

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

By

**SureshKumar Mohankumar
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Doctor of Philosophy
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University of New England**

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

| | |
|---|-------------|
| DECLARATION..... | I |
| ACKNOWLEDGEMENTS | III |
| LIST OF CONTENTS | IV |
| PUBLICATIONS ARISING FROM THIS THESIS..... | VIII |
| ANNOTATION..... | IX |
| LIST OF FIGURES | XII |
| LIST OF TABLES..... | XIX |
| ABSTRACT | XX |
| 1 GENERAL INTRODUCTION..... | 1 |
| 1.1 Introduction..... | 2 |
| 1.2 Glucose Homeostasis..... | 5 |
| 1.2.1 Glucose transport..... | 5 |
| 1.2.1.1 SGLT | 6 |
| 1.2.1.2 GLUT..... | 6 |
| 1.2.2 Glucose regulation..... | 8 |
| 1.2.2.1 Insulin | 11 |
| 1.2.2.2 Amylin | 18 |
| 1.2.2.3 Glucagon..... | 18 |
| 1.2.2.4 Incretin hormones | 19 |
| 1.2.3 Endogenous glucose production..... | 21 |
| 1.2.4 Hyperglycemia | 22 |
| 1.3 Diabetes mellitus..... | 23 |
| 1.3.1 Pathophysiology | 25 |
| 1.3.1.1 Insulin resistance..... | 26 |
| 1.3.1.2 β -cell dysfunction | 29 |
| 1.3.2 Complications..... | 30 |
| 1.3.3 Treatments..... | 31 |
| 1.3.3.1 Sulfonyl and non-sulfonylureas | 31 |
| 1.3.3.2 Biguanide..... | 34 |
| 1.3.3.3 Peroxisome proliferators-activated receptor-gamma (PPAR γ) activators (Thiazolidinediones) | 36 |
| 1.3.3.4 Alpha-glucosidase inhibitors | 38 |
| 1.3.4 Limitations | 38 |

| | | |
|------------|---|-----------|
| 1.4 | Traditional antidiabetic plants..... | 39 |
| 1.4.1 | <i>Gymnema sylvestre</i> R.Br (GS)..... | 40 |
| 1.4.2 | <i>Trigonella foenum-graecum</i> Linn (TFG)..... | 44 |
| 1.4.3 | <i>Curcuma longa</i> Linn. (CL)..... | 50 |
| 1.4.4 | <i>Pterocarpus marsupium</i> Roxb. (PM)..... | 53 |
| 1.5 | Summary..... | 60 |
| 1.6 | Aim | 62 |
| 2 | GENERAL METHODS AND MATERIALS..... | 63 |
| 2.1 | Chemicals and reagents | 64 |
| 2.2 | Plant materials and extract preparation | 64 |
| 2.3 | Animal ethics | 65 |
| 2.4 | <i>In vitro</i> bioassay | 65 |
| 2.4.1 | Animals and tissue preparation..... | 65 |
| 2.4.2 | Tissue culture assay..... | 66 |
| 2.5 | <i>In vivo</i> bioassay..... | 67 |
| 2.5.1 | Animals and Plasma sample collection | 67 |
| 2.6 | Glucose analysis..... | 67 |
| 2.7 | Insulin (Radioimmunoassay)..... | 68 |
| 2.7.1 | Culture media sample..... | 68 |
| 2.7.2 | Plasma sample | 69 |
| 2.8 | SDS-PAGE..... | 69 |
| 2.8.1 | Protein detection..... | 70 |
| 2.8.2 | Glycoprotein detection | 70 |
| 2.9 | Data analysis..... | 70 |
| 3 | PRELIMINARY <i>IN VITRO</i> SCREENING | 71 |
| 3.1 | Introduction..... | 72 |
| 3.2 | Materials and methods | 74 |
| 3.2.1 | General methods..... | 74 |
| 3.2.2 | Effect of plant extract on muscle glucose uptake and pancreas insulin secretion | 74 |
| 3.3 | Results | 75 |
| 3.3.1 | Glucose uptake by mouse muscle tissues <i>in vitro</i> | 75 |
| 3.3.2 | Insulin secretion from mouse pancreas tissues <i>in vitro</i> | 76 |
| 3.4 | Discussion..... | 78 |
| 4 | EFFECTS OF CLE AND PME ON TISSUES INVOLVED IN GLUCOSE HOMEOSTASIS | 81 |

