The Effects of Herbal extracts and compounds on the Glucose metabolism in HepG2 cells

By

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Abstract

The aim of this study is to investigate the effects of herbal substances on glucose metabolism in HepG2 cellular models, this project's main objective was to evaluate and compare the metabolic activities of HepG2 cells following treatment with herbal compounds and botanical extracts like phloretin, ouabain, berberine, metformin, rebaudioside-A, and *Stevia* extracts, goldenseal extracts, goat's rue extracts, and *Gymnema* extracts.

In the screening tests, changes in glucose uptake in response to treatment with extracts and individual compounds were recorded. Extracts of goldenseal (*Hydrastis candensis L*.) and the alkaloid berberine induced remarkable glucose uptake, and consumption, compared with the other compounds and extracts. And demonstrated significant glucose uptake ($p<0.05$, $p<0.01$) and consumption activity ($p<0.05$, $p<0.001$) when compared to the biguanide drug metformin ($p<0.01$). As a result, goldenseal ethanolic extracts (10µL/mL) and berberine (10µM) were identified as the key candidates of the research to investigate glucose uptake using flowcytometry, Glucose transporter-1, estimation of glycogen content and glucose release, and seahorse metabolic analysis.

In glucose uptake studies using flowcytometry, berberine augmented 3.4 times more glucose uptake, goldenseal stimulated 2 times, and metformin upsurges 1.8 times compared to the control ($p<0.001$). In the glucose transporter-1 expression assays, goldenseal, and berberine were augmented the expression 1.7 times and 1.4 times compared to the control ($p<0.05$). Berberine was upregulated 2.4 times, metformin twice, and goldenseal twice in glycogen synthesis studies after long-term treatment ($p<0.05$). In glucose release assays, berberine, metformin, and goldenseal releasing 50%, 30%, and 23% less glucose compared to the control ($p<0.05$). The Seahorse XF analysis, which is more in line with the literature, shows that the three treatments increase the rate of lactate production ($p<0.01$) while also improving glucose metabolism. When compared to the pure compound alkaloid berberine, goldenseal extract was found to be less effective in improving glucose metabolism. Nonetheless, both appear promising for the development of new oral anti-diabetic agents.

***Key words*:** Hypoglycaemic herbs, Goldenseal, 2-NBDG uptake, Glucose transporter-1, Seahorse XF analysis.

Declaration

I declare that, while registered as a candidate for this degree, I have not registered as a candidate for any other academic institution's award. Except where otherwise stated, the work contained in this thesis is based on my own research and has not been submitted for any other award at this or any other university.

Signed,

Kiran Kumar Kandunuri

(August 2023).

Dedication

This thesis is dedicated to my father Mr Mallikarjun Kandunuri and my mother Mrs Vijayalakshmi Kandunuri, for without their continuous support, boundless love, and encouragement this thesis would never have been completed.

Kiran Kumar Kandunuri

(August 2023).

Contents

Chapter 2: Materials and Methods

Chapter 3: Screening of the target herbal compounds and extracts

List of figures

List of tables

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Kiran Kumar Kandunuri

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Abbreviations

ACE: Angiotensin-converting enzyme

ADP: Adenosine Diphosphate

AGE: Agarose gel electrophoresis

AIM: Ancient Indian Medicine

Akt: Acute transforming retrovirus thymoma

AMPK: 5'-Adenosine monophosphate-activated protein kinase

ANOVA: Analysis of variance

ATP: Adenosine Triphosphate

BMI: Body Mass Index

BSA: Bovine serum albumin

cAMP: cyclic Adenosine Monophosphate

CRP: C-reactive protein

CVD: Cardiovascular Diseases

DKA: Diabetic ketoacidosis

DMEM: Dulbecco's Modified Eagle Medium

DPBS: Dulbecco's Phosphate-Buffered Saline

DTT: Dithiothritol

ECAR: extracellular acidification rate

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

ER: Endoplasmic Reticulum

ETC: electron transport chain

FBS: Fetal Bovine Serum

FCCP: Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone

FFAs: Free fatty acids

GAD65: Glutamic acid decarboxylase 65

GADA: Glutamic acid decarboxylase autoantibodies

GIP: Gastric inhibitory polypeptide/ Glucose-dependent insulinotropic polypeptide

GLP-1: Glucagon-like peptide-1

GLUT: Glucose transporter protein

GRP: Glucose regulated proteins

GSK-3: Glycogen Synthase kinase-3

GTT: Glucose tolerance test

HbA1c: Haemoglobin A1C

HDL: High Density Lipoprotein

HepG2: Human hepato cellular carcinoma cells

HGP: Hepatic glucose production

HHS: Hyperosmolar hyperglycaemic state

HILIC: Hydrophilic interaction liquid chromatography

HI-RPMI: Heat inoculated RPMI

HLA: Human Leucocyte Antigen

HMG-CoA: 3-hydroxy-3-methlglutaryl coenzyme A

HNF4: Hepatic nuclear factor 4 alpha

HPLC: High performance liquid chromatography

HRP: Horse Radish Peroxidase

HSD: Honest significant difference

IA-2A: Insulinoma-associated- protein 2 autoantibodies A

IA-2β: Insulinoma-associated-protein 2 autoantibodies β

IAA: Insulin autoantibodies

IAPP: Amyloid polypeptide intermediates

ICA: Islet cell cytoplasmic autoantibodies

IFNγ: Interferon gamma

IFT: Impaired fasting glucose

IGF: Insulin like growth factor

IGT: Impaired glucose tolerance

IR: Insulin receptor

IRS: Insulin receptor substrate

KOH: Potassium hydroxide

LDL: Low-density lipoprotein

mAb: Monoclonal antibodies

MELAS syndrome: Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like syndrome

MHC: Major histocompatibility system

MODY: Maturity onset diabetes of the young

MTT: Thiazolyl blue tetrazolium bromide

$NADP^{+:}$ Nicotinamide adenine dinucleotide phosphate

2 NBDG: 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2- deoxy-d-glucose

OCR: oxygen consumption rate

OGTT: Oral glucose tolerance test

PBS: Phosphate-buffered saline

PI: Propidium iodide

PI3K: Phosphatidylinositol 3 Kinase

PI3-K: Phosphatidylinositol 3-kinase

PI3K-PKB: Phosphoinositide-3-kinase–protein kinase B

PKC: Protein kinase C

PPAR: Peroxisome proliferators-activated receptors

PPAR-gamma: Peroxisome proliferators-activated receptor gamma agonists

RP: Reverse phase

RPMI: Roswell Park Memorial Institute medium

SDS-PAGE: Sodium dodecyl sulfate – polyacrylamide gel electrophoresis

SGLT: Sodium dependent Glucose Transporters

T1D: Type 1 diabetes

T2D: Type 2 diabetes

TBST: Tris Buffered Saline with Tween-20

TCM: Traditional Chinese Medicine

TE: Tris EDTA

Tm: Melting temperature

Chapter 1: Introduction and Literature Review

1.1. Background

Diabetes mellitus is a chronic multifactorial disorder which occurs due to the disruption of carbohydrate, lipid, and protein metabolism. It is generally characterized by abnormally elevated blood glucose levels, a condition known as hyperglycaemia. Compared to type 1 diabetes, type 2 diabetes is thought to be more prevalent. About 90% of instances of diabetes in the UK are type 2 diabetes, while only 10% are type 1 diabetes (Whicher, O'Neill, and Holt, 2020). These are elaborately discussed and analyzed in the Section 1.5. This research has mainly focused on alternative solutions, such as herbal remedies, for the treatment of type 2 diabetes. There are now four kinds of oral anti-hyperglycaemic drugs used in mainstream therapy for the management of type 2 diabetes, excluding insulin therapy. These include glucosidase inhibitors, biguanides, sulfonylureas, and thiazolidinediones. Each of these drug types has advantages and disadvantages that are specifically explored in Section 1.7.

A change in diet, an active lifestyle and regular exercise could significantly improve metabolic management when type 2 diabetes is at the borderline level, but these solutions have a less significant influence during advanced stages of the disease. However, some herbal alternatives which are frequently used in Ancient Indian Medicine (AIM), Ayurveda, and Traditional Chinese Medicine (TCM) were demonstrated (Wang *et al.*,2013) to offer symptomatic relief and facilitate the prevention of secondary complications of the disease. Some herbs instigated the regeneration of the pancreatic beta cells while improving insulin sensitivity, and some other herbal compounds exhibited antioxidant and cholesterol lowering properties. Therefore, the application of herbal compounds in treating diabetes could potentially improve normoglycaemia and further reduce relevant complications. In addition to this, herbal compounds are known for their affordability, efficacy, and safety with relatively fewer side effects. For instance, metformin – a biguanide class of drug known for its relatively lower toxicity and efficacy in lowering glucose levels – did indeed originate and was developed from an herb known as goat's rue (*Galega officinalis*). All these aspects of the study are discussed individually and analyzed in the Section 1.9, which focuses on herbal therapy for diabetes mellitus, and in the Section 1.5, which deals with diabetes mellitus.

The human hepatoma HepG2 cells employed in this work are often used as *in vitro* alternatives to primary human hepatocytes (Donato, Tolosa, and Gomez, 2015). The glucose absorption and metabolism of these cells, which have been chosen as a model for liver or hepatocytes, are similar to those of human liver cells, with cell-specific metabolic pathways and mechanisms of action, and they are the most extensively used in drug metabolism studies.

Glucose is stored as glycogen in the liver. Glycogen converts to glucose during hypoglycaemic conditions. Also, glucose can be synthesized from noncarbohydrate precursors like pyruvate, amino acids, and glycerol by gluconeogenesis, which maintains blood glucose concentrations, for example during fasting conditions and intense physical exercise (Andany, 2016). Thus, these intrinsic properties made this cell line a vital component of the study.

To create and study a model to demonstrate the efficacy of anti-diabetic herbal compounds, it is essential to study prominent mechanisms such as glucose uptake activity, glucose consumption, and accumulation of glycogen and other metabolic functions. By considering the substantial role played by these mechanisms in a multifactorial metabolic syndrome like diabetes, target phytochemicals such as berberine, metformin, phloretin, ouabain, and rebaudioside-A and Goldenseal (*Hydrastis canadensis* L.), Gurmar (*Gymnema sylvestre* (Retz.) R.Br. ex Sm.), Goat's rue (*Galega officinalis* L.), *Stevia* (*Stevia rebaudiana Bertoni*) extracts were chosen to trigger these mechanisms in the above specified cellular models for this research project.

1.2. Aims and objectives

The overall aim of this project is to identify the effect of traditional herbal compounds on glucose metabolism and anti-diabetic properties in cultured HepG2 cells, and to establish biochemical evidence for their efficacy. To accomplish this aim and establish the mechanism of anti-diabetic action on HepG2 cell metabolism, the following objectives were attempted in this research project:

•The effect of a variety of plant secondary metabolites such as an alkaloid (berberine), a flavonoid (phloretin), a cardiac glycoside (ouabain), a steviol glycoside (rebaudioside-A), as well as extracts of *Hydrastis canadensis* L, *Galega officinalis* L, *Gymnema sylvestre* (Retz.) R.Br. ex Sm.), and *Stevia rebaudian Bertoni*, on glucose uptake properties were assessed, and compared to a biguanide class of drug metformin.

•The effect of the target herbal extracts and individual compounds on glucose consumption properties was measured.

•The effects of the target herbal extracts and individual compounds on glycogen synthesis were investigated.

•The effect of the target herbal extracts and individual compounds on the glucose release property was investigated.

•The Seahorse metabolic analyzer was used to investigate mitchondrial respiration and glycolysis in treated cells.

1.3. Novelty

The herbs used in this research have a long history of application in Ayurveda, Traditional Chinese Medicine, and African and Western herbal medicine. Although they have long been renowned for their efficacy in providing symptomatic relief for diabetics, their application often suffers due to the lack of biochemical evidence and a definite understanding of their mechanisms of action in the literature available. This project attempts to fill the research gap by providing evidence and elucidating the mechanisms of the action of the target herbal compounds.

Previous investigations using various cell lines that had been published served as the basis for the study for this thesis. When searching through academic search engines like PubMed the following search terms were used: *Gymnema* and Sugar uptake (An *et al.*,2020 ; Wang *et al.*, 2014; Kumar, Mani, and Mani, 2010), *Stevia* and Sugar uptake (Bayat *et al.*, 2020 ; Rizzo *et al.*, 2013 ; Bhaskar, Madhav, and Chinnamma, 2015 ; Myint *et al.*, 2020), *Galega* or Goat's rue and Sugar uptake (Lui *et al.*, 2010), and Berberine and Sugar uptake(Chang, Chen, and Hatch, 2016 ; Tu, Zhu, and Zhou, 2018 ; Xu *et al.*, 2014 ; Zhang *et al.*, 2014; Zhang *et al.*, 2018). Additional search terms used to find pertinent papers included polyherbal formulations, diabetes, herbal hypoglycaemic formulations, anti-diabetic herbal drugs, anti-diabetic medicinal plants, complementary therapies, and alternative therapies.

Also,the names of 17 prominent anti-diabetic ayurvedic herbs i.e., *Asparagus racemosus* L.(Liliaceae) / Shatavari, *Azadirachta indica* L. (Meliaceae) / Arishtha / Neem, *Berberis aristate* L.(Berberidaceae) / Daruharidra, and *Capparis decidua* L.(Capparaceae) / Kareera, *Cinnamon zeylaniucm* L.(Lauraceae) / Tvak / Dalchini, and *Cocconia indica* L.(Cucurbitaceae) / Bimbi / Tundika / Kundru, *Emblica officinalis* L.(Euphorbiaceae) /

Amla,and *Gymnema sylvestre* (Retz) R.Br exSm.(Apocyanaceae) / Madhunashini / Gurmar, *Momordica Charntia* L.(Cucurbitaceae) / Karavella / Karela, and *Pterocarpus marsupium* L. (Fabaceae) / Vijayasar, *Plantago psyllium* L.(Plantaginaceae) /ovata Isabgol, and *Syzygium cumini* L.(Rutaceae) / Jambu / Badijamun, *Strychonos nux-vomica* L.(Loganiaceae) / Vishamushti / Kulcha,and *Tinospora cordifolia* L.(Menispermaceae) / Guduchi / Gurcha, *Trigonella foenum-graecum* L.(Leguminosae) / Medhika / Chandrika / Fenugreek, and *Catharanthus roseus* L.(Apocyanaceae) / Nitya kalyani / Sada bahar, *Withania Somnifera* L.(Solanaceae) / Ashwagandha were looked up using academic search engines including the Cochrane library, PubMed, Medline, Allied and Complementary Medicine, Ovid MEDLINE, BIOSIS Previews, EMBASE, and Medline.

1.4. Content of the thesis

Chapter 1 provides introduction and a detailed review on diabetes mellitus, and a detailed discussion of the application of herbal therapies for treating diabetes and analyzes the pros and cons of herbal therapies. Presents a report on the target phytocompounds of this study. Discusses the key mechanistic studies and cellular models used in this research.

Chapter 2 provides details of materials and methods applied for basal glucose uptake studies, glucose consumption, glycogen assay studies, glucose release studies and metabolic studies.

Chapter 3 provides screening of the target herbal compounds and extracts.

Chapter 4 provides metabolic properties of the key herbal compounds and extracts.

Chapter 5 provides discussion, and conclusions.

1.5. Diabetes Mellitus

1.5.1. General overview of the disease

Diabetes mellitus is a metabolic disorder that occurs when abnormal insulin levels, or abnormal insulin responsiveness, disrupt carbohydrate metabolism which maintains a healthy level of blood glucose and glucose homeostasis in blood plasma. The healthy level for blood plasma glucose in adult males is in the range of 4.0 to 5.4 mmol/L for pre-prandial levels, and up to 7.8 mmol/L after a meal (Diabetes UK, 2023). Plasma glucose levels outside this normal range are an indicator of glucose imbalance in the blood plasma. A persistently elevated level of blood plasma sugar in the human body is referred to as hyperglycaemia. Such elevated glucose levels are not healthy. In a similar manner, hypoglycaemia refers to the condition wherein the glucose levels in the body are too low. Although a combination of multifactorial metabolic disorders, diabetes is often predominantly characterized by hyperglycaemia, insulin resistance, or defects in insulin secretion. A non-communicable disease, diabetes is accounts for 4 million deaths worldwide in 2017 (Fact sheet, IDF Diabetes Atlas, 2017).

Chronic hyperglycaemia refers to persistently elevated blood glucose levels. In the long term, it leads to physiological and functional damage of different organs such as blood vessels, eyes, heart, kidneys, and the nervous system. Factors involved in the pathogenesis and the development of the disease include the destruction of the pancreatic beta cells by specific auto-immune processes which consequently disrupt insulin secretion and leads to insulin resistance. In general, disruption in insulin secretion and deficiency in insulin activity coexist and are often interrelated. This peculiar status always creates an ambiguity about the root cause of elevated blood glucose levels, the hyperglycaemic state, in diabetic patients. Inadequate insulin secretion or insulin inactivity disrupts the metabolism of adipose, carbohydrate and proteins in relevant organs and tissues. Disrupted insulin action occurs due to inadequate insulin secretion, and this makes tissue response weaker in the complex insulin receptor signalling pathway (RSSDI textbook of diabetes mellitus, 2017).

The symptoms of elevated blood glucose levels include frequent urination (i.e., polyuria) particularly at night, excessive thirst (i.e., polydipsia), increased appetite (i.e., polyphagia), recurrent infections such as urinary tract infections, foot infections, gangrenous cholecystitis, rhinocerebral mucormycosis, and malignant external otitis (Casqueiro *et al.*,2012), dry mouth, blurred vision, and weight loss. Short-term complications such as

hypoglycaemia and diabetic ketoacidosis (DKA) or a hyperosmolar hyperglycaemic state (HHS) can occur. Diabetic ketoacidosis is acute and life threatening. It occurs when the body is not capable of using blood glucose due to the insufficiency or absence of insulin. In this scenario, it breaks down the adipose tissue as an alternative source of energy. This process releases ketone bodies acetoacetate and β-hydroxybutyrate as a byproduct of fatty acid metabolism. Extremely elevated blood glucose levels, often 40 mM or over, causes non-ketotic hyperosmolar syndrome which creates a high risk of complications such as coma and death in elderly patients (Bell, 2018).

If left untreated over an extended period, elevated blood glucose levels lead to complications such as diabetic retinopathy which can potentially cause visual impairment, diabetic nephropathy which can lead to renal impairment, peripheral neuropathy which damages the peripheral nervous system and often causes the risk of foot ulcers, diabetic wounds, and amputations, and neurogenic arthropathy (Charcot Joints) (Dixon *et al.*,2017). On the other hand, diabetic autonomic neuropathy disrupts signals between the brain and the autonomous nervous system, affecting involuntary body functions, causing gastrointestinal, genitourinary, cardiovascular symptoms, and sexual dysfunction (Freeman, 2014). Elevated blood pressure and disrupted lipid metabolism is frequently noticed in diabetes patients, and it often increases the prevalence of atherosclerotic cardiovascular, peripheral arterial, and cerebrovascular diseases (Ruiz *et al.*,2017)

1.5.2. Prevalence of diabetes mellitus

The global prevalence of diabetes has reached epidemic proportions and causing 4 million deaths in 2017. The percentage of people with type 2 diabetes is mounting up in most countries, and 352 million people are on the brink of risk of developing type 2 diabetes in 2017. Roughly 425 million adults will have diabetes by 2017, and this will upsurge to 629 million by 2045. The majority of these (79%) belong to low- and middle-income countries between the age group of 40 and 59 years. Also, one in two people are undiagnosed (212 million). Diabetes causing healthcare expenditure approximately USD 727 billion dollars; it is 12% of the overall healthcare spending on adults in 2017. In 2017 more than 1,106,500 children were diagonosed with type 1 diabetes, and 1 in 7 live births (about 21 million) were affected by gestational diabetes (Fact sheet, IDF Diabetes atlas, 2017).

1.5.2.1. India

The comparative prevalence of diabetes in India, as estimated in 2017, in the age group 20-79 was 10.4 %, with 73 million of the adult population diagnosed with diabetes and an estimated 42 million adults having undiagnosed diabetes. In 2017 the average diabetes related expenditure per person was 119.4 USD, and diabetes related deaths were 997,803 (Fact sheet, IDF Diabetes atlas, 2017).

1.5.2.2. United Kingdom

As estimated in 2017, the proportional incidence of diabetes in UK in adults (age group 20-79) is 4.3 %, comprising 2.7 million adult diabetic patients. Undiagnosed diabetic adults were estimated at 0.51 million, and an average diabetes related spending per person was 4,988.6 USD. The mortality of diabetic patients in 2017 was 14,593 (Fact sheet, IDF Diabetes atlas, 2017).

1.5.3. Types of diabetes mellitus

Diabetes mellitus has been classified into two major categories: type 1 diabetes and type 2 diabetes. Type 1 diabetes occurs due to the lack of insulin secretion caused by dysfunctional pancreatic cells. Clinical detection of type 1 diabetes involves the identification of serological evidence of the pathological processes of the autoimmune system, and the factors that influence the pancreatic islets associated with genetic markers. Type 2 diabetes is the most common and it occurs due to the body's resistance to insulin activity or the inadequate supply of insulin by pancreatic cells (Groop and Pociot, 2014).

1.5.3.1. Prediabetes / borderline diabetes

In type 2 diabetes, before it is clinically diagnosed in a patient at borderline or a prediabetic stage, a degree of elevated blood glucose levels leads to the physiological and functional damage of target organs and tissues. Due to the lack of significant clinical symptoms, these changes may be unnoticed clinically for a long period. In such situations, borderline patients exhibit abnormal carbohydrate metabolism, and this can be evaluated by measuring plasma glucose levels in the fasting state or by an A1C test (i.e., haemoglobin A1C, HbA1c, or glycohemoglobin test), which can measure a person's average plasma glucose levels over the last three months (Stoppler, 2014).

In the United Kingdom prevalence of prediabetes is 27% and are likely prone to the risk of developing type 2 diabetes (Barry, 2017). One among every three individuals is prone to borderline diabetes, which is around 35.3% of the adult population of England (Mainous III *et al.*,2014). The increasing number of patients with recorded diagnosis of borderline diabetes has become a global concern as it carries an exhaustive impending burden on healthcare (Mainous *et al.*,2014).

Prediabetes is closely linked with obesity and characterized by elevated blood sugar levels that are slightly higher than normal, and this is known as the "grey area" between the ranges of normal blood glucose levels and diabetic levels. At this point, diabetes can be triggered, progress, regress, or remain the same. Consequently, the degree of hyperglycaemia reveals the severity of the underlying metabolic processes and suggests the required treatment more than the nature of the underlying mechanism itself. Borderline diabetes is often referred to as impaired glucose tolerance (IGT) due to the elevated sugar levels observed soon after eating, or impaired fasting glucose (IFT) due to the elevated blood sugar levels recorded after a period of fasting. Borderline diabetes is a critical stage in the advancement of diabetes, and proactive care can delay the disease or even halt the progression of type 2 diabetes. At this point, changes in lifestyle and nutrition intake can help turn it around (Tay, 2015).

Prediabetes can be triggered in anyone, irrespective of their race, age, or gender. However, Afro-Caribbean, South Asian and Native American populations are more susceptible due to their genetic predispositions. Risk factors of borderline diabetes include a history of diabetes in the family and biologically close relatives, overweight, elevated blood pressure, elevated levels of triglycerides, low levels of high-density lipoprotein (HDL), ageing, and high birth weight (Bansal, 2015).

1.5.3.2. Diagnosis of prediabetes

Blood tests such as fasting blood glucose test, glycated haemoglobin (HbA1c) test, or a simple dipstick urine examination are used to diagnose prediabetes. The following test parameters are used to indicate the occurrence of borderline diabetes: fasting blood sugar: 5.5 mM to 6.9 mM, HbA1c: 42 to 47 mmol/mol (6.0 to 6.4%). The normal amount of glucose in urine is 0 to 0.8 mmol/L - a higher measurement could be a sign of diabetes. Results over the upper limits of the ranges are thought to be borderline for diabetes, and further investigation is indicated to determine the chance of developing type 2 diabetes. If urological examination of HbA1c shows results below 42 mmol/mol (6.0%), but symptoms

of diabetes persist, further investigations such as oral glucose tolerance test (OGTT) are recommended (Bansal, 2015).

1.5.3.3. Preventing advancement of borderline diabetes in to type 2 diabetes

The positive side of the borderline diabetes is that it works like a wakeup alarm, by adopting a healthy lifestyle and including physical exercise, weight reduction efforts, and healthy dietary habits, prediabetes can be reversed (Bansal, 2015).

1.5.4. Classification of diabetes mellitus and different classes of glucose regulation in disease occurrence and development

The classification of the types of diabetes that affects an individual is generally dependant on the symptoms present during the diagnosis, and many of them generally do not fall into a specific single type due to symptomatic resemblance. For instance, a pregnant woman who is diagnosed with gestational diabetes is likely to continue to be a post-delivery hyperglycaemic and may later be diagnosed as having type 2 diabetes. In some diabetic individuals, after the application of large doses of exogenous steroids, their blood glucose levels may become normal. Once they stop taking glucocorticoids, elevated blood glucose levels may recur after a few years, and they could be determined as diabetic individuals again. This condition may be caused by recurrent episodes of pancreatitis. In another example, an individual who is suffering from high blood pressure and has been treated with thiazides may tend to express elevated glucose levels after a few years due the exacerbation of thiazides. Therefore, for the physician and the patient, it is more important to understand the pathogenesis of the hyperglycaemia and to treat it accordingly and effectively, rather than simply categorizing the diabetes (Baynes, 2015).

1.5.4.1. Immune-mediated (type 1) diabetes mellitus

Type 1 diabetes can occur at any age, even at the age of 8 and 9, but it generally occurs during childhood and adolescence. This type of diabetes is also called the insulin-dependent diabetes or juvenile-onset diabetes. Type 1 diabetics accounts for 5–10% of the total population of diabetics, which also includes type 2, gestational and borderline classes. Type 1 diabetes occurs due to absolute insulin deficiency which results from a cellular-mediated autoimmune destruction of pancreatic beta cells. Immune markers of beta cell destruction include islet cell cytoplasmic autoantibodies (ICA), insulin autoantibodies (IAA), glutamic acid decarboxylase autoantibodies (GADA), glutamic acid decarboxylase

65 (GAD65), insulinoma-associated protein 2 autoantibodies A (IA-2A), and insulinoma-associated protein 2 autoantibodies β (IA-2β) and have been shown to be involved in the pathogenesis of autoimmune diabetes. In diagnosis, any one or often more than one of these autoantibodies could be found initially in 85–90% of type 1 diabetic individuals during the 12 hours fasting hyperglycaemia detection test (Krishna and Srikanta, 2015).

Type 1 diabetes is strongly associated with the loci of genes such as human leukocyte antigen (HLA) system with linkage to DQA and DQB genes, and this major histocompatibility system (MHC) is often influenced by the DRB genes. Also, these HLA-DR/DQ alleles are major genetic determinants and could potentially be either predisposing or protective (Redondo, Steck, and Pugliese, 2017).

In type 1 diabetics, the progression rate of beta cell destruction varies in different age groups. It is rapid in infants and children, whereas it is slow in adults. Diabetic ketoacidosis may appear in type 1 diabetics, especially in children and teenagers during an extreme condition like when the body totally fails to use blood glucose due to the lack of insulin, and it breaks down adipose tissue as an alternative source of fuel. This process derives a by-product called ketone bodies, hydroxybutyrate and acetoacetate. In some type 1 adult diabetics, with suboptimal glycaemic control after fasting, there tends to appear a rapid change in the form of increase in blood glucose levels which frequently leads to ketoacidosis due to excess production of ketone bodies, during bacterial infection or cellular stress. While in some other adult patients, residual beta cells continue to function at their peak, preventing ketoacidosis for a long time. However, they eventually rely on exogenous insulin application for optimal glycaemic control and are susceptible to ketoacidosis (Savage *et al.*,2010).

Ketoacidosis is an advanced stage of the disease with an indication of trace insulin levels or the complete absence of insulin hormones, as expressed by low or untraceable levels of plasma C-peptide. Studies have revealed that maintaining elevated levels of C-peptide is indirectly connected to insulin levels, which eventually leads to glycaemic control. Therefore, the detection of C-peptide levels in plasma serum is one among the several methods for classifying diabetes, and C-peptide is also used as a marker of beta cell function (Diabetes Care, 2017).

Autoimmune destruction of insulin-producing pancreatic beta cells by a beta cell-specific autoimmune process has various factors of genetic susceptibility, in addition to environmental triggers such as dietary habits, obesity, viral infection, environmental

toxins, and psychological or physical stress. Early cessation of breast-feeding for infants below six months of age has also been associated with the increased risk of developing type 1 diabetes. But the association is poorly defined and has been postulated to play a causative role in genetically predisposed individuals (Dunger and Todd, 2008; Knip *et al.*,2010). And they are further susceptible to other autoimmune complaints such as Addison's disease, celiac sprue, Graves' disease, Hashimoto's thyroiditis, vitiligo, autoimmune hepatitis, pernicious anaemia, and myasthenia gravis (Van den *et al.*,2009).

1.5.4.2. Idiopathic diabetes mellitus

The American Diabetes Association proposed two subcategories for type 1 diabetes mellitus: type 1A or immune-mediated diabetes (IDM) and type 1B or idiopathic diabetes. It has been revealed in Afro-American and Asian patients, as well as in Hispanic and native Americans, European, and Mediterranean patients (Guarnotta *et al.*,2018), that in general, those who suffer from type 1 diabetes have no evidence of influence of autoimmunity, the absence of beta cell autoimmune markers, permanent insulinopenia, and prone to ketoacidosis, and the pathogenesis remains unknown (Catarino *et al.*,2020). And have poorly defined aetiologies, such as permanent insulinopenia, which predisposes them to episodic ketoacidosis with fluctuating levels of insulin deficiency. As a result, the need for insulin replacement therapy in affected patients may fluctuate. However, this type of diabetes affects only a small percentage of type 1 diabetics. It is genetically predisposed but not linked to HLA (Diabetes Care, 2017).

1.5.5. Type 2 diabetes

This category includes about 90–95% of people with diabetes. Both adult-onset diabetes and non-insulin-dependent diabetes are terms used to describe it. Type 2 diabetics typically display insulin resistance and, in rare instances, relative insulin deficiency in the early stages of the disease. And these individuals do not depend on the application of exogenous insulin throughout their lifetime to bring down elevated blood glucose levels to optimal glucose levels. There are several known and unknown triggers that induce type 2 diabetes, but trigger-specific aetiologies are still not properly defined. In this form of diabetes, unlike type 1 diabetes, pancreatic beta cells are at an absolute intact and safe mode, and do not undergo autoimmune destruction. Therefore, causes of type 2 diabetes are different from that of type 1 (Piero, Nzaro, and Njagi, 2014), but according to Böni-Schnetzler and Meier (2019), increased innate immune cells, cytokines, and chemokines harm pancreatic

islets in chronic conditions. In some cases, beta cell mass reduction is the primary cause in the aetiology of type 2 diabetes.

There are some people who have insulin resistance but do not have diabetes, and vice versa. This is because insulin resistance plays a crucial role in the development of type 2 diabetes; yet there are also some people who have diabetes but do not have insulin resistance. However, deficiencies in the insulin signalling pathway are responsible for the development of type 2 diabetes because they stop glucose from accessing muscle and adipose tissue. In this instance, however, the specific factors that contribute to insulin resistance have not been thoroughly examined. However, the processes that have been highlighted include oxidative stress, inflammation, insulin receptor mutations, endoplasmic reticulum stress, and mitochondrial malfunction (Yaribeygi *et al.*,2019).

Often type 2 diabetics are obese individuals, and obesity itself is a predominant factor of insulin resistance. Individuals, who appear normal in terms of body weight, might have excessive active fat in the form of visceral fat which is accumulated around a few important internal organs such as the liver, pancreas, and intestines (Gastadelli, 2008). During the influence of external triggers such as infection and stress, ketoacidosis may occasionally occur in type 2 diabetes.

In type 2 diabetes, an upsurge in blood glucose tends to develop slowly and gradually at the primary stage of the disease, and the individual does not express any classic symptoms of the disease. Therefore, it often goes unnoticed for many years during clinical examinations. But such undiagnosed patients are always prone to developing macro and micro vascular complications. In type 2 diabetics, insulin levels may appear to be optimal or rise, the elevated plasma glucose levels in these individuals would be expected to result in elevated insulin levels with proper pancreatic beta cell function. Therefore, insulin secretion tends to be defective or inadequate to recompense insulin resistance.

Predominant factors of developing type 2 diabetes are obesity, ageing and sedentary lifestyles. Losing a few stones may increase insulin sensitivity, and the body may respond well to medication, but the recurrence of insulin resistance has been noticed as soon as the body restores visceral fat. It is often seen in women before they are affected with gestational diabetes, and in patients who are suffering from high blood pressure or dyslipidaemia, and it is subjective to different racial and ethnic subgroups based on their genetic predisposition to the disease. Also, there is evidence that type 2 diabetes has a strong genetic basis despite its complexity (Diabetes Care, 2017) (Ref: Section 1.6.4).

1.5.6. Other types of diabetes mellitus

Maturity onset diabetes of the young (MODY) is a "monogenic diabetes" caused by mutations in 14 independent autosomal dominant genes that can be inherited from either parent, according to Online Mendelian Inheritance in Man (OMIM). MODY-2 and MODY-3 are the most common forms. MODY disrupts the beta cell function and is often characterized by impaired insulin secretion with minimal or no defects in insulin action, which leads to the onset of hyperglycaemia before the age of 25, and it is thus called the maturity onset diabetes of the young (Anık *et al.*,2015).

The most common types of MODY are caused by mutations in HNF-1-alpha, HNF-4-alpha, HNF-1-beta and Glucokinase. HNFs are hepatic transcription factors referred to as hepatocyte nuclear factors, which are a group of phylogenetically unrelated transcription factors that regulate the transcription of a diverse group of genes into proteins. And HNFs are not only expressed in the liver but also, in other tissues and organs, such as the pancreas and the kidney, playing important roles in regulating the development and functions of these tissues (Hwee *et al.*,2018).

The less common forms of MODY result from mutations in other transcription factors, including insulin promoter factor-1 (IPF-1) and NeuroD1. A common type of MODY, MODY-2, is associated with mutations in the glucokinase gene on chromosome 7p, which create a defective glucokinase molecule. Glucokinase is an enzyme which metabolizes glucose molecule into glucose-6-phosphate. During this process, insulin producing beta cells get stimulated and insulin secretion is instigated. Therefore, glucokinase acts as the “glucose sensor” for pancreatic beta cells. Thus, the presence of the defective glucokinase gene results in higher blood glucose levels to stimulate normal levels of insulin secretion from defective pancreatic beta cells (Bishay and Greenfield, 2016).

1.5.6.1. Neonatal diabetes

Neonatal diabetes is a hyperglycaemic condition noticed in infants at around 6 months of age, and it may not fall under type 1 diabetes. Its effect can be transient or permanent. A defect in ZAC/HYAMI imprinting due to a defective gene causes transient neonatal diabetes. Whereas permanent neonatal diabetes is caused by activating mutations of the KCNJ11 gene encoding the Kir6.2 subunit of the β-cell K^+ ATP channel, which is linked with GCK (glucokinase), INS (insulin), and ABCC8 (ATP-binding, regulator subunit of the K^+ channel). It is potentially treatable with sulfonylureas. There is also a mitochondrial

form of diabetes, in which most diabetics are prone to deafness in later stages of the disease, because of the point mutation that occurs in mitochondrial DNA at position 3,243 in the $tRNA^{leu}$ gene which involves an A-to-G transition. A similar lesion can be found in mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like syndrome (MELAS syndrome), though diabetes is not a part of the syndrome (Bryan and Bryan, 2008).

The inheritance of traits such as the inability to convert proinsulin to insulin has been found in some populations, and this genetic abnormality occurs in an autosomal dominant fashion. The consequential glucose intolerance is minimal. And in a few families, the occurrence of mutant insulin molecules along with consequentially impaired receptor binding has been found in connection with autosomal inheritance and found to be minimally impaired, or at times even participated in normal glucose metabolism (Bryan and Bryan, 2008).

