EVALUATION OF ANTIDIABETIC AND ANTIOXIDANT PROPERTIES AND METABOLITE PROFILING OF *MOMORDICA CHARANTIA* FRUIT USING METABOLOMICS APPROACH

BY

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ABSTRACT

Momordica charantia Linn (Cucurbitaceae) has been widely commercialized based on traditional usage as an antidiabetic product. However, the scientific evidence of its antidiabetic activity is not sufficient. Hence, the major aims of this research were to evaluate the antidiabetic activity of *M. charantia* fruit through proton-nuclear magnetic resonance (^{1}H-NMR) spectroscopy based metabolomics, to investigate its mechanism of action, to profile the identified antioxidants as antidiabetic agents through liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) based metabolomics, and to develop a validated regression model using fourier transform infra-red spectroscopy (FT-IR) based fingerprinting. Initially, the fruit was extracted by the solvent with different ethanol in water concentration (0, 20, 40, 60, 80, 100%, v/v). Then, the extracts were subjected to different *in vitro* assays. The 80% aqueous ethanolic extract possessing the highest *in vitro* bio-activity was chosen for further *in vivo* antidiabetic evaluation utilizing ^{1}H NMR based metabolomics approach. *In vitro* dipeptidyl peptidase-4 (DPP-IV) inhibitory and 3T3-L1-cell glucose uptake activities were also tested for the same extract to explain its mode of action in relation to the metabolomics results. LC-MS and GC-MS based metabolomics approaches were applied to profile the bioactive compounds present in the extract. Finally, a validated calibration model was developed using FT-IR based fingerprinting. The 80% ethanolic extract showed a high inhibitory activity on 1, 1-diphenyl-2 picrylhydrazyl (DPPH) radical and high ferric reducing antioxidant power, but failed to exhibit inhibitory activity against α-glucosidase and xanthine oxidase enzymes. Thereby, the antidiabetic activity of this extract was further evaluated using streptozotocin obese-diabetic induced rats (STZ ob-db). The results showed that the administration of the 80% ethanolic extract at 300 mg/kg bw for 4 weeks significantly ($P < 0.05$) reduced the blood glucose level and normalized the blood lipid profile of rats. However, the data obtained from the metabolomics showed that the metabolite profiles of the serum and urine of rats could not be fully normalized by the 80% ethanolic extract and metformin. The identified biomarkers in serum and urine were 2-hydroxybutyrate, leucine, adipate, alanine, acetate, succinate, 2-oxoglutarate, dimethylamine, creatine, creatinine, betaine, glucose, taurine, phenylacetylglycine, allantoin and hippurate. Furthermore, it was found to ameliorate the energy metabolism through the improvement of 3T3-L1-cell glucose uptake and inhibition DPP-IV but not through the inhibition of *α*-glucosidase and xanthine oxidase. This extract displayed strong antioxidant activities which further showed a positive correlation to antidiabetic activity. LC-MS and GC-MS based metabolomics approaches helped to identify several antioxidants in this extract such as ascorbic acid, margarolic acid, brevifolincarboxylic acid, quercetin 3-O-glycoside, kuguacin H, cucurbitacin E, 3-malonylmomordicin I, goyaglycoside G, gentiobiose, glucose, galactonic acid, palmitic acid, galactose, mannose, and fructose. Finally, the validated regression model based on the FT-IR based fingerprinting has been successfully developed for the first time through this study in regard to predict the antioxidant activities of the new set of the extracts of *M. charantia*. In conclusion, this study showed that the *M. charantia* fruit extract has a great potential to be efficaciously used in the management of diabetes.