| | | |
|------------|--|------------|
| 4.1 | Introduction | 82 |
| 4.2 | Material and methods | 84 |
| 4.2.1 | General methods | 84 |
| 4.2.2 | Insulin secretion <i>in vitro</i> | 84 |
| 4.2.3 | Muscle glucose uptake <i>in vitro</i> | 84 |
| 4.3 | Results | 85 |
| 4.3.1 | Insulin secretion <i>in vitro</i> | 85 |
| 4.3.1.1 | Effect of CLE on insulin secretion | 85 |
| 4.3.1.2 | Effect of PME on insulin secretion | 88 |
| 4.3.2 | Muscle glucose uptake <i>in vitro</i> | 89 |
| 4.3.2.1 | Effect of CLE on muscle glucose uptake | 89 |
| 4.3.2.2 | Effect of PME on muscle glucose uptake | 92 |
| 4.4 | Discussion | 95 |
| 5 | BIOASSAY-GUIDED PURIFICATION OF PME | 100 |
| 5.1 | Introduction | 101 |
| 5.2 | Material and methods | 104 |
| 5.2.1 | General methods | 104 |
| 5.2.2 | Data analysis | 104 |
| 5.2.3 | Preliminary purification studies | 105 |
| 5.2.3.1 | Aqueous extraction | 105 |
| 5.2.3.2 | Organic solvent extraction | 105 |
| 5.2.3.3 | Ultrafiltration | 106 |
| 5.2.3.4 | Sephadex G-25 column chromatography | 108 |
| 5.2.3.5 | Trypsin digestion | 109 |
| 5.2.3.6 | Ion exchange chromatography | 110 |
| 5.2.4 | Bioassay-guided purification of PME | 111 |
| 5.2.5 | Molecular weight determination | 113 |
| 5.2.6 | Peroxidase activity | 113 |
| 5.2.7 | Purification of #SK/PME/07 | 114 |
| 5.2.7.1 | Affinity Chromatography | 114 |
| 5.2.7.2 | PVPP treatment | 114 |
| 5.2.7.3 | Isoelectric focusing | 115 |
| 5.2.7.4 | Chloroform –Methanol precipitation | 115 |
| 5.2.8 | Dose response curve | 116 |
| 5.2.9 | N-terminal Sequencing | 116 |
| 5.3 | Results | 117 |
| 5.3.1 | Preliminary purification studies | 117 |
| 5.3.1.1 | Aqueous extraction | 117 |
| 5.3.1.2 | Organic solvent extraction | 118 |
| 5.3.1.3 | Ultrafiltration | 119 |
| 5.3.1.4 | Sephadex G-25 column chromatography | 121 |
| 5.3.1.5 | Trypsin digestion | 122 |
| 5.3.1.6 | Ion exchange chromatography | 123 |
| 5.3.2 | Bioassay-guided purification of PME | 125 |
| 5.3.3 | Molecular weight determination | 127 |
| 5.3.4 | Peroxidase activity | 128 |
| 5.3.5 | Purification of #SK/PME/07 | 129 |
| 5.3.5.1 | Affinity Chromatography | 129 |