1.5.6.2. Genetic disorders of insulin action

Sometimes the mutations of the insulin receptors cause metabolic abnormalities such as modest hyperglycaemia, hyperinsulinemia, and severely elevated hyperglycaemia. Previously, this syndrome was known as type A insulin resistance (Young *et al.*,2005). In some patients, these mutations may trigger an abnormal skin disorder such as acanthosis nigricans. In women, these mutations tend to make them prone to virilised and enlarged cystic ovaries. In paediatric syndromes such as Leprechaunism and the Rabson-Mendenhall syndrome, a mutated insulin receptor gene with altered function subsequently induces life threatening insulin resistance. Those infants affected with Leprechaunism and the Rabson syndrome have characteristic facial features and fatalities in infancy, while those affected with the Mendenhall syndrome are associated with dental, nail, and pineal gland hyperplasia. In syndromes like insulin resistant lipoatrophic diabetes, no significant changes in the structure or function of the insulin receptor have yet been found, and the disease is attributed to the location of the lesion(s) in the post receptor signal transduction pathways (Young *et al.*,2005).

1.5.6.3. Exocrine pancreatic disorders

Damage can occur to the pancreas to the point of exocrine pancreatic insufficiency by any means of pathogenic processes such as pancreatitis, trauma, infection, pancreatectomy, and pancreatic carcinoma, and can lead to diabetes. In the case of pancreatic cancer, extensive

damage of the pancreas has been noticed, and on the contrary, in adenocarcinoma, a small portion of affected area of the pancreas can induce diabetes, which indicates a different mechanism than the simple reduction in beta cell mass. Diseases such as cystic fibrosis and hemochromatosis at advanced stages tend to damage pancreatic beta cells and disrupt insulin secretion. In fibrocalculous pancreatopathy, individuals experience abdominal pain radiating to the back, and pancreatic calcifications are noticed during X-ray studies. Also, pancreatic fibrosis and calcium stones in the exocrine ducts have been found at autopsy (Nieto and Bastidas, 2016).

1.5.6.4. Endocrinopathy associated diabetes mellitus

Hormones such as somatotropin, glucagon, epinephrine, and cortisol antagonize insulin's antiproteolytic action on skeletal muscles in humans. The presence of elevated amounts of these hormones induces diabetes in individuals with defective insulin secretion and elevated blood glucose levels, as well as in those with hormonal disorders such as acromegaly, Cushing’s syndrome, pheochromocytoma and glucagonoma. Elevated glucose levels would tend to normalise after surgical removal of the malignant tumours (American Diabetes Association, 2013).

1.5.6.5. Drug induced diabetes

Application of drugs such as corticosteroids, thiazide diuretics, beta-blockers, antipsychotics, and statins induce a form of secondary diabetes. Indeed, it is a consequence of having another health condition. These drugs may precipitate diabetes by either upholding insulin resistance or by downgrading insulin secretion, and in this case the role of pancreatic beta cell dysfunction, is not yet exposed (Rehman *et al.*,2011). Vacor is a toxic compound generally used as a rodenticide. A study on the effect of vacor on endocrine and autonomic functions revealed that ingestion of vacor induces diabetes mellitus and can permanently damage pancreatic beta cells (Karam *et al.*,1980; Diabetes Care, 2017). And similar effects are noticed upon the application of intravenous pentamidine. Drugs such as nicotinic acid and hormones like glucocorticoids could possibly induce insulin resistance. Alpha interferon is a bio therapeutic agent commonly used to treat certain types of malignant tumours, and individuals who are on alpha interferon medication tend to develop diabetes due to the induction of islet antibodies and insulin deficiency (American Diabetes Association, 2013).

1.5.6.6. Virus induced diabetes mellitus

Viral agents such as coxsackievirus B, cytomegalovirus, adenovirus, mumps, and congenital rubella can potentially cause beta cell destruction, while affected individuals exhibit Human Leucocyte Antigen (HLA) and immune markers which are specific to type 1 diabetes (Morgan and Richardson, 2014).

1.5.6.7. Unfamiliar types of immune mediated diabetes

Systemic lupus erythematosus and acanthosis nigricans are autoimmune disorders in which the former produces anti-insulin receptor antibodies sporadically and the latter produces them prolifically. Individuals who produce elevated titers of the Glutamic Acid Decarboxylase Autoantibodies (GAD) tend to develop an autoimmune disorder of the central nervous system, called as the stiff man syndrome. Individuals who are suffering from this disorder are most likely to develop diabetes. Anti-insulin receptor antibodies tend to bind to the insulin receptor and prevent insulin from binding to target receptors in corresponding tissues, therefore eventually causing diabetes. Whereas, in some other individuals, these antibodies could act as insulin agonists after binding to the target receptor and promote elevated blood glucose levels. Chromosomal disorders such as Down syndrome, Turners syndrome and Klinfelter syndrome tend to induce diabetes by creating insulin resistance, and therefore this kind of diabetes is known as type B insulin resistance. In Wolfram syndrome, an autosomal recessive gene induces insulin deficiency. The absence of pancreatic beta cells is frequently noticed after surgery or autopsy (American Diabetes Association, 2013).

1.5.6.8. Gestational diabetes mellitus (GDM)

It is a type of diabetes that affects women during pregnancy and the presence of elevated blood glucose levels and insufficient insulin supply have been noticed, which prevents glucose transport into the cells. And it is well defined as glucose intolerance developed during pregnancy as the expectant mother must produce extra insulin to meet her baby's needs. The mother’s body will have too much sugar in blood plasma, and she may develop gestational diabetes. In the post-partum period, the sugar levels may revert to normal, but in some cases the mothers are likely to continue to have impaired glucose tolerance or develop frank diabetes after few years (Szymańska *et al.*,2008). Transient gestational diabetes is an undeniable possibility for the subsequent development of type 2 diabetes. However, in most individuals, GDM would be resolved automatically after the birth of the

child or extends for about six weeks, whereas in some individuals it may persist and reinforce into type 2 diabetes category (Marathe, Gao, and Close, 2017).

In all the above sections, the various forms of diabetes have been discussed due to their symptomatic similarities, and an overview has been presented. From here onwards, the focus of this study will only deal with type 2 diabetes. The pathophysiology, aetiology, mainstream treatment, complications, and alternative solutions of type 2 diabetes will be discussed in detail in the following sections.

1.6. The focus of the research--type 2 diabetes mellitus

Typically, type 2 diabetes develops in middle age or later. One of the disease's common causes is obesity. Symptoms are frequently minor, nonexistent, or go unnoticed. It indicates a relative rather than an absolute lack of insulin, and insulin resistance is a major contributing factor in type 2 diabetes. Even with anti-diabetic medication, resistance to ketosis has been proven to be a progressive illness. At diagnosis, tissue damage is frequently apparent.

The pathophysiological basis of the disease is a combination of impaired beta cell functions along with marked increase in peripheral insulin resistance at receptor or post receptor levels and increased hepatic glucose output production. The circulatory levels of insulin and C-peptide may be variable from hyper to normal insulinemic levels in most of the patients. Type 2 diabetes is further sub classified into obese and non-obese types. Coma is rare in type 2 diabetics, but it may result from extreme hyperglycaemia and hyperosmolarity. Ketoacidosis can occur in fulminating illnesses in situations like severe demand for insulin supply. However, spontaneous ketosis does not occur, and lactic acidosis is rarely seen (Baynes, 2015).

1.6.1. Pathophysiology of type 2 diabetes

A relative deficiency of endogenous insulin in the presence of impaired insulin action tends to increase hepatic glucose output and decrease insulin-mediated glucose uptake because of post-receptor defects in muscle tissue. As it is a heterogeneous syndrome, defects could be varied from person to person and possibly between populations.

1.6.2. Insulin resistance in type 2 diabetes

The term “insulin resistance” was coined to describe type 2 diabetes. Insulin is a hormone and a growth factor, which deals with metabolism of carbohydrates, lipids, and proteins, and plays a key role in cell growth and cell regulation. When a body fails to respond to a given amount of insulin to transport plasma glucose into cells, it is called insulin resistance. Insulin resistance starts in a person prior to the actual onset of type 2 diabetes. Obesity and family history are couple of factors that insulin resistance starts and develops. Insulin is necessary for the transport of blood glucose into muscle cells and adipose tissues. The insulin receptors on these cells, upon activation, enable muscle cells and fat cells to promote glucose uptake from the blood plasma. With factors such as obesity, muscle cells do not respond to the insulin, and then the pancreas produce more insulin than normal, and the muscles continue to be resistant. As long as an individual produces enough insulin to rise above this resistance, the blood glucose level remains normal. Once the pancreas fails to maintain normal blood glucose, the sugar level starts to rise after meals at the initial stages of the disease, and eventually in the fasting state too (Figure 1). At this stage of the disease, overt diabetes will occur, and it is also a risk factor for cardiovascular diseases.

1.6.3. Aetiology of type 2 diabetes

The mass of the pancreatic beta cells in type 2 diabetics is normal or increased in the primary stage of the disease, and in the subsequent stages of the disease, the pancreatic mass will be reduced to 50%, or abnormal beta cells, amyloid deposits, are seen. At the time of diagnosis, 80% of the gland will be destroyed due to an increase in beta cell mass apoptosis. In malnutrition related diabetes mellitus in the Indian subcontinent, fibrosis, shrinkage of glands and ductal dilatations are seen, and the beta cell mass is reduced **(**Zheng, Ley, and Hu, 2018) (Figure 1).

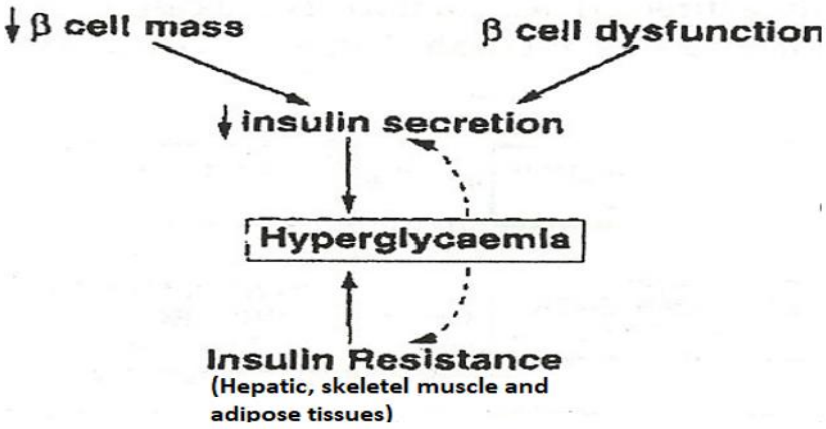


Figure 1: Diagrammatic representation of the contribution of both insulin resistance and reduced insulin secretion to the hyperglycaemia of type 2 diabetes (Raman, 2009).

1.6.4. Genetics in type 2 diabetes

A parent who has type 2 diabetes raises their child's risk of developing the disease by 50%, and the risk rises even more if both parents have the disease. As a result, understanding the patient's family history is critical in determining the likelihood of the disease manifesting itself. Most ethnic groups are thought to have a polygenic inheritance. Many candidate genes are ruled out, including the insulin receptor. Researchers focused on the regulation of the insulin gene during the genetic study of type 2 diabetes. Obesity-related insulin resistance is a risk factor for disease development and related difficulties (Weijing *et al.*,2014). Although 70 to 83 susceptibility genes were identified at a genome-wide significant level, the genetic loci identified only account for about 10% of the disease's overall heritability. Furthermore, the relationship between these genetic loci and disease occurrence, prognosis, and progression remains elusive (Wang *et al.*,2016; Sun, Yu, and Hu, 2014).

Massive amounts of data on the genetics of type 2 diabetes have been generated by genome-wide association studies (GWAS). The GWAS emerged against the backdrop of genotyping arrays covered by common single nucleotide polymorphisms (SNPs), which were used in a variety of cohorts that merged to form significant international consortia. As a result, a list of genetic loci influencing type 2 diabetes and quantitative glycemic traits is starting to emerge. More than 100 loci have been linked to type 2 diabetes and insulin resistance, and while the rs7903146 SNP has a stronger effect than the other TCF7L2 variants, the stated SNP has only a minor effect (odds ratio ~1.4). While causal variants have been identified for many of these loci, a significant portion of the heritability of these

phenotypes remains unknown. Nearly 100 loci have been linked with type 2 diabetes, accounting for ~10%–15% of the genetic predisposition to the disease (Florez *et al.*,2018).

1.6.5. Influence of the diet, behaviour, and environment

Lowglycemic diets have been proven to have beneficial effects; also, the majorities of type 2 diabetics are not physically active enough and adopt sedentary lifestyle, which increases their risk of developing type 2 diabetes. Regular exercise and active lifestyle play an important role in the prevention and maintenance of type 2 diabetes. Daily workouts decrease insulin resistance, increasing the number of receptors, sensitivity, and level of insulin, thereby promoting glucose uptake in the tissues (Ajala *et al.*,2013; Najafipour *et al.*,2017) (Figure 2).

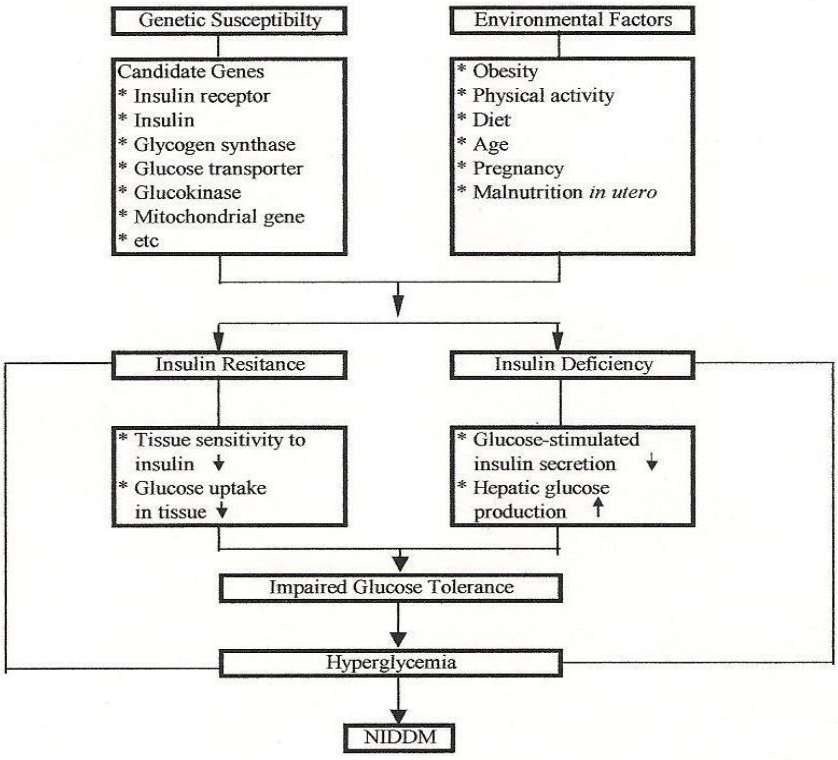


Figure 2: Progressive pathogenesis of type 2 diabetes mellitus (Defronzo, 2004)

1.7. Treatment of diabetes

Oral anti-diabetic drugs are used as a first step of pharmacological therapy when diet and exercise fail to achieve good glycaemic control in patients with type 2 diabetes. These drugs were classified by their actions into 1. Hypoglycaemic agents and 2. Anti-hyperglycaemic agents. This classification reflects the difference in their chemistry and their mode of action, and various drug options available for treating type 2 diabetes

effectively. These include sulfonylurea drugs, meglitinides, biguanides, alpha-glucosidase inhibitors, thiazolidinediones, and combinatorial drugs.

Surgical options such as pancreas transplantation and islet cell transplantations are a new approach of the treatment (Juan *et al.*,2016). It has been demonstrated that people who are severely obese can see improvements in their ability to maintain stable blood sugar levels after undergoing gastric bypass surgery or other weight loss procedures, such as bariatric surgery. This surgery is also an excellent treatment for those who are overweight and suffer from type 2 diabetes (Dixon *et al.*,2011).

1.7.1. Insulin therapy for type 2 diabetes

1.7.1.1. Role of endogenous insulin

Insulin facilitates glucose transfer across cell membranes in tissues. Insulin is synthesised and secreted by beta cells of islets of Langerhans. Insulin gene expression and its regulation is located on short arm of chromosome 11. Insulin secretion from beta cells has two phases. The first phase is solely contributed by insulin stored inside beta cells and occurs within half a minute of rise in glucose levels. The second phase starts about 15 minutes later and continues for more than an hour. This phase mostly comprises newly synthesized insulin. In diabetics, the first phase is abolished, and the second phase is delayed and pronounced. Glucose is a stimulus for insulin secretion. Glucose acts on the beta cells by activating glucokinase enzyme that facilitates phosphorylation of glucose to glucose-6-phosphate. The glucose receptors located on the surface stimulates intracellular mechanisms after entry of the glucose into the cells. Prompt and immediate response depends on the release of the second signal, cyclic-AMP. Thus, glucose-stimulated insulin release is biphasic in nature (Fu, Gilbert, and Liu, 2013).

1.7.1.2. Treatment by application of exogenous insulin

Acute conditions such as difficulty in controlling hyperglycaemic levels with oral antidiabetic agents, insulin therapy may be initiated as an augmentation or replacement therapy, but in the long-term treatment, titration of insulin is bound to fail in controlling elevated blood glucose levels and the relevant complications. Application of biguanides such as Metformin is likely to continue along with exogenous application of insulin, due to its efficacy in reducing mortality and cardiovascular events in obese diabetic individuals (Petznick, 2011).

1.7.1.3. Concerns about exogenous insulin application

Predominant side effects include pain in the case of injection therapy. Weight gain occurs due to anabolic effects of insulin, which promotes excessive appetite as a sign of defensive eating caused by insulin-promoted hypoglycaemia, and resultant caloric retention due to decreased glycosuria. In severe conditions, insulin-stimulated hypoglycaemia may have implications such as increased risk of dementia and cardiac arrhythmia (Petznick, 2011).

1.7.1.4. Novel alternatives to exogenous insulin

Extensive research has been going on in the alternative usage of incretins in the treatment. These are a group of gastrointestinal hormones which stimulate pancreatic beta cells after meals and promote the secretion of endogenous insulin **(**Kim and Egan, 2008). Insulin like growth factor (IGF) or somatomedin is a peptide hormone that mainly promotes growth, and it is also being noticed that it has some ability to decrease elevated blood glucose levels. However, more clarity is needed to confirm this further (Rajpathak *et al.*,2009). Glucagon-like peptide-1 (GLP-1) receptor analogs, or agonists such as exenatide, liraglutide and albiglutide acts like incretin mimetics and promotes insulin secretion with a lower risk of causing acute hypoglycaemia, when compared to insulin therapy, sulfonylureas and meglitinides (Garber, 2011).

1.7.1.4.1. Exenatide Extended-Release: It is a glucagon-like peptide-1 receptor agonist, recently have been using for the treatment of type 2 diabetes mellitus. It can be injected subcutaneously once weekly, and dose titration is not needed. In a clinical trial (rand omized, controlled trials), application of adjunctive exenatide extended release (2 mg once weekly), for 24-30 weeks considerably promoted glycaemic control and reduced bodyweight in inadequetly controlled type 2 diabetes patients, and this effect sustained up to 6 years of treatment. Addition of exanatide ER to oral antidiabetic agents provided better glycaemic control than applying exenatide twice daily, and it was generally well tolerated, with a low/no risk of hypoglycaemia. The common side effects are headache, gastrointestinal troubles, and injection-site reactions (Syed and Mc Cormack, 2015).

1.7.2. Oral anti-diabetic agents: Classification, mechanism of action and concerns

There are six different classes of oral anti-diabetic agents that are currently being used in mainstream therapy; these are discussed below briefly and exclusively in terms of core concepts (Figure 14).

1.7.2.1. Class 1: Sulfonylurea drugs

Molecular formula: $CH_3N_2O_3S$ (Figure 3); **Molecular weight:** 123.1 g/mol

Examples: Tolazamide (Figure 4), chlorpropamide, gliclazide, and glimepiride

Molecular structure:

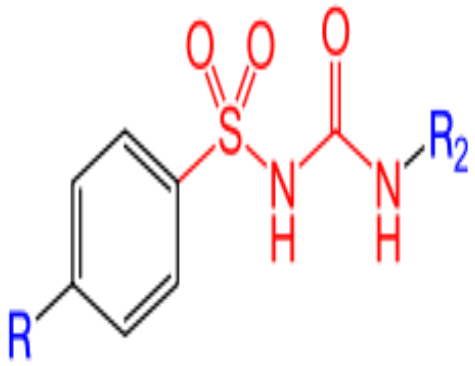


Figure 3: Structure of sulfonylureas showing the sulfonylurea backbone in red, and the side chains that distinguish each compound in blue (Deruiter, 2003).

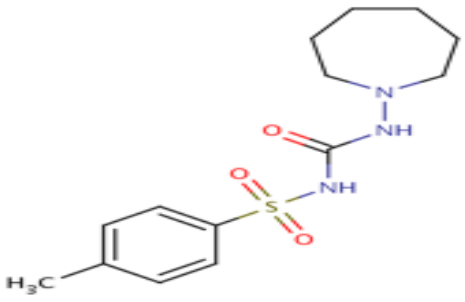


Figure 4: Tolazamide (Soliman, 1983)

Mechanism of action: Studies have clearly delineated the different modes of action of sulfonylureas during acute and chronic phases of therapy. The kinetics of insulin secretion involves four stages: synthesis, transport, storage, and release. Sulfonylureas are likely to bind to ATP-sensitive potassium-channel receptors on the pancreatic cell surface, reducing potassium conductance and causing depolarization of the membrane. Depolarization stimulates calcium ion influx through voltage-sensitive calcium channels, raising the intracellular concentrations of calcium ions which in turn induces the secretion, or

exocytosis of insulin (Krishnan, 1995; Shuck, 1994; Yano, 1994; Imming, 2006; and Overington, 2006).

Concerns: Appropriate use of sulphonylureas drugs generally has a positive effect. The range of recognized adverse effects is limited, and they are usually minor and reversible. Some effects like weight gain and other dose-related idiosyncratic reactions such as the alcohol flush are more common in older patients, and additive hypotensive effects may occur too (Krishnan, 1995; Shuck, 1994; Yano, 1994; Imming, 2006).

1.7.2.2. Class 2: Meglitinides (glinides)

Molecular formula: $C_{17}H_{16}ClNO_4$ (Figure 5); **Molecular weight:** 333.7 g/mol

Examples: Repaglinide (Prandin) (Figure 6), nateglinide (Starlix), and mitiglinide (Glufast)

Molecular structure:

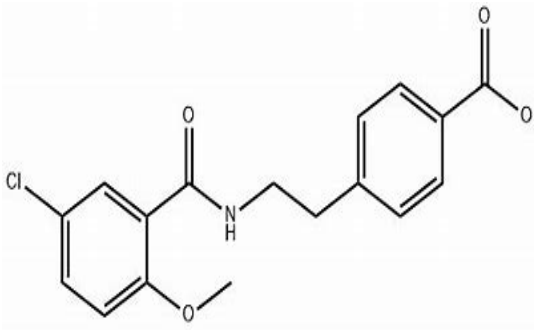


Figure 5: Structure of meglitinides (Deruiter, 2003).

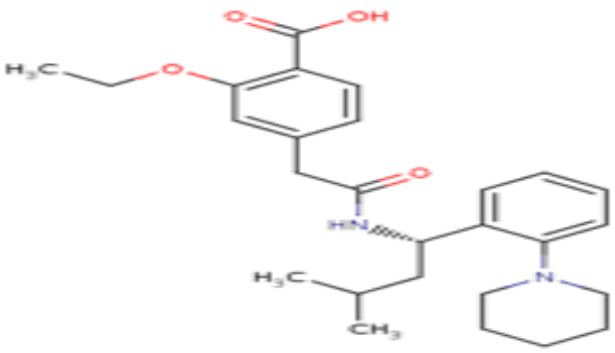


Figure 6: Repaglinide (Culy, 2001)

Mechanism of action: Repaglinide binds to the ATP sensitive potassium channels in the β-cell. This potassium channel blockade depolarizes the β-cell, which leads to an opening of calcium channels. The resultant increased calcium influx induces insulin secretion. The

ion channel mechanism is highly tissue selective with a low affinity for heart and skeletal muscle (Culy, 2001).

Concerns: Meglitinides are generally well tolerated. Contra indications of this drug are like those for sulphonylureas. When compared with long acting sulphonylureas, the risk of hypoglycaemia appears to be low if a meal is missed and the appropriate dose is skipped. Weight gain has been reported with repaglinide usage, but it has generally been modest. Other common side effects of repaglinide usage include back pain and upper respiratory tract infections (Rodolfo *et al.*,2013; Otonkoski *et al.*,1999; Meissner *et al.*,1999; Tanizawa, 2000; Hu, 2000).

1.7.2.3. Class 3: Biguanides

Molecular formula: $C_2H_7N_5$ (Figure 7); **Molecular weight:** 101.11 g/mol

Examples: Metformin (Figure 8), phenformin, and buformin

Molecular structure:

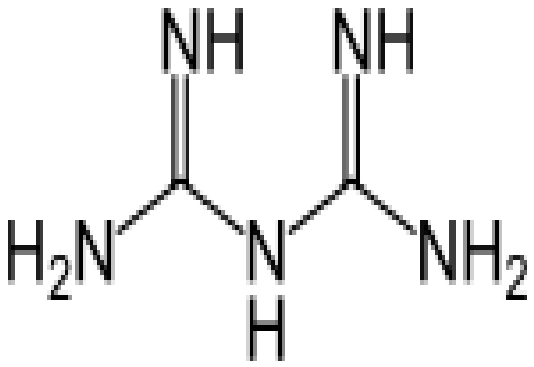


Figure 7: Molecular structure of a biguanide (Deruiter, 2003).

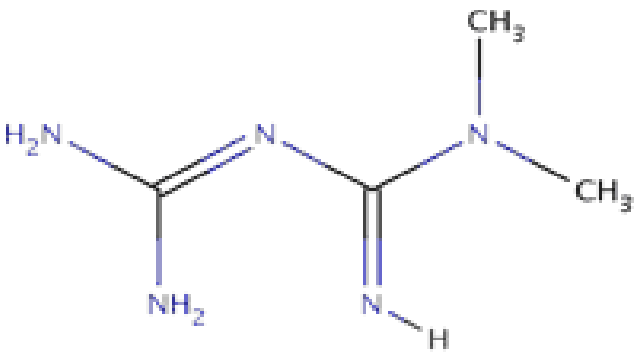


Figure 8: Metformin (Grzybowska, 2011)

Mechanism of action: Metformin's pharmacological mechanisms of action are different from that of other classes of oral anti-hyperglycaemic agents. Metformin decreases hepatic glucose production, decreases intestinal absorption of glucose, and improves insulin sensitivity by increasing peripheral glucose uptake and utilization (Grzybowska, 2011).

Concerns: The major side effects are gastrointestinal. About 20% of users experience anorexia, nausea, vomiting, diarrhoea, and abdominal discomfort, making them the most common side effects. And one also can include indigestion, headache, and diarrhoea (Marchesini *et al.*,2001; Ungar, 1957; Lord, 2003; and Nair, 2004).

1.7.2.4. Class 4: Alphaglucosidase inhibitors

Molecular formula: $C_{10}H_{21}NO_7$ (Figure 9); **Molecular weight:** 267.2 g/mol

Examples: Voglibose (Figure 9), and miglitol (Figure 10)

Molecular structure:

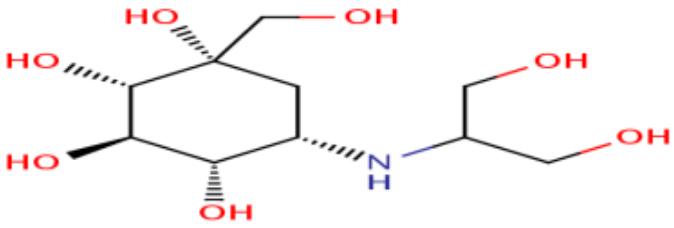


Figure 9: Molecular structure of voglibose (Deruiter, 2003)

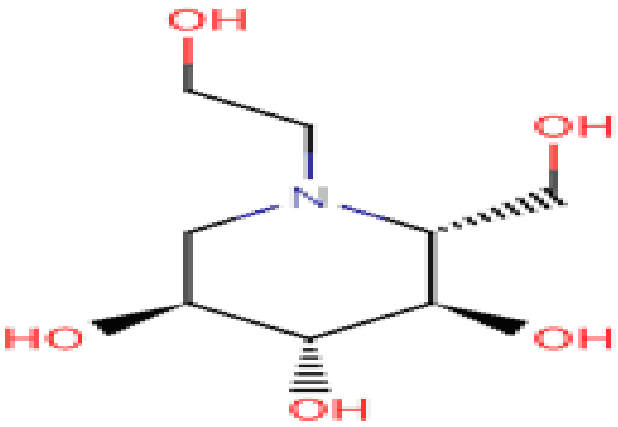


Figure 10: Miglitol (Sels *et al.*,1999)

Mechanism of action: By inhibiting the Alpha-glucosidase enzyme, carbohydrates are not broken down as efficiently and glucose absorption is delayed (Sels *et al.*,1999).

Concerns: Due to pharmacological actions of the drugs, the major common side effects are gastrointestinal in nature, and the most frequent side effects are flatulence, diarrhoea, and abdominal discomfort. All these symptoms tend to regress as a patient gets used to the drug, and after a few months of the therapy, the abdominal pain and discomfort tend to become insignificant (Nair *et al.*,2004; Danielsen, 1987; Naim, 1988; and Nichols, 1998).

1.7.2.5. Class 5: Thiazolidinediones

Molecular formula of 2, 4- Thiazolidinedione: $C_3H_3NO_2S$ (Figure 11); **Molecular weight:** 117.1g/mol

Examples: Rosiglitazone (Figure 12), pioglitazone, lobeglitazone, and troglitazone

Molecular structure:

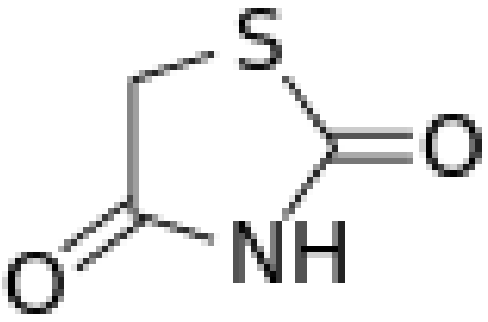


Figure 11: Molecular structure of Thiazolidinedione agents (Deruiter, 2003).

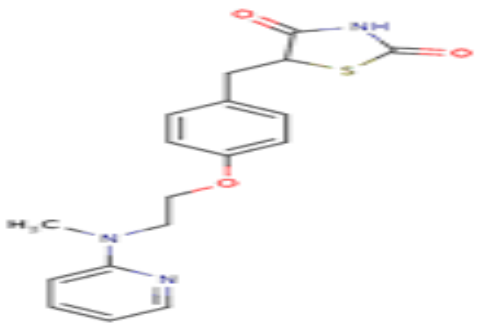


Figure 12: Rosiglitazone

Mechanism of action: Rosiglitazone acts as an agonist at peroxisome proliferator activated receptors (PPAR) in target tissues for insulin action such as adipose tissue,

skeletal muscle, and liver. Activation of PPAR-gamma receptors regulates the transcription of insulin-responsive genes involved in the control of glucose production, transport, and utilization. In this way, rosiglitazone enhances tissue sensitivity to insulin (Hamann *et al.*,1999; Gampe *et al.*,2000).

Concerns: The major side effects in the long-term usage include fluid retention, congestive heart failure (CHF), and liver disease (Sarraf *et al.*,1999; Hamann *et al.*,1999; Caira *et al.*,2000; Gampe *et al.*,2000). Rosiglitazone was frequently prescribed to patients in the past few decades; however, due to rising concerns regarding the drug's safety, this practise has since been discontinued.

1.7.2.5.1. Sodium glucose Cotransporter-2(SGLT2) inhibitors (Gliflozins or Flozins):

SGLT2 inhibitors such as canagliflozin, empagliflozin, and dapagliflozin improve elevated blood glucose levels by blocking glucose reabsorption in the proximal convoluted tubule of the kidney and by increasing glycosuria. Also, addresses other symptoms such as excretion of sodium in the urine i.e., early natriuresis, and promotes vascular health, reduces plasma volume, and improves high blood pressure (Cowie and Fisher, 2020).

Concerns: The common side effects are back ache, balanoposthitis, dizziness, dyslipidaemia, discontuous ketoacidosis, hypoglycaemia, urinary disorders, and infections. And uncommon sideffects includes, dry mouth, weight loss, constipation, genital pruritus, hypovolaemia, and vulvovaginal pruritus. Also, rare side effects include angioedema and Fournier’s gangrene (Cowie and Fisher, 2020).

1.7.2.6. Class 6: Drugs combinations

Example: Metformin and Rosiglitazone (Figure 13); **Molecular Formula:** $C_{26}H_{35}ClN_8O_7S$; **Molecular weight:** 639.1 g/mol

Mechanism of action: Rosiglitazone maleate is a member of the thiazolidinedione class of antidiabetic agents and is a PPAR-gamma agonist. It improves glycaemic control by improving insulin sensitivity while reducing circulating insulin levels. Metformin hydrochloride is a member of the biguanide class of antidiabetic agents. It decreases hepatic glucose production, reduces the intestinal absorption of glucose, and increases peripheral glucose uptake and utilization. Unlike sulfonylureas, metformin does not produce hypoglycaemia in either patients with type 2 diabetes or normal subjects and does

not cause hyperinsulinemia. With metformin therapy, insulin secretion remains unchanged, while fasting insulin levels and day-long plasma insulin response may decrease (Nawrocka and Starczewski, 2007).

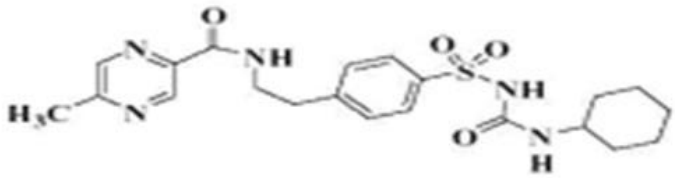
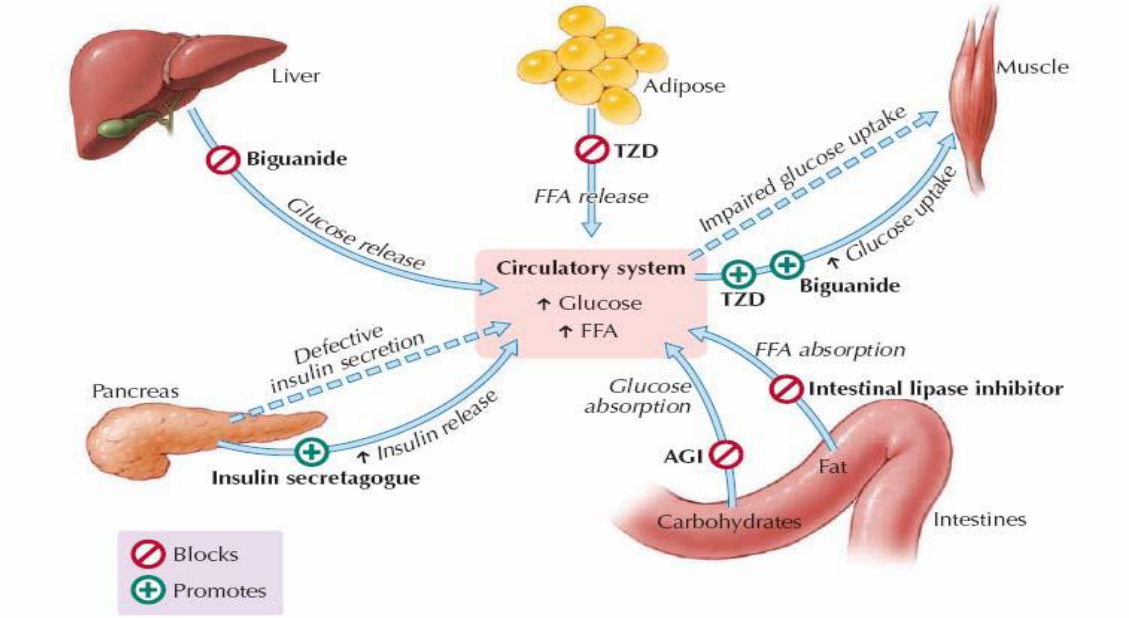


Figure 13: This structure is a combination of Rosiglitazone maleate and Metformin hydrochloride formed by exchanging hydrogen bonds.

Concerns: Drug combinations like metformin and rosglitazone can cause menstrual problems in women (Venkatesan, 2001).



TZD = thiazolidinedione; FFA = free fatty acid; AGI = α-glucosidase inhibitor

Figure 14: Major target organs and mechanism of actions of orally administered anti-hyperglycaemic agents in type 2 diabetes (Cheng, 2005).