خلاصة البحث

ثمرة المومرديكا تشارانتينا من الفصيلة القرعية, يتم تسويقه بشكل واسع كمنتح لعلاج مرض السكر. ولكن الأدلة العلمية على نشاطه المضاد لداء السكر ليست كافية . لذلك, الهدف الرئيس لهذه الدراسة التحقق من فعليته لعلاج داء السكري باستخدام الميتابولوميات مطيافية الرنين النووي المغناطيس (H-1 NMR)لاكتشاف الية عمله, التعرف على مضاة الاكسدة التي تعمل مضادة لداء السكر باستخدام الميتابولوميات المعتمدة على طريقة قياس الطيف الكتلي-الاستشراب السائل (LC-MS) وطريقة قياس الطيف الكتلي-الاستشراب الغازي (GC-MS) وهدفت أيضا لتطوير نموذج انحدار باستخدام التبصيم المعتمد على تحويل فورييه مادون الأحمر (FT-IR). تم استخلاص الثمار بنقعها في محاليل الإيثانول والماء المختلفة التركيز(0، 20، 40، 60، 80، 100%، v/v) تم اختبار المستخلصات وتقييم نشاط هذه المكونات في اختبارات خارج الجسم الحي .(in vitro) بعد ذلك تم اختيار المستخلص 80% ذي النشاط الأعلى لاختبار تقييم النشاط المضاد لداء السكري داخل الجسم الحي باستخدام الميتابولوميات المعتمدة على الـ H-NMR1. تم وضع المستخلصات في اختبارات تثبيط انزيم دايبيبتيل بيبتايديس-4 (DPP[IV]) واختبار أخذ خلايا T3-L13 للجلوكوز لشرح آلية تأثيرها مع نتائج الميتابولوميات. أما لميتابولوميات المعتمدة على الـ LC-MS و GC-MS فقد استُعملت لتوصيف المركبات النشطة بيولوجيا في المستخلصات. وأخيرا تم تطوير نموذج مثبَت الصحة باستخدام التبصيم المعتمد على الـ FT- IR أظهرت مستخلصات الـ 80% من الإيثانول نشاطا تثبيطيا عاليا على جذور 1-دايفينيل-2 بيكريلهايدرازيل وكان لها قوة مضادة للأكسدة(AOX) في إرجاع الحديديك، ولكن لم تكن فعالة في تثبيط إنزيم ألفا-جلوكوسيديس(AGI) وإنزيم زانتين أوكسيديس. ولذلك تم تقييم النشاط المضاد لداء السكري لهذا المستخلص باستخدام الجرذان المصابة بالسكري بالتسمين وبمركب ستريتوزوتوسين. أظهرت النتائج أن إعطاء المستخلص بجرعة 300 مج/كج من وزن الجسم 4 أسابيع خفض مستويات السكر في الدم بشكل ملحوظ ($P<0.05$) وأعاد مستوى الدهون في دماء الجرذان إلى مستواها الطبيعي. لوحظ نفس الأثر مع عقار الميتفورمين والأتوفاستاتن.. أثبتت أن المستخلص حسن الطاقة، والأحماض الأمينية، والبيورين، والكرياتينين، والمرارة، وأيض مكروبات القناة الهضمية. بالإضافة إلى أنه أصلح أيض الطاقة من خلال تحسين خلايا T3-L13 للجلوكوز ومن خلال تثبيط إنزيم DPP-IV ولكن من خلال (AGI) تم إثبات أن لدى هذا المستخلص نشاطا (AOX) والذي يرتبط إيجابيا بالنشاط المضاد لداء السكري. تستنتج هذه الدراسة أن مستخلصات ثمرة المومرديكا تشارانتينا تعتبر علاجا واعدا لمرض السكري.

APPROVAL PAGE

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DEDICATION

To my beloved father Mr. Perumal, my dearest mother Late Mrs. Meenachee and my loving family