| | | |
|------------|---|------------|
| 5.3.5.2 | PVPP treatment..... | 130 |
| 5.3.5.3 | Isoelectric focusing..... | 131 |
| 5.3.5.4 | Chloroform-Methanol precipitation..... | 132 |
| 5.3.6 | Dose response curve..... | 134 |
| 5.3.7 | N-terminal sequencing..... | 136 |
| 5.4 | Discussion..... | 136 |
| 6 | ANTIDIABETIC PROPERTIES OF #SK/PME/07..... | 143 |
| 6.1 | Introduction..... | 144 |
| 6.2 | Material and methods..... | 146 |
| 6.2.1 | General methods..... | 146 |
| 6.2.2 | <i>In vitro</i> studies..... | 146 |
| 6.2.2.1 | Insulin secretion in vitro..... | 146 |
| 6.2.2.2 | Glucose uptake in vitro..... | 147 |
| 6.2.2.3 | In vitro data analysis..... | 148 |
| 6.2.3 | <i>In vivo</i> studies..... | 149 |
| 6.2.3.1 | Experiment 1..... | 149 |
| 6.2.3.2 | Experiment 2..... | 150 |
| 6.2.3.3 | Experiment 3..... | 151 |
| 6.2.3.4 | In vivo data analysis..... | 152 |
| 6.3 | Results..... | 153 |
| 6.3.1 | <i>In vitro</i> studies..... | 153 |
| 6.3.1.1 | Insulin secretion in vitro..... | 153 |
| 6.3.1.2 | Glucose uptake in vitro..... | 159 |
| 6.3.2 | <i>In vivo</i> studies..... | 163 |
| 6.3.2.1 | Experiment 1..... | 163 |
| 6.3.2.2 | Experiment 2..... | 166 |
| 6.3.2.3 | Experiment 3..... | 171 |
| 6.4 | Discussion..... | 178 |
| 6.4.1 | <i>In vitro</i> studies..... | 178 |
| 6.4.2 | <i>In vivo</i> studies..... | 185 |
| 7 | GENERAL DISCUSSION..... | 190 |
| | REFERENCES..... | 204 |
| | APPENDIX..... | 244 |

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 | <i>Curcuma longa</i> |
| CLE | <i>Curcuma longa</i> 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 | <i>Gymnema sylvestre</i> |
| GS₃ and ₄ | Gymnemic acids 3 and 4 |
| GSE | <i>Gymnema sylvestre</i> 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 |
| °C | Degree celsius |
| PBS | Phosphate buffer saline |
| PI3-kinase | Phosphatidylinositol 3-kinase |
| PKA | Protein kinase A |
| PKB | Protein kinase B |
| PKC | Protein kinase C |
| PM | <i>Pterocarpus marsupium</i> |
| PME | <i>Pterocarpus marsupium</i> Extract |
| PPAR-γ | Peroxisome proliferators-activated receptor-gamma |
| PVPP | Insoluble polyvinylpyrrolidone |
| 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 | <i>Trigonella foenum-graecum</i> |
| TFGE | <i>Trigonella foenum-graecum</i> 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

| | |
|---|----|
| Figure 1-1 Glucose homeostasis: roles of insulin, glucagon, amylin, and GLP-1..... | 9 |
| Figure 1-2 Cross section of the pancreas | 10 |
| Figure 1-3 Metabolic hypothesis of glucose-stimulated insulin release from β -cells..... | 13 |
| Figure 1-4 Effect of insulin on glucose uptake and metabolism..... | 15 |
| Figure 1-5 Molecular mechanism of insulin-stimulated glucose transport..... | 16 |
| Figure 1-6 Overview of the pathogenesis of type 2 diabetes mellitus | 25 |
| Figure 1-7 The site of action of sulfonylurea and non-sulfonylurea insulin secretagogues. | 32 |
| Figure 1-8 Metformin activates AMPK in liver and muscle to improve glucose and lipid metabolism in type 2 diabetes..... | 35 |
| Figure 1-9 Mechanism of action of thiazolidinediones | 37 |
| Figure 1-10 <i>Gymnema sylvestre</i> R. Br..... | 41 |
| Figure 1-11 <i>Trigonella foenum-graecum</i> Linn (TFG) Seeds | 45 |
| Figure 1-12 <i>Curcuma longa</i> Linn. (CL) rhizomes | 50 |
| Figure 1-13 <i>Pterocarpus marsupium</i> Roxb (PM) heartwood. | 53 |
| Figure 3-1 Comparative effect of plant treatments on glucose uptake (\blacktriangle) and on insulin secretion (\bullet) in folds with respect to control under hyperglycemic culture condition <i>in vitro</i> | 77 |