1.8. The need for new and cost-effective treatments

As mentioned in the previous section, the Sulfonylureas class of drugs tend to cause hypoglycaemia and weight gain. Some secretogogues like repagilinide do not stimulate insulin secretion when there is normoglycaemia (Bell, 1998). Drugs like those of the meglitinides class cause common side effects like back pain, low blood sugar, and upper

respiratory infections (Hu, 2000). The Biguanides class of drugs, like metformin causes sideeffects such as gastrointestinal problems, nausea, cramps, diarrhoea, and lactose intolerance, and vitamin B-12 deficiency too can be seen over a period (Bailie, 1992). Alpha glucosidase inhibitors may make one prone to hepatic necrosis, inhibiting a digestive enzyme alpha-glucosidase which leads to digestive disorders and intestinal gas formation (Lebowitz, 2005). The major side effects with thiazolidinedioes are fluid retention, weight gain, and anaemia (Olefsky, 2000). Combinatorial agents such as the metformin and rosiglitazone duo cause menstrual disturbances in women (Venkatesan, 2001).

In the light of all these issues which are likely to cause a range of further health effects (Haq, 2004; Aronson, 2009), along with expensive manufacturing practices and price with current mainstream drugs, there is an urgent need to promote and investigate alternative antidiabetic herbal compounds, which are economical with fewer side effects (Grover, 2002), when compared to current mainstream antidiabetic agents. In the following section, an overview on antidiabetic herbal therapy is presented, and antidiabetic herbal compounds are explored and analysed in detail.

1.9. General overview on anti-diabetic herbal therapy

In the previous section, the mainstream treatments for type 2 diabetes and their adverse effects were explored in detail. In contrast, in this section, the efficacy of the individual herbs, and the synergistic effects of crude extracts and polyherbal formulations in treating diabetes are explored and analysed in detail. Although the ancient Indian medicine Ayurveda (AIM) and traditional Chinese medicine (TCM) are well known and practised in most of the developing nations, there are also other herbal therapies used by aboriginals in different parts of the world such as Africa, Australia, and the continent of North America (e.g., Cree herbal therapy prevalent among some native Canadian groups). However, though literature review has shown that the herbal compounds used in this research are covered and practiced in AIM, TCM and Western medical systems, suggesting an overlap between the systems, but predominantly used in Ayurveda (i.e., berberine and its relevant compounds, *gymnema* and *stevia*), this review focusses mainly on antidiabetic agents used in the Ayurveda.

Herb-based drug discovery is an ever-growing industry that is seriously engaged in large scale screening of therapeutic herbs to check their efficacy and pharmacological properties. Therapeutic herbs and their applications for the treatment of various diseases have predated recorded history. Most mainstream drugs have indeed originated from herbal sources. For instance, the popular oral anti-diabetic agent metformin is originally derived as galegine from the leaves of goat's rue (*Galega officinalis* L.), aspirin from willow (Salix) bark, digoxin from foxglove (Digitalis), morphine from opium poppy (*Papaver somniferum* L.) and quinine from cinchona (*Cinchona officinalis* L.) bark.

Mainstream drugs and herbal agents exhibit slight resemblances in their properties but differ in their therapeutical application and methodology (Vickers, Zollman, and Lee, 1999). Generally, herbal therapists use intact herbs, crude extracts or polyherbal formulations which are indeed a composition of multiple constituents, and claim that these extracts have a synergistic effect when compared to the isolated, precise, active principle of the herb, on the comprehensive well-being of the patient, and also claim that toxicity is reduced due to the buffering effect of the composition of multiple ingredients in crude extracts (Vickers, Zollman, and Lee, 1999).

The herbs used in Ayurveda are proven to provide symptomatic relief and prevent secondary complications of diabetes. Though mainstream treatment is effective in controlling the disease, it is expensive and has adverse effects. Ayurveda formulations

could regenerate beta cells in islets of Langerhans and overcome insulin resistance, while possessing antioxidant and cholesterol lowering properties (Hannan *et al.*,2007; Hannan *et al.*,2012; Shailey and Basir, 2012; Khosla *et al.*,2006; Singh and Gupta, 2007). The present review is focussed on characteristics of prominent anti-diabetic herbs and their hypoglycaemic activities that have shown proven cellular, pre-clinical, or clinical evidence. Polyherbal formulations from multiple herbal extracts are also discussed. Herbal compounds that demonstrate significant hypoglycaemic properties are emphasised and the mechanisms of the active constituents have been enlisted. The promising nature of herbs is discussed in contrast to mainstream therapy, and the reasons for a majority the herbs not being explored to their full potential will then be discussed. Therefore, it is important to highlight their potential and ascertain their therapeutic properties and benefits.

In recent years, people are increasingly resorting to medicinal herbs as an alternative to mainstream therapy due to the perception as it is a natural remedy for diabetes (Modak *et al.*,2007). However, only limited herbs have been researched and characterised, to validate their efficacy in comparison to that of contemporary synthetic counterparts. This review covers recent and updated research on the efficacy, side effects, and mechanisms of action of hypoglycaemic herbs in *in vitro*, *in vivo* and *ex vivo* systems.

1.9.1. Contemporary anti-diabetic drugs

In diabetes patients, either ß-cell disruption in pancreas will lead to a decrease in insulin secretion, or other cells in the body develop insulin resistance. The major stimulants for beta cell disruption are cytokines and gluco-lipo toxicity (Wang, Guan, and Young, 2007; Kim and Yoon, 2011). Contemporary anti-diabetic drugs tend to establish glucose homeostasis in two ways: by stimulating pancreatic beta cells as they promote insulin secretion, or by promoting glucose uptake by tissue cells by acting as insulin additives (Kokil *et al.*,2010). There are different types of oral anti-diabetic agents such as insulin secretagogues (e.g., sulfonylureases and meglitinides), insulin sensitizers such as Metformin (e.g., biguanides and thiazolidinediones) and α-glucosidase inhibitors (e.g., miglitol and acarbose). Injectable glucagon-like peptide analogs and agonists such as exenatide, liraglutide and DPP-4 inhibitors will encourage GLP-1 serum concentration and slowdown glucose absorption in the intestines (Krentz and Bailey, 2005; Anagnostis *et al.*,2011; Iltz *et al.*,2006; Neumiller, 2009; Scheen, 2011).

However, hypoglycaemic drugs have adverse side effects, such as sudden fall in blood sugar levels, lactic acidosis, idiosyncratic liver cell injury, neurological deficit, digestive

disorders, headache, giddiness, and even mortality in some cases (David, 2002). Application of external insulin does not always match the cell demands to function properly. Oral drugs moderately tend to correct limited ill effects caused by type 2 diabetes. They do not establish complete glucose homeostasis; and with long-term application, insulin resistance is observed. These disadvantages with current anti-diabetic drugs encourage patients to adapt to alternative therapies such as Ayurveda.

1.9.2. Ayurveda

Ayurveda is a Sanskrit word meaning knowledge: Veda translates to science, and Ayur means life. The wisdom of ancient Hindu saints and healers was orally transmitted for centuries, and finally Ayurvedic scriptures were preserved in Sanskrit and grew into a health science to promote a comprehensive health system rather than just curing ailments. There are eight main branches in Ayurveda: General Medicine (*Kaya chikitsa*), Paediatrics (*Kaumara bhruthya chikitsa*), Psychiatry (*Bhutha vidhya*), Otolaryngology (i.e., ENT), Ophthalmology and Dentistry (*Shalakya chikista*), Surgery (*Shalya chikitsa*), Toxicology (*Agada tantra*), Rejuvenation Therapy (*Rasayana chikitsa*), and Sexual Vitality (*Vajeekarana chikitsa*) (Chopra and Doiphode, 2002). The Ayurvedic scripture descended from "*Atharvaveda*", which was existed between 1000 and 1500 BC, and was described by a physician named Atreya and a surgeon, Dhanvantari. *Charaka samhita* (1000 BC), *Sushruta samhita* (100 AD) and *Bhela samhita* are principal, early texts in Ayurveda literature and practice. Ayurveda pharmacopeia contains comprehensive descriptions of over 1,500 herbs and 10,000 formulations. *Madhav nidan* (800 AD) is a diagnostic compilation that provides over 5,000 signs and symptoms of diseases (Mishra, Singh, and Dagenais, 2001).

1.9.2.1. Ayurveda treatment for diabetes

According to the Ayurveda philosophy, all living and non-living things that exist in nature comprise five basic elements: air, water, earth, fire, and space (ether). In human beings, these five elements exist in the form of three humors or *doshas*: the *vata* quality refers to the elements of space and air, *pitta* to fire and water, and *kapha* to water and earth. These three humors control biological, pathophysiological, and psychological functions of living beings—an imbalance in these humors is prone to illnesses. The Sanskrit name for diabetes is *madhumeha* (madhu means sweetness, and meha means excessive urination), and common therapy for diabetes begins with the assessment of humor imbalance (*dosha*).

Usually, in urinary disorders *kapha* is ineffective, whereas in diabetes *vata* is often intensified. The treatment would thus be focussed on to restoring harmony between *vata* and *pitta.*

Hypoglycaemic herbs are the key compounds in Ayurvedic formulations for treating diabetes. Polyherbal formulations are widely used in Ayurveda and would have synergistic effects on patients by providing comprehensive health benefits (Parasuraman, Thing, and Dhanaraj, 2014). In Ayurvedic herbal therapy, whole herbs or herbal mixtures are used with the perception that the remedy should work as a whole to cure the ailment. Ayurveda pharmacopeia did not suggest any form of isolation, separation, or purification of active compounds that should deliver the targeted result with a defined mechanism. The intact herb shows its efficacy when it is used in its natural form individually, or in herbal mixtures to create a stronger potency while all the parts of the herb are working collectively as a cure for the disease. This process is comparable to that of whole foods being processed by the human digestive system to absorb the required nutrients rather than consuming isolated nutrients and vitamins.

In general, there are many compounds that exist in each herb, and among them, only a few may be effective in the treatment of type 2 diabetes. Different extraction methods may yield different active ingredients, and different parts of plant produces different types of active ingredient profiles and at various concentrations. For instance, *Galega officinalis*, which is widely used to derive metformin, has high concentrations of galegine in its reproductive parts as compared to its stems and leaves (Oldham *et al.*,2011).

Ethno-botanical studies have identified and recorded more than 1,200 species of hypoglycaemic plants around the world, which have been distributed into 725 different classes. There are 20 prominent herbs used for the treatment of diabetes, and among them 17 are prescribed in the Ayurveda (the individual herbs are summarized below, identified using the botanical / Sanskrit / Hindi / family names) (Marles and Farnsworth, 1995).

1.9.3. Prominent anti-diabetic herbs used in Ayurveda

Most of the prominent anti-diabetic herbs are vegetables and spices, and a few are of wild origin. However, limited active constituents of these herbs have been well screened, characterised, and authenticated for their effectiveness based on a systematic cellular, animal, and clinical studies in order to be compared with mainstream drugs to demonstrate their potential anti-diabetic activity.

1.9.3.1. *Asparagus racemosus* L. (Liliaceae) / Shatavari

Classification: Kingdom: Plantae, **Clade:** Angiosperms, **Clade:** Monocots, **Order:** Asparagales, **Family:** Asparagaceae, **Subfamily:** Asparagoideae, **Genus:** Asparagus, **Species:** *A. racemosus*, **Binomial name**: *Asparagus racemosus* L.

Compounds of *Asparagus racemosus* root extracts (Figure 15) exert insulinotropic activity. In a study, lower doses of the *Asparagus* extracts such as ethanolic extract, hexane, chloroform, and ethyl acetate fractions of the *Asparagus racemosus* root promoted the lowering of elevated blood glucose levels. However, it did not enhance insulin secretion. Whereas high doses of the extract had a pronounced effect on insulin output in isolated, perfused rat pancreas, isolated rat islet cells, and clonal beta-cells (Hannan *et al.*,2007; Hannan *et al.*,2012).

Figure 15: *Asparagus racemosus*; the plant has an adventitious root system with tuberous roots; the root extracts are mainly used in Ayurveda, following a regimen of processing, and drying (Hannan *et al.*,2012).

1.9.3.2. *Azadirachta indica* L. (Meliaceae) / Arishtha / Neem

Classification: Kingdom: Plantae, **Clade:** Angiosperms, **Clade**: Eudicots, **Clade:** Rosids, **Order:** Sapindales, **Family:** Meliaceae, **Genus:** Azadirachta, **Species:** *A. indica,* **Binomial name:** *Azadirachta indica* L.

Azadirachta indica A. Juss, is commonly known as the Indian lilac tree. Studies have shown that its aqueous extracts encourage antioxidant protection in alloxan-induced diabetic rats (Shailey and Basir, 2012). Leaf extracts and seed oil (Figure 16) counteracted the high levels of glucose concentration in the blood and increased the peripheral glucose utilisation in normal and diabetic rabbits, and this effect is similar to that of glibenclamide (Khosla *et al.*,2000). Leaf extracts have been demonstrated to be ulcer protective and produce healing effects in normal and diabetic rats. The mechanism was studied on a number of parameters, including offensive acid-pepsin secretion in four-hour pylorus ligation, pentagastrin (PENTA, 5 μg/kg/hr)-stimulated acid secretion and gastric mucosal proton pump activity, and defensive mucin secretion including the life span of gastric mucosal cells in rats (Dorababu *et al.*,2006). The leaf extract has been observed to produce anti-hyperglycaemic activity in streptozotocin diabetic rats without altered serum cortisol level (Gholap and Kar, 2004). Meliacinolin is a derivative of neem which behaves as an insulin sensitizer, and improves renal function, lipid profile, and antioxidant activity. As diabetes is a multifactorial disease, meliacinolin may be interacting with multiple targets involved in diabetes pathogenesis. α-Glucosidase and α-amylase inhibitors lower the levels of post-prandial hyperglycaemia and inhibit the absorption of carbohydrates (Gutierrez and Guzman, 2012).

A polyherbal formulation known as Dianex, made from the aqueous extracts of *Azadirachta indica, Gymnema sylvestre, Eugenia jambolana, Momordica charantia, Cassia auriculata, Aegle marmelose, Withania somnifera,* and *Curcuma longa,* was screened for hypoglycemic activity in normal and streptozotocin-induced diabetic mice. Dianex was well tolerated in experimental mice at higher doses (up to 10 g/kg in mice, acute toxicity up to 2.5 g/kg in rats, sub-acute toxicity studies for 30 days), and did not show any toxic manifestation (Mutalik *et al.*,2005).

Beta-sitosterol is a steroid obtained from *Azadirachta indica,* which may be source of its hypoglycaemic activity (Mukherjee *et al.*,2006). In a clinical study of patients with type 2 diabetics, it was observed that the consumption of the powdered part, aqueous extract, and

alcoholic extract of *Azadirachta indica* at high doses for 14 days resulted in hypoglycaemic activity (Waheed, Miana, and Ahmad, 2006).

Figure 16: ***Azadiracta Indica*** leaves, fruits (seeded drupes), and seeds, bark and oil are used in Ayurveda, but mainly leaf extracts are used in studies to check its anti-diabetic and other pharmacological properties (Satyanarayana *et al.*,2015).

1.9.3.3. *Berberis aristata* L. (Berberidaceae) / Daruharidra

Classification: Kingdom: Plantae, (Unranked): Angiosperms, (Unranked): Eudicots, **Order:** Ranunculales, **Family:** Berberidaceae, **Genus:** Berberis, **Species:** *B. aristata*, **Binomial name:** *Berberis aristata* L.

The plant is predominantly found in the Himalayas (Figure 17). The principle constituents are alkaloids such as berberine, berbamine, aromoline, karachine, palmatine, oxycanthine, and oxyberberine (Chakravarthi, Dhar, and Siddiqui, 1950; Rahman and Ansari, 1983; Chatterjee, 1951). A study shows that prominent constituents like berberine encourage glucose-stimulated insulin secretion rather than basal insulin secretion in a dose-dependent fashion in rat's pancreatic islets (Ko *et al.*,2005).

It can improve glucose-stimulated insulin secretion in rat islets, and possibly exerts the insulinotropic effect aided by hepatic nuclear factor 4 alpha (HNF4) and glucokinase, which is different from sulphonylureas (Wang *et al.*,2008). To stimulate glucose uptake activity, application of 50 µM berberine along with 0.2 nM insulin in 3T3-L1 adipocytes have shown that the level of insulin increased by 10 nM, which pronounced the efficacy of

berberine. It increased glucose transporter-4 translocation into the plasma membrane by enhancing insulin signalling pathways and the insulin receptor substrate-1-phosphoinositide 3 Kinase-Akt. Application of berberine in Min6 cells improved insulin secretion and proliferation with the help of enhanced insulin / insulin-like growth factor-1 signalling cascade. These observations were proving the efficacy of berberine as an insulin sensitizing and insulinotropic agent (Ko *et al.*,2005). In addition, it is shown that berberine, when compared to other phytochemicals like phloretin, ouabain, and metformin, demonstrates remarkable non-insulin mediated glucose uptake activity in human hepatoma cells (Kandunuri and White, 2012). It has been noted that berberine has more glucose uptake activity than metformin.

Figure 17: ***Berberis aristata***'s stem, roots, and fruits are used in Ayurveda, the root bark contains the bitter alkaloid berberine, which has been studied for its antidiabetic and other pharmacological properties (Potdar, Hirwani, and Dhulap, 2012).

1.9.3.4. *Capparis decidua* L. (Capparaceae) / Kareera

Classification: Kingdom: Plantae, **Clade**: Angiosperms, **Clade:** Eudicots, **Clade:** Rosids, **Order:** Brassicales, **Family:** Capparaceae, **Genus:** Capparis, **Species:** *C. decidua*, **Binomial name:** *Capparis decidua* L.

This plant can be found throughout India, especially in dry conditions (Figure 18). The hypoglycaemic effect of the herb was seen in alloxanized rats fed with 30 percent extracts of *Capparis decidua* fruit powder for a duration of three weeks. This extract also reduced alloxan-induced lipid peroxidation significantly in erythrocytes, kidney, and heart. *C.*

decidua was also found to alter superoxide dismutase and catalase enzyme levels to reduce oxidative stress (Yadav, Sarkar, and Bhatnagar, 1997). It also exhibited hypolipidaemic activity (Agarwal and Chauhan, 1988).

Figure 18: ***Capparis decidua*** tree with fruits; its fruits, roots, and bark are used to concoct different medicines in Ayurveda (Sharma *et al.*,2010).

1.9.3.5. *Cinnamon zeylaniucm* L. (Lauraceae) / Tvak / Dalchini

Classification: Kingdom: Plantae, **Clade:** Angiosperms, **Clade:** Magnoliids, **Order:** Laurales, **Family:** Lauraceae, **Genus:** Cinnamomum, **Species:** *C. Zeylaniucm*, **Binomial name:** *Cinnamomum Zeylaniucm* L.

Cinnamon (Figure 19) has an insulinotropic effect which helps in storing glucose in the form of glycogen. In a study using rats, cinnamon caused an increase in a compound IRS-1, which helps in increasing glucose uptake in muscle tissues. It is also known to increase the transporter mechanisms (GLUT-4) that improve glucose uptake from the bloodstream and into tissues (Anand *et al.*,2010; Qin *et al.*,2009).

The effects of cinnamon and diabetes were published in the journal *Diabetes Care* in 2003 by Khan and his colleagues. This study on type 2 diabetes was conducted with 60 people around the age of 50. The patients were divided into six groups of 10 patients, and Groups 1 through 3 were treated with 1, 3, or 6 grams of cinnamon daily, while Groups 4 through 6 were given placebo. The treatment was conducted for 40 days. The researchers had analysed fasting glucose, LDL cholesterol, triglycerides, and total cholesterol. The results revealed that no significant changes were observed in the placebo group over the 40-day period. While in the cinnamon groups, there were reductions in the levels of fasting

glucose (reduced by 18 percent, to 29 percent), triglycerides (reduced by 23 percent, to 30 percent), LDL cholesterol (reduced by 7 percent, to 27 percent), and total cholesterol (reduced by 12 percent, to 26 percent). Also, high-dose, long-term consumption of cinnamon was found to be safe. A similar study was performed in Germany where 79 patients with type 2 diabetes were examined. In this study, half the patients received placebo the rest received 3 grams of cinnamon daily for a duration of four months. In this study, no difference between the two groups was reported for LDL or HDL cholesterol, triglycerides or HgbA1c, but fasting glucose levels dropped by about 7 percent more in the group receiving cinnamon (Mang *et al.*,2006). In another study, 25 post-menopausal women with type 2 diabetes were treated with 1.5 grams of cinnamon daily for six weeks. The results indicated that cinnamon was not associated with a significant change in insulin sensitivity, glucose tolerance, or cholesterol profile. In a similar manner, another study concluded that cinnamon did not improve HgbA1c, fasting glucose, or blood lipids in patients with either type 1 or type 2 diabetes (Vanschoonbeek, 2006).

There is evidence that cinnamon extracts contain components that improve insulin action; however, the effects of cinnamon on non-insulin stimulated glucose uptake require further investigation. A study reported the effects of cinnamaldehyde on the glucose transport activity of glucose transporter-1 (GLUT-1) in L929 fibroblast cells under both basal conditions and conditions where in glucose uptake is activated by glucose deprivation. The result concluded that cinnamaldehyde has a dual action on the glucose transport activity of GLUT-1. Under basal conditions, it stimulates glucose uptake and reaches a 3.5-fold maximum stimulation at 2.0 mM. However, cinnamaldehyde also inhibits the activation of glucose uptake by glucose deprivation in a dose-dependent manner. Further to these experiments with cinnamaldehyde, analogs have shown that these activities are fully dependent on α, β-unsaturated aldehyde structural motif in cinnamaldehyde. The inhibitory, but not the stimulatory activity, of cinnamaldehyde was maintained after a wash-recovery period. Pre-treating cinnamaldehyde with thiol-containing compounds, such as β-mercaptoethanol or cysteine, blocks the inhibitory activity of cinnamaldehyde. The results suggest that cinnamaldehyde inhibits the activation of GLUT-1 by forming a covalent link to target cysteine residue(s). This dual activity suggests that cinnamaldehyde is not a major contributor to the anti-diabetic properties of cinnamon (Plaisier *et al.*,2011).

Figure 19: ***Cinnamon zeylaniucm L.*** Bark, dried flowers, and powder, and other parts such as leaves, flowers, fruits, and roots, have medicinal properties which are used in Ayurveda and Traditional Chinese Medicine (Ranasinghe *et al.*,2012).

1.9.3.6. *Cocconia indica* L. (Cucurbitaceae) / Bimbi / Tundika / Kundru

Classification: Kingdom: Plantae, **Division:** Magnoliophyta, **Class:** Magnoliopsida, **Order:** Cucurbitales, **Family:** Cucurbitaceae, **Genus:** Coccinia, **Species:** *C. indica*, **Binomial name:** *Cocconia Indica* L.

Clinical study has shown that by consuming dried extract of about 500 mg/kg of *Coccinia indica* (Figure 20) for as long as six weeks would significantly increase insulin concentration. In a similar manner, consumption of the plant extract also proved to be beneficial in animal subjects due to insulin secretion or influence of enzymes (Kamble *et al.*,1998).

Figure 20: ***Cocconia Indica*** flowers and fruits; It's a creeper, its young shoots and fruits have culinary and medicinal uses; Studies revealed that compounds in

the plant have glycemic control activity, and involves in glucose metabolism (Kaushik *et al.*,2017).

1.9.3.7. *Emblica officinalis* L. (Euphorbiaceae) / Amla

Classification: Kingdom: Plantae, **Division:** Angiospermae, **Class:** Dicotyledonae, **Order:** Geraniales, **Family:** Euphorbiaceae, **Genus:** Emblica, **Species:** *officinalis Geartn*, **Synonym:** Phyllanthus emblica Linn, **Binomial name**: *Emblica Officinalis* L.

Amla tree (Figure 21) is commonly found in the mixed deciduous forests of Indian subcontinent; it is a rich source of antioxidants and deeply nourishes the tissues. It is found to be a potent rejuvenator that promotes tissue health by detoxifying them. It also supports the proper function of digestive acids. The active constituents of Amla extracts stimulate insulin secretion, improve insulin action, inhibit protein glycation, and starch digestion (Nain *et al.*,2012).

Figure 21: *Emblica officinalis* leaves and fruits (Berrys); every part of the plant has therapeutic value, but fruits are the most widely used. The polyphenols such as tannins and flavonoids found in the Berrys exert major bioactivities (Yadav *et al.*,2017).

1.9.3.8. *Gymnema sylvestre* L. (Asclepiadaceae) / Madhunashini / Gurmar

Classification: Kingdom: Plantae, **(Unranked):** Angiosperms, **(Unranked):** Eudicots, **(Unranked):** Asterids, **Order**: Gentianales, **Family:** Apocynaceae, **Subfamily:** Asclepiadoideae, **Genus:** *Gymnema*, **Species:** *G. sylvestre*, **Binomial name:** *Gymnema sylvestre* L.

Gymnema sylvestre (Figure 22) is one of the most sought-after herbs for the treatment of diabetes. The leaves are used for preparing the herbal medicine. The early documentation of hypoglycaemic action of *Gymnema* dates to late 1920s. The crude extracts and dihydroxy gymnemic triacetate (isolated compound) show hypoglycaemic effects in diabetic rats induced with streptozotocin (Daisy, Eliza, and Farook, 2009). This effect is induced by gymnemic acids that can delay glucose intake into the bloodstream. The sugar molecule absorption by the intestine is prevented since gymnemic acid molecules fill the receptors located in the absorptive external layers of the intestine. The crude extract which contains dihydroxy gymnemic helps release insulin by stimulating the regeneration of the remaining beta cells (Kanetkar, Singhal, and Kamat, 2007).

Studies have also shown that aqueous extracts of *Gymnema sylvestre* leaves can stimulate insulin secretion in mice cells and isolated human islets *in vitro* (Al-Romaiyan *et al.*,2012). They also revealed that the plasma glucose level and beta cell mass and insulin levels, when measured in the healthy rats with a dose of dihydroxy gymnemic triacetate were not changed, further suggesting that it is a normoglycaemic compound. Another study indicates that hydroxy gymnemic triacetate, isolated from *Gymnema sylvestre*, signifies a promising contender for alternative medicine in the management of diabetes mellitus, since it lowers blood glucose levels and biochemical parameters in STZ induced diabetic animals (Daisy, Eliza, and Farook, 2009). Extensive clinical trials on human subjects are required for further exploring the prospects of the compound being used as a drug.

Gurmar powder, which is prepared from *Gymnema sylvestre* leaves, is one of the main Ayurvedic products used for the treatment of diabetes. It correlates the metabolic activities of liver, kidney, and muscle tissues, stimulates insulin secretion, and exerts a blood glucose lowering effect. It also inhibits the intestinal absorption of sugars, thereby preventing blood sugar variations and it is also a diuretic and cardiac stimulant. It is also a sugar buster, which when taken prevents the taste buds from absorbing sugar molecules which discourages glycosuria (Paliwal, Kathori, and Upadhyay, 2009; Modak *et al.*,2007).

Figure 22: ***Gymnema sylvestre*** is a woody, climbing plant, commonly found in the tropical forests of central and southern India. The leaves are used for medicinal applications, i.e., chewing the leaves destroys the sweet tasting ability, thus it commonly called as "gurmar" in Hindi, which means "sugar destroyer" (Paliwal, Kathori, and Upadhyay, 2009; Modak *et al.*,2007).

1.9.3.9. *Momordica Charntia* L. (Cucurbitaceae) / Karavella / Karela

Classification: Kingdom: Plantae, **(unranked):** Angiosperms, **(unranked):** Eudicots, **(unranked):** Rosids, **Order:** Cucurbitales, **Family:** Cucurbitaceae, **Genus:** Momordica, **Species:** *M. charantia***, Binomial name:** *Momordica charantia* L.

Studies have shown that the application of *Momordica charantia* (Figure 23) extract causes substantial lowering of peripheral blood glucose and elevates the level of plasma insulin in diabetic rats. This effect was due to the regeneration of pancreatic beta cells in treated rats. Known anti-diabetic phytochemicals such as momordicin, charantin, and insulin-like protein galactose binding lectin were isolated from different parts of the plant and the separated compounds proved to possess insulin-mimetic activity (Raman and Lau, 1996). Aqueous extracts of unripe green fruits, partially displaying insulin release activity in isolated beta cells of obese diabetic mice, indicated that the insulin stimulation and release action is the effect of perturbations of beta cell membranes (Ahmed *et al.*,1998; Abdollahi *et al.*,2011). *Momordica charantia* promotes the renewal of partial beta cells in the pancreas or promotes the recovery of partially damaged beta cells, and further stimulates insulin secretion (Hafizur, Kabir, and Chishti, 2011). Due to its intrinsic nature of pancreatic beta cell regeneration, physicians are prescribing active constituents of this herb as an adjuvant for type 2 diabetes patients (Singh and Gupta, 2007; Nagy, Bastawy and Abdel-Hamid, 2012).

Bitter gourd powder plays an important role in Ayurveda for the treatment of diabetes. It contains principles like bitter glycosides, saponins, alkaloids, reducing sugars, phenolics, oils, free acids, polypeptides, sterols, 17amino acids including methionine, and a crystalline product named p-insulin. It is documented for its hypoglycaemic activity along with antihaemorrhoidal, astringent, stomachic, emmenagogue, hepatic stimulant, anthelmintic, and blood purifier activities. It promotes immunity against general infections and helps in the lowering of blood and urine sugar levels (Grover and Yadav, 2004).

Figure 23: ***Momordica Charntia*** **L.** leaves and un-ripened fruits, which are oblong in shape with a pockmarked and warty exterior, and a hollow cross section filled with medium to large flat seeds and pith. The whole fruit, seeds, leaves, and vines have been used in Ayurveda and Traditional Chinese Medicine for centuries (Chaturvedi, 2012).

1.9.3.10. *Pterocarpus marsupium* L. (Fabaceae) / Vijayasar

Classification: Kingdom: Plantae, **(unranked):** Angiosperms, **(unranked):** Eudicots, **(unranked):** Rosids, **Order:** Fabales, **Family:** Fabaceae, **Subfamily:** Faboideae, **Tribe:** Dalbergieae, **Genus:** Pterocarpus, **Species:** *P. marsupium*, **Binomial name:** *Pterocarpus marsupium* L.

Active principles like epicatechin, pterosupin, marsupin, and pterostilbene would promote insulin sensitivity (Grover, Vats and Yadav, 2005). It is evident that the presence of tannates in the extract is the main factor for anti-diabetic activity. Pancreatic beta cell regeneration has been observed after the application of flavonoid fractions (Chakravarty *et al.*,1980). Active compounds like marsupin, pterosupin, and liquiritigenin demonstrated antihyperlipidemic activity (Jahromi and Ray, 1993). Epicatechin promotes insulin secretion, insulin release and adaptation of proinsulin to insulin in *in vitro* experiments.

Epicatechin increases glycogen contents of a rat's diaphragm in a dose-dependent manner and encourages oxygen uptake in adipose cells and tissues of different organs by its insulin-mimicking nature (Ahmad *et al.*,1989).

In a multi-centre clinical trial, Vijayasar was studied for its glucose lowering effects, and to determine adverse side effects compared with a standard pharmacological agent tolbutamide, for the treatment of type 2 diabetes. It is concluded that Vijayasar (Figure 24) is as effective as tolbutamide in the management of type 2 diabetes without any adverse side effects (ICMR study group, 2005).

Figure 24: ***Pterocarpus marsupium*** leaves and round-kidney shaped pods; these are broadly winged and hold one seed which is kidney-shaped; The crude extracts of Vijayasar displayed marked improvement on oral glucose tolerance post sucrose load in normal rats, except aqueous fraction, all other extracts showed improvement on oral glucose tolerance post sucrose load on streptozotocin (STZ)-induced diabetic rats (Mishra *et al.*,2013).

1.9.3.11. *Plantago psyllium* L. (Plantaginaceae) / *ovata* Isabgol

Classification: Kingdom: Plantae, **Clade:** Angiosperms, **Clade:** Eudicots, **Clade:** Asterids, **Order:** Lamiales, **Family:** Plantaginaceae, **Genus:** Plantago, **Species:** *P. ovata*, **Binomial name:** *Plantago ovata* L.

Psyllium husk is obtained from plants in the genus Plantago, and it is mainly cultivated in India (Figure 25). It is commonly used as a food and nutritional supplement due to its high quantities of both soluble and insoluble fibre. Research has proven a variety of benefits of

psyllium, such as lipid control (Rudkowska, 2012) and cholesterol-lowering in type 2 diabetes patients (Uehlecke, Ortiz, and Stange, 2008). Researchers have published works that show that fiber deficiency may be a common contributor to both diabetes and coronary artery diseases (Wallstrom *et al.*,2012). For patients with elevated cholesterol level, which is a common condition in type 2 diabetic patients, *psyllium*, which contains high levels of both soluble and insoluble fibre, offers cholesterol-lowering benefits. It also has a less known side effect such as promoting insulin resistance, and is relatively cheaper (Grover *et al.*,2011). A dosage of 10 to 20 g of *psyllium* is recommended for daily consumption (Moreyra, Wilson, and Koraym, 2005). Researchers at the department of paediatrics, University of Zaragoza, Spain, have revealed that children and adolescents benefit from *psyllium*'s blood sugar and cholesterol-lowering effects (Moreno *et al.*,2003). A study conducted with type 2 diabetes patients showed that supplementing *psyllium* to regular meal might be an added benefit for those people who are suffering from elevated post-prandial glucose concentrations even after taking regular antidiabetic medications (Bajorek and Morello, 2010).

Figure 25: *Plantago psyllium* L. plant with numerous, tiny, white, flowering shoots. Tiny capsules hold the seeds that open when the plant is mature, the husk is used in culinary and medical purposes as it contains soluble and insoluble fibre, and it has displayed lipid control and cholesterol-lowering in type 2 diabetes patients (Rudkowska, 2012 ; Uehlecke, Ortiz, and Stange, 2008).

1.9.3.12. *Syzygium cumini* L. (Rutaceae) / Jambu / Badijamun

Classification: Kingdom: Plantae, **Clade:** Angiosperms, **Clade:** Eudicots, **Clade:** Rosids, **Order:** Myrtales, **Family:** Myrtaceae, **Genus:** Syzygium, **Species:** *S. cumini*, **Binomial name:** *Syzygium cumini* L.

Syzygium cumini (Figure 26) is used extensively as a traditional medicine to treat diabetes in India. Studies have been carried out to isolate and identify the putative anti-diabetic compound from the *S. cumini* seed, and mycaminose is a compound isolated from the seed extract. Mycaminose (50 mg/kg), ethyl acetate, and methanol are compounds extracted from the *S. cumini* seed (200 and 400 mg/kg), which were used to evaluate the anti-diabetic activity against streptozotocin (STZ)-induced diabetic rates. The results showed significant ($p<0.05$) reduction in blood glucose levels. The standard drug, Glibenclamide (1.25 mg/kg) also produced a significant ($p<0.05$) reduction in blood glucose levels against STZ-induced diabetic rats. Bark extract stimulates the development of insulin-positive cells from the pancreatic duct epithelial cells (Kumar *et al.*,2008).

Figure 26: *Syzygium cumini* leaves and fruits; It's an evergreen tree and all parts of the tree are used in the Ayurveda. The tree bears fruits during the summer season and is of both dietary and medicinal use i.e., bark decoction is used in treating diabetes mellitus in Ayurveda (Perera, Ekanayake and Ranaweera, 2017).

1.9.3.13. *Strychonos nux-vomica* L. (Loganiaceae) / Vishamushti / Kulcha

Classification: Kingdom: Plantae, **Clade:** Angiosperms, **Clade:** Eudicots, **Clade:** Asterids, **Order:** Gentianales, **Family:** Loganiaceae, **Genus:** Strychnos, **Species:** *S. nux-vomica*, **Binomial name:** *Strychnos nux-vomica* L.

Strychnos nux-vomica (Figure 27) contains strychnine and brucine as its main compounds. It also contains minor alkaloids such as protostrychnine, vomicine, n-oxystrychnine, pseudostrychnine, isostrychnine, chlorogenic acid, and a glycoside. The extract also produces a significant increase in super oxide dismutase (SOD), catalase and total protein (TP) levels and a decrease in lipid peroxidation (LPO), total cholesterol, serum creatinine and blood urea nitrogen (BUN) levels in alloxan-induced diabetic rats, which proves antioxidant characteristics of the extract. The seeds are used to treat diabetes, asthma, and reduced sexual activity, and to improve appetite. In a study where extracts of hydroalcoholic, aqueous extracts were evaluated for their antidiabetic activity based on extractive yield and phytoconstituents, in alloxan-induced diabetic rats using gliclazide as standard, and *S. nux-vomica* extracts show anti-hyperglycaemic activity in these animals. The antidiabetic activity of the methanolic extract of *Strychnos nuxvomica* evaluated in normal and alloxan-induced diabetic rats showed increased body weight and decreased blood glucose levels, proving that the extract exhibited significant anti-diabetic activity. The results also indicated a dose-dependent effect. The antidiabetic activity may be due to the increased uptake of glucose by the tissue, or by an increase in pancreatic beta cell function, or the inhibition of glucose uptake by the intestine (Bhati *et al.*,2012).