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LIST OF ABBREVIATIONS

^{1}H NMR	Proton Nuclear Magnetic Resonance Spectroscopy
A	Absorbance
AAE	Ascorbic Acid Equivalent
ACUC	Animal Care and Use Committee
AGEs	Advanced Glycated End Products
AI	Atherogenic Index
AMD	Aminopyrine-N-Demethylase
AMPK	Adenosine-5-Monophosphate Kinase
ANH	Aniline Hydroxylase
BMI	Body Mass Index
BW	Body Weight
cAMP	Cyclic Adenosine Monophosphate
CDO	Cysteine Dioxygenase
CMC	Carboxymethylcellulose
CPMG	Carr-Purcell-Meiboom-Gill
CSAD	Cysteine Sulfinic Acid Dehydrogenase
DM	Diabetes Mellitus
DMEM	Dulbecco's Modified Eagle Media
DMSO	Dimethyl Sulfoxide
DPP(IV)	Dipeptidyl Peptidase 4
DPPH	1,1-Diphenyl-2-Picrylhydrazine
ESI	Electrospray Ionization
FBS	Fetal Bovine Serum
FPG	Fasting Plasma Glucose
FRAP	Ferric Reducing Antioxidant Power
FTIR-ATR	Fourier Transform Infra-Red-Attenuated Total Reflectance
G6PD	Glucose-6-Phopshate –Dehydrogenase
GAD65	Glutamate Decarboxylase 65
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GCMS	Gas Chromatography Mass Spectrometry
GDM	Gestational Diabetes Mellitus
GIP	Gastric Inhibition Polypeptide
GLP 1	Glucagon-Like Peptide 1
GPC	Glycerophosphocholine
GPR91	G protein–coupled receptor-91
GST	Glutathione S-Transferase
HbA1C	Haemoglobin A1C
HDL	High Density Lipoprotein
HFD	High Fat Diet
IA	Islet Autoimmunity
ICRACU	Integrated Centre for Research Animal Care and Use
ID	Inner Diameter
JOD	Juvenile Onset Diabetes
LDL	Low Density Lipoprotein
LOD	Limit of Detection

LOQ	Limit of Quantification
MSTFA	N-Methyl-N- (Trimethylsilyl) Trifluoroacetamide
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
MVDA	Multivariate Data Analysis
NDA^+	Nicotinamide Adenine Dinucleotide
NFB	Nuclear Factor Beta
NIDDM	Non-Insulin Dependent Diabetes Mellitus
NIST	National Institute of Standards Technology
NMR	Nuclear Magnetic Resonance Spectroscopy
OB-DB	Obese-Diabetic
OGTT	Oral Glucose Tolerance Test
OPLS	Orthogonal Partial Least Square
PAG	N-Phenylcetylglycine
PBS	Phosphate Buffer Saline
PLE	Pressurized Liquid Extraction
PLS-DA	Partial Least Square Discriminant Analysis
PNPG	*p*-Nitrophenyl-α-D-Gluucopyronase
PPHG	Post Prandial Hyper Glycemia
PW	Pulse Width
RAPD	Random Amplified Polymorphic DNA
RAGEs	Receptor Advanced Glycated End Products
RAS	Renin-Angiotensin System
RD	Relaxation Delay
RIN	Rat Insulinoma
SD	Standard Deviation
SD	Standard Deviation
SEM	Standard Error Mean
STZ	Animal Care and Use Committee
STZ	Streptozotocin
STZ-OBDB	Streptozotocin-Obese Diabetic
T2	Transverse Relaxation Time
TAGE	Toxic Advanced Glycated End Products
TCA	Tricarboxylic Acid
TIC	Total Ion Chromatogram
TPTZ	2,4,6-tris (2-Pyridyl)-s-Triazine
TSP	Trimethylsilyl Propionic Acid
UCP	Uncoupling Protein
UV	Ultraviolet
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Plant-based traditional medicine always stands as a golden mark to exemplify the outstanding phenomenon of symbiosis. Natural products from the medicinal plants have been the popular alternative for the treatment of various diseases and primary health care. Verma et al. (2008) stated that 80% of people in developing countries is still dependent on traditional herbal medicine derived from plants and animals for their primary and secondary health issues. Herbal medicines have been practiced since antiquity by traditional medicine practitioners. It has been continuing in practice until today because of its cultural beliefs in many parts of the world and promising biomedical properties. Furthermore, owing to rising medical treatment cost as well as the emergence of new diseases, prevailing focus on an idea of evidence-based medicine in recent decades have been greatly addressed (Narins, 2000). According to Kim et al. (2015), it is forecast that, by the year 2050, the total global herbal drug market will increase to US$ 5trillion.