| | |
|--|------------|
| Figure 4-1 Effect of CLE (0-100 μ l) on insulin secretion from mouse pancreatic tissues at hyperglycemic (12 mM glucose) culture condition over 30 minute (Acute) incubation <i>in vitro</i> | 86 |
| Figure 4-2 Effect of CLE (0-100 μ l) on insulin secretion from mouse pancreatic tissues over chronic incubation (24 hours) <i>in vitro</i> | 87 |
| Figure 4-3 Effect of PME on insulin secretion from mouse pancreas tissues over chronic incubation (24 hours) <i>in vitro</i> | 89 |
| Figure 4-4 Effect of CLE (0-10 μ l) on <i>in vitro</i> glucose uptake by mouse abdomen muscle tissues under hyperglycemic culture condition over chronic incubation (24 hours) | 90 |
| Figure 4-5 Effect of CLE (5 μ l), insulin (100 nM), wortmannin (100 nM), CLE combined with wortmannin and insulin with wortmannin on <i>in vitro</i> glucose uptake by mouse abdomen muscle tissues under hyperglycemic culture condition over chronic incubation (24 hours)..... | 91 |
| Figure 4-6 Effect of PME (0-100 μ l) on glucose uptake by abdomen muscle tissues under hyperglycemic culture condition over chronic incubation (24 hours) <i>in vitro</i> | 92 |
| Figure 4-7 Effect PME (10 μ l), insulin (100 nM), wortmannin (100 nM), PME combined with wortmannin and insulin with wortmannin on <i>in vitro</i> glucose uptake by mouse abdomen muscle tissues under hyperglycemic culture condition over chronic incubation (24 hours) | 94 |
| Figure 5-1 Organic solvent extraction of FPME with ethyl acetate and butyl alcohol.. | 106 |
| Figure 5-2 Ultrafiltration of FPME through 10 and 30k molecular mass cut-off membrane..... | 107 |
| Figure 5-3 Sephadex G-25 column chromatography fractionation of FPME..... | 109 |

| | |
|---|------------|
| Figure 5-4 Anion exchange chromatography fractionation of FPME | 111 |
| Figure 5-5 Bioassay-guided fractionation of PME | 113 |
| Figure 5-6 Effect of PME and BPME on A- glucose uptake by mouse muscle tissues and B- insulin secretion from mouse pancreatic tissues over chronic incubation under hyperglycemic culture conditions. | 117 |
| Figure 5-7 Effect of PME, EA, Aq.EA, BA and Aq.BA fractions on A- glucose uptake by mouse muscle tissues and B- insulin secretion from mouse pancreatic tissues over chronic incubation under hyperglycemic culture conditions. | 119 |
| Figure 5-8 Effect of 10KR, 10KE, 30KE and 30 KR fractions on A- glucose uptake by mouse muscle tissues and B- insulin secretion from mouse pancreatic tissues over chronic incubation under hyperglycemic culture conditions. | 120 |
| Figure 5-9 Sephadex G-25 fractionation of PME; Sx1, Sx2, ...Sx10 fractions on A- glucose uptake by mouse muscle tissues and B- insulin secretion from mouse pancreatic tissues over chronic incubation under hyperglycemic culture conditions. | 122 |
| Figure 5-10 Trypsin digestion of PME; +Trypsin and -Trypsin samples on A- glucose uptake by mouse muscle tissues and B- insulin secretion from mouse pancreatic tissues over chronic incubation under hyperglycemic culture conditions. | 123 |
| Figure 5-11 Anion exchange chromatography of PME; 0M, 0.25M, 0.5M, 0.75 M and 1M samples on A- glucose uptake by mouse muscle tissues and B- insulin secretion from mouse pancreatic tissues over chronic incubation under hyperglycemic culture conditions. | 124 |