Figure 27: ***Strychonos nux-vomica*** tree with medium ripened fruits; It is a medium-sized tree and leaves are ovate, It is a major source of alkaloids strychnine and brucine, derived from the seeds inside the round, green to orange fruit and bark also contains brucine and other toxic compounds, also seeds contain minor alkaloids such as, protostrychnine, vomicine, n-oxystrychnine, pseudostrychnine, isostrychnine, chlorogenic acid, and a glycoside. Thus, seeds used traditionally to treat diabetes, asthma, aphrodisiac and to improve appetite (Bhati *et al.*,2012).

1.9.3.14. *Tinospora cordifolia* L. (Menispermaceae) / Guduchi / Gurcha

Classification: Kingdom: Plantae, **Clade:** Angiosperms, **Clade:** Eudicots, **Order:** Ranunculales, **Family:** Menispermaceae, **Genus:** Tinospora, **Species:** *T. cordifolia*, **Binomial name:** *Tinospora cordifolia* L.

Guduchi (Figure 28) is a widely used in Indian medicine for treating diabetes mellitus. It is a large, glabrous, deciduous climbing shrub. The primary constituents of the herbs are aretinosporine, tinosporide, tinosporaside, cordifolide, cordifol, heptacosanol, clerodanefurano diterpene, diterpenoid furanolactone tinosporidine, columbine and beta sitosterol (Upadhyay *et al.*,2010).

It is documented that the daily consumption of either alcoholic or aqueous extract of *T. cordifolia* decreases the blood glucose level and increases glucose tolerance in rodents. The oral administration of root extracts of Guduchi for about six weeks in alloxan-induced diabetic rats caused a decrease in glucose levels in blood and urine, and a decrease of lipids

in serum, brain, and in tissues, as well as an increase in body weight. The aqueous extract at a dose of 400 mg/kg could cause significant anti-hyperglycaemic effect in animal models, its effect was equivalent to only one unit/kg of insulin (Prince, Menon, and Gunasekaran, 1999; Sinha *et al.*,2004).

Figure 28: ***Tinospora cordifolia*** is a large, deciduous extensively spreading climbing shrub with several elongated twining branches and alternate simple leaves, the leaves are mainly used for medicinal purposes, and every other part of the plant also used in various medications in Ayurveda (Prince, Menon, and Gunasekaran, 1999; Sinha *et al.*,2004).

1.9.3.15. *Trigonella foenum-graecum* L. (Fabaceae) / Medhika / Chandrika / Fenugreek

Classification: Kingdom: Plantae, **Clade:** Angiosperms, **Clade:** Eudicots, **Clade:** Rosids, **Order:** Fabales, **Family:** Fabaceae, **Genus:** Trigonella, **Species:** *T. foenum-graecum*, **Binomial name:** *Trigonella foenum-graecum* L.

4-Hydroxyisoleucine forms almost 80 percent of the free amino acids in fenugreek seeds (Figure 29), and exhibits insulin-stimulating characteristics (Broca *et al.*,1999). The prominent isomer of 4-hydroxyisoleucine, which is a typical branched-chain amino acid, that derived from fenugreek seeds, is responsible for the effects on glucose and lipid metabolism in experimental rats and proves to increase glucose stimulated insulin release by isolated islet cells in rats, mice and humans (Grover, Yadav, and Vats, 2002; Modak *et al.*,2007; Broca *et al.*,1999; Haeri *et al.*,2012). *In vitro* and *in vivo* studies have shown that 4-hydroxyisoleucine promotes glucose-induced insulin release (Saxena and Vikram, 2004).

The fibre content in seeds may encourage insulin sensitivity by slowing down carbohydrate metabolism, which leads to reduced insulin levels and lowered blood glucose. The anti-hyperglycaemic effect of the seed extracts, powder, gum of fenugreek seeds, and its leaves, are related to slow gastric emptying because of its high fibre content and the inhibition of carbohydrate digestive enzymes and stimulation of insulin secretion (Chauhan *et al.*,2010). Syndrex is an Ayurvedic product used for the treatment of diabetes, in which the main ingredient is germinated fenugreek extract. It exerts activities like lowering blood sugar in people with diabetes. It promotes slow absorption of sugars in the intestines and stimulates insulin (Kaczmar, 1998).

Figure 29: *Trigonella foenum-graecum* L. leaves and seeds are used in culinary purposes and crude extracts and isolated compounds such as 4-Hydroxyisoleucine used in research and thereupetic purposes (Haeri *et al.*,2009; Haeri *et al.*,2011).

1.9.3.16. *Catharanthus roseus* L. (Apocynaceae) / Nitya kalyani / Sada bahar

Classification: Kingdom: Plantae, **Clade:** Angiosperms, **Clade:** Eudicots, **Clade:** Asterids, **Order:** Gentianales, **Family:** Apocynaceae, **Genus:** Catharanthus, **Species:** *C. roseus*, **Binomial name:** *Catharanthus roseus* L.

A pre-clinical study revealed that the administration of methanolic extracts of *Vinca rosea* (Figure 30) in alloxan-induced diabetic rats for 20 days and the methanolic extract at a high dose (500 mg/kg) exerted significant anti-hyperglycaemic activity, increased body weight, urea, and cholesterol levels of the experimental rodents. Hepatic hexokinase enzymes were significantly ($p<0.01$) increased and glucose 6- phosphatase and fructose 1, 6- bisphosphatase were significantly ($p<0.05$) decreased by the application of

Catharanthus roseus leaf in diabetic rats when compared to normal rats (Jayanthi *et al.*,2010).

Figure 30: ***Catharanthus roseus*** flowers and leaves; In Ayurveda the extracts of its roots and shoots, are used. In Traditional Chinese Medicine, extracts from it have been used to treat diabetes and other diseases (Tiong *et al.*,2013).

1.9.3.17. *Withania Somnifera* L. (Solanaceae) / Ashwagandha

Classification: Kingdom: Plantae, **Clade:** Angiosperms, **Clade:** Eudicots, **Clade:** Asterids, **Order:** Solanales, **Family:** Solanaceae, **Genus:** Withania, **Species:** *W. somnifera*, **Binomial name:** *Withania somnifera L.*

Researchers have reported that *Withania somnifera* (Figure 31) improves insulin sensitivity in an experimental model of type 2 diabetes, where type 2 diabetes rats were fed with 200 to 400 mg/kg of *Withania somnifera* once a day for five weeks. The results have showed significant increase in blood glucose, glycosylated haemoglobin (HbA1c) and serum insulin levels in the controlled group of rats. HbA1c is an indicator of blood glucose regulation and it is normal when blood glucose levels are carefully regulated. *Withania somnifera*-fed rats showed elevated levels of blood glucose, HbA1c while insulin was reduced. In the same animal model, an oral glucose tolerance test showed significant improvement in glucose tolerance in the rats that were fed *Withania somnifera*. In this study, the control rats (rats not given *Withania somnifera*) experienced a decrease in insulin sensitivity. However, diabetic rats given *Withania somnifera* experienced a significant improvement in the insulin sensitivity index. The study concludes that an aqueous extract of *Withania somnifera* normalizes hyperglycaemia in non-insulin-dependent diabetes mellitus rats by improving insulin sensitivity (Anwer *et al.*,2008).

Figure 31: ***Withania somnifera*** fruits and leaves, it's root and leaf extracts are used to treat diabetes (Gorelick *et al.*,2015).

1.9.4. Polyherbal formulations

Combination of multiple extracts of antidiabetic herbs is used as a single drug of choice rather than an individual herb these are commonly known as polyhedral formulation. Some of the well-known polyherbal formulations are Ayush-82, D-400, Diabecon, Dianex, Epiinsulin, Pancreatic tonic, Diacare and Diabeta.

The characteristics of the individual formulations are given below. Since these formulations are commercial products, the actual ingredients that makes the formulations (i.e., percentage of combination of each herb) remains unknown due to trade secrets, and a limited number of formulations are available to the public.

1.9.4.1. Ayush-82 and D-400

Ayush-82 is a blend of four herbs: *Syzygium cuminii*, *Momordica charantia*, leaves of *Gymnema sylvestre*, and the seeds of *Mangifera indica* (Chowdhary and Dua, 1998; Pand ey, Rajagopalan, and Chowdhary, 1995). Whereas D-400 is a combination of *Pterocarpus marsupium, Ficus glomerulata, Gymnema sylvestre, Momordica charantia, Ocimum sanctum, Eugenia jambolana* and *shilajit* (Maji and Singh, 1995).

These herbal drugs exercise glucose-lowering effects, and these are prominent polyherbal formulations in Ayurveda. Further study is needed to consolidate their effectiveness.

1.9.4.2. Abraga Chendooram

It is a combination of *abragum* (purified black mica, 80 g), *vengaram* (dehydrated borax, 0.5 g), *Saranaiver charu* (juice of root of *Trianthema decandra Linn*), *Adathodaielai charu* (juice from the leaves of *Adhatoda zeylanica Linn*), *Alam Vizhuthu*, and *Kudineer* (root of *Ficus benghalensis Linn*). During studies, it has shown hypoglycaemic activity and provides an insight for further investigations (Sankar and Aggarwal, 1998; Shankar and Singhal, 1995).

1.9.4.3. Diabecon

It is a combination of *Gymnema sylvestre, Pterocarpus marsupium, Glycyrrhiza glabra, Casearia esculenta, Syzygium cumini, Asparagus racemosus, Boerhavia diffusa, Sphaeranthus indicus, Tinospora cordifolia, Swertia chirata, Tribulus terrestris, Phyllanthus amarus, Gmelina arborea, Gossypium herbaceum, Berberis aristata, Aloevera, Triphala, Commiphora wightii, shilajit, Momordica charantia, Piper nigrum, Ocimum sanctum, Abutilon indicum, Curcuma longa, and Rumex maritimus.* It reduces long term diabetic complications and acts like an insulin analog, and discourages glycated haemoglobin levels, encourages repair and regeneration of pancreatic beta cells, and efficiently promotes free radical scavenging, thereby protecting beta cells from oxidative stress. In addition, it increases C peptide levels, peripheral utilization of glucose, enhances hepatic and muscle glucagon levels, normalizes the microalbuminurea, and regulates the lipid profile (Kundu and Chattaerjee, 2010).

1.9.4.4. Dianex

It is made from aqueous extract of *Azadirachta indica*. Beta-sitosterol is a steroid obtained from *Azadirachta indica* for its hypoglycaemic property (Mutalik *et al.*,2005).

1.9.4.5. Epi insulin

It is a combination of epicatechin and benzopyran. Epicatechin stimulates elevated insulin secretion by promoting the cAMP content of islets of Langerhans. It raises cathepsian activity which converts proinsulin to insulin. Due to its insulin mimicking nature, it promotes osmotic hemolysis and inhibits Na/K ATPase activity in red blood cells. It promotes glucose and lipid metabolisms, encouraging comprehensive health to the patient. Although the use is limited to the treatment of type 2 diabetes, it can also be used as an

adjuvant for the treatment of type 1 diabetes to reduce the amount of external insulin administration. Due to its gentle hypoglycaemic nature, prescribers generally recommend this polyherbal drug as preventive medication for potential diabetes patients, along with current oral anti-diabetic drugs for established patients (Modak *et al.*,2007).

1.9.4.6. Pancreatic tonic 180cp

It is a mixture of *Pterocarpus marsupium, Gymnema sylvestre, Momordica charantia, Syzygium cumini, Trigonella foenum graceum, Azadirachta indica, Ficus acemosa, Aegle marmelos,* and *Cinnamomum tamala,* and is generally used as a nutritional supplement (Rao, Salem, and Gleason-Jordan, 1998).

1.9.4.7. Sandan podia

The main ingredient in this formulation is *Tinospora cordifolia* sugar (six parts) and each part of remaining herbs such as *Santalum album* (sandalwood) sawdust, *and ropogon citratus* (lemongrass) root, *Vitiveria zizanioides* (vetiver) root, *Syzygium aromaticum* (clove) flower bud, *Anacyclus pyrethrum* (pyrethrum) root, and purified *Shilajit*. The administration of this drug has resulted in symptomatic relief in type 2 diabetes patients (Shankar and Singhal, 1994).

1.9.4.8. Diacare

It is a mixture of *Sanjeevan mool, Himej, Jambu beej, Kadu, Namejav,* and *Neem chal*. It has a bitter taste and improves glucose metabolism. Diabetes Daily Care was formulated for type 2 diabetics and contains all the natural ingredients prescribed in Ayurveda for the treatment of diabetes for optimal response. It can be used for the treatment of both insulin-dependent and non-insulin-dependent diabetics. The effects can be observed within 90 days of consumption, and ailments are cured within a stretch of a year and half. Patients depending on insulin can switch to this medicine. The course duration for the treatment has six phases, and each phase lasts for 90 days (Modak *et al.*,2007).

1.9.4.9. Diabeta

It is a blend of *Gymnema sylvestre, Vinca rosea* (periwinkle), *Curcuma longa* (turmeric), *Azadirachta indica* (neem), *Pterocarpus marsupium* (kino tree), *Momordica charantia* (bitter gourd), *Syzygium cumini* (black plum), *Acacia Arabica* (black babhul), *Tinospora cordifolia*, *Zingiber officinale* (ginger). The formulation of this medicine is based on

Ayurvedic scriptures, and it is further investigated through modern clinical trials and research methods. Diabeta promotes the comprehensive health of patients and gives symptomatic relief from weakness, giddiness, leg pain, body aches, polyuria, and pruritus. It not only works as an anti-diabetic agent, but also aids in activities such as immunomodulating, antihyperlipidimics, hepatoprotective and anti-stress. It corrects and prevents degenerative complications of diabetes with its effective mechanism of action on omnious octet. It can be used as an adjuvant for mainstream drugs to prevent resistance to oral hypoglycaemic drugs (Modak *et al.*,2007).

1.9.4.10. *Triphala* tablets and *Kadal Azhinjil* Choornam

It contains *triphala*, and the bark and roots of *Salacia chinensis*. Hypoglycaemic effects on application have been reported (Sivaprakasam *et al.*,1984).

1.9.4.11. MA-471

Formulation of this drug is consisting of a combination of the following herbs: *Enicostemaa littorale, Phyllanthus niruri, Eugenia jambolana, Melia azadirachta* (indica), *Terminalia arjuna, Aegle marmelos,* and *shilajit*. This mixture exerts a significant hypoglycaemic activity and hypolipidemic activity, even in those patients who developed have resistance to oral antidiabetic drugs (Sircar *et al.*,1996).

1.9.4.12. M-93

It is a mixture of four herbs: *Aegle marmelos* (bilva), *Azadirachta indica* (neem, nimba), *Ocimum sanctum* (tulsai), and *Piper longum* (kalimircha). After application, it has had a positive effect, and has no known side effects (Kumar and Kumar, 1995). As discussed in the previous sections, individual, intact herbs or herbal mixtures are being used in Ayurveda for the treatment of diabetes and its complications. Although many literatures are available, the research on anti-diabetic Ayurvedic herbal compounds are still lagging in the study of active constituents and mechanisms of action of individual herbs or polyherbal formulations. It is vital to unveil the active compounds and their molecular activity, as it is important to investigate the mechanism of action by using appropriate models to analyse the therapeutic efficacy of that herb / formulation and standardise the product.

1.9.5. Known active antidiabetic herbal principles

Generally, phyto chemicals are secondary metabolites of plants which are produced for self-defence against invading pathogens. Secondary metabolites such as alkaloids, amino acids, alkyl disulphides, flavonoids, glycopeptides, polysaccharides, peptides, triterpeniods, steroids, xanthone, lipids, phenolics, coumarins, iridoids, inorganic ions, and guanidines are known to have antidiabetic activity. The following phyto constituents are reported to have significant antidiabetic activity: aminoacids like hypoglycin A and hypoglycin B; alkaloids like catharanthine, leurosine, lochnerine, arecoline, and vindoline; pinitol, epicatechin, bengalenoside, anemarans (A,B,C,D), atractans (A,B,C), dioscoran (A,B,C,D,E,F), ephedrans (A,B,C,D,E), glycoproteins (moran A), mucilage, nimbidin, peptides (Pinsulin),S-methyl cysteine sulphoxide, S-allyl cysteine sulphoxide, and rographolide, allicin (thio-2-propene-1-sulfinic acid S-allyl ester), shamimin, beta vulgarosides I–IV, glycoside of leucopelargonidin and leuco delphinidin, mangiferin, marsupsium, pterosupin, pterostilbene, salacinol, swerchirin, trigonelline, berberine, harmane, norharmane, pinoline, quercetin, chlorogenic acid, hesperidin, naringin, epigallocatechin gallate, charantin, galactomannan, lactuca in C, furofuran lignan, lactucaside, beta-sitosterol, gymnemic acid IV, elatosides (E,G,H,I), oleanolic acid, kalopanax saponin A, hederagenin, cryptolepine, Shamimin, chamaemeloside, momordin Ic, scoparianosides A, B, and C, trihydroxyoctadecadienoic acids, kaempferol glucosides, caffeoyl glucoside, bakuchiol, swerchirin, thysanolactone, bellidifolin, kolaviron, escins (Ia, Ib, IIa, IIb, and IIIa), kotalanol, fagomine, 4-O-beta-D-glucopyranosylfagomine 3-O-beta-Dglucopyranosylfagomine, 3-epifagomine, myrciacitrins I and II, myrciaphenones A and B, 4-hydroxybenzoic acid, senegin II, Z-senegasaponins a and b, E and Z-senegasaponins, E and Z-senegins (II, III, and IV), paeoniflorin, 8-debenzoylpaeoniflorin, prunin, coutareagin, masoprocol, and oleanolic acid glycosides, ginsenoside, senticoside A, boussingoside, momordin, tormentic acid, ursolic acid, panaxan, laminaran, coixan, pachymaran, lithosperman, trichosan, saciharan, abelmosan, kakonein, flavone C-glycoside, icariin, neomyrtillin, sappanchalcone, caesalpin P, 3-deoxysappanone, protosappanin A, brazilin, hyperin, berberine, anisodamine, multiflorine, 1-deoxynojirimycin, acarbose, voglibose, and ferulic acid. There are several patented anti-diabetic compounds available: cryptolepine, maprouneacin, 3β, 30-dihydroxylupen-20(29)-en-2-one, harunganin, vismin, and quinones SP18904 and SP18905. Herbal

principles such as nordihydroguaiaretic acid (ndga), was proved to lower cholesterol levels in diabetic mice (Jerald, Joshi, and Jain, 2008; Daniel and Norman, 2001).

1.9.6. Known mechanism of actions of active herbal principles

The antidiabetic activity of herbal compounds exerts a variety of mechanisms. The mechanisms of action of herbal anti-diabetics could be grouped as:

- Mechanistic actions similar to those of adrenergic compounds; sympathomimetic (i.e., Adrenomimeticism), pancreatic beta cell potassium channel blocking, cAMP (2nd messenger) stimulation (Marles and Farnsworth, 1996).
- Inhibition in renal glucose reabsorption (Eddouks *et al.*,2002).
- Stimulation of insulin secretion from beta cells of islets or / and inhibition of insulin degradative processes (Pulok *et al.*,2006).
- Reduction in insulin resistance (Pulok *et al.*,2006).
- Providing certain necessary elements like calcium, zinc, magnesium, manganese, and copper for the beta-cells (Mohamed *et al.*,2006).
- Regenerating and / or repairing pancreatic beta cells (Mohamed *et al.*,2006).
- Increasing the size and number of cells in the islets of Langerhans (Mohamed *et al.*,2006).
- Stimulation of insulin secretion (Esmaeili and Yazdanparast, 2004).
- Stimulation of glycogenesis and hepatic glycolysis (Miura, 2001).
- Protective effect on the destruction of the beta cells (Kim *et al.*,2003).
- Improvement in digestion along with reduction in blood sugar and urea (Krishnan, 1968).
- Prevention of pathological conversion of starch to glucose (Sepha and Bose, 1956).
- Inhibition of β -galactocidase and α–glucocidase activity (Sharma and Mujumdar, 1990).
- Cortisol lowering activities (Gholap and Kar, 2004).
- Inhibition of alpha-amylase activity (Heidari, Zareae, and Heidarizadeh, 2005).
- Preventing oxidative stress that is possibly involved in pancreatic ß-cell dysfunction found in diabetes (Hideaki *et al.*,2005).
- Promoting glucose uptake activity in hepatoma cells (HepG2) (Kandunuri and White, 2012).

The above list shows the different mechanisms of actions of different phyto constituents and their sites of action, similar to mechanism of actions of mainstream oral anti-diabetic agents (Jarald *et al.*,2008).

1.9.7. Discussion

Diabetes is a metabolic disorder; whose predominant feature is the persistent presence of elevated sugar levels in the peripheral blood stream. This is due to inadequate amounts of insulin or insulin resistance which leads to metabolic failure of carbohydrates, lipids, and proteins. The use of herbal remedies (i.e., Ayurveda) for treating diabetes has been in practice for a long time in countries like India. There are several potent anti-diabetic herbs used in Ayurveda and herbal therapy for treating type 1 and type 2 diabetes patients.

1.9.8. Comparison between herbal therapies over mainstream therapy

1.9.9. Disadvantages of mainstream therapy

The prospect of identifying new, herbal treatments for type 2 diabetes broadens the range of treatments available, as well as identifying local, cheaper alternatives to western medicine. Synthetic drugs such as metformin contain hepato toxins, which lead to chemical-driven liver damage (i.e., idio syncretic hepatotoxicity) (Cone, Bachyrycz, and Murata, 2010). Sulfonylureas (i.e., Tolazamide) tend to cause hypoglycaemia and weight gain. Some secretogogues like repagilinide do not stimulate insulin secretion when there is normoglycaemia. Drugs like Meglitinides (Repaglinide) causes side effects like back pain, low blood sugar levels, and upper respiratory tract infections. Biguanides (i.e., Metformin) shows its ill effects on gastrointestinal tract with nausea, cramps and diarrhoea and develops lactose intolerance and causes vitamin B-12 deficiency over a period. Alpha glucosidase (i.e., Miglitol) inhibitors may lead to hepatic necrosis and inhibits a digestive enzyme known as alpha-glucosidase which causes digestive disorders and intestinal gas formation. The side effects with Thiazolidinedioes (i.e., Rosiglitazone) are fluid retention, gaining excessive weight and anaemia. Drug combinations like metformin and rosglitazone causes menstrual problems in women. These disadvantages with current anti-diabetic drugs encourage diabetic patients to adapt to alternative therapies such as Ayurveda because of its affordability and it is proven to cause less side effects (Joslin diabetes centre, 2015; David, 2002).

1.9.10. The advantages and disadvantages of hypoglycaemic herbs and polyherbal formulations

Ayurveda herbal drugs are known to have following advantages: Since Ayurveda herbal formulations constituted mainly plant extracts or the use of intact herbs, they are rich in antioxidants and essential nutrients required to respond body's natural recovery process and provide comprehensive health and Ayurveda therapies generally provide relief without adverse effects even after prolonged administration. In a clinical study the general well-being of the volunteers has been improved and none of them complained of any physical disorder (Murthy *et al.*,2010; Udupa and Singh, 1993).

Herbal drugs are cheaper than synthetic drugs. It's because plant-based herbal ingredients and extracts are used to make herbal drugs, while expensive chemicals are used to produce synthetic drugs. As Ayurvedic herbal formulations are made from natural products, it produces no allergic reactions or counter-indications. However, there is no proven record that everyone who takes herbal medicine will benefit in the same manner as another patient the end results may vary.

1.9.10.1. Ayurvedic herbal drugs' known disadvantages

Herbs collected in the wild are unsafe for consumption since unknown active compounds could be deposited in the intact plants. Incorrect identification of important herbs may result in non-effective or even toxic compounds being administered to the patient. Herbal medications tend to interact with mainstream medicines and may lead to further complications. Therefore, it is always advisable to consult a doctor before switching to alternative treatments while using mainstream medicines. Though herbs are often effective, they are not a panacea for all kinds of diseases.

One of the known issues with Ayurveda treatment is heavy metal toxicity in the formulations (Umrani and Paknikar, 2011). As there is limited health regulation on the use of Ayurvedic anti-diabetic products, heavy metals such as lead, arsenic, thallium, and mercury are often incorporated for their ascribed therapeutic properties and to enhance potency, which often leads to clinically significant heavy-metal poisoning risk to the patient (Chopra and Doiphode, 2002; Gogtay *et al*,2002). As an example, in a clinical observation in London, four different cases where anti-diabetic Ayurvedic medication was consumed have been studied. The first case involves an elderly patient suffering from encephalopathy with the highest blood lead concentration that has been associated with

survival. This has been regarded as a common theme with Ayurvedic medicines from previous reports of Ayurveda related lead poisoning (Dargan *et al.*,2008). Summary of each case is presented below:

Case 1 involves an Asian diabetic male aged 60, who is on Ayurvedic medication for the previous 6 – 8 years. Analysis of the medication revealed that it incorporated 6.8 percent of lead by weight. Due to lead toxicity, he had been suffering from poor mobility, increasing confusion, and anaemia (haemoglobin 8.7 g/dL). His blood examination showed basophilic stippling. His health further worsened within the span of 12 hours with symptoms such as increasing drowsiness and inattentiveness, hypoventilation, and seizures. After the administration of tracheal intubation and ventilation, his convulsions were controlled with benzodiazepines and barbiturates. Lead concentration in his blood was at strikingly elevated levels of 15.88 μmol/L (3290 μg/L). After triple chelation therapy, his blood lead concentrations fell to 8.76 μmol/L (1815 μg/L) at 2 weeks, 3.16 μmol/L (655 μg/L) at four weeks and 0.87 μmol/L (180 μg/L) at 12 weeks. After six weeks of intensive treatment and respiratory track placement (tracheostomy), his kidney and liver functioned in a regular mode. During this period, he developed common associated complications such as pneumonia and urinary sepsis. After removing the external tracheal tube, he suffered from a severe sensory and extensor motor polyneuropathy of limbs. A nerve conduction evaluation test revealed that there is a significantly impaired mixed motor, sensory, and primarily axonal peripheral neuropathy. After a six-week period of in-patient neurorehabilitation, in three years his blood lead concentration dropped to 0.5 μmol/L (105 μg/L) and he was able to move and lead a routine life without any long-term neural complications (Dargan *et al.*,2008).

Case 2 involves an Asian diabetic female, aged 57, who was on a couple of Ayurvedic medications suffering from clinical symptoms like tiredness, lethargy, and anaemia (haemoglobin 6.9 g/dL). Her blood film revealed basophilic stippling. Lead concentration in her blood was at 5.9 μmol/L (1220 μg/L). Analysis of her Ayurveda drugs showed that one had no substantial lead whereas the other accounted for 6.8 percent. After she underwent a couple of course treatments, each of 19-day durations, with the drug 2,3-Dimercaptosuccinic Acid (DMSA) (10 mg/kg, eight hourly for five days, followed by 10 mg/kg, 12 hourlies, for 14 days). She showed improvement and her haemoglobin levels surged up to 12.6 g/dL, and her blood lead concentration reduced to 1.2 μmol/L (250 μg/L) (Dargan *et al.*,2008).

Case 3 involves an Asian diabetic patient with high blood pressure, aged 65, and he was anaemic (haemoglobin 9.2 g/dL). He has been on ten different Ayurvedic medications for a year. An analysis of the medications revealed that they contained 1.47 percent lead. Before using these tablets, he had normal haemoglobin (13.5 g/dL). For the past six months, he has been suffering from chest pain, abdominal pain, constipation, and lethargy after strainful physical work. Lead concentration in his blood was 6.58 μmol/L (1360 μg/L). He has undergone a 19-day course of oral DMSA. Further examinations revealed that his symptoms were mitigated, and his post-chelation blood lead concentration was 1.11 μmol/L (230 μg/L) (Dargan *et al.*,2008).

Case 4 involves an Asian diabetic person, aged 59, who suffered with stomachache, lethargy, and anaemia (haemoglobin 8.4 g/dl). Basophilic stippling was observed in his blood film. Lead concentration in his blood was 4.1 μmol/L (850 μg/L). He is on two Ayurvedic tablets for diabetes which contain 1.56 percent and 2 percent lead. A couple of months after withdrawing the Ayurvedic medicines, his blood lead concentration had reduced to 3.5 μmol/L (725 μg/L), and haemoglobin had improved to 11.5 g/dL. However, his symptoms persisted. He went through a 19-day course of DMSA, after which his symptoms settled and his blood lead concentration reduced to 1.76 μmol/L (365 μg/L) (Dargan *et al.*,2008).

There is no significant population data available on toxic element incorporation and heavy metal poisoning in Ayurveda formulations. It is likely to be under reported/recognised, mainly as lead and mercury poisoning exerts non-specific symptoms. Therefore, it is vital to investigate and quantify the incidence and possible risk of heavy metal poisoning.

1.9.11. Reasons for herbal medicine to be poorly accepted by mainstream medicine

The reasons for the lack of general public's interest in herbal medicine and the drive for its incorporation into mainstream medicine are summarised as follows:

1. There is no proper scientific evidence for safety, efficacy and understanding of herbal medicines. Therefore, patients undergoing herbal treatment might be unknowingly exposing themselves to the risks of toxicity or overdose. Due to the absence of a clear understand ing of the mechanisms of the apparent therapeutic effect, the progression of many promising herbs has hindered to date. Polyherbal compounds contain mixture of herbs, and each herb potentially contributes hundreds of chemical ingredients and most of them are presented in extremely low concentrations. Further laboratory investigations are recommended in order to increase concentrations of targeted active principles in intact plants, or in hairy root cultures that need further attention by using elicitor technology. Separation and purification of targeted active principles and structural elucidation of unknown compounds of every possible combination are both impractical and expensive in terms of time and research expenses (Vlietinck, Pieters, and Apers, 2009).

2. Regulation and quality control challenges: Large proportions of the side effects that result from the consumption of herbal medications can be attributed to the poor quality of herbal drugs, some resulting from the raw ingredients that were contaminated, and the others are intended incorporation of heavy metals in order to enhance their potency. These adverse effects may be the results of errors in incorrect identification of the herb, poor manufacturing practices, and the lack of standardisation, contamination of final products, substitution, incorrect dosage, or preparation. Therefore, there is an urgent need to implement proper cultivation, harvestation, and production processes, and adapting modern analytical techniques and international regulation to enhance the quality of herbal drugs (Marcus and Grollman, 2002).

3. Variation in source herbal materials and resultant products: the ingredients of herbal formulations are often associated with environmental factors such as light, temperature, soil quality, seasons, the time of harvest, and the age of the plant (Marcus and Grollman, 2002). And man-made variations in source herbs emphasize the necessity for appropriate product standardization of herbal drugs (Li *et al.*,2008).

4. False perception in the safety of herbal drugs: There is a perception among the general public that traditional medicinal products are safer since they are prepared from natural sources without involving much processing (Calixto, 2000). Ironically, plant-derived phytochemicals are secondary metabolites and these are not always benign molecules and many of them express toxicity on application (Gurib, 2006). Traditional medicines must be judiciously prescribed and used in the same way as mainstream medication so that there is a clear awareness of potential herb–herb and herb–drug interactions.

5. Venture capital investors are discouraged by the high level of risk and huge investment involved in this industry. In comparison to mainstream drugs, it takes an average span of 12 years to grow in the herbal drug industry and it requires large investments (Tofte, Per Molgaard and Winther, 2012). Also, they are not ready to invest the funds needed for analysing and standardizing herbal drugs. Product development processes would take significantly longer time periods and incur greater costs due to the complex nature of the mixtures in herbal preparations. Screening each of the constituents or combinations of the phytochemicals in the complex mixture as it is done for mainstream medicines would not be pragmatic and needs further investment. Technological adaptations and the processes are also expensive. It is not possible to take out proprietary rights over a tree or its fruit in order to prevent others from growing a particular plant for personal use (McIntyre, 1999).

Regardless of the above-mentioned limitations, by employing proper scientific methods and tools, and further investigation of well-known anti-diabetic herbs in traditional medicine may provide a new understanding for valuable resources for innovative, evidence-based, and effective treatment solutions for diabetes.

1.9.12. Conclusion

In Ayurveda treatments single intact herbs or polyherbal formulations are used as drugs for the management of type 2 diabetes. As discussed previously, there are few known setbacks in the application of Ayurvedic medicines, such as the incorporation of heavy metals like lead, mercury, arsenic, and thallium, in order to improve efficacy and potency of the drug. It is a common problem in traditional medicines like Ayurveda and Traditional Chinese Medicine. However, there is no adverse effect in using individual herbs in the right quantity and furthermore, studies have revealed that some herbs show excellent hypoglycaemic activity in cellular, preclinical. and clinical studies. However, further work

is required to study the active ingredients that can be isolated, purified and elucidated, and study their mechanism of action. Mainly pre-clinical studies include oral glucose tolerance tests and common experimental models would be streptozotocin and alloxan-induced diabetic mice or rats. Limited studies are performed to investigate the anti-diabetic phytochemicals to unveil their mechanism of actions. Although most of the anti-diabetic Ayurvedic herbs exhibit hypoglycaemic / anti-hyperglyceamic, it is observed in the laboratory studies that the degree of efficacy varies in chronic diabetes and its associated complications. In conclusion, herbs have been a natural source of remedy for the treatment of diabetes for a long time; still, the active compounds of many herbs have not been well characterized till date. Further investigations must be carried out to evaluate the mechanisms of action of Ayurveda herbs with antidiabetic and insulino-mimetic activity. Though there is a perception that generally herbs are safe to consume, many herbal formulations are not safe due to the issues discussed previously. Therefore, toxicity studies should be elucidated to be standardise for medication purposes. It is thus extremely beneficial for mankind, to isolate, purify, and elucidate structures and studies on cellular, preclinical, and clinical studies of Ayurveda hypoglycaemic herbs, which may lead to innovation for better alternatives for the treatment of type 2 diabetes in terms of efficacy and affordability.

1.10. Target Phytocompounds of the Study

Following on from the preceding section, in this section target phytocompounds used in the study are introduced together with a brief explanation of the considerations that lead to their application in this research project. Additionally chromatographic analysis of shade dried leaves of *Stevia* was prepared in the UK is presented to estimate the predominant active components, which are likely to vary depending on the climatic conditions and geographical conditions where they have been cultivated.

1.10.1. Phloretin

Molecular formula: $C_{15}H_{14}O_5$; **Molecular weight:** 274.27 g/mol.

Source of Phloretin: Apple tree leaves, pear fruit and Manchurian apricot (Manchurian Apricot, 2014).

Classification: Kingdom: Plantae, **Clade:** Angiosperms, **Clade:** Eudicots, **Clade:** Rosids, **Order:** Rosales, **Family:** Rosaceae, **Genus:** Malus, **Species:** *M. pumila*, **Binomial name:** *Malus pumila* L.

Phloretin (Figure 33) is a flavonoid like compound; it is described as a dihydrochalcone (Phloretin, 2014) a type of natural phenol, and commonly found in apple tree leaves (Figure 32) and pear fruit. Flavonoids are ubiquitous secondary metabolites of plants and possess varied useful biochemical and high antioxidant properties compared to other antioxidants such as vitamins C and E. The important structural aspects of radical scavenging and chelating activity of the flavonoids are mainly governed by their chemical structure, due to their reliance on the flavan nucleus, the number, positions, and types of substitutions, and also offer promising validations for existing differences in their structure and activity relationships. For instance, multiple hydroxyl groups in the structure facilitate substantial antioxidant, chelating, and pro-oxidant activity, whereas Methoxy groups enable adverse steric effects and promote lipophilic efficiency and membrane partitioning during cell division. A double bonded carbonyl group in the heterocycle or polymerization of the nuclear structure contributes towards a more stable flavonoid radical through conjugation and electron delocalization. Therefore, it is evident that there is strong link between flavonoid structure and its activity (Heima, Tagliaferro, and Bobilyaa, 2002). Also due to their potential beneficial properties such as antiviral, anti-allergic, antiplatelet, anti-

inflammatory, and antitumor activities, flavonoids gained considerable attention from the research fraternity (Pietta, 2000).

Figure 32: ***Malus pumila*** **L.** fruits and leaves; phloretin is a dihydrochalcone (a natural phenol), commonly found in apple tree leaves (Picinelli, Dapena, and Mangas, 1995).

