHMS)) have shown that there has been an increasing trend in the reported prevalence of DM for almost the past two decades since 1996 to 2015 (Tee & Yap, 2017). In this matter, wogonin and its chemical analogs isolated from the leaves of *T. indica* and *T. scandens* could be the candidates to tackle aforementioned problem associated with T2DM. Wogonin is chosen in this study due to the fact that it has been investigated by previous study as one of the major phytoconstituents in the leaves of *T. indica* that may be responsible for the antidiabetic effect (Ahmed et al., 2012). However, the mechanism behind this study remains to be extensively unexplored.