Identification, Bioassay-guided Isolation & Pharmacological Properties of an Antidiabetic Active Compound(s) from Traditional Medicinal Plants

By

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Declaration

I certify that the substance of this thesis does not contain any work that has been previously published or written, except where due reference is made in the text. It has not already been submitted for any degree and is not being currently submitted for any other degree.

I certify, to the best of my knowledge, any help received in preparing this project and any sources used, have been acknowledged in this report.

Sureshkumar Mohankumar

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List of Contents

Publications arising from this Thesis

Research Papers

Mohankumar. S., McFarlane. JR., *An Aqueous extract of Curcuma longa (Turmeric) rhizomes stimulates insulin release and mimics insulin action on tissues involved in glucose homeostasis* in vitro., (submitted to Phytotherapy Research)

Mohankumar. S., McFarlane. JR., *Effect of aqueous extracts of some Ayurvedic medicinal plants on cellular glucose metabolism and insulin secretion* in vitro. (submitted to Journal of Ethnopharmacology)

Conference Abstracts

Mohankumar. S., McFarlane. JR., 2006, *Effect of an Australian native plant, Emu bush extracts on cellular glucose metabolism under basal and hyperglycemic conditions* in vitro 19th World Diabetes Congress, Cape Town, South Africa.

Mohankumar. S., McFarlane. JR., 2006, *A Bioassay Guided Evaluation of Some Ayurvedic Medicinal Plants*. ADS & ADEA, Annual scientific meeting, Gold Coast, Australia.

Mohankumar. S., McFarlane. JR., 2006, *Effect of Aqueous extract of turmeric on pancreas function under basal and hyperglycemic conditions* in vitro. SBB&MS Postgraduate Conference, University of New England, Armidale, Australia.

Mohankumar. S., McFarlane. JR., 2005, *Turmeric stimulates insulin secretion from the mouse pancreas* in vitro. ADS & ADEA, Annual scientific meeting, Perth, Australia.

Annotation

Standard chemical symbols and SI units are used without definition

ADP	Adenosine diphosphate
ACTH	Adreno corticotropic hormone
AICR	5-amino imidazole-4-carboxamide-1-b-D-ribofuranoside
AMPK	Adenosine monophosphate-activated protein kinase
ANNOVA	Analysis of variance
ANP	Atrial natriuritic peptic
ATP	Adenosine triphosphate
ATPase	Adenosine monophosphatase
AUC	Area under curve
BA	Butyl alcohol
cAMP	Cyclic adenosine monophosphate
CL	*Curcuma longa*
CLE	*Curcuma longa* Extract
coA	Coenzyme A
CON A	Concanavalin A
CPM	Counts per minute
CRF	Corticotrophin releasing factor
CSHS	Charcoal striped horse serum
CV	Cardio vascular
DBI	Diazepam-binding inhibitor
DMEM	Dulbecco's modified Eagle's medium
DPP-4	Dipeptidyl peptidase-4
EA	Ethyl acetate
ELISA	Enzyme-linked immunosorbent assay
FFA	Free fatty acids
GAD	Antibodies against glutamic acid
GHRH	Growth hormone releasing hormone
GIP	Glucose-dependent insulinotropic peptide
GLP	Glucagon-like peptide
GLUT	Facilitative glucose transporter
GS	*Gymnema sylvestre*
GS_3 and $_4$	Gymnemic acids 3 and 4
GSE	*Gymnema sylvestre* Extract
GTT	Glucose tolerance test

HbA1c	Glycosylated hemoglobin
HDL	High density lipoprotein
IA-2	Insulinoma associated autoantigen-2
IAA	Insulin autoantibodies
IAPP	Islet Amyloid polypeptide
ICA	Islet cell antibodies
IDDM	Insulin-dependent diabetes mellitus
IL-6	Interleukin-6
IR	Insulin receptor
IRS	Insulin receptor substrate
IV	Intravenous
IVGTT	Intravenous glucose tolerance test
kDa	Kilodalton
LDL	Low density lipoprotein
NEFA	Non-esterified free fatty acid
NHMRC	National health and medical research council
NIDDM	Non-insulin-dependent diabetes mellitus
ºC	Degree celsius
PBS	Phosphate buffer saline
PI3-kinase	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PM	*Pterocarpus marsupium*
PME	*Pterocarpus marsupium* Extract
PPAR-γ	Peroxisome proliferators-activated receptor-gamma
PVPP	Insoluble polyvinylpolypyrrolidone
PYY	Peptide YY
RIA	Radioimmuno assay
RPM	Rotation per minute
SDS-PAGE	Sodium dodecyl sulphate-poly acrylamide gel electrophoresis
SGLT	Sodium dependent glucose transporter
SREBP	Sterol-regulatory-element-binding protein
STD	Standard
STZ	Streptozotocin
SUR	Sulphonylurea receptor
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TFG	*Trigonella foenum-graecum*
TFGE	*Trigonella foenum-graecum* Extract
TMB	3,3',5,5' tetramethylbenzidine

TNF- α	Tumor necrosis factor- alpha
TRH	Thyrotropin-releasing hormone
UGDP	University group diabetes project
UKPDS	United Kingdom prospective diabetes society
VLDL	Very low density lipoprotein
WHO	World health organization
α-cells	Alpha-cells
β-cells	Beta-cells

List of Figures

List of Tables

Abstract

Recent trends in diabetes treatments show an increasing interest in traditional systems of medicine. Ayurveda, a traditional Indian system medicine, advocates a wide range of medicinal plants to treat diabetes. Although there have been numerous studies on extracts from these medicinal plants that demonstrate antidiabetic activity, scientific studies directed to the isolation, purification and identification of active ingredients responsible for the hypoglycemic activity and also the modes of action of these extracts/active ingredient(s) on glucose homeostasis have been often inconclusive or lacking except for a few cases. The aim of this present study was to identify and isolate a potent antidiabetic compound(s) from some extensively advocated Ayurvedic antidiabetic plants such as *Trigonella foenum-graecum* Linn (TFG), *Pterocarpus marsupium* Roxb (PM), *Gymnema sylvestre* R.Br (GS) and *Curcuma longa* Linn (CL).