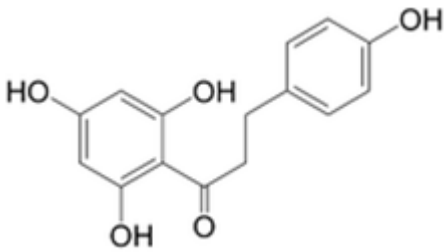


Figure 33: Molecular structure of Phloretin (Shen *et al.*,2017).

The effect of flavonoids such as phloretin, apigenin, genistein, and daidzein on rogen sensitive and insensitive prostate cancer cells in which glycolytic metabolism differs, stimulation of GLUT-4 and GLUT-1 and glucose uptake has been noticed, and this indicates different effects of phloretin depending on the type of cell (Menendez *et al.*,2014).

In a research cellular glucose influx in pancreatic beta cells with a self-referencing micro biosensor-based nanomaterials study, the phloretin promoted maximum inhibition with remaining non-oscillating flux, signifying that, the glucose transporters which were not inhibited by phloretin were likely promoting leftover non-oscillatory uptake. Whereas the impaired uptake via GLUT-2 may be promoting oscillation loss in type 2 diabetes (Shi *et al.*,2011). By considering the above varying properties of phloretin in conjunction with glucose transport all over the tissues, it would be worth testing glucose uptake property of

phloretin. Thus, phloretin was selected as one among the four target phytochemicals to conduct a comparative study on glucose uptake mechanism against Human hepatocellular carcinoma (HepG2) cells.

1.10.2. Ouabain octahydrate

Molecular formula: $C_{29}H_{44}O_{12}$; **Molecular weight:** 728.77 g/mol.

Source of Ouabain: *Acokanthera ouabaio* tree bark, wood, and roots.

Classification: Kingdom: Plantae, **(unranked):** Angiosperms, **(unranked):** Eudicots, **(unranked):** Asterids, **Order:** Gentianales, **Family:** Apocynaceae, **Genus:** Acokanthera, **Species:** *A. ouabaio*, **Binomial name:** *Acokanthera ouabaio* L.

Ouabain (Figure 35) is a toxic cardiac glycoside also known as g-strophanthin and is exclusively found in the mature fruits and bark of long stemmed, woody vines such as *Strophanthus gratus* and Acokanthera *ouabaio* (Figure 34), which are predominantly found in West Africa, distributing to Asia, from Southern India to New Guinea and further extended to Southern China (World Checklist of Selected Plant Families, 2014).

Being a cardiac glycoside, ouabain contains a glycoside (a sugar molecule) that influences the contractile force of the cardiac muscle by increasing cardiac output by increasing the force of contraction. Thus, it is often being used for the treatment of congestive heart failure and cardiac arrhythmia (Singh and Rastogi, 1970). However, there are limited research papers available on the application of ouabain for its hypoglycaemic activity, one of such papers had been revealed a significant decrease in plasma glucose levels in dogs (Triner *et al.*,1968), other than this there is any cellular work was not being noticed previously by employing ouabain.

Figure 34: Leaves and inflorescence of ***Acokanthera ouabaio****;* the bark, wood, and roots and other parts of *Acokanthera* contain cardiotonic glycosides i.e., acovenoside A and ouabaïne (World Checklist of Selected Plant Families, 2014).

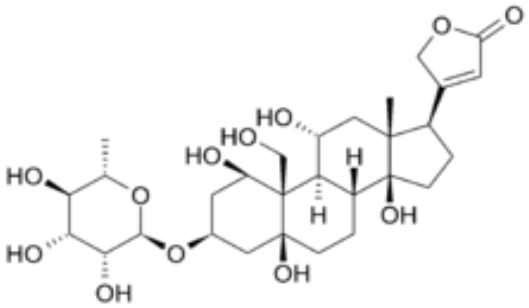


Figure 35: Molecular structure of Ouabain (Blaustein, Juhaszova, and Golovina, 1998)

Respecting Triner's thought-provoking experimental observations, it would be interesting to conduct cellular studies further to unveil the efficacy of ouabain in promoting glucose uptake. Therefore, ouabain was chosen as one among the four target phytochemicals to conduct a comparative study on glucose uptake mechanism against Human hepatocellular carcinoma (HepG2) cells.

1.10.3. Berberine chloride hydrate

Molecular formula: $C_{20}H_{18}NO_4^+$; **Molecular weight:** 371.81 g/mol.

Source of Berberine: It is a quaternary ammonium salt from the protoberberine group of isoquinoline alkaloids. It is commonly found in plants of *berberidaceae* family, in essential, *Berberis aquifolium* (oregon grape), *Berberis vulgaris* (barberry), *Berberis aristata* (tree turmeric)], *Hydrastis canadensis* (goldenseal), *Xanthorhiza simplicissima* (yellow root), *Phellodendron amurense* (amur cork tree), *Coptis chinensis* (chinese gold thread), *Tinospora cordifolia*, *Argemone mexicana* (prickly poppy) and *Eschscholzia californica* (californian poppy) (Cicero and Baggioni,2016). Please refer Sections 1.9.3.3 and 1.10.5 for more details. Berberine (Figure 36) is frequently used in various forms in ancient Indian medicine Ayurveda and Traditional Chinese Medicine (TCM) to treat diabetes and other ailments. It is usually available in the rhizomes, bark, stems, and the roots of plants of Berberidaceae family. Anti-diabetic properties of berberine were elucidated in the previous Section 1.9.3.3.

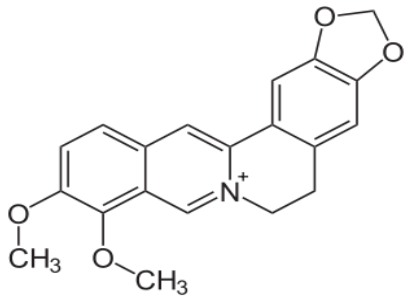


Figure 36: Molecular structure of Berberine (Yin *et al.*,2007; Wang *et al.*,2011; Gulfraz *et al.*,2008))

Based on the observations on the efficacy of berberine in previous studies (Yin *et al.*,2007; Wang *et al.*,2011; Gulfraz *et al.*,2008), it was chosen as one among the four test compounds.

1.10.4. Metformin hydrochloride

Molecular formula: $C_4H_{11}N_5$; **Molecular weight:** 165.6 g/mol.

Source of Metformin: The herbal plant *Galega officinalis* L. (Fabaceae) (Goat's rue). Please refer Section 1.10.7 for other details.

Metformin (Figure 37) is a biguanide class of drug and currently it is highly sought-after oral agent for the treatment of type 2 diabetes, a comprehensive description can be found in Section 1.7.2.3. Originally metformin was extracted from goat's rue (*Galega officinalis*) in the form of galegine and further purified as metformin, additional discussion can be found in Section 1.10.7.

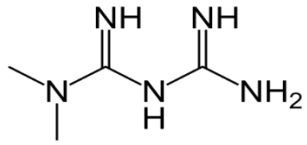


Figure 37: Molecular structure of Metformin (Grzybowska,2011)

Being a first line drug for the treatment of type 2 diabetes, it predominantly influences 5' AMP-activated protein kinase (AMPK) and protein kinase C (PKC) and promotes glucose uptake in skeletal muscle cells and adipocytes in both diabetic subjects and animal models (Turban *et al.*,2012), and in an animal study it promoted glucose transport and influenced Glucose transporter-1 (GLUT-1) and Glucose transporter-4 (GLUT-4) in myocardiocytes (Fischer *et al.*,1995).

The idea that AMPK mediates the glucose uptake property of metformin was challenged by loss-of-function and genetic association link studies, which gave an insight on the AMPK-independent effects of the metformin that include the mitochondrial actions in the liver was thought to be the primary site of action of metformin, and displayed similar properties of the counter regulatory hormone glucagon (Rena, Pearson, and Sakamoto, 2013). Therefore, by considering above observations, metformin was selected in this research as a positive control to evaluate efficacy of other test compounds.

1.10.5. Goldenseal

Classification: Kingdom: Plantae, **Clade:** Angiosperms, **Clade:** Eudicots, **Order:** Ranunculales, **Family:** Ranunculaceae, **Subfamily:** Hydrastidoideae, **Genus:** Hydrastis L, Species: *H. canadensis*, **Binomial name:** *Hydrastis Canadensis* L.

Goldenseal, also known as orangeroot or yellow puccoon, is a perennial herb (Figure 38). Commonly found in southeastern Canada and the eastern United States. It appears as thick, yellow knotted rootstock, and it has a purpulish hairy stem above ground and yellow below ground (*Hydrastis Canadensis*, 2017; Foster and Duke, 2000).

During the European colonization, goldenseal was in extensive use as a multipurpose herbal drug among certain Native American tribes of North America. They used it to treat various health conditions such as skin diseases, ulcers, and gonorrhoea (Benjamin and Barton,2017), It contains the isoquinoline alkaloids such as berberine, berberastine, hydrastine, hydrastinine, tetrahydroberberastine, canadine, and canalidine (Weber *et al*,2003). Goldenseal promotes lowering of blood glucose levels in type 2 diabetic people. Active principles such as berberine may be responsible for this effect. In a preliminary study, daily intake of 1-gram berberine as a food supplementation for two months significantly reduced pheripheral blood glucose levels in type 2 diabetes people (University of Michigan Health System,2015).

Figure 38: Godenseal herb with its rhizome, reproductive and asexual parts; the rhizome is mainly used in herbal preparations (*Hydrastis Canadensis*, 2017, and Foster and Duke, 2000).

1.10.6. *Gymnema sylvestre* L.

Please refer Section 1.9.3.8.

1.10.7. Goat's rue

Classification: Kingdom: Plantae, **(Unranked):** Angiosperms, **(Unranked):** Eudicots, **(Unranked):** Rosids, **Order:** Fabales, **Family:** Fabaceae, **Subfamily:** Papilionoideae, **Genus:** Galega**, Species:** *G. officinalis*, **Binomial name:** *Galega officinalis* L.

Galega officinalis is known as French lilac, goat's-rue, galega, professor-weed or Italian fitch (Figure 39), it is commonly found in Middle East, Europe, and western Asia. It has been cultivated as a forage crop and as green manure, can also be used as an ornamental and a bee plant (*Galega officinalis,* 2014).

Goat's rue was used as herbal tonic in traditional herbal medicine in medieval Europe for its purported effect of hyperglycemia and diuresis, also, to treat bubonic plague, worms, and snake bites. Guanidine is a substance, extensively found in Goat's rue, it has peripheral

blood glucose lowering capacity. Studies on guanidine, biguanides and related compounds led to the discovery of the commonly used biguanide class of drug, metformin. Goat's rue has been analyzed for its active substances, such as galegine, hydroxygalegine, guanidine and several guanidine derivatives, such as 4-hydroxygalegine flavones, flavone glycosides, kaempferol, and quercetin. Research on galegine and related/similar molecules in the first half of the 20th century led to the development of oral antidiabetic drugs (Bailey, 2004).

Figure 39: Inflorescence of Goat's rue herb; it is a natural source of galegine, the whole plants or leaves or inflorescence are used to produce metformin and other therapeutic preparations, but the reproductive parts (i.e., inflorescence) have high concentrations of galegine compared to its stems and leaves (Oldham *et al.*,2011).

1.10.8. Rebaudioside – A

Molecular Formula: $C_{44}H_{70}O_{22}$ **; Molecular Weight:** 951.0 g/mol

Source of Rebaudioside-A: The sweet heb *Stevia rebaudiana bertoni* L. (Section 1.10. 9).

It is a steviol glycoside (Figure 40), and contains just glucose and no other simple sugars such as fructose and galactose, it has four glucose molecules with the central glucose of the triplet linked to the main steviol structure at hydroxyl group, and the residual glucose at its carboxyl group forms an ester bond, and it is 200 times sweeter than cane sugar.It inhibits α-glucosidase enzyme with half maximal inhibitory concentration (IC50), of 35.01 μg/ml , at this concentration it can inhibit ATP-sensitive K+-channels, also, stimulate the insulin secretion from MIN6 cells in a dose- and glucose-dependent manner, which is mediated via inhibition of ATP-sensitive K+-channels and requires the presence of high glucose (Adari *et al*,2016 ; Abudula *et al.*,2008 ; Williams *et al.*,2009).

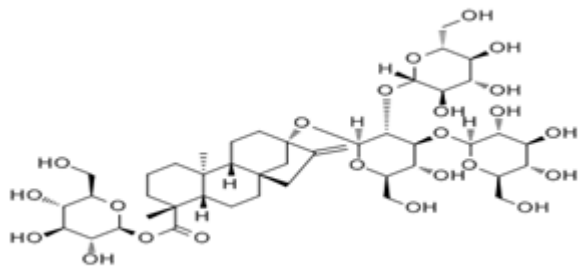


Figure 40: Chemical structure of Rebaudioside- A (Adari *et al.*,2016)

1.10.9. *Stevia rebaudiana Bertoni*

Classification: Kingdom: Plantae, **Clade:** Angiosperms, **Clade:** Eudicots, **Clade:** Asterids, **Order:** Asterales, **Family:** Asteraceae, **Genus:** *Stevia*, **Species:** *S. rebaudiana*, **Binomial name:** *Stevia rebaudiana Bertoni*

Stevia rebaudiana Bertoni is a perennial shrub, belongs to *Asteraceae* family, also known as a sweet leaf (Figure 41). The genus *Stevia* originated from Paraguay, Brazil and Central America. It is generally found in areas with 500-3500m altitude in addition of 1500 to 1800mm yearly rainfall. The growth is favoured in the regions with temperature ranging from -6^{o}C to +43^{o}C (De Oliveira *et al.*,2004). Though it is 250-300 times sweeter than the cane sugar, the plant is being consumed traditionally by the inhabitants of Paraguay and Brazil for centuries, as a zero-calorific sweetener (Achi *et al.*,2000). It is also used in medicinal teas as a treatment for common ailments such as heart burn and gastro-oesophageal reflux (Vanek, Nepovim, and Valicek, 2001). Although several species of genus *Stevia* exist, only *Stevia rebaudiana B*ertoni gives the sweetest essences named stevioside and rebaudioside-A (Savita *et al.*,2004). It is generally available in the form of fine white powder, extracted from *Stevia* leaves (Elkins, 1999).

The main interest of the plant's secondary metabolites is steviol glycosides such as stevioside and rebaudioside-A. Stevioside is recognized to be helpful in the treatment of type 2 diabetes by reducing post-prandial blood glucose levels and promoting glucose metabolism (Gregersen *et al.*,2004). The United States Food and Drug Administration (FDA) and The European Food Safety Authority's (EFSA) which is a scientific Panel on additives, have worked on the possibilities of steviol glycosides as a replacement for common sugar (Rich, 2012).

Figure 41: *Stevia* leaves; mainly leaves are used in the culinary preparations, herbal preparations, and extracts (Savita *et al.*,2004; Elkins, 1999).

1.10.9.1. Active compounds in *Stevia* leaf

1.10.9.2. Steviol glycosides

The Joint Expert Committee for Food Additives (JECFA) 63rd annual meeting (JECFA, 2006) published a statement that steviol glycosides are the chemical constituents of *Stevia rebaudiana Bertoni* which contains at least ten varieties of glycosides, of which the most prominent compounds are stevioside and rebaudioside-A. The report published at the meeting states that more than 95% of the evaluated material containing glycosylated derivatives of steviols are stevioside, rebaudioside-A and C and dulcoside-A with minor quantities of rubausoside, steviolbioside and rebuioside-B, D, E and F. In addition, the meeting published tentative specifications for commercially available steviol glycosides powder, such as it should contain a minimum of 95% of a mixture of glycosides (i.e., stevioside, rebaudioside-A, C, and dulcoside-A). Similarly, the overall percentage of stevioside and rebaudioside-A content should be maintained as 70% out of 95% of total steviol glycosides. and the meeting recommended a temporary acceptable daily intake (ADI) of 2mg/kg by weight (bw) for Steviol glycoside based on studies conducted on rats using a safety factor of 200 (based on non-observable/observed effect level (NOEL) for Stevioside of 970 mg/kg bw/day or 383 mg/kg bw/day).

1.10.9.3. Classification of Steviol glycosides

Steviol glycosides are obtained by extracting *Stevia* leaves with hot water and followed by solvent purification of the water-soluble extract (ion exchange resins may also be incorporated in the purification process) (Steviol glycosides, 2008). The primary Steviol glycosides are stevioside and rebaudioside-A; rebaudioside-C and dulcoside-A are secondary glycosides. The names, chemical formula, formula weight, and the structural formula for each steviol glycoside (Figure 42) are summarised below:

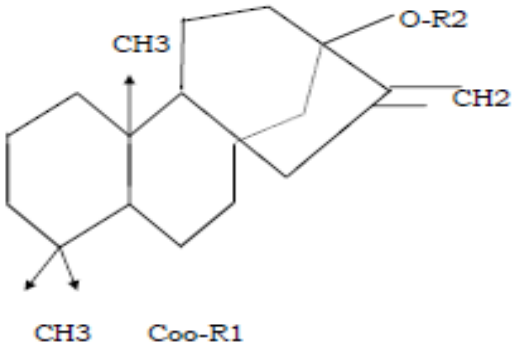
Stevioside ($C_{38}H_{60}O_{18}$; 804.88): 13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy] kaur-16-PM-18-oic acid β-Dglucopyranosyl ester (Steviol glycosides, 2008).

Rebaudioside-A ($C_{44}H_{70}O_{23}$; 951.03): 13-[(2-O-β-D-glucopyranosyl-3-β-Dglucopyranosyl-β-D-glucopyranosyl] oxy.kaur-16-PM-18-oic acid β-Dglucopyranosyl ester (Steviol glycosides, 2008).

Rebaudioside-C ($C_{44}H_{70}O_{22}$;967.03): 13- [(2-O-α-L-rhamnopyranosyl-3-O—β Dglucopyranosyl-β-D-glucopyranosyl -β- D-glucopyranosyl-β-D-glucopyranosyl] oxy. kaur-16-PM-18-oic acid β-D- glucopyranosyl ester (Steviol glycosides,2004).

Dulcoside-A ($C_{38}H_{60}O_{17}$; 788.88): 13-[2-O-α-L-rhamnopyranosyl-β-Dglucopyranosyl] oxy. kaur-16-PM-18-oicacid β-D- glucopyranosyl ester (Steviol glycosides,2004).

The chemical structures of each Steviol glycosides are shown in Figure 5.



Compound Name	R1	R2
Stevioside	β-Glc	β-Glc-β-Glc (2 → 1)
Rebaudioside A	β-Glc	β-Glc-β-Glc (2 → 1) β-Glc (3 → 1)
Rebaudioside C	β-Glc	β-Glc-α-Rha (2 → 1) β-Glc (3 → 1)
Dulcoside A		β-Glc-α-Rha (2 → 1)

Figure 42: Structural formula of Steviol Glycosides (Steviol glycosides, 2008)

Steviol (R1=R2=H) is the aglycone of the Steviol glycosides. Glc and Rha represent, respectively, glucose and Rhamnose sugar moieties.

1.10.9.4. Major active compounds in *Stevia* leaf grown in temperate climate

The Steviol glycoside concentration and composition in the leaves are the main quality measures of *Stevia* crude extracts. Stevioside and rebaudioside-A are the major compounds found in *Stevia* leaves and these glycosides determine the quality of the "taste". When there are elevated levels of stevioside in the leaves, a "liquorice" after taste is expressed, whereas elevated levels of rebaudioside-A express an enriched sweet taste and a reduced after taste. However, the concentration of these glycosides varies from cultivator to cultivator depending on geographical conditions and climatic conditions during cultivation, and in general plants grown in a temperate climate like summertime in the UK express elevated levels of stevioside in their leaves, whereas plants cultivated during spring generally express elevated levels of rebaudioside-A (Stevia.net, 2015; Stevia info, 2015; Saifi *et al.*,2014). In this research, 10 cm grown plantlets of *Stevia*, were purchased from Norfolk Herbs, Blackberry Farm, Dillington, Dereham, Norfolk, NR19 2QD (Norfolk Herbs, 2015).

1.11. Key mechanistic studies and cellular models of the research

1.11.1. General overview on cell-based assays for drug discovery screening

To discover new drugs, a methodological approach is required to optimize time and resources. Conventionally, to study the hypoglycaemic activity of target compounds in preclinical *in vivo* studies, animal models are employed, whereas clinical studies are being performed in patients with diabetes. While *in vivo* studies are essential to unveil the efficacy of new antidiabetic agents, they reveal lesser information about the mechanistic actions when compared to cell based *in vitro* assays. The advantages of cell-based assays are that a researcher can directly manipulate culturing conditions, such as using nutrient media of their choice and antibiotics as experiment demands. And the target cells can be refrained from the impact of physiological and biochemical influence of supporting organs and tissues, as is originally done in a living system. And rapid, large-scale screening of new compounds is possible at the preliminary stage of the drug discovery process, and these assays are relatively economical, also ethically controversial animal testing can be reduced to a minimum. The cell-based study detailed in this thesis focuses on different features of non-insulin-mediated glucose metabolism that occur following the application of botanical extracts and compounds with medicinal properties.

1.11.2. Glucose uptake mechanism

Glucose is a vital source of fuel for all cells, tissues, and organs, and especially the brain. The homeostatic mechanisms that control glucose levels in the blood are evolved to ensure supply of glucose to the brain, and to avoid the hyperglycaemic conditions that develop when glucose levels are persistently above about 10 mM.

Blood glucose levels are regulated by the gastrointestinal tract (splanchnic area), pancreatic β cells, pancreatic α cells, kidneys, muscle cells, brain, adipose tissue, and liver (octet organs). These organs control the uptake and release of glucose from the blood usually within the range of 70 to 99 mg/dL (about 3.9 to 5.5 mM; Wardlaw and Haml, 2007). However, it is being observed that occasionally in a healthy individual glucose levels raise

above 140 mg/dL (8.3 mM) after consumption of a carbohydrate rich meal (Guyton and Hall, 2006).

While the liver synthesises glucose, the muscle tissue consumes most of the peripheral glucose, and the kidneys' play a role in glucose metabolism and excretion. As Glucose regulation and homeostasis is a multi-organ process, coherence and coordination between octet organs is essential. In contrast, when the glucose homeostasis disrupts, these octet organs turn to be detrimental and contribute towards, decreased incretin effect, decreased insulin secretion, increased glucagon secretion, increased glucose reabsorption and production, decreased glucose uptake, insulin resistance, increased lipolysis/inflammation and increased hepatic glucose production. Therefore, these eight players together known as "ominous octet" (Defronzo, 2009), but in general insulin, glucagon and incretins are known as major players. Thus, the following subsection discusses different regulation methods involved in glucose homeostasis.

1.11.2.1. Hormonal regulation of Glucose Homeostasis

1.11.2.1.1. The role of Insulin and Glucagon in regulating blood glucose

The pancreatic β cells produce insulin in response to elevated blood glucose levels. Insulin reduces elevated blood glucose through different mechanisms like i) stimulating glucose uptake into tissues, ii) converting glucose into glycogen in the liver and muscle, iii) inducing fat storage by converting glucose into fatty acids. In contrast pancreatic alpha cells produce glucagon hormone, when there are lower levels of blood glucose occurs. Glucagon stimulates the conversion of glycogen, into glucose through a process called glycogenolysis, in which breakdown of glycogen (n) to glucose-1-phosphate and glycogen (n-1) would be normally promoted. Glucagon also encourages synthesis of glucose through gluconeogenesis, in which new glucose is derived from lactic acid and certain amino acids. As a result, the liver discharges glucose into the blood stream rapidly as the situation demands and promotes a homeostatic elevation of glucose levels in the blood (Tortora and Grabowski, 2003) (Figure 43).

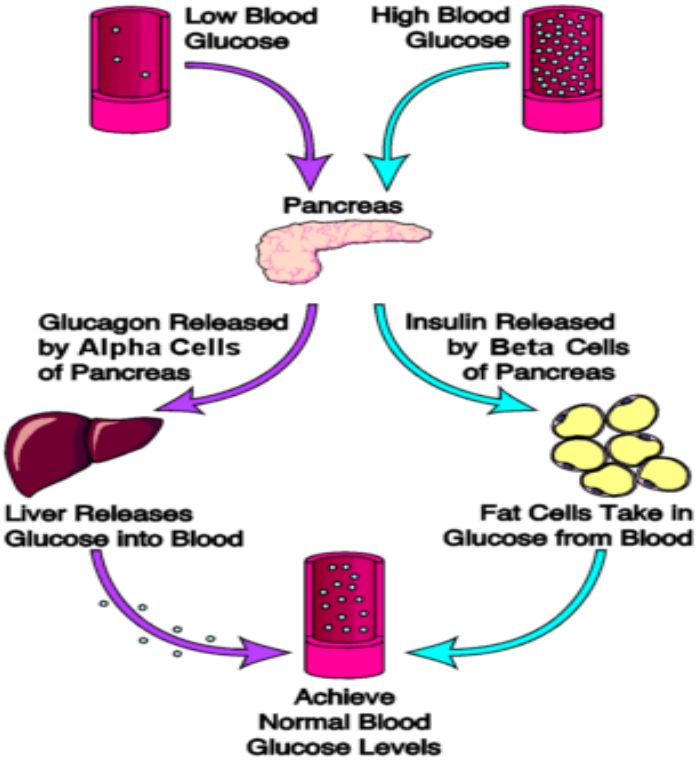


Figure 43: Regulation of blood glucose levels by Glucagon and Insulin (Norman, 2016)

1.11.2.1.2. The role of Incretin hormone in regulating blood glucose

The incretins are a group of glucose dependent metabolic peptide hormones that influence the regulation of blood glucose levels. Insulin secretion, and glucagon secretion to some extent, is controlled by principle incretin hormones glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide / glucose-dependent insulinotropic polypeptide (GIP). Incretins are produced by the intestinal cells, and essentially induce disposal of glucose, which is formed due to ingestion of a carbohydrate rich meal. GLP-1 down regulates glucagon secretion from alpha cells, and both incretin peptides stimulate glucose-dependent insulin secretion via activation of G protein-coupled receptors on β cells. Incretins appear only after carbohydrate ingestion, and whenever concentrations of carbohydrates in the blood reach such an extent which stimulates insulin secretion. Therefore, whenever blood glucose levels are reduced, levels of incretins also decrease with lower insulin secreting activity (Drucker and Nauck, 2006; Porte, Sherwin, and Baron, 2003; Drucker, 2006; Ahren, 2003).

1.11.2.3. The process of Glucose uptake/transport into cells

Glucose cannot diffuse itself across cell membranes and it must enter cells through transport proteins known as GLUTs (Tortora and Grabowski, 2003; Guyton, 2006; Wright, Hirayama and Loo, 2007). There are 12 glucose transporters found till the date, the Glucose transporter-4 (GLUT-4) is the major transporter for muscle, adipose tissue, and the heart. Insulin promotes uptake of glucose into these tissues by stimulating a translocation of GLUT-4 from intracellular vesicles to the plasma membrane and increases the facilitated diffusion of glucose into these tissues through GLUT-4 (Tortora and Grabowski, 2003; Uldry and Thorens, 2004).

The actions of insulin are mediated by the insulin receptor, which comprises 4 subunits, 2 alpha subunits situated exterior to the cell membrane, and 2 beta subunits that project through the cell membrane into the cell cytoplasm. The mechanism of glucose uptake into the cells comprises the following phases. Insulin binds to the alpha subunits, the beta subunits which have tyrosine kinase activity undergo auto phosphorylation. The tyrosine kinase gets activated further and causes phosphorylation of insulin receptor substrate (IRS) proteins, and PI-3-Kinase gets activated due to the action of phosphorylated IRS, that attaches to the phosphatidylinositol (PI) 3-kinase. Thus, activated PI-3-kinase stimulates translocation of glucose transporter 4 (GLUT-4) and enhances glucose uptake (Guyton and Hall, 2006; Porte *et al.*,2003; Peterson and Shulman, 2006).

In contrast, the brain cells (glial and neuron cells), also liver cells use other glucose transporters GLUT-1, 2, 3 and 8. These glucose transporters are constitutively expressed on the cell membrane surface, and these are independent of insulin. Therefore, glucose transport can be facilitated spontaneously without insulin intervention. After entering the cells, glucose will be phosphorylated by glucokinase in the liver, or hexokinase in other cells, to glucose-6-phosphate, which prevents reverse diffusion of glucose out of the cell due to lower concentration gradients. Following phosphorylation, glucose enters the glycolytic pathway or transforms into glycogen or fat deposits (Guyton and Hall, 2006; Tortora and Grabowski, 2003; Uldry and Thorens, 2004; Defronzo, 2004).

Impaired glucose transport decreases glucose absorption in muscle cells in type 2 diabetes (Cline *et al.*,1999). In mammalian cells, facilitative glucose transport systems are comprised of a wide group of membrane proteins that are located ubiquitously on the plasma membrane and are responsible for mediating regulation of glucose uptake across

the cell membranes (Yamamoto *et al.*,2011). Glucose transport into cells occurs through two different systems, namely, sodium glucose transport systems (SGLTs) and sodium independent glucose transport systems (GLUTs). In SGLTs sodium ion transport is coupled with active glucose transport, whereas in GLUTs sodium independent glucose transport occurs (Wood and Trayhurn, 2003).

GLUT family members: GLUTs are uniport glucose transporters and are structurally related to sodium glucose transporters. They provide transport across the cell membranes driven by a concentration gradient (Yamamoto *et al.*,2011). GLUTs are integral membrane glycoproteins, and they occur in several different forms, GLUT isoforms 1-14. Generally, the GLUT-1 isoform is expressed in all tissues but is predominantly present in the brain and red blood cells. It promotes basal glucose uptake and is encoded by the SLC2A1 gene of the solute carrier 2 family (Fukumoto *et al.*,1988; Mueckler *et al.*,1985).

GLUT-1, expressed on HepG2 cells, was the first glucose transporter to be cloned (Fukumoto *et al.*,1988; Mueckler *et al.*,1985). The GLUT-2 isoform is present in liver, beta cells of pancreas, proximal tubules of kidney, retina, and small intestine. It promotes glucose sensing in these key tissues and is encoded by the SLC2A2 gene (Fukumoto *et al.*,1989). The GLUT-3 isoform is present largely in brain cells and nerve cells, and at low levels in cardiac muscle tissue, liver tissue, kidney cells, and in placental cells. It complements GLUT-1 in tissues wherever and whenever additional energy is needed and is encoded by the SLC2A3 gene (Kayano *et al.*,1988).

The GLUT-4 isoform, encoded by SLC2A4, is present as the main form in skeletal muscle tissue, cardiac muscle tissue and adipose tissue. In contrast to other forms, it is an important insulin responsive form of GLUT, in which insulin stimulates expression of GLUT-4 on the plasma membrane by inducing a translocation from intracellular vesicles to the plasma membrane (Buse *et al.*,1992; Chiaramonte *et al.*,1993; Choi *et al.*,1991; Fukumoto *et al.*,1989). GLUT-5 isoform majorly presents in intestinal absorptive cells (i.e., enterocytes), and can be found at lower levels in skeletal muscular tissue, brain tissue, and adipose tissue. It also functions as a fructose transporter and is encoded by the SLC2A5 gene (Concha *et al.*,1997; Kayano *et al.*,1990).

GLUT-6 isoform displays close similarity with GLUT-9, and it commonly occurs in tissues such as spleen, brain, and leukocytes. Its function is not clearly understood to date, and it is encoded by the SLC2A6 gene (Doege *et al.*,2000a). The GLUT-7 isoform is found

in liver and works as a microsomal glucose transport protein in gluconeogenesis (Waddell *et al,*1992). GLUT-8 is found in testicular tissue, brain tissue, and blastocysts, and is also known as GLUTX1, and its function remaining unclear (Carayannopoulos *et al.,*2000; Doege *et al.,*2000b; Ibberson *et al.,*2000). GLUT-9, found in kidney and liver at low levels, is also known as GLUTX, has no known function (Phay *et al.,*2000). GLUT-10 is found in pancreatic and liver tissue, but its role is unclear (Wylie *et al.,*2001).

The functions of GLUTs 11 and 12, both found in cardiac tissue are not clear (Doege *et al.,*2001). GLUT-12 is also found in prostrate tissue (Rogers *et al.,*2002). GLUT-13 is found in brain tissue and performs as a proton-coupled myo-inositol transporter (Uldry *et al.,*2001). Lastly GLUT-14 has close similarity to GLUT-3 and is found in testicular tissue (Wu and Freeze, 2002).

GLUT isoforms transport monosaccharides such as D-glucose and structurally similar molecules but are not likely to allow L-glucose transport due to its structural difference (Cura and Carruthers, 2012). Unlike sodium ion dependent transporters, these facilitative transporters dynamically collect glucose with varying regulatory functions, different kinetic properties and cell surface expression in different organs, and thereby provide an integrity between glucose uptake, metabolism and insulin signal generation and response. Due to the presence of GLUT-1, 2, 3 and 5 in liver cells, nerve cells, kidney cells and erythrocytes, an unregulated and concentration gradient based glucose uptake takes place (Augustin, 2010; Cura, and Carruthers, 2012). In adipose and muscle tissues GLUT-4 promotes regulated glucose uptake (Augustin, 2010; Cura and Carruthers, 2012). Whereas, in the pancreatic β-cells, GLUT-2 glucose transporters promote a rapid glucose uptake irrespective of the extracellular sugar levels (Schuit *et al.,*2001).

Bidirectional transport of the glucose can occur, since it is passively transported along a concentration gradient (Augustin, 2010; Cura and Carruthers, 2012). For this reason, it is important that glucose is rapidly converted to glucose-6-phosphate once inside the cell, which maintains the concentration gradient in favour of glucose uptake. In contrast, Na+ dependent transporters (SGLTs) promote active glucose uptake against a concentration gradient using Na+ gradient. There are two isoforms of SGLTs. SGLT1 is mainly found in intestinal tissue and promotes glucose and galactose uptake, and SGLT2 occurs in kidney tissue and promotes glucose uptake from the proximal tubule (Augustin, 2010; Cura and Carruthers, 2012). Therefore, it is evident that these proteins regulate the glucose transport between extracellular and intracellular compartments, in that way maintains a continuous

source of this vital energy supply between the cells (Yamamoto *et al.*,2011; Cura and Carruthers, 2012).

In diabetes disruption of glucose uptake into peripheral tissues occurs due to insulin resistance, which leads to accumulation of excessive glucose in the peripheral blood circulation. As a result, this study concentrated on non-insulin mediated glucose metabolism, using different pharmacological options to increase the rate of cellular glucose uptake (Ball et al., 2002).

Conventionally, glucose uptake studies are performed with radiotracers such as 2-deoxy-D-[14C] glucose or 2-deoxy-D-[3H] glucose, which have shortcomings associated with handling and disposal of radioactivity. Furthermore, measuring glucose uptake straightaway by using radioactive tracers in single, viable cells is not possible (Yamada *et al.*,2000). Therefore, in this study a glucose uptake assay has been used which involves incubation of HepG2 cells with a fluorescent D-glucose analog 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-d-glucose (2-NBDG) followed by microplate reading and flow cytometry detection of fluorescence emitted by the HepG2 cells. In this method 2NBDG used in a flow cytometry application allows direct and more sensitive measurement of relative fluorescent density, i.e., synonymous to glucose uptake by the cell (Chenhui *et al.*,2005). This assay can be employed over a range of applications to detect or screen potential drug candidates and provides an understanding of the mechanisms involved in accumulation and consumption of glucose and its metabolism in experimental cellular models (Chenhui *et al.*,2005).

As human hepatocellular carcinoma cells (HepG2 cells) can respond to insulin, these can be used to model anti-diabetic activity (Zou *et al.*, 2005; Leira *et al.* ,2002) as assayed by glucose uptake, potential anti-diabetic activity of the target phyto-compounds were assessed by observing glucose uptake by the HepG2 cells. The factors contributing to the application of HepG2 cells as a model cell line of this research were also described in Section 1.12.1. Since they have known histories of anti-diabetic activity (Section 1.10), phytochemicals phloretin, ouabin, berberine, and metformin, rebaudioside – A, and extracts of goldenseal , *gymnema* , goat's rue and *stevia* were used, and their efficacies have been discussed in Section 1.10.

1.11.3. Glucose utilization, storage, and excretion

The consumption, production, storage, and excretion of glucose by various tissues are discussed in the following sections.

1.11.3.1. Muscle tissue/cells: Most of the glucose absorption by peripheral tissues occurs in muscle tissue (75% to 80%). When glucose enters muscle cells, it is used in the glycolytic and oxidative phosphorylation pathways to generate ATP. When energy demands are met, the excess glucose can be converted to glycogen and stored for later usage. In the skeletal striated muscle cells glycogen synthesis is regulated by an enzyme, glycogen synthase, and insulin stimulates glycogen synthase enzyme in two different pathways which leads to dephosphorylation. The primary pathway is to trigger the phosphatases that dephosphorylate the glycogen synthase enzyme, whereas the secondary pathway is to inhibit the kinases that are contributing to phosphorylation. Furthermore, the PI 3-kinase pathway plays a key role in GLUT-4 activation and has a major role in insulin action by stimulating glycogen synthase (Guyton and Hall, 2006; Porte, Sherwin and Baron, 2003; DeFronzo, 2004).