Momordica charantia is a popular medicinal plant of the Cucurbitaceae family that is widely available in local market. It is known as ‘peria katak’ in Malay and bitter melon or bitter gourd in English (Premila and Lisa, 2007). It also has been reported to possess antidiabetic, antimicrobial, antioxidant, antiviral and antitumor activities (Grover and Yadav, 2004; Wu and Ng, 2008). Numerous studies have been reported on antidiabetic properties of *M. charantia* fruit extract using various rat models since 1950’s (Raman and Lau, 1996; Basch et al., 2003; Ojewole et al.,

2006). However, previous studies only analyzed some targeted biomarkers such as glucose and insulin to evaluate the efficacy of this fruit. It causes a bias interpretation of the results since the metabolism in diabetes is very complex. Our knowledge is still limited to reveal all metabolite alterations responsible to cause diabetes. Thus, the best approach to evaluate the efficacy of the sample is to analyze all possible metabolites in biofluids using a new holistic approach known as metabolomics. Metabolites profile of an organism reflects significant information about its physiological status. Another advantage of using this approach is the possibility to identify new biomarkers causing a particular disease, and to reveal the mode of action of herbs in the treatment of this disease (Gabrielsson et al., 2006). Some examples of this specific work are the application of NMR-based metabolomics to evaluate antidiabetic activity of natural *Centella asiatica* (Maulidiani et al., 2016), *Phyllanthus niruri* (Mediani et al., 2016), *Andrographis paniculate* (Akthar et al., 2016), and green tea (Zhang et al., 2013).

In recent years, the major obstacle faced by the herbal industries is overlooked findings of many possibly bioactive natural compounds during drug discoveries based on bioassay guided fractionation approach. It is because of the fact that the single bioactive agent found to be present in low abundance in natural sources and biological effect could arise due to a synergistic action of multiple bioactive ingredients in a single source or from a multiple source in a particular formulation (Williamson, 2001). Currently, metabolomics approach has been practiced to overcome the bottlenecks in the identification of bioactive compounds in medicinal herbs (Wang et al., 2006). It can help to rationalize the therapeutic superiority of many plant extracts over single isolated constituent. It identifies and quantifies multiple targets in order to obtain an overview of all compound classes

and brings an important insight into the natural product by linking putative bioactivity with some compounds in a targeted plant (Fiehn, 2002; Newman and Cragg 2007).

Identification of antioxidants in *M. charantia* fruit is important since these compounds correlate with antidiabetic activity through suppression of glycation of proteins, inactivation of enzymes, and alteration in structural functions of collagen basement membrane of pancreatic β-cell (Nirmala et al., 2011). Some antioxidants have been identified in this fruit, but there is a complete lack of information from the existing literature on the correlation of these compounds with an antidiabetic activity. Moreover, the antioxidant activity of the fruit crude extract does not confirm precisely about the total number of compounds responsible for an antioxidant effect of the fruit crude extract since all the existing studies had been based on the antioxidant activities of the different individual compounds. It is a well-known fact that the bioactivity of an individual compound may differ when these compounds are within the complex matrix of the plant material. For example, it is quite common that the antioxidant activity of a sample can disappear when it is fractionated, phenomenon attributed to possible synergistic effects with other components of the sample (Kuhlisch et al., 2015). Thus, the reported antioxidant compounds may not be solely or individually responsible for the antioxidant activity of the whole fruit. One way of detecting all the compounds related to antioxidant activity is to use metabolomics approach (Verpoorte et al., 2009).

The analytical methods that are used in metabolomics are varied. No single analytical instrument is available up to date to detect all range of compounds with high sensitivity and resolution. Thus, the use of different analytical instruments is highly recommended to detect as much as possible bioactive compounds in a sample.