An in-house developed *in vitro* tissue culture-based bioassay method was employed in the present study to determine the effects of plant extracts on insulin secretion from mouse pancreas tissues and on glucose uptake by mouse skeletal muscle tissues under both normoglycemic (5mM glucose) and hyperglycemic (12mM glucose) culture conditions. The results from our preliminary study indicated that all these plant extracts have beneficial effects on glucose homeostasis either by stimulating insulin or enhancing glucose uptake or activating both. In terms of their comparative effects on tissues that regulate glucose metabolism, the aqueous extracts of plants, PM and CL, were found to

be more potent when compared with other studied aqueous extracts of plants TFG and and GS, within culture conditions.

The potential dose dependent effects of both *Curcuma longa* extract (CLE) and *Pterocarpus marsupium* extract (PME) on insulin secretion only at hyperglycemic culture condition but not in normoglycemic conditions revealed that these treatments do not provoke hypoglycemia under basal physiological condition and also indicated that these treatments may have β-cell metabolism augmenting or protecting or regenerating active constituents. Similarly, CLE and PME treatments in the absence or presence of insulin on mouse skeletal muscle tissues *in vitro* showed a dose-dependent glucose uptake activity. In contrast to CLE, the lack of significant potentiation of glucose uptake activity of PME in the presence of insulin (saturating dose) and inhibition of activity in the presence of wortmannin revealed that the constituents of PME may have insulin like or mimicking active constituents. These results prompted us to identify the hypoglycemic constituent(s) of PME.

A bioassay-guided purification was performed on the PME in order to isolate the bioactive compound(s) responsible for antidiabetic activity. The preliminary results indicated that: 1) Boiling destroyed both glucose uptake and insulin secretion activity of PME. 2) It remains potent even after ethyl acetate and butyl alcohol extraction. 3) The potent activity was evident in the molecular weight ranges between the >10 kDa and <30 kDa. 4) Trypsin digestion destroyed the activity of PME.

Bioassay-guided purification yielded a potent antidiabetic fraction, #SK/PME/07. Electrophoresis of non-reduced #SK/PME/07 on SDS-PA gels showed two major fuzzy protein bands at ≤27kDa and ≤20kDa. Gels stained to detect the presence of glycoprotein showed positive and the poor resolution and fuzzy appearance of bands, suggested that these proteins are heavily glycosylated. In addition, #SK/PME/07 showed a negative test for peroxidase activity, which indicated that #SK/PME/07, has no peroxidase molecules. The results from our Con A chromatography of #SK/PME/07 indicated that the glycoproteins of #SK/PME/07 have high affinity towards Con A. However, the appearances of cross linkage of #SK/PME/07 with Con A proteins resulted in abandonment of this method of purification. In a further experiment, isoelectric focusing (ISF) of #SK/PME/07 indicated that #SK/PME/07 may have two or more negatively charged isoforms of glycosylated protein(s). Absence of significant potentiation of activity of #SK/PME/07 after treating with an insoluble polyvinylpolypyrrolidone (PVPP) suggested that #SK/PME/07 either contains no tannins or absence of their association with the active protein molecules of #SK/PME/07. The precipitate that was obtained after chloroform-methanol precipitation of #SK/PME/07 showed a fuzzy protein band at 27kDa. Interestingly, the effect of this PPT on insulin secretion and glucose uptake was more potent than the #SK/PME/07, which supports our previous findings that the hypoglycemic constituent(s) of #SK/PME/07 is/are proteins.

An *In vitro* study to elucidate the pharmacological mechanisms underlying the insulin-releasing and sensitizing effects of #SK/PME/07 revealed that #SK/PME/07 exhibited insulin-releasing effects, partially by mimicking the effects of sulphonylureas on insulin

secretary pathway and partially having additional actions on either β-cell nutrient metabolism or second-messenger pathways or insulin and proinsulin biosynthesis and stimulates glucose uptake activity, by dominantly mimicking insulin action on PI3-kinase pathway coupled with additional beneficial effects on other secondary pathways.

Interestingly, insulinotropic and insulin sensitizing effect of #SK/PME/07 *in vitro* is maintained *in vivo* and accounts for the improvement of glucose tolerance. Three daily intravenous administrations of #SK/PME/07 had prolonged effects on insulin secretion as well as on glucose clearance in non-diabetic normal sheep,using both young (2 years old) and old (5-6 years old), pointing out that #SK/PME/07 may be considered as a novel potential antidiabetic drug.

In many ways, use of #SK/PME/07 to combat the adverse effects of hyperglycemia appears to be beneficial by enhancing the target area directly associated with the normal glucose regulatory processes. The results provide evidence that the principal effects are prolonged by many hours to days, unlike the numerous currently available antidiabetic drugs which over stimulate the β-cells or pose a risk of hypoglycemia.