1.11.3.2. Hepatocytes/Liver cells: Although glucose uptake occurs in hepatocytes without mediation of insulin, insulin has a vital role in the regulation of glucose consumption and synthesis by the liver. During the fed state, in which circulating levels of both glucose and insulin will increase, insulin stimulates glycolysis, which enhances glucose uptake. Insulin will also increase glycogen synthesis, and excess glucose will be converted to fat after glycogen stores are replenished, a process aided by insulin's impact on acetyl CoA carboxylase. During starvation glucose concentrations lower, insulin levels fall, and glucagon levels increase. Glucose homeostasis is maintained by breakdown of glycogen, a process triggered by glucagon. Under conditions of more extreme starvation, gluconeogenesis is activated through phosphoenolpyruvate carboxykinase (Guyton and Hall, 2006; Porte, Sherwin, and Baron, 2003; DeFronzo, 2004).

1.11.3.3. Adipose/Fat tissue: In general, extra glucose will be converted to fatty acids in the liver and stored as triglycerides in adipose tissue when glucose levels are high and glycogen stores are full. Fat tissue accounts for only about 5% of basal glucose transport. Insulin has a significant role in promoting triglyceride synthesis and storage. Insulin activates lipoprotein lipase in the capillary walls of fat tissue, that breakdown triglycerides further into fatty acids and promote fatty acid uptake by adipocytes, and inside of the

adipocytes they again revert into triglycerides for the storage purpose. Insulin inhibits hormone sensitive lipase inside of the adipocytes; thus, it prevents triglycerides from being turned into fatty acid by the process of hydrolysis, and thereby it prevents fatty acids entry into the blood stream. Also, insulin promotes glucose transport into adipocytes, to synthesise glycerol, which is essential to form triglycerides in combination with fatty acids (Guyton and Hall, 2006; Defronzo, 2004).

1.11.3.4. Kidney cells: Kidney cells are involved in glucose homeostasis through three mechanisms, glucose uptake, gluconeogenesis, and reabsorption of glucose at the level of proximal convoluted tubule. When blood glucose levels exceed more than 180 mg/dL, kidneys promote glucose excretion through the urine, of which about 90% is reabsorbed by the segment1 (S1) sodium glucose cotransporters 2 (SGLT2). In segment 2 (S2), the SGLT2 and glucose transporter 2 both are presented and supports glucose reabsorption, while around 10% of remainder glucose is reabsorbed in the segment 3 (S3) where SGLT1 and glucose transporter 1 are presented. However, as glucose concentration in the filtered urine raises further, which often goes beyond the brink of glucose reabsorption, more glucose will be lost in the urine, and causes fatigue in diabetic individuals (Guyton and Hall, 2006; Wright, Hirayama, and Loo,2007; Katsuno *et al.*, 2007; Ghani and Defronzo, 2008).

1.11.4. Glucose consumption studies

A blood glucometer is a device generally employed for the measurement of glucose levels in the blood samples of diabetic people. In this research it was used to measure the changes in the glucose levels in the long term treated (i.e., goldenseal, *gymnema*, goat's rue, and *stevia* ethanolic extracts and rebaudioside-A, berberine and metformin and controls) cell culture medium. A detailed description is provided in a relevant Section, Chapter -2 (Materials and Methods).

1.11.5. Protein estimation studies

Bradford protein assay was employed to quantify the protein content in both short-term (1 hr) (i.e., goldenseal glycerite and ethanolic extracts, gymnema ethanolic extracts and *stevia* aqueous extracts) and long-term (48 hr) treated cells (i.e., goldenseal, *gymnema*, goat's rue, *stevia* ethanolic extracts and rebaudioside-A, berberine,metformin and controls), and the mean values of triplicates of protein content was used to divide the

mean values of triplicates of glucose uptake levels and glucose consumption levels, thereby,glucose uptake levels and consumption levels of the single cell was estimated. A detailed description is provided in a relevant Section, Chapter -2 (Materials and Methods).

1.11.6. Cytotoxicity studies

A cytotoxicity study has been performed to assess the cell viability after the long-term treatment with optimal concentrations of the target phytocompounds and extracts. A detailed description is provided in a relevant Section, Chapter -2 (Materials and Methods).

1.11.7. Estimation of Glycogen levels in the samples

Glycogen is the primary short-term molecule, which functions as a form of energy storage, and synthesized mainly in the liver and muscle (Sections 1.11.2.1, 1.11.3.1 and 1.11.3.2). It is a branched glucose polymer, in α-1, 4 linkages, with branching via α-1, 6 linkages. Irregular use of glycogen and disruption in glycogen synthesis is commonly found in diabetes. The glycogen assay was employed to measure glycogen levels in the long-term treated samples (Amoasii *et al.*, 2016). A detailed description is provided in a relevant Section, Chapter -2 (Materials and Methods).

1.11.8. Seahorse metabolic analysis

Any live cell requires Adenosine triphosphate (ATP) which is commonly referred as “molecular unit of currency” of intracellular energy transfer. Mitochondrial oxidative phosphorylation and glycolytic metabolism are the vital steps in ATP synthesis, however, energy requirements and capacity of the cell and fluctuations in the energy levels are closely regulated. Glycolysis and oxidative phosphorylation are also part of the biosynthetic requirements, as they generate intermediates for protein, DNA, and lipid biosynthesis. Therefore, fluctuations in energy metabolism are coupled to cellular functions such as cell division, differentiation, and activation. Therefore, quantification of glycolysis and oxidative phosphorylation rates may be useful to estimate physiological status of the cells in diabeteic patients.

Metabolic analysis assay quantifies oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) simultaneously, the former is an indicator of mitochondrial respiration, where as the latter is mainly the outcome of glycolysis. Cellular respiration (OCR) and glycolysis (proton excretion) causes rapid and measurable changes in absorbed

oxygen levels and free protons in a 'transient microchamber' of the instrument, and are measured every few seconds by solid state sensor probes located 200 microns above the cell monolayer. The instrument continues to measure the concentrations until the rate of change is linear and then calculates the slope to determine OCR and ECAR, respectively. Therefore, this study explored OCR and ECAR levels in the long term treated cells with target phyto compounds (de Moura and Van Houten, 2014; Gibert, McGee, and Ward,2013; Raftery, Jayasundara, and Di Giulio,2017).

1.11.9. Glucose release studies

In this research, the released glucose levels in the "glucose free and phenol red free" cell culture medium of long-term treated cells have been measured. A detailed description is provided in a relevant Section, Chapter -2 (Materials and Methods).

1.12. Target mammalian cellular models of the research

1.12.1. Human hepatocellular carcinoma (Hep G2) cells

HepG2 cells originate from the hepatocellular carcinoma tissue of a 15-year-old Caucasian male. HepG2 cells have 55 chromosomes and appear epithelial in morphology. HepG2 cells usually express plasma proteins like albumin, fibrinogen, and plasminogen, and heterodimeric lipid transfer proteins like microsomal triglyceride transfer protein. They are adherent, robust and behave like epithelial cells, grow as monolayers and aggregate in small clusters (HepG2 cells, 2015). HepG2 cells grown in 3-D porous polystyrene scaffolds exhibited clear morphological appearance and expressed numerous bile canaliculi that were rarely seen in cultures grown on 2-D. As 3-D cultures enhance cell structure and improve functionality during toxicology studies, this method of application is potentially useful in drug testing and toxicology studies (Bokhari *etal.*,2007). By considering the above features of HepG2 cells, and because of their well-known competence to respond to insulin and insulin mimicking compounds, they are routinely used to model anti-diabetic action as assayed by glucose uptake experiments, and in this research HepG2 cells are chosen (Zou *et al.*,2005; Wu *et al.*,2013).

Chapter 2: Materials and Methods

2.1. Chromatographic analysis of *Stevia rebaudiana Bertoni* leaf

2.1.1. Materials

In this study, plantlets of *Stevia* (10 cm) were bought from Norfolk Herbs, Blackberry Farm, Dillington, Dereham, Norfolk, NR19 2QD (Norfolk Herbs, 2015). Plants were purchased in spring 2012 and initially placed on a windowsill (Figure 44) to protect them from excessive cold. Later in the summer plants were transferred into bigger pots and placed in an open environment (Figure 45).

Figure 44: *Stevia* herbs grown on the windowsill at temperate climate in Northwest of UK.

Figure 45: *Stevia* plants grown in an open environment in mid-spring and summer (4 to 5 months) season in Northwest of UK.

2.1.1.1. Shade drying of *Stevia* leaves

In the last week of October, *Stevia* leaves were harvested and underwent a shade drying process. The principles for the shade drying are the same as for sun drying (Fruit and vegetable processing, 2013), and shade drying is carried out essentially for fruits and herbs which tend to lose their colour and turn brown if they are exposed to direct sunlight. *Stevia* has a natural rich green colour and gives a more attractive finish-product when they are dried in the shade. *Stevia* leaves were dried in the shade with full air circulation (Figure 46); normally shade drying takes more time than drying in full sunlight.

Figure 46: *Stevia* leaves while shade drying.

After few weeks of shade drying, the leaves were completely lost moisture content. Broken and discoloured leaves were removed, and only fine quality leaves were collected for further processing. By using gentle application of a mortar and pestle, a fine powder of *Stevia* leaves was prepared (Figure 47).

Figure 47: Fine powder of *Stevia* leaves

After obtaining the fine powder of *Stevia* leaves, a chromatographic analysis was performed to quantify the principal glycosides content for the assessment of its effect on glucose uptake mechanism in HepG2 cells.

2.1.1.2. High Performance Liquid Chromatographic (HPLC) - MS analysis of shade-dried *stevia* leaf extract

High-performance liquid chromatography or high-pressure liquid chromatography is a technique in separation chemistry, which is applied to separate, to identify, and to quantify each component in a composition. The analysis of a complex mixture of steviol glycosides in *stevia* leaf extract is a complicated task and often encounters challenges like poor accuracy and poor peak separation in the chromatogram with application of amino and RP-18 columns which are commonly used (Dacome *et al.,* 2005; Pol *et al.,* 2007; Hearn and Subedi, 2009; Kolb *et al.,* 2001; Geuns, 2010; Clos *et al.,* 2008; Gardana *et al.,* 2003). Also, amino columns need longer equilibration periods, so these are not applicable to mass spectrometric (MS) detection, whereas RP-18 executes poor selectivity for quantitatively predominant glycosides such as stevioside and rebaudioside-A in *stevia* leaves and relevant products. To overcome issues like these, reverse phase (RP) and hydrophilic interaction liquid chromatography (HILIC) were employed (Rieck *et al.,* 2010; Zimmermann *et al.,* 2011) and rebaudioside-A was used as an internal standard in this analysis.

HILIC columns are a modified form of normal phase liquid chromatography which are partially overlaps with ion chromatography and reversed phase liquid chromatography and uses hydrophilic stationary phases with reversed-phase type eluents. Once components in the mixture of steviol glycosides are separated using these special columns, they can be identified by application of UV and MS, whereas components in UV peaks can be identified by mass spectrometry (Gardana *et al.*, 2003; Choi *et al.*, 2002; Pol *et al.*, 2007). In this experiment when the detector expresses a peak, it can be simultaneously diverted to a mass spectrometer. There it would express a fragmentation pattern that could be compared against a computer database of already existing or known patterns. The identity of a large range of known and unknown compounds can be found without need of retention times.

2.1.1.2.1. Materials

LC/MS grade solvents and additives such as, water (Optigrade, Promochem, Wesel, Germany), acetonitrile (Mallinckrodt Baker, Deventer, The Netherlands), and formic acid (Fluka/Sigma Aldrich, Steinheim, Germany) were used in the elution process (Zimmermann,2011).

2.1.1.2.2. Methods

Preparation of test sample and the standard: The samples were extracted by a mixture of acetonitrile and water (1+1). 300 mg of the powdered samples were extracted with 3 mL of the extraction mixture in an unltrasonic bath. After centrifugation, the supernatant was transferred in a 10 mL-volumetric flask. The extraction of the same sample was repeated two times (i.e., in total three extraction steps) and the supernatants collected in the same volumetric flask, which was then filled up to 10 mL (Rieck *et al.*, 2010) and the standard pure rebaudioside-A (Wako Chemicals, Neuss, Germany, purity >99%) was dissolved in water/acetonitrile (50:50, v/v).

Application of HPLC/MS: A Waters (Acquity, USA) ultra-performance liquid chromatographic system was employed for HPLC/MS detection, comprising a binary pump, auto sampler (injectable volume of the sample: 1 or 10 μL for single ion recording or product-ion scan, correspondingly), column oven set at 40°C, diode-array detector, scanning from 190 to 400 nm, along with a TQD triple quadrupole mass spectrometer with electrospray interface operating in negative mode. Hydrophilic interaction liquid chromatography columns (125mm×2mm) (Nucleodur, Macherey-Nagel, Germany), were

employed to carry out the separation process with water (A) and acetonitrile (B) as eluents and these are acidified by adding 0.1% (v/v) formic acid.

A flow rate of 0.45 mL/min was used with gradient elution: 0–10 min, 7% A; 10–15 min, 7–16% A; 15–17 min, 16–20% A; 17–20 min, 20% A; 20–22 min, 20–7% A; 22–26 min, 7% A (Zimmermann, 2011). Further to this preparation, the mass spectrometer was manually tuned by employing pure rebaudioside-A solution, to establish working parameters: capillary voltage of −2.0 kV, cone voltage of 28.0V, extractor voltage of 3.0V, radio-frequency voltage of 1.10V, source temperature of 150°C, desolvation temperature of 400°C, cone gas (nitrogen) flow of 50 L/h, and desolvation gas (nitrogen) at a flow rate of 800 L/h, whereas in tandem mass spectrometry mode, the inert gas argon was used for collision purposes and its flow rate was 0.3 mL/min (Zimmermann, 2011).

The test sample was mostly analyzed in selected ion recording mode operation, recording known and hypothetical m/z values (i.e., mass number/ charge number) of the different glycosides. After identifying the peaks of known compounds, these are examined by application of product-ion scans at lower (20 V) [a], medium (40 V) [a], and elevated collision energies (60 V) [a,] the entire setup was systematically controlled by Mass Lynx 4.1 software (Zimmermann, 2011). Volt[a] represents a unit of energy. In the Mass Lynx software, the potential difference would be measured in volts, and this enhances acceleration of the ions in the fragmentation cell, this energy is called ‘collision energy’, and it is a device-specific value. On the other hand, whether this collision energy should be considered high or low depends on the device and the analyte (Zimmermann, 2011).

2.2. Glucose uptake studies in cultured human hepatocellular carcinoma cells (HepG2 cells)

2.2.1. Materials

The molecular structures and glucose uptake properties of the phytochemicals listed below have been discussed in Chapter 1, Section 1.10.

2.2.1.1. Phloretin (Ph) : Sigma-Aldrich, ≥99% purity ; synonyms: β-(4-Hydroxyphenyl)-2,4,6-trihydroxypropiophenone,2′,4′,6′-Trihydroxy-3-(4-hydroxyphenyl)propiophenone,3-(4-Hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl)-1-propanone; CAS Number 60-82-2; molecular formula $C_{15}H_{14}O_5$, molecular weight 274.27 g/mol. Stored at 2 - 8 °C, appears as white to off white powder; colour in solubility state appears faint yellow to dark yellow, solubility/turbidity is clear at 50 mg/ml in MeOH (Phloretin,2015).

2.2.1.2. Ouabain octahydrate (Ou) : Sigma-Aldrich, ≥95% purity, HPLC grade; synonyms: 1β,3β,5β,11α,14,19-Hexahydroxycard-20(22)-enolide 3-(6-deoxy-α-L-mannopyranoside), acocantherine, G-strophanthin; CAS Number 11018-89-6; molecular formula $C_{29}H_{44}O_{12} \cdot 8H_2O$, molecular weight 728.77 g/mol. Stored at room temperature, appears as white to off white powder; colour in solubility state appears faint yellow to no colour, solubility/turbidity is clear to slightly hazy and dissolves 50 mg/ml in hot purified water (Ouabain, 2011).

2.2.1.3. Berberine chloride hydrate (Ber) : Sigma-Aldrich, ≥90% purity, synonym: natural yellow 18, CAS Number 141433-60-5, molecular formula $C_{20}H_{18}ClNO_4 \cdot xH_2O$, and molecular weight 371.81 g/mol (anhydrous form), can be stored at room temperature, appears in natural yellow colour, available in powder form, colour in solubility state appears yellow to light yellow, solubility/turbidity is slightly yellow and soluble in methanol and dimethyl sulfoxide (DMSO), and very slightly soluble in cold water, whereas sparingly soluble in hot water, available in crystalline powder form (Berberine chloride hydrate, 2015).

2.2.1.4. Metformin hydrochloride (Met): Sigma-Aldrich, laboratory grade chemical, CAS Number 1115-70-4, molecular formula $C_4H_{12}ClN_5$, molecular weight 165.6 g/mol; it can be stored at room temperature, appears in crystalline white colour, available in powder form, soluble in water, 95% alcohol, insoluble in ether and chloroform (Metformin hydrochloride, 2014, 2015).

Based on the literature target phytochemicals were initially madeup as 100x stock solutions in DMSO and used at the following working concentrations, DMSO (1%) (Wilkening, Stahl, and Bader, 2003; Tam *et al.,* 1997), phloretin (200 µM), ouabain (10 µM), berberine (10 µM), and metformin (1000 µM). And DMSO was also used as a solvent control (Ref: Section 2.2.2.1.7).

2.2.1.5. Goldenseal Glycerite: Liquid crude extracts of Goldenseal glycerites, Herb Pharm Company, Williams, Oregon, U.S.A.

Ingredients: Certified organic vegetable glycerin (non-palm), distilled water and goldenseal extractives. Non-GMO & Gluten-Free, contains 20% glycerol. Potency: Dry herb / menstruum ratio: 1: 5; Extraction rate 140 mg of fresh herb per 0.7 ml. It was prepared from the rhizome and rootlet of *hydrastis canadensis* plants which are certified organically grown. To assure optimal extraction of goldenseal's bioactive compounds, the rhizome and rootlet are hand-dug in the autumn, are thoroughly cleaned and carefully shade-dried, and are then thoroughly extracted in certified organic non-palm glycerin. Certified organic by Organic Certifiers (Goldenseal glycerite, 2018).

To apply in the experiments, 0.312 µL/mL, 0.625 µL/mL, 1.25 µL/mL, 2.5 µL/mL, 5 µL/mL, 10 µL/mL concentrations were prepared by performing doubling serial dilutions in RPMI-1640 full media, and an untreated control was used to compare the effect of the different concentrations. 0.2% (v/v) glycerol was used as solvent control.

2.2.1.6. Goldenseal Tincture: Liquid crude extracts from "Napiers the Herbalists" company, Edinburgh. It contains 60% ethanol as solvent and 40% dry herb (Goldenseal tincture, 2018). To apply in the experiments, 0.312 µL/mL, 0.625 µL/mL, 1.25 µL/mL, 2.5 µL/mL, 5 µL/mL, 10 µL/mL concentrations have been prepared by performing doubling dilutions in RPMI-1640 full media, and an untreated control was used for comparison. 0.6% (v/v) alcohol was used as solvent control.

2.2.1.7. Goat's rue Tincture: Liquid crude extracts from "Napiers the Herbalists" company, Edinburgh. *Galega officinalis* is extracted in sugar beet ethanol (alcohol). The part used is the herb. It contains 45% ethanol as solvent and 55% dry herb (Goat's rue tincture, 2018). To apply in the experiments, 0.312 µL/mL, 0.625 µL/mL, 1.25 µL/mL, 2.5 µL/mL, 5 µL/mL, 10 µL/mL concentrations have been prepared by performing doubling dilutions in RPMI-1640 full media, and an untreated control was used for comparison. 0.45% (v/v) alcohol was used as solvent control.

2.2.1.8. *Gymnema* Glycerite: The active principle in *Gymnema* leaves is gymnemic acid. It exists in the form of gymnemogen or deacylgymnemic acid. Molecular structure of gymnemic acid is shown in Figure 48.

A commercial crude liquid extract of *Gymnema sylvestre* was purchased from Nature's Answer, Hauppauge, New York, U.S.A (Nature's answer, 2010). It contains a minimum of 75 mg of gymnemic acids in 300 mg of serving and guarantees that the constituents of the extract are in the same synergistic ratios as in the plant (*Gymnema* leaf, 2010). This *Gymnema* crude extract contains 50% of glycerol and is free from alcohol. However, during production of the crude extract, alcohol has been used for extraction purposes along with water and natural extractants. Finally, by using cold bio chelated proprietary extraction process alcohol and other extractants have been removed (*Gymnema* leaf, 2010).

To apply in the experiments, 0.312 µL/mL, 0.625 µL/mL, 1.25 µL/mL, 2.5 µL/mL, 5 µL/mL, 10 µL/mL concentrations have been prepared by performing doubling dilutions in RPMI-1640 full media, and an untreated control was used for comparision.

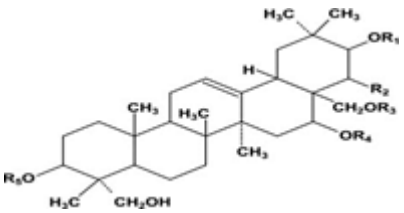


Figure 48: Molecular structure of gymnemic acid.

2.2.1.9. *Gymnema* Tincture: Liquid crude extracts from "Napiers the Herbalists" company, Edinburgh, Scotland. *Gymnema sylvestre* is extracted in sugar beet ethanol (alcohol). The part used is the leaf. It contains 45% Ethanol as solvent and 55% dry herb (Gymnema tincture, 2018). To apply in the experiments, 0.312 µL/mL, 0.625 µL/mL, 1.25 µL/mL, 2.5 µL/mL, 5 µL/mL, 10 µL/mL concentrations have been prepared by performing doubling dilutions in RPMI-1640 full media, and an untreated control was used for comparision. 0.45% (v/v) alcohol was used as solvent control.

2.2.1.10. *Stevia* leaf extracts (aqueous): *Stevia* liquid concentrate is made from the whole leaves and is concentrated in a base of purified water. It was purchased from Planetary herbals, Soquel, California, USA (*Stevia* extracts, 2018). To apply in the experiments, 0.312 µL/mL, 0.625 µL/mL, 1.25 µL/mL, 2.5 µL/mL, 5 µL/mL, 10 µL/mL concentrations have been prepared by performing doubling dilutions in RPMI-1640 full media. And an untreated control was used for comparision.

2.2.1.11. *Stevia* leaf extracts (Ethanolic): Liquid crude extracts from "Nutramedix" company, Jupiter, Florida, USA. *Stevia* leaves have been extracted in ethanol (20-24%) (*Stevia* tincture, 2018). To apply in the experiments, 0.312 µL/mL, 0.625 µL/mL, 1.25 µL/mL, 2.5 µL/mL, 5 µL/mL, 10 µL/mL concentrations have been prepared by performing doubling dilutions in RPMI-1640 full media. And an untreated control was used for comparision. 0.24% (v/v) alcohol was used as solvent control.

2.2.1.12. Rebaudioside –A : (Sigma-Aldrich, ≥96% pure, HPLC), synonym: (4α)-13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)-oxy]kaur-6-en-8-oic acid β-D-glucopyranosyl ester, CAS Number 58543-16-1, molecular formula $C_{44}H_{70}O_{23}$, and molecular weight 967.01, can be stored at room temperature, appears in white colour, available in powder form, colour in solubility state appears as clear solution, soluble in in water, ethanol and their binary mixtures (Rebaudioside-A , 2018) .

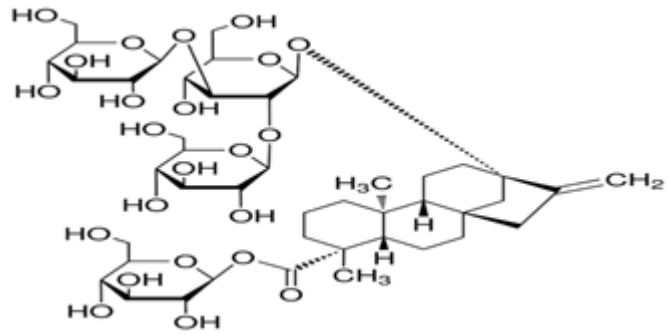


Figure 49: Rebaudioside –A

To apply in the experiments, 10 mg/mL solution was prepared in full medium, and from that 0.312 µL/mL, 0.625 µL/mL, 1.25 µL/mL, 2.5 µL/mL, 5 µL/mL, 10 µL/mL concentrations have been prepared by performing doubling dilutions in RPMI-1640 full media. And an untreated control was used to check the affect of the different concentrations.

2.2.1.13. Solvent controls: 0.6% (v/v), 0.45 % (v/v) and 0.24% (v/v) ethanol solutions were used as solvent controls.

2.2.2. Methodology

2.2.2.1. Culture of HepG2 cells

Following from Section 1.12.1, Chapter 1, HepG2 cells were kindly provided by the Institute of Liver Studies, King's College Hospital, London. HepG2 cells were maintained

in the Dulbecco's modified eagle's medium (DMEM) supplemented with 10% foetal bovine serum, 100units/mL penicillin, 100 µg/mL streptomycin, and 2mM L-glutamine (Invitrogen, CA, USA) and incubated at 37 °C within a humidified atmosphere of 5% CO_2 in air. Cells were allowed to grow up to 75% confluence. Glucose uptake assay was performed on growing cells and on confluent cells, and for shorter and longer durations, to study varying glucose uptake properties with varying cell density (Bader *et al.*, 1994).

2.2.2.1.1. Harvesting cells for sub-culture

HepG2 cells were seeded at a density of 1 - 5 x 10^4 cells / mL, in 6 mL full medium in a 25 cm^2 cell culture flask. Confluent monolayers formed within two to three days and cells were sub-cultured once a week in a ratio of 1:5 to 1:10 as follows. Medium was removed and cells rinsed with about 2.0 mL PBS/trypsin-EDTA (described below), per 25 cm^2 flask; most of the trypsin-EDTA was discarded. After incubation for around 10 minutes at 37 °C, cells gradually became rounded and eventually detached from the bottom of the flask; at this stage as it was essential to check the progress of the trypsin digestion and to quench the digestion, full medium was added, and all cells were detached. The cells were washed with 5 mL full medium and diluted into new flasks with full medium.

2.2.2.1.2. Standard full medium

Standard mammalian cell culture media were used. RPMI 1640 with 10% foetal calf serum (FCS), 2mM glutamine, and DMEM, with NEAA (non-essential amino acids), 1 mM sodium pyruvate, 2 mM glutamine, 10% foetal calf serum were employed. Following the recipe mentioned in Section 2.2.2.1, after taking care at every stage to prevent the contamination, application of antibiotics was stopped.

2.2.2.1.3. Full medium with alternative metabolic fuels

Media with defined energy substrates are often used in experiments with HepG2 cells in which a different sugar or other metabolic fuel is used instead of glucose. Almost all commercial supplies of RPMI 1640 or DMEM contain glucose, but some glucose-free formulations are available and were used to prepare media with controlled levels of glucose or galactose. Galactose DMEM was made with glucose-free DMEM (Invitrogen Cat. no. 11966-025) 500 mL, foetal calf serum 50 mL, NEAA (100x) 5 mL, sodium pyruvate (100 mM) 5 mL and galactose (0.5 M) 11 mL to give a final concentration of 9.63 mM. Galactose solution was prepared freshly by dissolving 1.35 g in 15 mL de-ionised water and sterilised by filtration. An equivalent glucose DMEM was prepared with

glucose-free DMEM (Invitrogen Cat. no. 11966-025) 500 mL, foetal calf serum 50 mL, NEAA (100x) 5 mL, sodium pyruvate (100 mM) 5 mL and glucose (0.5 M) 11 mL. Glucose solution was prepared freshly by dissolving 1.35 g in 15 mL de-ionised water and sterilised by filtration.

2.2.2.1.4. Trypsin-EDTA

A standard type of solution was used to detach the adherent cells from the flask or dish surface. Prepared with 200 ml $Ca^{2+}$and Mg^{2+}-free PBS sterilised by autoclaving, plus 2 ml sterile 0.5M EDTA pH7.5 and 20 ml of 10x trypsin. All media were prepared with de-ionised water suitable for HPLC.

2.2.2.1.5. Counting cells using a counting chamber (Haemocytometer)

50 – 100 µL of freshly harvested cells were transferred to a 1.5 mL eppendorf tube and one volume of trypan blue dye added and mixed gently. Cells that excluded trypan blue were live; cells that took up trypan blue were dead. The haemocytometer was prepared by ensuring the silvered surface was clean and placing a clean coverslip over the grid. 20 µL of cells was applied to the haemocytometer in the groove on the side. Two aliquots were applied over two separate counting grids (Figures 50 and 51).

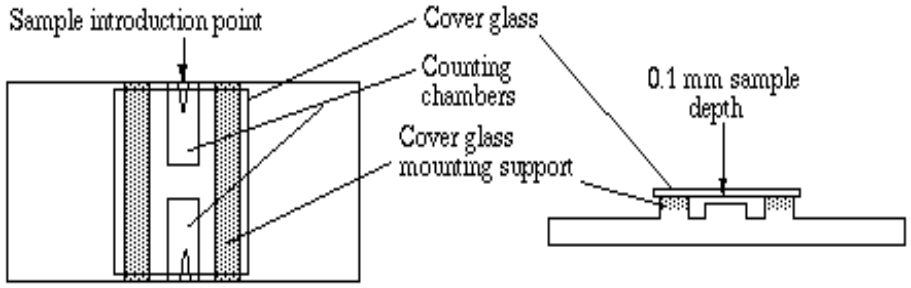


Figure 50: Haemocytometer

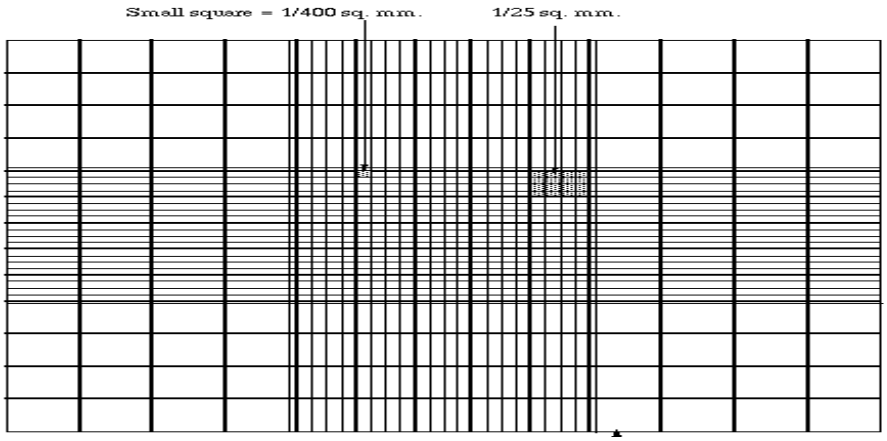


Figure 51: The grid in the Haemocytometer

The grid comprises nine large squares, as shown in the Figure 51. Cells were counted in the middle large square where the lines were intersected to form a grid of 25 squares within the large square; each of the 25 squares was made up of 16 small squares. The total number of cells in the middle large square was counted, if a cell overlapped the boundary was included, if it was overlapped the top or right-hand side of the boundary was also considered, but the cells were ignored which were overlapping the bottom or left-hand side of the boundary.

Calculation: The area of the central large square was 1.0 mm^2, and the sample depth was 0.1 mm. Therefore, the volume of the cells over the central large square was 0.1 mm^3 or 10^{-4} mL. If the total cell counts across the central large square = T. Cell concentration = T x 2 x 10^4 cells / mL. Here the factor of 2 was a dilution factor because the cells were diluted with one volume of Trypan blue.

The importance of cell counting: Most experiments involving the cells require an accurate and consistent measurement of the cell number at the start. This is because the cells grow and sometimes behave differently when grown at different cell densities. HepG2 cells were normally seeded at 5 x 10^4 cells/mL. This was a low density that allows the cells to grow further two or three days before becoming confluent and essentially trypan blue stains dead cells, which appear faint or dark blue within the grid. In this research, 90% cell viability was maintained throughout the study (i.e., % viability= live cell count/ total cell counts x100).

2.2.2.1.6. Glucose uptake assay

The probe 2-NBDG is a fluorescent glucose analogue used to monitor glucose uptake in live cells. In a study the intracellular fate of 2-NBDG in E. coli cells was explored. After uptake of 2-NBDG by E. coli cells, 2-NBDG converted to a 2-NBDG metabolite which was also fluorescent, and then the 2-NBDG metabolite was transformed into non-fluorescent forms. Later the 2-NBDG metabolite converted back to 2-NBDG into its original form by G6 Pase and at the same time released inorganic phosphate. This established the feasibility of using 2-NBDG for glucose uptake studies and viability assessment of various living systems. Therefore 2-NBDG can act as an indicator of cell viability based on glucose uptake activity (Yoshioka *et al.*,1996). Although sensitive to its environment 2-NBDG fluorescence typically displays excitation/emission maxima of ~465/540 nm and can be visualized using optical filters designed for fluorescein. 2-NBDG (2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-deoxyglucose); Molecular formula:

$C_{12}H_{14}N_4O_8$.; Molecular weight: 342.26, CAS no /Name: 186689-07-06/D-Glucose, 2-deoxy-2- (7-nitro-2, 1, 3-benzoxadiazol-4-yl) amino) – (Figure 52), soluble in water and recommended solvent was methanol.

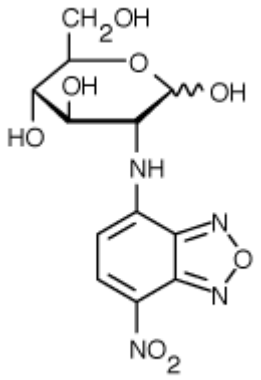


Figure 52: Chemical structure of 2-NBDG

2.2.2.1.7. Procedure for 24-well plate format

HepG2 cells were seeded in a 24-well plate (Costar) at 5 x 10^4 cells / mL, and 0.5 mL of the cells per well in medium containing 11 mM glucose. The cells were left to enter the growth phase overnight and were then treated with 5 µL volumes of test reagents in each well, including ouabain (10 µM) (Ozdemir *etal.*, 2012), phloretin (200µM) (Guo *etal.*, 2005; Wu *etal.*, 2009), metformin (1000 µM) (Zang *etal.*, 2004), and berberine (10 µM) (Yin *et al*,2002; Liu *et al*,2011). After administration, concentrations of working solutions were 100-fold diluted from the stock. The solvent control was DMSO (1%). Cells were incubated for a further 1 hour or 24 hours treatment.

To measure glucose uptake after treatment, medium was removed from the wells and a 100 µL aliquot of 2-NBDG medium or 100 µL aliquot of solvent control was added to each well. The cells were incubated for 15 minutes at 37 °C with 2-NBDG or solvent control. The medium was then removed, and the cells were washed with 0.5 mL PBS. 0.5 mL PBS was added to each well and the fluorescence with λ_{ex} 485 nm, λ_{em} 530 nm, was measured in an Omega fluostar plate reader. The microplate reader settings for the bottom of the well were 200 counts per well, and gain 1000 (although this was changed according to the signal).

2-NBDG solution: 2-NBDG was supplied from Invitrogen as a 5 mg aliquot (Catalogue no. N13195; mw = 342.26). It was dissolved in 1.46 mL methanol to give 10 mM stock solution, and stored at -80 °C. For each 24-well plate 15 µL 2-NBDG stock solution was added to 1.5 mL glucose-free DMEM (Invitrogen 11966-025) and for the solvent control 15 µL methanol was added to 1.5 mL glucose-free DMEM, here solvent control was employed to check the main working functionality of 2- NBDG (plan in Table 1). As 2-NBDG tends to transport intracellularly through the same GLUTs as glucose, it was possible that competitive inhibition of 2-NBDG uptake by D-glucose could occur (Hassanein *et al*,2011). To avoid this effect, which would reduce the sensitivity of the assay, it was necessary to use glucose free medium to prepare the 2-NBDG medium and the solvent control.

Plan for 24-well plate: Each row of six wells had one type of treatment, or control treatment. At the glucose uptake step, half of the wells were treated with solvent control, and the other half of the wells were treated with the 2-NBDG medium. This meant that each treatment or control had three replicates (Table 1).