| | |
|---|------------|
| Figure 5-12 Bioassay-guided fractionation of PME; QSE, QSR, QSR/10KE, QSR/10KR and #SK/PME/07 fractions on A- glucose uptake by mouse muscle tissues and B- insulin secretion from mouse pancreatic tissues over chronic incubation under hyperglycemic culture conditions. | 125 |
| Figure 5-13 Coomassie R250 stained SDS-PAGE of marker proteins (STD) and #SK/PME/07. The arrow indicates the two major protein bands of #SK.PME/07. | 127 |
| Figure 5-14 Glycoprotein stained SDS-PAGE of horseradish peroxidase and #SK/PME/07. | 128 |
| Figure 5-15 Purification of #SK/PME/07; PME, #SK/PME/07, CAB and CAUB fractions on A- glucose uptake by mouse muscle tissues and B- insulin secretion from mouse pancreatic tissues over chronic incubation under hyperglycemic culture conditions. | 130 |
| Figure 5-16 #SK/PME/07 and ISF fractions on A- glucose uptake by mouse muscle tissues and B- insulin secretion from mouse pancreatic tissues over chronic incubation under hyperglycemic culture conditions. | 131 |
| Figure 5-17 #SK/PME/07, PPT and SN fractions on A- glucose uptake by mouse muscle tissues and B- insulin secretion from mouse pancreatic tissues over chronic incubation under hyperglycemic culture conditions. | 133 |
| Figure 5-18 Coomassie R250 stained SDS-PAGE of #SK/PME/07 and PPT. The arrow indicates the major protein bands of #SK.PME/07 and PPT. | 134 |
| Figure 5-19 Dose response curve of PME and #SK/PME/07 A) on insulin secretion from mouse pancreas tissues and B) on glucose uptake by mouse skeletal muscle tissues over chronic incubation under hyperglycemic culture conditions. | 135 |

| | |
|--|------------|
| Figure 6-1 Effect of glucose (5 and 12 mM) on insulin secreting activity of #SK/PME/07 and tolbutamide (0.1 mM) from mouse pancreatic tissues <i>in vitro</i> | 154 |
| Figure 6-2 Effect of control (PBS), tolbutamide, diazoxide and tolbutamide + diazoxide (TOL+DIA) on insulin secretion from mouse pancreas tissues under hyperglycemic culture condition (12 mM glucose) in absence or presence of #SK/PME/07 | 155 |
| Figure 6-3 Effect of control (PBS), arginine and KCl on insulin secretion from mouse pancreas under hyperglycemic culture condition (12 mM glucose) in absence or presence of #SK/PME/07 | 156 |
| Figure 6-4 Effect of #SK/PME/07 on insulin secretion and synthesis by pancreatic tissues over 24 hours under hyperglycemic glucose (12 mM) and followed by 24 hours under basal glucose (5 mM) | 158 |
| Figure 6-5 Effects of glucose (5 and 12 mM) on glucose uptake activity of #SK/PME/07 and metformin (10 mM) by mouse muscle tissues <i>in vitro</i> | 159 |
| Figure 6-6 Effect of control (PBS), insulin (100 nM), metformin (10 mM), wortmannin (100 nM) insulin +wortmannin (INS+WOR), insulin + metformin (INS+MET) and metformin +wortmannin (MET+WOR) on glucose uptake by mouse skeletal muscle tissues incubated for 24 hours in hyperglycemic condition (12 mM glucose) in absence or presence of #SK/PME/07 | 161 |
| Figure 6-7 Effect of three daily intravenous administrations of PME (50 ml) and PPME (0.5 ml) on plasma glucose clearance in normal sheep. | 164 |
| Figure 6-8 Effect of three daily intravenous administrations of PME (50 ml) and PPME (0.5 ml) on plasma insulin in normal sheep | 165 |

