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

By

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(Master of Pharmacy)

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A thesis submitted for the degree of Doctor of Philosophy of the 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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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)


Conference Abstracts


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


 Annotation

Standard chemical symbols and SI units are used without definition

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adreno corticotropic hormone</td>
</tr>
<tr>
<td>AICR</td>
<td>5-amino imidazole-4-carboxamide-1-b-D-ribofuranoside</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>ANNOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ANP</td>
<td>Atrial natriuritic peptic</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine monophosphatase</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under curve</td>
</tr>
<tr>
<td>BA</td>
<td>Butyl alcohol</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CL</td>
<td><em>Curcuma longa</em></td>
</tr>
<tr>
<td>CLE</td>
<td><em>Curcuma longa</em> Extract</td>
</tr>
<tr>
<td>coA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>CON A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts per minute</td>
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<tr>
<td>CRF</td>
<td>Corticotrophin releasing factor</td>
</tr>
<tr>
<td>CSHS</td>
<td>Charcoal striped horse serum</td>
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<tr>
<td>CV</td>
<td>Cardio vascular</td>
</tr>
<tr>
<td>DBI</td>
<td>Diazepam-binding inhibitor</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DPP-4</td>
<td>Dipeptidyl peptidase-4</td>
</tr>
<tr>
<td>EA</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acids</td>
</tr>
<tr>
<td>GAD</td>
<td>Antibodies against glutamic acid</td>
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<tr>
<td>GHRH</td>
<td>Growth hormone releasing hormone</td>
</tr>
<tr>
<td>GIP</td>
<td>Glucose-dependent insulinotropin peptide</td>
</tr>
<tr>
<td>GLP</td>
<td>Glucagon-like peptide</td>
</tr>
<tr>
<td>GLUT</td>
<td>Facilitative glucose transporter</td>
</tr>
<tr>
<td>GS</td>
<td><em>Gymnema sylvestre</em></td>
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<tr>
<td>GS3 and 4</td>
<td>Gymnemic acids 3 and 4</td>
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<td><em>Gymnema sylvestre</em> Extract</td>
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<tr>
<td>GTT</td>
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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  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
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor- alpha</td>
</tr>
<tr>
<td>TRH</td>
<td>Thyrotropin-releasing hormone</td>
</tr>
<tr>
<td>UGDP</td>
<td>University group diabetes project</td>
</tr>
<tr>
<td>UKPDS</td>
<td>United Kingdom prospective diabetes society</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
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<tr>
<td>α-cells</td>
<td>Alpha-cells</td>
</tr>
<tr>
<td>β-cells</td>
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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 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.