Table 1: Plan for 24-well plate

	Solvent Control			**2-NBDG Medium**			**Av intrinsic fluorescence**	**Av Adjusted fluorescence**
A	14017	13752	13720	15319	22240	17874	13830	4648
B	13758	13746	13266	27195	25078	23230	13590	11578
C	13534	13348	13290	26454	21846	23839	13391	10656
D	13437	13234	13322	22171	20778	27929	13331	10295

The sample data presented here is fluorescence at excitation/ emission maxima of λ_{ex} 485 nm and λ_{em} 530 nm. These values are triplicates of samples. Solvent control expresses intrinsic fluorescence of the samples, whereas 2-NBDG treated samples express fluorescence of 2- NBDG uptake. To obtain average adjusted fluorescence, average intrinsic fluorescence values were subtracted from average fluorescence values of the 2-NBDG treated medium.

2.2.2.1.8. Procedure for 24-well plate format (short-term treatment; performed in 2017)

HepG2 cells were seeded in a 24-well plate (Costar) at 5 x 10^4 cells / mL, and 0.5 mL of the cells per well in full medium containing 11 mM glucose. The cells were left to reach the confluency and the media was removed, the cells were then treated with 0.5 mL volumes of varying concentrations of test reagents (0.312 µL/mL, 0.625 µL/mL, 1.25 µL/mL, 2.5 µL/mL, 5 µL/mL, 10 µL/mL) in each well, for each of the different test plates, including goldenseal glycerite, goldenseal tincture, *gymnema* glycerite and *stevia* aqueous extracts. The untreated control was distilled water. Cells were incubated for a further 1-hour treatment, and glucose uptake was measured as explained in Section 2.2.2.1.7.

Plan for 24-well plate: The cells in the first three wells of the first column were treated with distilled water (untreated control), and cells in three wells of columns 2 to 6 were treated with concentrations 10 µL/mL to 0.625µL/mL. The first three wells of the bottom row were allocated for background fluorescence and the cells in other three wells in the same row were treated with 0.312µL/mL concentrations.

2.2.2.1.9. Procedure for 96-well plate format (long-term treatment; performed in 2017)

Same procedure as Section 2.2.2.1.8 has been employed, but in this case the cells have been treated for 48 hour with target ethanolic extracts such as Goldenseal tincture, Goat's rue tincture, *Gymnema* tincture, *Stevia* ethanolic extracts and Rebaudioside –A, Berberine, and Metformin was used as positive control, and solvent controls were 0.6% (v/v), 0.45 % (v/v),0.24 % (v/v) ethanol solutions, and DMSO (1%). For the aqueous extracts, distilled water was used as a solvent control.

Plan for 96-well plate: In a 96 well plate the top and bottom rows and the first and last columns were left unused. Columns 2 to 7, in an each column three wells were treated with varying concentrations 10 µL/mL to 0.312µL/mL of the target compounds, and the three wells in column 8 was allocated for berberine, three wells in column 9 was allocated for metformin, three wells in columns 10 and 11 were allocated for solvent controls 0.6% (v/v), 0.45 % (v/v) and the last three wells in column 11 were allocated for solvent control 0.24 % (v/v) ethanol solutions. The remaining wells were organised for DMSO (1%) and for the untreated control.

2.2.2.1.10. Glucose uptake assay by flow cytometry

HepG2 cells were cultured in six separate flasks (Corning, surface area 25 cm^2) until they reached confluency, then treated for 48 hours with test compounds goldenseal ethanolic extracts (10µL/mL), berberine (10 µM), and positive control metformin (1000 µM), solvent controls DMSO (1%) and ethanol (0.6 % (v/v)) and an untreated non-fluorescent negative control. At the end of the treatment, medium was removed, and the cells were washed once with glucose free medium. The cells were trypsinised and collected, followed by quenching with ice cold glucose free medium in six, 15ml tubes. Cells were treated with 400µL of 0.1 mM 2-NBDG and incubated for 15 minutes.

The cells were collected in 18 eppendorf tubes (1.5 mL) and spun down at 800g for 5 minutes for settling down at the bottom of the tube. Medium was removed from the tube and 1.0 mL ice cold PBS was added. The cells were spun down at 5000rpm for 20 seconds. PBS was removed from the tube and 1.0 mL ice cold PBS was added and the cells were suspended. The cells were analysed with a Guava flow cytometer controlled using Guava Express Plus software, version 4.1 (Merck Millipore). The HepG2 cells which took up 2-NBDG displayed fluorescence with excitation and emission at the wavelength of the fluorescence λ ex=485 nm and λ em= 535 nm respectively, and the effect was measured in the channel used to detect fluorescence (Glucose uptake cell-based assay kit, Cayman chemicals, 2014).

2.3. Glucose consumption studies

A blood glucometer is a device generally employed for the measurement of glucose levels in the samples of diabetic people. In this research it was used to measure the changes in the levels of glucose in the long-term treated (48 hr) cell culture medium. In this study the SD codefree glucometer was used, it was manufactured by SD Biosensor Incorporation, Korea. It has a “glucose oxidase biosensor”, and measures with a range of 0.6 ~ 33.3 mM. The test strips require 0.9 µL of liquid sample for a single measurement. The tip of the inserted test strip into the glucometer absorbs required volume of the treated cell culture medium by capillary action upon contact of the sample.

A glucometer works based on the glucose oxidase- peroxidase method, glucose oxidase is an enzyme extracted from the growth medium of a fungus *aspergillus niger*. It catalyses

the oxidation of beta-D-glucose present in the blood plasma to convert into D-glucono -1,5 – lactone, and releases hydrogen peroxide (H_2O_2) as byproduct; the lactone is then gradually hydrolysed to D-gluconic acid. The hydrogen peroxide produced is then broken down to oxygen and water by a peroxidase enzyme. Oxygen then react with an oxygen acceptor such as ortho toluidine which itself converted to a coloured compound, the amount of which can be measured colorimetrically.The inbuilt colorimeter, which works on the basis of Beer Lambert's law, by which it calculates the concentration of glucose by determining the absorbancy/ optical density of the sample solution i.e., concentration of glucose in the test sample = (absorbancy of test/absorbancy of standard)*concentration of standard, The standard is programmed into the machine while manufacturing.The result displays in mmol/L. An in-built calculator measures the values and displays on the screen (Tonyushkina and Nichols, 2009).

Figure 53: SD Biosensor Glucometer and a pack of blood glucose test strips

The samples of the long-term treated (48 hr) confluent cells with varying concentrations (0.312 µL /mL to 10 µL /mL) of test compounds, goldenseal ethanolic extracts, *gymnema* ethanolic extracts, goat's rue ethanolic extracts, *stevia* ethanolic extracts , rebaudioside –A ,and berberine (10 µM), metformin (1000 µM) , and negative control DMSO (1%) and solvent controls 0.6% (v/v) ethanol , 0.45 % (v/v) ethanol , and 0.24 % (v/v) ethanol and untreated cell culture medium and RPMI-1640 medium were tested. Calculation of the glucose consumption per single cell was measured by dividing the mean value of triplicates of each treatment by mean value of triplicates of protein content. And then the

relative levels of glucose consumption at different concentrations were compared with glucose consumption rate of untreated control and RPMI-1640 medium.

2.4. A. Bradford protein assay

Both short-term (1 hr) and long-term (24hr or 48 hr) treated cells were employed in protein quantification assays, and the mean values of triplicates of protein content was used to divide the mean values of triplicates of glucose uptake levels and glucose consumption levels, thereby, to estimate glucose uptake levels and consumption levels of the single cell. The cell lysates were prepared by adding 0.1 mL of 1.0 M NaOH to each well and rocked them thoroughly for 15 minutes to ensure lysis of the cells. In case of frozen stored plates, after thawing, the samples in well were thoroughly mixed by pipetting before taking an aliquot for assay.

Preparation of the standards: BSA stock solution was prepared in water (10 mg/mL) and checked the absorbance at 280 nm, which should be 0.667 for 1.0 $mg.ml^{-1}$. 50 µL of each standard was used, in triplicate. Standards were prepared as shown in the below table.

Table 2: Different concentrations of BSA standard solution

Concentration of BSA (µg/µL)	Volume of BSA stock (µL)	Volume of Water (µL)
0	0	1000
5	5	995
10	10	990
20	20	980
40	40	960
60	60	940
80	80	920
100	100	900

Assay procedure: 50 µL of each cell lysate was taken per well in a 96 well plate, and each lysate assayed in triplicates, and applied 50 µL of BSA protein standard per well and each standard assayed in triplicates. Added 150 µL of Pierce Bradford reagent by using 8-

channel pipette, and mixed Bradford reagent and sample in a plate reader by shaking for 10 seconds, and left at room temperature for 15 minutes, and then read absorbance at 595 nm in Omega fluostar plate reader.

In protein estimation experiments standard curve preparation is essential; thus, the known properties of the standard solution were measured, and graph was plotted, this typical standard curve allows the similar or same properties to be estimated for the unknown or test samples by interpolation of the standard curve. Therefore, the samples with already known properties are considered as the standards and the relating graph is the standard curve. Standard curves are generally used to estimate the concentration of proteins or nucleic acids such as DNA. This property measurement generally takes place in terms of parameters such as absorbance, optical density, luminescence, fluorescence, and radioactivity. In this study absorbance was considered as the major parameter to estimate accurate protein content in the lysates.

2.4. B. MTT cytotoxicity assay

The MTT assay is a colorimetric assay. NAD(P)H-dependent cellular oxidoreductase enzymes may reflect the number of viable cells in a culture and can reduce the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan, which is insoluble and purple in colour. Generally, these assays are performed in the dark since the MTT is a light sensitive agent. After long-term treatment on confluent HepG2 cells, to check the effect of the optimal concentrations of the target phytocompounds MTT assays have been performed.

Assay procedure for 24 well plates (Nunc cell culture plates)

Thiazolyl blue tetrazolium bromide (MTT) (Sigma-Aldrich, Poole, UK), was dissolved in PBS (5 mg/ml). 100 µl of MTT solution was added to each well of cells in a 24 well plate. After 2 hours incubation at 37^0C the medium was removed and 500 µl of DMSO was added and the cells were incubated further 30 minutes. And the absorbance was read at 570 nm and 670 nm in a plate reader (Fluostar omega microplate reader).

2.5. Statistical analysis

The experimental data are presented as mean ± standard deviation (S.D) of the mean of n independent measurements. Statistical significance was determined as the difference from

the vehicle control to target treatments by employing one-way ANOVA with Bonferroni's multiple comparison post hoc tests. Also, Student's t-test was employed, $p>0.05$(not significant), $*p<0.05$, $**p<0.01$, $***p<0.001$, and $****p<0.0001$.

2.6. Western blotting analysis for detection of GLUT-1

The steps in western blotting technique to detect expression of GLUT-1 protein in HepG2 cells are summarised below.

2.6.1. SDS polyacrylamide gel electrophoresis

2.6.1.1. Preparation of samples

The HepG2 cells were cultured in four 75 cm^2 flasks (Costar); 12 mL of cells with a density of 2.5 x 10^5 cells/mL were seeded in each flask, and incubated until they reach confluence. They were treated for long-term (48 hour) with test compounds goldenseal ethanolic extract (10µl/ml), berberine (10 µM), solvent controls DMSO (1%) and 0.6% ethanol (v/v). After treatment cells were trypsinised and harvested and quenched with 1mL of fresh full medium and collected in 1.5mL microfuge tubes. The cells were centrifuged at 1000 rpm for 5 minutes, the supernatants removed, and 1 mL of PBS added to wash the cells. The cells were centrifuged at 1000 rpm for 5 mins and the supernatants removed. To each pellet, 100 µl of RIPA buffer (lysis buffer) containing a cocktail of protease inhibitors (Sigma-Aldrich) was added to solubilise the membranes. Cells were re-suspended by pipetting and left on ice for 10 minutes to lyse. The lysate was spun at 12000rpm for 5 minutes. Lysate supernatant was transferred to new tubes. 50 µl of 2x SDS-PAGE sample buffers (see below for the composition) was added to 50 µl of lysate. The samples were heated at 95 °C to denature the proteins and stored at -20 °C.

Composition of sample buffer: 2X buffer was prepared: 250 mM Tris pH 6.8, 4% SDS, 20% glycerol, 0.005% bromophenol blue, 20% β- mercaptoethanol added freshly for each preparation to 0.8 ml of 2.5X stock solution of the other components.

2.6.1.2. Preparation of the running gel

A 12% running gel was prepared by mixing 4mL of acrylamide monomer solution (30% w/v), 2.50 mL of 4X running buffer (1.5M Tris-HCl; pH 8.8), 0.10mL of 10% (w/v) SDS, 3.30 mL of dH2O, 0.10 mL of 10% (w/v) APS (freshly prepared ammonium perodoxisulfate), 0.01mL TEMED. The mix was applied to the sandwich of electrophoresis plates assembled in a gel caster, to a level about ¾ full, and covered with 100 µL butanol saturated with water and left for 20 min for polymerisation. At the end butanol was removed from the surface.

2.6.1.3. Preparation of the stacking gel

4% concentration of stacking gel was prepared by mixing 0.665mL of acrylamide monomer solution (30% w/v), 0.625 mL 8X stacking buffer (1.0M Tris-HCL; pH 6.8), 0.05 mL 10% (w/v) SDS, 3.625 mL dH_2O, 0.1mL APS (10% w/v), and 0.010 mL TEMED. The stacking gel solution was added on to the running gel in the electrophoresis plates, a 10 teeth comb inserted and left for polymerisation for 20 minutes.

2.6.1.4. Preparation of gel apparatus

The gel unit containing glass, plastic plate and spacers were removed from the dual gel caster and washed thoroughly with de-ionised water and placed against the upper buffer chamber of the electrophoresis apparatus (SE250, Hoefer Scientific instruments) oriented with the glass plate looking outside and secured to the unit with 2 red clamps. Running buffer (10 mL SDS 10%, 3.0 g Tris base, and 14.4 g glycine to 1 L with dH_2O) was added to the upper buffer chamber until it covers the comb. The comb was removed slowly from the stacking gel in order not to damage the wells. A 25 or 50 µL glass syringe, or flatten pipette tips, were used to load the samples and controls into the wells (same volume is maintained in each well), 25 µL of goldenseal and berberine treated samples and controls were loaded into the wells; 5 µL of BioRad markers were used. The left-over wells were filled with SDS-PAGE sample buffer.

2.6.1.5. Running the gel

The safety interlock lid was placed on the gel electrophoretic unit and leads were connected to the mains. The gel was run at 10mA constant current (power supply, POWER PAC 300, BioRad) until the samples migrated into the running gel, when the current was increased to 20mA. The run was completed when the dye reached the bottom of the running gel.

2.6.1.6. Western blotting

2.6.1.6. A. Electro-blotting

The spacers were taken out gently and glass plate was removed smoothly within the gel unit. The aluminium plate along with the gel was dipped in the Towbin transfer buffer (TTB: 3.03g Tris, 14.41g glycine, 10mL 10% (w/v) SDS, up to 800mL with H_2O, 200mL methanol) for about 5-10 minutes. The stacking gel was separated from the running gel from the plastic plate. Four pieces of chromatography paper (Whatman, 3MM) were cut to take the same shape as the sponges for the blot. A piece of nitrocellulose membrane was also cut (e.g., Hybond-C extra, Amersham) just bigger than the size of the running gel. All the papers, sponges and nitrocellulose membrane were wetted with TTB. The transfer sandwich was arranged in the following order: grey side of the cassette, sponge, two pieces of chromatography paper, membrane, running gel, two pieces of chromatography paper, sponge, and black side of the cassette.

The entire transfer unit was assembled under TTB to minimise trapping air bubbles. The transfer unit was closed, and it was inserted into the transfer apparatus (TE22, Hoefer Scientific Instruments), the grey side with the positive pole (anode). The protein would go from the gel (- side) to the membrane (+ side). The tank of the transfer apparatus was filled with TTB. The leads of the transfer apparatus were connected to the power supply (EPS600, Pharmacia) and proteins transferred overnight with a constant voltage of 10V.

2.6.1.6. B. Staining of blotted proteins

After overnight transfer the membrane was washed in ultrapure water and positions of marker proteins were marked using indelible pens. To quantify relative amounts of protein that had been blotted from each sample, a temporary staining of the proteins was carried out after overnight transfer, using a reversible protein stain for nitrocellulose membranes (Thermo Scientific Pierce, cat. no. 024580). After staining an image of the membrane was taken using an UVItec image analyser and the stain was removed, following instructions in the kit. The membrane was finally washed with ultrapure water and used for immunodetection.

2.6.1.6. C. Immuno-detection of GLUT-1

I. Washing: the membrane was placed in a small plastic box and soaked in TBS (Tris-buffered saline: 100mM Tris-HCl pH 7.5, 0.9% (w/v) NaCl) for 5 -10 minutes.

II. Blocking: The membrane was rinsed with wash buffer (0.1% Tween-20 in TBS) and then blocked by incubation with blocking buffer with 3-6% (w/v) milk powder (Marvell) in wash buffer, to block the unoccupied protein binding sites. The membrane was incubated with rotation at room temperature for 30 minutes.

III. Incubation with primary antibody: The blocking buffer was discarded, and the membrane washed three times with wash buffer (0.1% Tween-20 in TBS). The wash buffer was poured off and the membrane incubated with the primary antibody solution containing rabbit anti-GLUT-1 antibody. The first antibody tried was from Santa Cruz (sc-7903 purchased from Bioscience UK) and was a polyclonal rabbit raised against amino acids 218-260 of human GLUT-1. It was expected to detect GLUT-1 as a 55 kDA protein in human cell lysates. A variety of conditions were tried at first, including 3% or 5% blocking buffer and dilutions 1:500, 1000, 2000. To minimise background staining a dilution of 1:1000 in 5% blocking buffer was used.

The first antibody did not give interpretable results (described below) and a second antibody was tried, abcam 15309 (abcam, UK). This was also a rabbit polyclonal antibody, raised against an undisclosed peptide of the human sequence from the C-terminus of the protein. It was used at a dilution of 1:500 in 4% blocking buffer.

The membrane was incubated with primary antibody solutiuon with constant rotation at room temperature for at least 2 hours. Alternatively, the membrane in primary antibody was left overnight at 4 °C then incubated for one hour with constant rotation at room temperature.

IV. Incubation with secondary antibody: The primary antibody solution was discarded, and the membrane was washed three times for 5 minutes in wash buffer. The wash buffer was poured off and the membrane incubated with the secondary antibody, donkey anti-rabbit IgG conjugated with horse radish peroxidase (Santa Cruz sc-2313, Bioscience, UK) at a 1:2000 dilution in blocking buffer. The membrane was incubated on a roating platform at room temperature for at least 1 hour.

V. Detection of secondary antibody

The horse radish peroxidase attached to the secondary antibody was detected using a chemiluminescent substrate prepared according to Thorpe *et al*. (1985).

Following incubation with secondary antibody, the membrane was washed with four times with wash buffer and rinsed once with TBS. Two solutions were freshly made. The first 50 µl luminol solution (250 mM in DMSO; stored -20 C), 22 µL coumaric acid (90mM in DMSO; stored at -20 °C) and 0.5 mL 1M Tris pH 8.5, were made up to 5 mL with water. The second comprised 3.2 µL hydrogen peroxide (30% w/v), added to 0.5 mL 1M Tris pH 8.5 made up to 5 mL with water.

The membrane was inserted into a plastic sleeve. 2mL of each solution was mixed and added to the membrane in the sleeve. The membrane was placed in a dark cupboard for 2 minutes, and then taken out; the excess luminescent substrate was squeezed out and replaced with a second lot. After further two minutes incubation in the dark, the excess reagent was squeezed out and the membrane taken to the camera dark box for measurement of luminescence. For image analysis an UVItec (Cambridge, UK) documentation system was used. Images were saved as tiff files and analysed further using image J software (imagej.nih.gov/ij).

2.7. Estimation of Glycogen

Several methodologies have been considered to establish a protocol that was compatible with a plate reader format and does not require the use of isotopes. Some date back to

1905, decribed in Seifter *etal.,* (1950), in which tissues were extracted with KOH by boiling, followed by precipitation of glycogen and detection using anthrone in concentrated sulphuric acid (Seifter *etal.,* 1950). Although the method was simple, the reagents used were particularly hazardous. A widely quoted study by Passonneau and Lauderdale (1974) compared three methods, one of which used a strong acid to extract and breakdown the glycogen to glucose, which is detected enzymatically to produce a fluorescent product, NADPH. This method was adopted.

Cell culture: In this study, confluent cells were used to produce measurable amounts of glycogen reliably. The cells were seeded in a 24 well plate, allowed to grow to confluency and then treated for 48 hours with target phytocompounds, Goldenseal ethanolic extract (10 µL/mL) and berberine (10 µM), and a positive control metformin (1000 µM), and the solvent controls DMSO (1%) and 0.6 % ethanol (v/v), and an untreated control. After 48-hour treatment, the cells were harvested and washed with ice cold PBS and resuspended in 1.0 mL PBS.

Assay procedure: The cells were transferred to 2.0 mL capped eppendorf tubes and spun at 5000 rpm for 10 seconds to pellet cells and PBS was removed. 0.5 mL 2.0 M HCL was added and mixed thoroughly, and the mixture incubated at 95-100 °C for 5 minutes. To ensure complete homogenisation of the pellet, mixtures were vortexed thoroughly and incubation at 95-100 °C continued for a further 55 minutes. The tubes were allowed to cool, and then spun at 5000 rpm for 10 seconds; distilled water was added to make the volume to 0.5 mL. To this mixture 0.5 mL of 2M NaOH was added, mixed in by vigorous vortexing, and then spun at 12000 rpm for 10 minutes. 100 µL aliquots were assayed in triplicate for glucose using the hexokinase / glucose-6-phosphate dehydrogenase (Sigma-Aldrich) (Table 3) in a white opaque 96 well plate. Levels of glucose were determined through the amount of NADPH generated, measured by fluorescence (Passonneau and Lauderadale, 1974).

Table 3: Solutions (Sigma-Aldrich) used in the master mix; * Hexokinase combined with glucose -6-phosphate dehydrogenase from baker's yeast (Sigma-Aldrich H8629-500UN); supplied as lyophilised powder, resuspend in 2x buffer (100 mM Tris, 4 mM MgCl2, 2mM DTT) then add 1 vol glycerol. Stored in a freezer.

Reagent	**Concentration**	**Vol reqd / ml** **Master mix**	**Final concentration**	**Storage**
Tris HCl pH 8.0	100 mM	500 µl	50 mM	Fridge
$MgCl_2$	100 mM	100 µl	10 mM	Fridge
DTT	100 mM	10 µl	1 mM	Freezer
ATP	10 mM	100 µl	1 mM	Freezer
NADP	10 mM	100 µl	1 mM	Freezer
Enzyme*	100 U/ml	10 µl	1 mM	Freezer
Water		180 µl	1 U/ml	Freezer

Glucose standards of 0, 0.78 µM, 1.56 µM, 3.12 µM, 6.25 µM, 12.5 µM, 25 µM, and 50 µM were used to calibrate the assay. 50 µL aliquots of sample or standards, 50 µL water and 50 µL of master mix was added to each well. The plate was coverd and incubated at 37^0C for 30 minutes. And the fluorescence was read with excitation 345 nm and emission 485 nm, with a gain of 1000, top optic (Fluostar Omega microplate reader).

Also, protein assays were performed, such that glycogen content of the mean values of the triplicates of the treated cells were divided by the mean values of the triplicates of the protein content of the treated cells, to estimate the glycogen content per cell.

2.8. Estimation of Glucose release from HepG2 Cells

In this assay, HepG2 cells grown to confluency in a 24 well plate was treated for 48 hours with target phytocompounds Goldenseal (10µL/mL), berberine (10µM), metformin (1000µM), and the controls DMSO (1%), ethanol (0.6% (v/v)), and distilled water (1%) as an untreated control. The cell culture medium was completely removed, washed, and

replaced with glucose free and phenol red free DMEM (Sigma-Aldrich). The plates were incubated for 1 hour at 37^0 C. The medium was collected and assayed for glucose following thre method described in Section 2.7. Cell protein content was measured by the Bradford assay to normalize the data.

2.9. Measurement of metabolic activity in treated HepG2 cells

2.9.1. Analysis of treated HepG2 cells metabolism and mitochondrial respiration

The Seahorse Bioscience XF24 high-throughput metabolic analyser (Massachusetts, USA) has been employed in this study to check mitochondrial function of the treated HepG2 cells in real time. The XF cell Mito stress Kit is an optimised solution and was used in this study to analyse mitochondrial activity, which was based on the rate of change in dissolved oxygen and pH in the cell culture media and simultaneously nearby viable adherent HepG2 cells cultured in a 24 well microtitre plate in real time mode. This method calculates the rate of treated HepG2 cellular metabolism in detail i.e., calculation of oxygen and proton exchange rate based on short periods of time (5 minutes) and requires only a small volume of medium.

The 24 optical fluorescent heads of the analyser were fixed in disposable sterile cartridges that are in a 24 well microtitre plate. As soon as the optical heads enter the wells and engrossed into the medium, they transmit optical signals independently and at the same time along with other optical heads. The assay runs every 2 to 5 minutes and produces the data for oxygen consumption rate (OCR) in pmol/min, which indicates mitochondrial respiration, and extracellular acidification rate (ECAR) in mpH/min that denotes glycolysis.

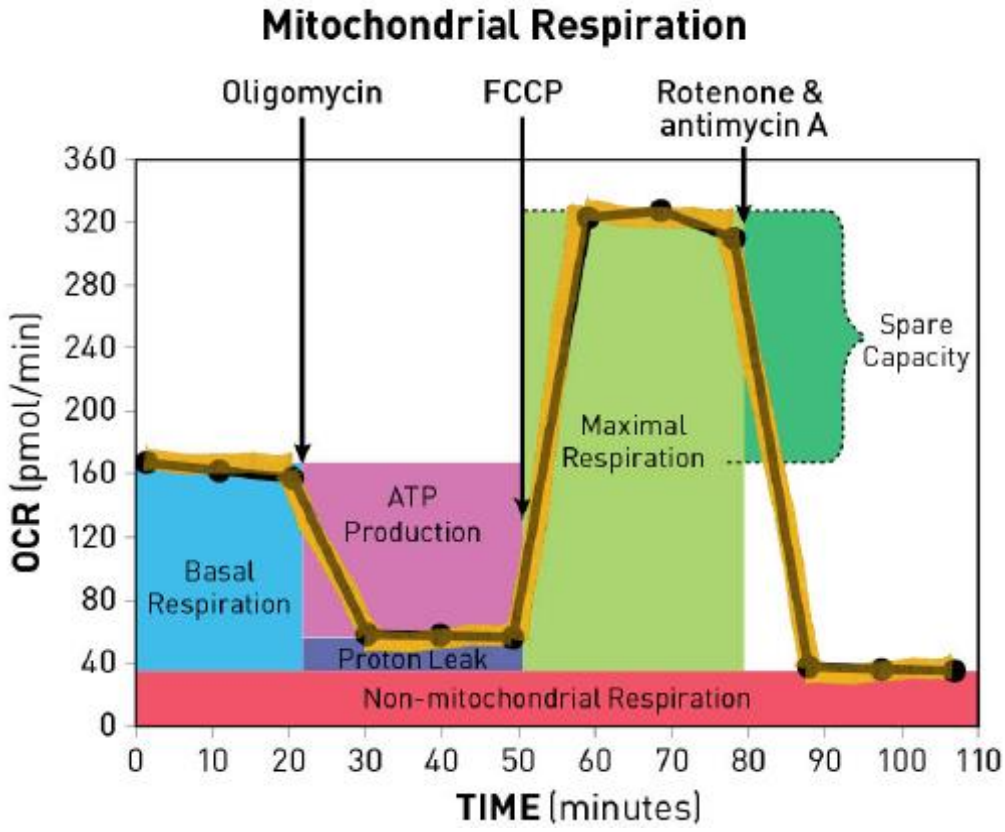


Figure 54: XF Cell Mito Stress Test. Sketch of the vital parameters of mitochondrial respiration. Sequential compound injections calculate basal respiration of the cells, ATP production and proton leak, maximal respiration and spare respiratory capacity, and non-mitochondrial respiration (Seahorsebio, 2018 retrieved from www.Seahorsebio.com)

There were 3 respiration modulators in the XF Cell mito stress experiment and works independently and explicitly on a specific target complex of the electron transport chain (ETC). The initial application of oligomycin inhibits ATP synthase (complex V) enzyme therefore, the oxygen consumption rate (OCR) in the mitochondrial respiration would fall considerably, and this effect was directly interconnected to ATP generation. Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) acts as the uncoupling modulator and that uncouples the potential of the mitochondrial membrane and dismisses the proton gradient across the membrane, therefore respiration was no longer linked to ATP formation and electron discharge by the electron transport chain was controlled by availability of the oxygen, and complex IV oxygen consumption was at its peak. The third modulator was made up of two components, rotenone, which acts as a complex I inhibitor, and antimycin A, which acts as a complex III inhibitor. This combination exclusively inhibits mitochondrial respiration and allows measurement of non-mitochondrial respiration. Also, the spare respiratory capacity of the cells, is the cells' response to increased energy

demand and supply, and it can be measured from the difference between maximal respiration and basal respiration of the cells, using the FCCP-stimulated OCR (Figure 55).

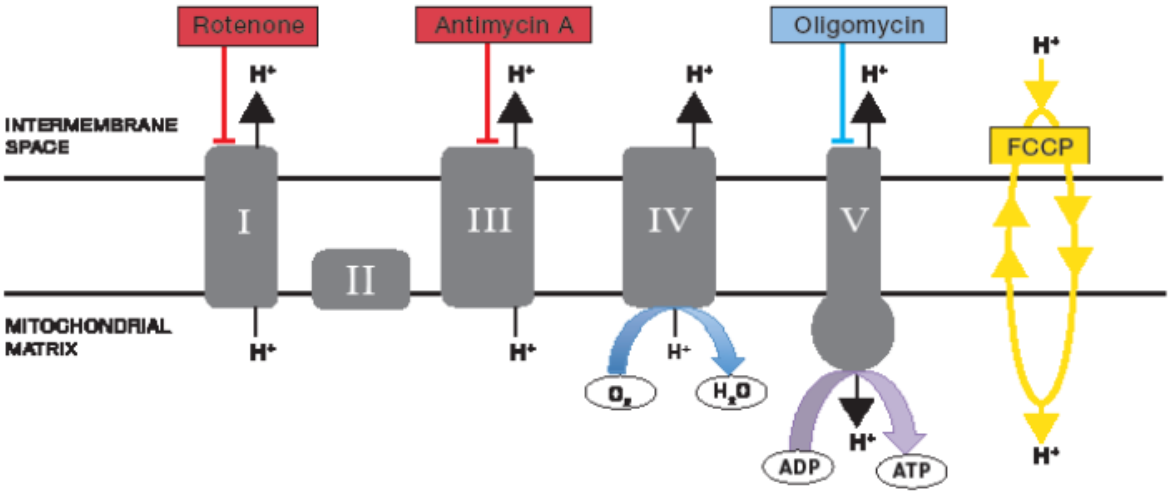


Figure 55: XF Cell Mito Stress experiment modulators of the electron transport chain. This picture denotes the complexes of the electron transport chain and the objective of the compounds in the XF Cell Mito Stress Test Kit. Oligomycin inhibits ATP synthase (complex V), FCCP uncouples oxygen consumption by ATP production, and rotenone/ antimycin A inhibit complexes I and III, correspondingly (Seahorsebio, 2018 retrieved from www.Seahorsebio.com).

2.9.2. Materials

Uridine, penicillin/streptomycin, DMEM, glucose, glutamine, sodium pyruvate, buffering agent and HEPES buffer (Sigma-Aldrich), trypsin (Invitrogen) and fetal bovine serum (Hyclone), and the other consumables and reagents were from Seahorse Bioscience (XF24-3 FluxPak cat. No. 102070-001; XF Cell Mito Stress Test Kit cat.No. 101706-100).

2.9.3. Cell culture and Treatments: Confluent HepG2 Cells were maintained in DMEM, 10% FBS + 22 mM glucose, in 75-cm^2 T-flasks in a controlled incubator at 37°C, 95% humidity, and 10% CO^2. Every three days, the cells were detached from the flasks by applying 0.25% solution of trypsin and further subcultured. The cells were less than 80% confluence at the time of subculture. In this study, cells were seeded at 30,000 cells/well in an XF24 cell culture microtitreplate. The cells were incubated with controls (1% DMSO, and 0.6% ethanol), and target phytocompounds goldenseal ethanolic extracts (10 µl/ml),

berberine (10 μM), and metformin (1000 μM) for 48 hours before the start of the experiment (Figure 56).

After 48-hour treatments, basal respiration of the cells, proton leak, and ATP turnover after applying oligomycin (1μM) to inhibit ATP formation, maximal mitochondrial respiratory capacity by adminstring FCCP (carbonyl cyanide 4-trifluoromethoxy phenylhydrazone) (1μM) as uncoupler, and non-mitochondrial respiration by applying rotenone and antimycine (1μM) to break mitochondrial respiration were measured (OCR).

Assay procedure: The cell culture media of the treated cells was removed from the 24 wellplates, and 500 μl of Seahorse media was added to each well and washed the cells, finally 525 μl of Seahorse media was added to each well and incubated for 45 minutes in the hood. In the Seahorse media glucose concentration was 10 mM and pyruvate concentration were 1 mM. At the end of the assay number of the cells was counted by employing crystal violet staining assay. The OCR readings were divided by the cell count per well (Crystal violet cytotoxicity assay Kit; BioVision, Inc., Milpitas, California, USA), with triplicate or quadruplicate samples.

Table 4: A 24–well microtitre plate design for the Seahorse metabolic analysis experiment. The target compounds include goldenseal (10μL/mL), berberine (10 μM) and metformin (1000 μM), and the controls include 1% DMSO (v/v), and 0.6% ethanol (v/v).

Background	1%DMSO (v/v)	1%DMSO (v/v)	1%DMSO (v/v)	0.6%Ethanol (v/v)	0.6%Ethanol (v/v)
Goldenseal (10 μL/mL)	Goldenseal (10 μL/mL)	Goldenseal (10 μL/mL)	**Background**	0.6%Ethanol (v/v)	Blank
Blank	Blank	**Background**	Metformin (1000 μM)	Metformin (1000 μM)	Metformin (1000 μM)
Berberine (10 μM)	Berberine (10 μM)	Berberine (10 μM)	Blank	Blank	**Background**

.

2.9.4. Crystal violet staining assay: This assay is a rapid and reliable method to quantify viable cells. The dead cells detach from the bottom of the wells, this character can be used to quantify viable cells by staining with crystal violet dye. It binds to proteins and DNA; therefore, the number of viable cells is directly proportional to the amount of crystal violet staining in a culture.

Materials: Paraformaldehyde (4%), Phosphate buffered saline (PBS), crystal violet (0.2%) (Triarylmethane dye), sodium dodecyl sulfate (5%) (Crystal violet cytotoxicity assay Kit; BioVision, Inc., Milpitas, California, USA).

Procedure: The media from the 24 well plates was aspirated, and 50 µl of PFA (4% Paraformaldehyde) was added to each well. After 10 minutes rocking, the PFA was aspirated, and the cells were washed with PBS. 25 µl of crystal violet (0.2%) was added to each well, and the cells were rocked for 10 minutes, and again washed with PBS. Then 100 µl of SDS (5%) was added to each well, and the cells were rocked for 5 minutes. 50 µl per well samples were aliquotted into a 96 well plate. The absorbance was read on a plate reader (Fluostar omega microplate reader) at 570 and 590 excitations.

Chapter 3: Screening of the target herbal compounds and extracts

The target herbal compounds of the study are phloretin, ouabain, berberine, metformin, and rebaudioside-A and the target extracts are *Stevia* leaf extracts, goat's rue extracts, *Gymnema* leaf extracts, goldenseal extracts. To check the efficacy of the compounds, and find out optimal concentrations of the extracts, screening experiments were performed, and the effect of the pure compounds was compared with the extracts. Based on the efficacy, the key compounds were identified, and a selected set of treatments are explored in the next series of experiments described in Chapter 4.

3.1. Chromatographic analysis of *Stevia rebaudiana Bertoni* leaf

Chromatographic analysis of the shade dried leaves of *Stevia* was performed to characterise the presence of component compounds which could be employed in the studies.

3.1.1. Results

The system was calibrated using rebaudioside-A (Wako Chemicals, Neuss, Germany, purity >99% and 97%) in water/acetonitrile (50:50, v/v). The standard calibration curve of rebaudioside A (Figure 56) was linear with a correlation coefficient r = 0.999327. As exhibited in the chromatogram (Figure 57), the peak of rebaudioside-A standard was identified with a retention time of 14.14 minutes.