Figure 6-9 Plasma glucose clearance after an IVGTT performed in saline and #SK/PME/07 (5 µl) treated young sheep at 2 yr of age. Mean (± S.E.) plasma glucose clearance in sheep on day 0 (A) when untreated, and on day 3, (B) day 21, (C) and day 25 (D) when treated..... **167**

Figure 6-10 Plasma glucose (mean ± S.E.) at 0, 60 and 120 minutes after an IVGTT of saline, and #SK/PME/07 treated groups (A). Mean (± S.E.) glucose AUC_(0-180 min) (min·mmol/L) in sheep, after an IVGTT performed before (day 0) and after (day 3, 21 and 25) treating with saline, and #SK/PME/07 (B). **168**

Figure 6-11 Plasma insulin levels after an IVGTT performed in saline and #SK/PME/07 (5 µl) treated young sheep (2 yr of age). Mean (± S.E.) plasma insulin in sheep on day 0 (A) when untreated, and on day 3, (B) day 21, (C) and day 25 (D) when treated..... **169**

Figure 6-12 Plasma insulin (mean ± S.E.) at 0, 15 and 30 minutes after an IVGTT of saline, and #SK/PME/07 treated groups (A). Mean (± S.E.) insulin AUC_(0-60 min) (min·mmol/L) in sheep, after an IVGTT performed before (day 0) and after (day 3, 21 and 25) treating with saline and #SK/PME/0. **170**

Figure 6-13 Plasma glucose clearance after an IVGTT performed in saline and #SK/PME/07 (5 and 15 µl) treated adult sheep (5-6 yr of age). Mean (± S.E.) plasma glucose clearance in sheep on day 0 (A) when untreated, and on day 3, (B) day 21, (C) and day 25 (D) when treated..... **172**

Figure 6-14 Plasma glucose (mean ± S.E.) at 0, 60 and 120 minutes after an IVGTT of saline, and #SK/PME/07 (5 and 15 µl) treated groups (A). Mean (± S.E.) glucose AUC_(0-180 min) (min·mmol/L) in sheep, after an IVGTT performed before (day 0) and

after (day 3, 21 and 25) treating with saline, and #SK/PME/07 (5 and 15 μ l) (B). Means indicated by an asterisk (*) are significantly different relative to the control group ($P < 0.05$) **174**

Figure 6-15 Plasma insulin levels after an IVGTT performed in saline and #SK/PME/07 (5 and 15 μ l) treated adult sheep (5-6 yr of age). Mean (\pm S.E.) plasma insulin in sheep on day 0 (A) when untreated, and on day 3, (B) day 21, (C) and day 25 (D) when treated. **175**

Figure 6-16 Plasma insulin (mean \pm S.E.) at 0, 15 and 30 minutes after an IVGTT of saline, and #SK/PME/07 (5 and 15 μ l) treated groups (A). Mean (\pm S.E.) insulin AUC_(0-60 min) (min.mmol/L) in sheep, after an IVGTT performed before (day 0) and after (day 3, 21 and 25) treating with saline and #SK/PME/07 (5 and 15 μ l). **176**

List of Tables

| | |
|---|-----------|
| Table 3-1 Effects of some Ayurvedic plant treatments on glucose uptake by mouse muscle tissues <i>in vitro</i> | 75 |
| Table 3-2 Effects of some Ayurvedic plant treatments on insulin secretion from mouse pancreas tissues <i>in vitro</i> | 76 |

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

Abstract

be more potent when compared with other studied aqueous extracts of plants TFG 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.

Abstract

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 ≤ 27 kDa and ≤ 20 kDa. 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 polyvinylpyrrolidone (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

Abstract

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, insulintropic 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.