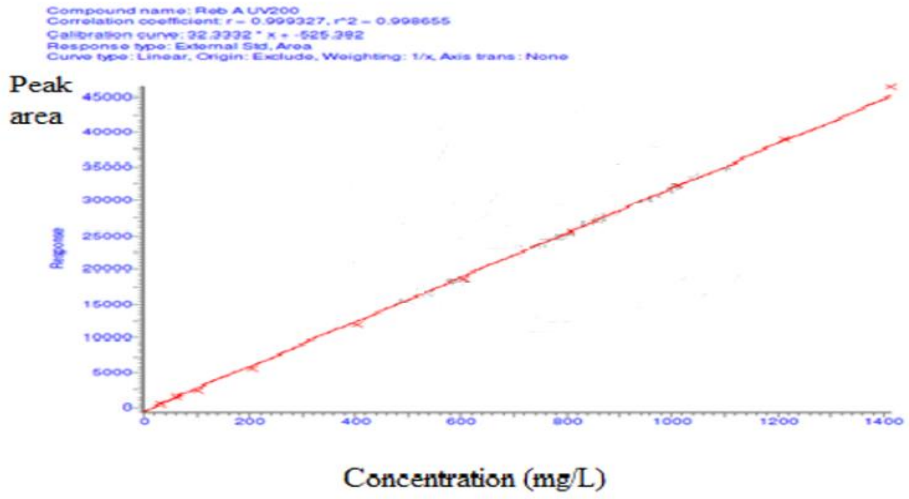


Figure 56: Calibration curve of the standard rebaudioside -A; It is a linear curve with a correlation coefficient r = 0.999327. Stock solutions of rebaudioside-A standards with

concentrations of 200, 400, 600, 800, 1000, 1200 and 1400 mg/L were used for the calibration curve. Triplicate determinations were carried out and the mean values were taken in drawing the calibration curve.

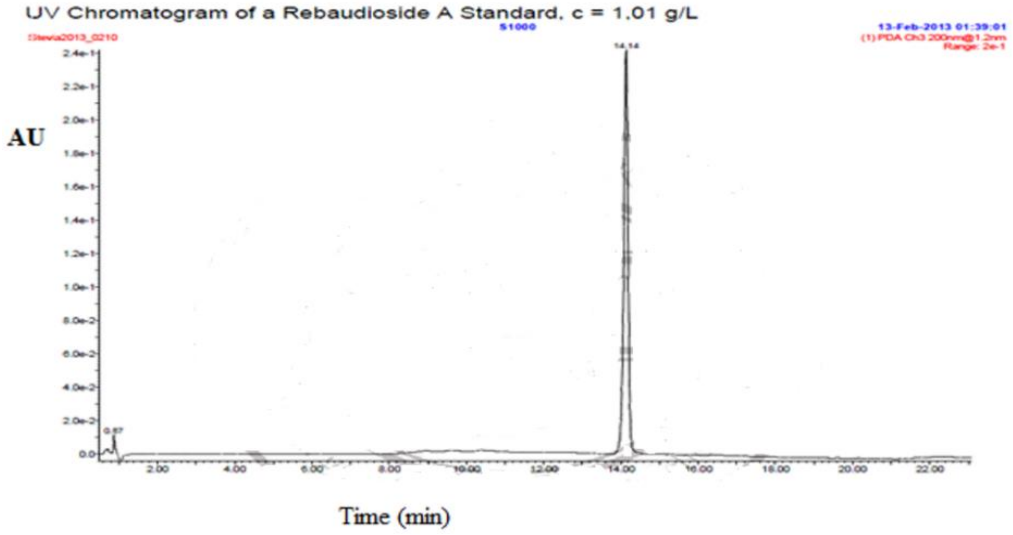


Figure 57: UV chromatogram of rebaudioside-A standard; As indicated, rebaudiosude-A containing peak was identified to be with retention time of 14.14 minutes.

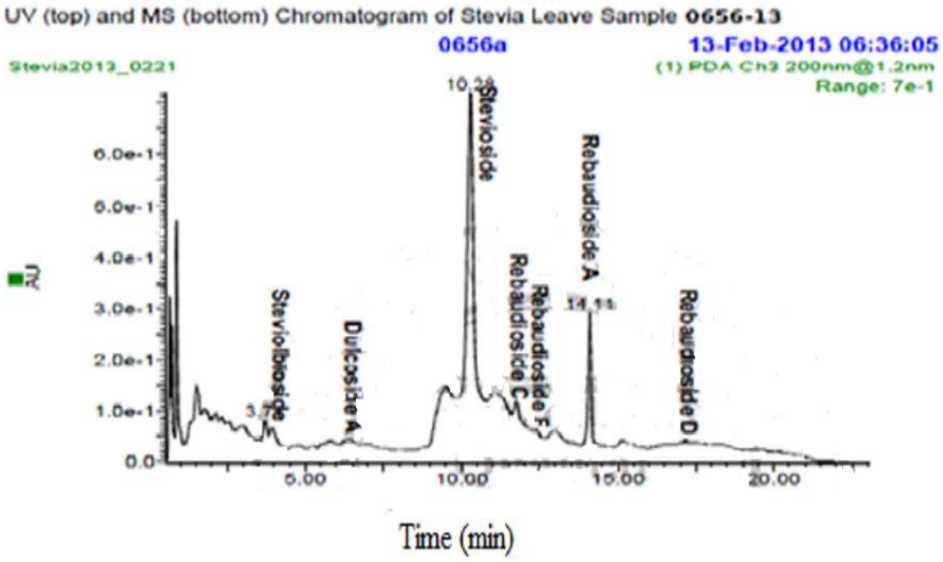


Figure 58: UV chromatogram of extracts of *Stevia* leaves. At a retention time of 10.28 min, stevioside is expressing a prominent sharp peak, which is also reflecting its abundance in the leaf extract. Following at 14.11 min. retention time rebaudioside-A is expressing a clear peak and indicating it as the second leading compound, and at 3.7 min. retention time steviolbioside is expressing a small peak due to its relative lower quantity.

The rest of the compounds also express blunt and faint peaks at different retention times and reflecting their relative lower quantities in the test sample.

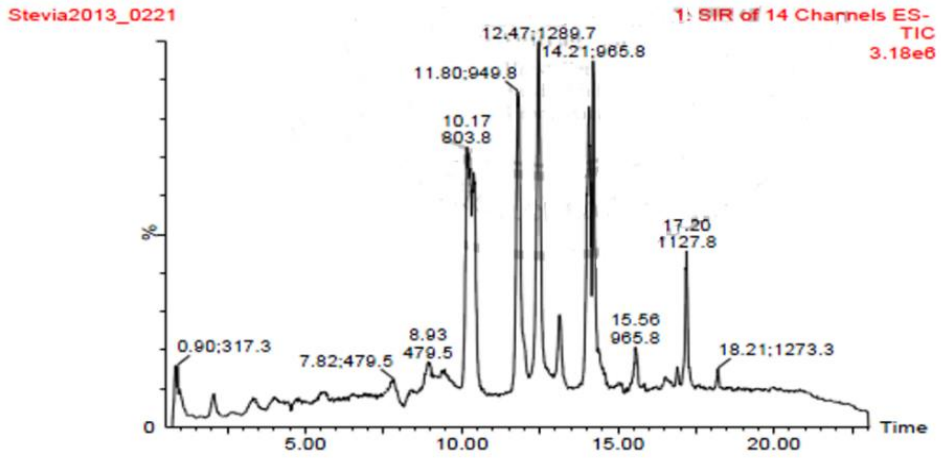


Figure 59: MS chromatogram of *Stevia* leaf extracts. Peaks of stevioside, rebaudioside-A, and rebaudioside-C, and other glycosides, and less distinct peaks with tailing (Zimmermann, 2011). The peaks were corresponding to the m/z ratio of their ions. A few glycosides partly underwent source fragmentation and as a result they can be seen as two or more mass peaks. For instance, stevioside can be seen at time points 12.47, 17.20 and 18.21 with respective m/z ratios 1289.7, 1127.8 and 1273.3. Whereas rebaudioside A can be seen at time points 11.80, 14.21 and 15.56 with respective m/z ratios 949.8, 965.8 and 965.8. And also, steviolbioside can be seen at time point 10.17 with an m/z ratio of 803.8.

In order to analyse the samples, the same method and apparatus was used as described by Zimmermann (2011). The analysis revealed uneven peaks with tailing, along with clear peaks, this could have occurred because of overloading of the MS detector, due to injection of a highly concentrated solution. The peaks were typically coordinated by the m/z of their $[M–H]^-$ ions and usually the peaks with the premier intensity were presumed to be the well-known compounds of that m/z (Zimmermann, 2011). The glycosides that behave abnormally in this scenario may be due to loss of a glucose moiety in the ion source.

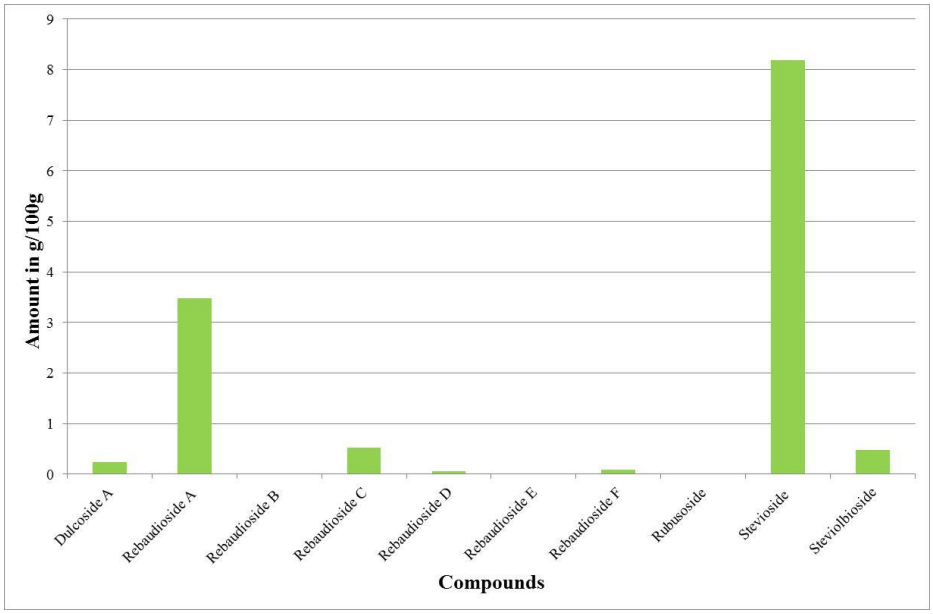


Figure 60: Steviol glycosides identified in *Stevia* leaf extracts. Stevioside (~ 8%) appears the most prevalent compound and rebaudioside- A (3.5%) appears the second prominent compound.

The total glycocosides content in the extracts of shade-dried leaves was 13.02 % (w/w). The predominant compound was stevioside, 8.18% (w/w). Other components found were: Rebaudioside-A, 3.47 % (w/w); Rebaudioside-B, < 0.01* % (w/w); Rebaudioside-C, 0.52 % (w/w); Rebaudioside-D, 0.06 % (w/w); Rebaudioside-E, < 0.01* % (w/w); Rebaudioside-F, 0.09 % (w/w); Rubusoside, < 0.01 % (w/w); Steviolbioside, 0.47 % (w/w); and Dulcoside-A, 0.24 % (w/w); * these values are equivalent to the detection limit. These data are presented in Figures 58, 59, 60.

3.2. Glucose uptake studies in cultured human hepatocellular carcinoma cells (HepG2 cells)

The testing compounds phloretin, ouabain, berberine, metformin, and rebaudioside-A, and *Stevia* leaf extracts, goat's rue extracts, *Gymnema* leaf extracts, and goldenseal extracts were employed in glucose uptake and glucose consumption studies to find out the optimal concentrations of the crude extracts, and to check the effect of the pure compounds compared with the crude extracts, and at the end of the study based on the efficacy, the most active compounds were selected for further investigation by flowcytometry based glucose uptake, western blotting analysis for GLUT-1 protein, glycogen synthesis experiments, glucose release experiments and the seahorse metabolic analysis (Chapter 4) .

3.2.1. Results

The glucose uptake experiments were performed on growing and confluent HepG2 cells, and these were exposed to the target phytochemicals for 1 hour and 24 hours of incubation time courses.

3.2.1.1. Effect of ouabain, phloretin, berberine and metformin on 2-NBDG uptake by HepG2 cells

2-NBDG acts as a fluorescent indicator of direct glucose uptake in HepG2 cells. The results for each experiment are expressed as net fluorescence, which was obtained by subtracting intrinsic fluorescence of cells, i.e., before adding 2-NBDG, from fluorescence after adding 2-NBDG. To show the average effect obtained based on a series of independent experiments, the net fluorescence value was normalised relative to net fluorescence of 2-NBDG uptake in cells treated with vehicle control DMSO (Figure 61). ANOVA with Bonferroni's multiple comparison post hoc analysis was performed on the normalised values to provide an indication of the significance of the differences between means in the data.

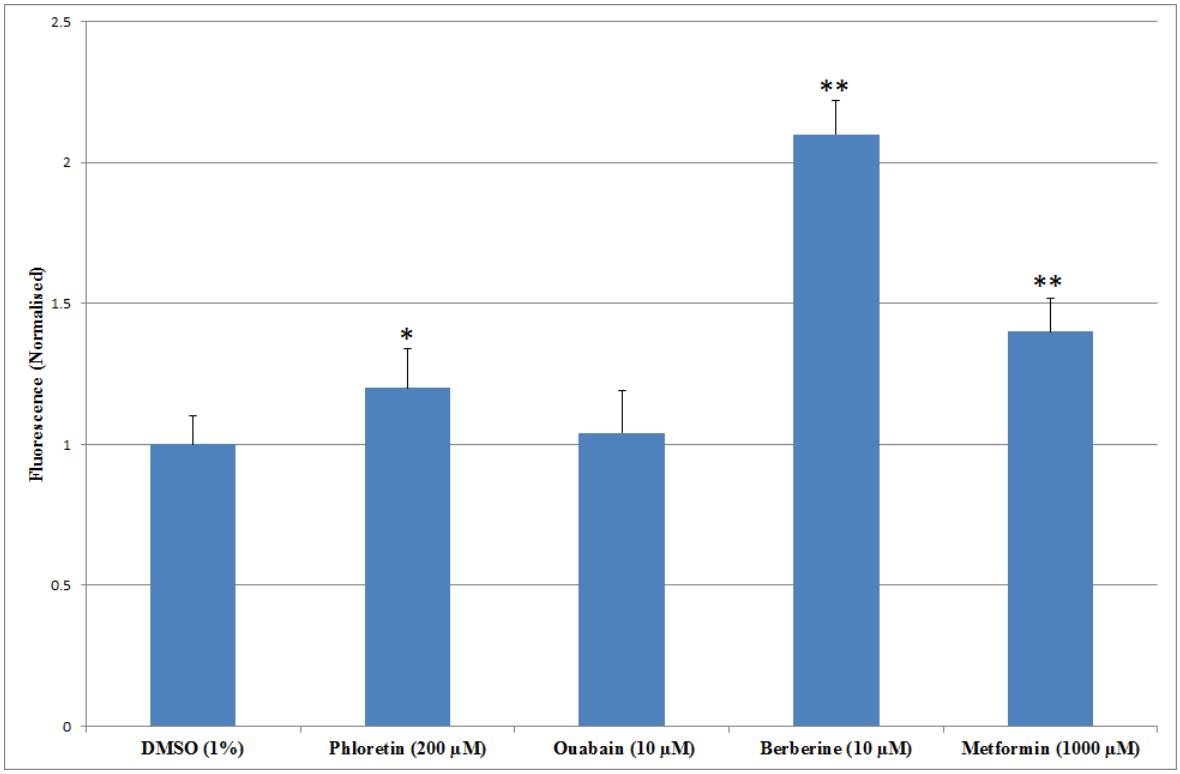


Figure 61: Effect of short-term treatment of selected phytochemicals on uptake of 2-NBDG by growing HepG2 cells. Data are changes in fluorescence normalised to DMSO vehicle control and are the mean +SD from seven experiments, and the treatments in each experiment were conducted in triplicate wells. Significant difference from vehicle control (DMSO) to target treatments were assessed by employing one-way ANOVA with Bonferroni's multiple comparison post hoc test, *$p<0.05$ and **$p<0.01$.

As shown in Figure 61, in growing HepG2 cells a 1 hr treatment with berberine induced stimulation, by 110% ($p<0.01$), of glucose uptake compared with control cells treated with DMSO only. Other phytochemicals gave more modest stimulations of 20% by phloretin ($p<0.05$) but no significant change in ouabain ($p>0.05$). Treatment with metformin, which is known to stimulate glucose uptake in liver, was also effective, producing a 40% increase in 2-NBDG uptake ($p<0.01$).

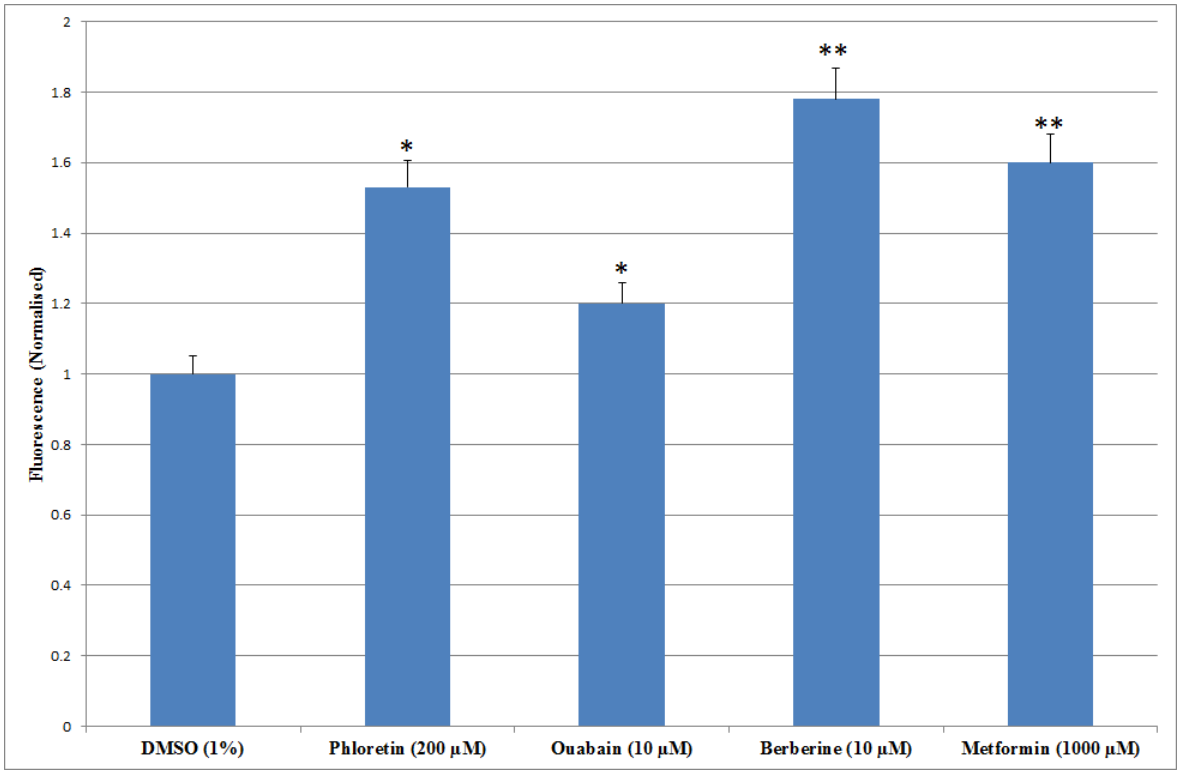


Figure 62: Effect of long-term treatment of target phytochemicals on uptake of 2-NBDG by growing HepG2 cells. Data are changes in fluorescence normalised to DMSO vehicle control and are the mean +SD from seven experiments, and the treatments in each experiment were conducted in triplicate wells. Significant difference from vehicle control (DMSO) to target treatments were assessed by employing one-way ANOVA with Bonferroni's multiple comparison post hoc test, *$p<0.05$ and **$p<0.01$.

As shown in Figure 62, in growing HepG2 cells after long-term treatment (i.e., 24 hours), berberine has shown stimulation of glucose uptake activity (78%) ($p<0.01$), followed by metformin (60%) ($p<0.01$), phloretin (53%) and finally ouabain (20%) ($p<0.05$), compared with control DMSO.

As early pre-clinical studies have shown, examining all variables may impede therapeutic efficacy in *in vivo* trials. Therefore, insulin was not used in these *in vitro* experiments. Also, an *in vitro* study investigated the link between glucose uptake and AMP-activated protein kinase (AMPK) phosphorylation by the phytochemicals naringenin and naringin in high glucose-treated HepG2 cells. This study discovered that naringenin and naringin are both positive modulators of AMPK activation, enhancing glucose uptake regardless of insulin stimulation in high glucose treated HepG2 cells (Dayarathne *et al.*, 2021).

A high-fat diet combined with a low-dose of streptozotocin injection induced type 2 diabetes in rats, and rats with 25 mg/kg streptozotocin followed by a normal diet feeding showed normalised blood glucose levels and pancreatic structure, indicating that a normal diet may help recovery from certain symptoms of type 2 diabetes (Guo *et al.,* 2018).

In a study using rats that had become streptozotocin-induced diabetics, swartz fruit from the Solanum torvum genus, which contains phenolic compounds showed anti-diabetic properties (Gandhi, Ignacimuthu and Paulraj, 2011). In a different investigation, streptozotocin-induced diabetic rats showed hypoglycaemic activity when exposed to an ethanolic extract of the fruit of Solanum torvum (Satyanarayana *et al.,* 2022). In a rodent trial, the treatment of red betel combo extract for 14 days (9mL/Kg body weight dosage) reduced the rat's blood glucose level by up to 55.42%, which was substantially different from the rat's blood glucose level on day 3 (Safithri, Bintang and Syefuddin, 2023). In each of the aforementioned cases, only the therapeutic interventions of the diet and phytoextracts were investigated; insulin was not administered.

Berberine induced glucose uptake by 78% after 24h (Figure 62), yet by 110% after only 1h (Figure 61), in these studies the outcome of the experiments indicated the efficacy of the targeted herbal compounds, as these initial screening of the targeted herbal compounds have been carried out just before the London Metropolitan University crisis-2012, as university’s sponsor licence was revoked, international students had not been permitted to enter the university to carryout studies, therefore, protein estimation studies did not carry out to normalize the outcome of the glucose uptake output, this issue has been resolved in further studies carried out in 2017-2019. And more so in light of the fact that this was just preliminary study, and a rapid initial screening was conducted to determine the efficacy of the target phytochemicals.

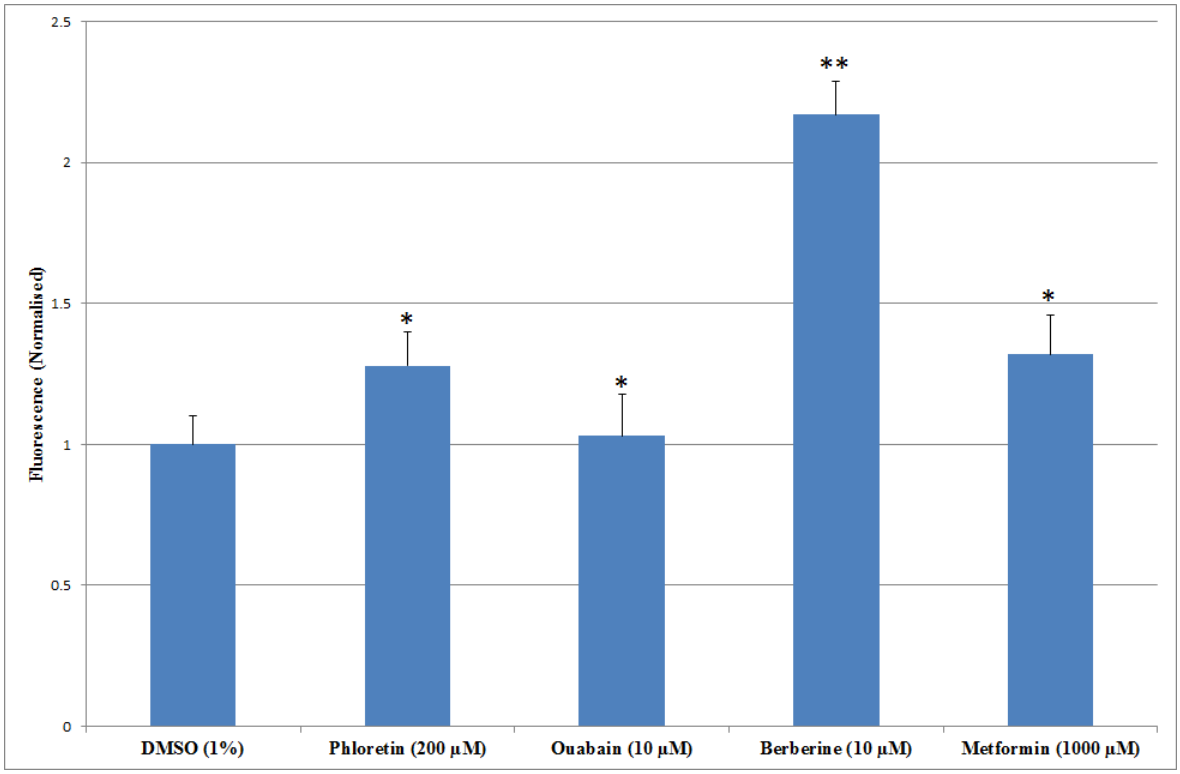


Figure 63: Effect of short-term treatment of target phytochemicals on uptake of 2-NBDG by confluent HepG2 cells. Data are changes in fluorescence normalised to DMSO vehicle control and are the mean +SD from seven experiments, and the treatments in each experiment were conducted in triplicate wells. Significant difference from vehicle control (DMSO) to target treatments were assessed by employing one-way ANOVA with Bonferroni's multiple comparison post hoc test, *$p<0.05$ and **$p<0.01$.

As shown in Figure 63, in confluent HepG2 cells for 1 hour treatment, berberine is showing glucose uptake activity (117%; $p<0.01$), followed by phloretin (28%), and ouabain has no effect and finally metformin showing 32% ($p<0.05$) of uptake activity compared with control DMSO.

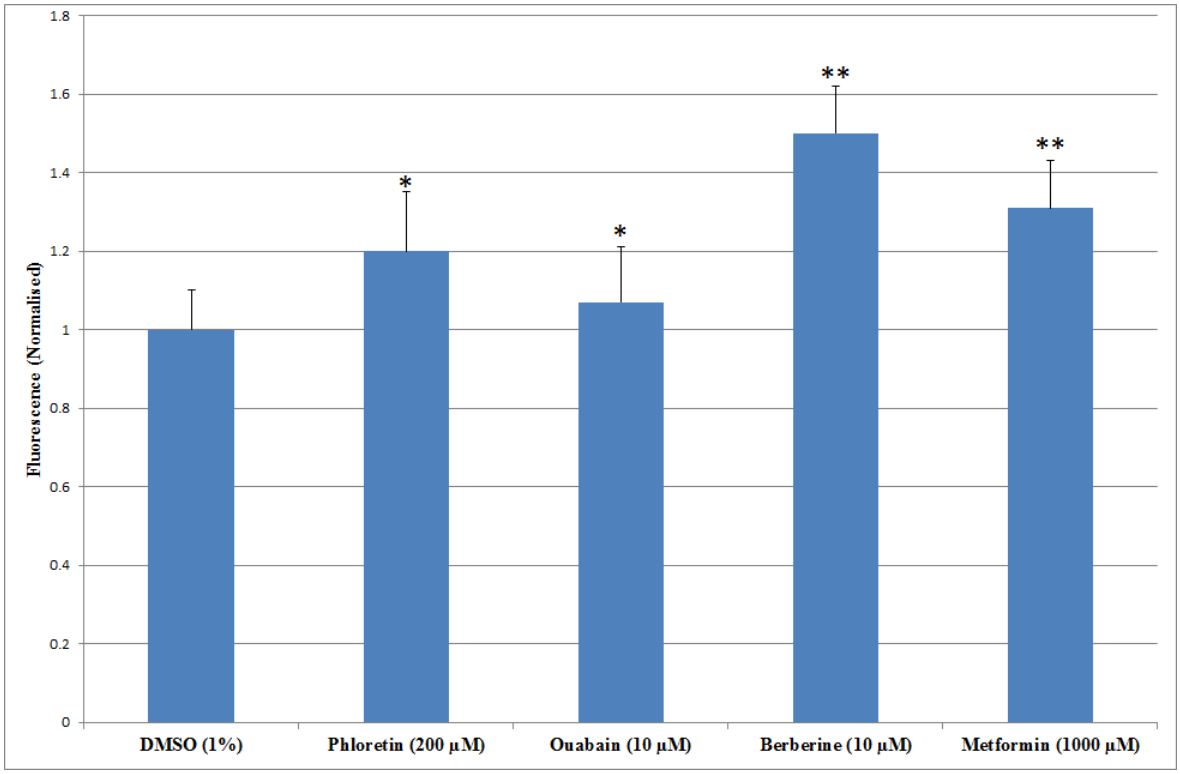


Figure 64: Effect of long-term treatment of target phytochemicals on uptake of 2-NBDG by confluent HepG2 cells. Data are changes in fluorescence normalised to DMSO vehicle control and are the mean +SD from seven experiments, and the treatments in each experiment were conducted in triplicate wells. Significant difference from vehicle control (DMSO) to target treatments were assessed by employing one-way ANOVA with Bonferroni's multiple comparison post hoc test, *$p<0.05$ and **$p<0.01$.

As shown in Figure 64, in confluent HepG2 cells for 24 hours treatment, berberine is showing a stimulation of 2-NBDG uptake activity (50% increase above DMSO vehicle control) ($p<0.01$), followed by phloretin (20%), ouabain (.07%), and finally metformin (31%) ($p<0.01$).

Across all four types of treatment (short-term, long-term, growing, and confluent cells) berberine showed the most significant stimulation of 2-NBDG uptake activity in comparison to the other phytochemicals. Where 110% more 2-NBDG uptake is observed compared to DMSO (Figure 61), in short-term treatment in growing cells. In long-term treatment in growing cells, 78% more 2-NBDG uptake is observed compared to DMSO. In short-term treatment in confluent cells, 117% more 2-NBDG uptake is observed compared to DMSO. Finally, in the long-term treatment on confluent cells, 50% more 2-NBDG uptake is observed compared to DMSO. In general metformin showed the next highest

stimulation of uptake compared to berberine, with more modest effects shown by phloretin, and ouabain has little effect relatively.

Further to the above research on the anti-diabetic efficacy of the berberine, research has been extended and investigated further in comparison to the other crude extracts (i.e., goldenseal, *Gymnema*, goat's rue and *Stevia*) and pure compounds (i.e., rebaudiosude-A and metformin), and performed exclusively on confluent HepG2 cells for 1 hour and 48 hours of incubation time courses.

3.2.1.2. Effect of goldenseal glycerite, goldenseal ethanolic extracts, *Gymnema* glycerite and *Stevia* aqueous extracts on 2-NBDG uptake in HepG2 cells after short-term treatment (1 hr)

The confluent HepG2 cells were exposed to goldenseal glycerite (Section 1.10.5), goldenseal ethanolic extracts (Section 1.10.5), *Gymnema* glycerite (Section 1.9.3.8) and *Stevia* aqueous extracts (Section 1.10.9) for 1 hour treatment. These herbs were chosen based on their antidiabetic properties discussed in the Chapter 1, Section 1.10.

3.2.1.2.1. Effect of goldenseal glycerite and goldenseal ethanolic extracts on 2-NBDG uptake in HepG2 cells in the short-term treatment

In order to show the average effect, from the values obtained based on a series of independent experiments, the mean value of the net fluorescence was divided by the mean values of the protein content of the treated cells, and then net fluorescence was normalised to net fluorescence of 2-NBDG uptake of the untreated control (Figure 65). ANOVA with Bonferroni's multiple comparisons post hoc analysis was performed on the normalised values to provide an indication of the significance of the differences between means in the data.

The first set of experiments in Section 3.2.1.1 was performed in 2012, HepG2 cells were seeded in a 24-well plate (Costar) at a density of 5 x 10^4 cells / mL: 0.5 mL volume of the cells per well. The cells were left to enter the growth phase or confluency and were then treated with 5 µL volumes of test reagents in each well. In this case, "London Metropolitan University Crisis-2012", was not permitted to carry out experiments to estimate the protein content of the treated cells. However, due to the precise seeding density, the overall effect was convincing as discussed in the Chapter 1, Sections 1.9.3.3, 1.10.1, 1.10.2, 1.10.3, 1.10.4.

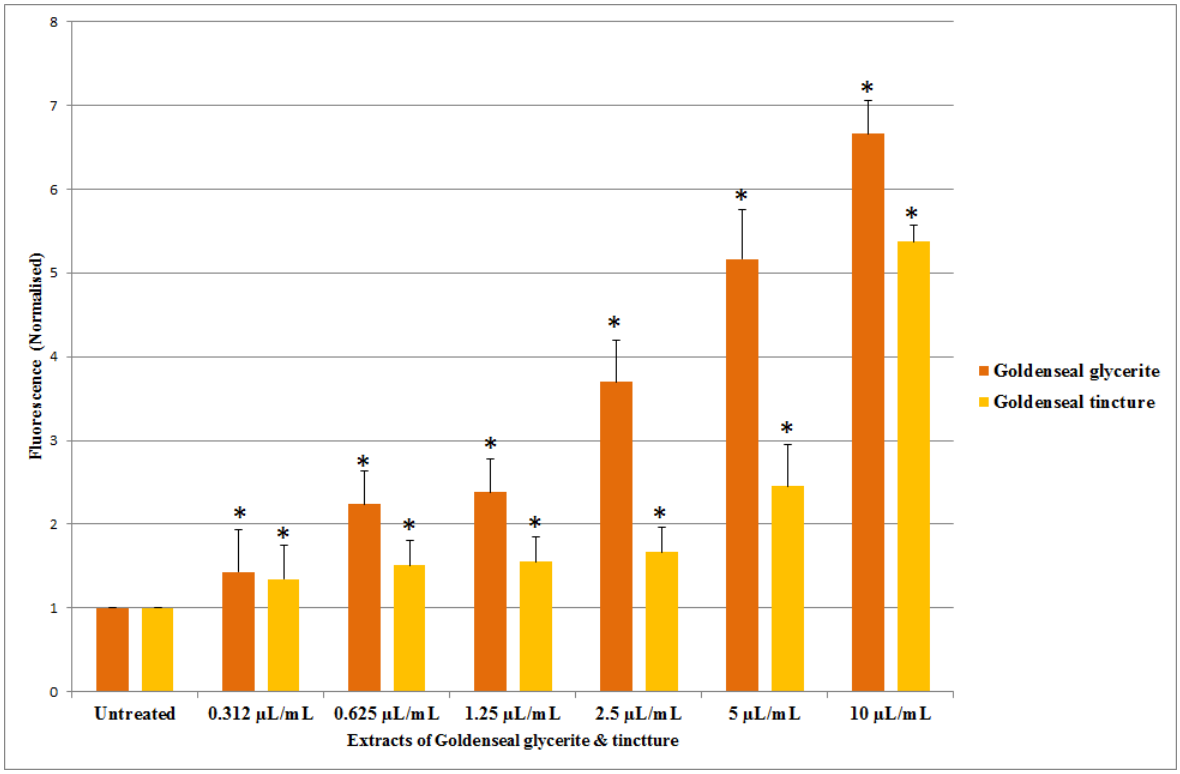


Figure 65: Effect of a short-term treatment of Goldenseal herbal extracts on uptake of 2-NBDG by confluent HepG2 cells. Data are changes in fluorescence normalised for protein content, and normalised to untreated control, and are the mean + SD from three experiments with goldenseal glycerite extract and five experiments with goldenseal ethanolic extracts. The treatments in each experiment were conducted in triplicate wells. Significant difference from untreated control to target treatments were assessed by employing one-way ANOVA with Bonferroni's multiple comparison post hoc test, *$p< 0.05$.

As shown in Figure 65, in confluent HepG2 cells a 1 hr treatment with different concentrations of goldenseal glycerites and ethanolic extracts stimulated glucose uptake, i.e., at the top concentration (10 µL/mL), by 6.5 times with goldenseal glycerites ($p<0.05$), and 5.4 times with goldenseal ethanolic extracts ($p<0.05$), compared with untreated cells. At lower concentrations (0.312 µL/mL to 5 µL/mL) glucose uptake was stimulated modestly by the ethanolic extract, but consistently higher at the same dilutions by the glycerite extract.

3.2.1.2.2. Effect of *Gymnema* glycerite extracts on 2-NBDG uptake in HepG2 cells after short-term treatment

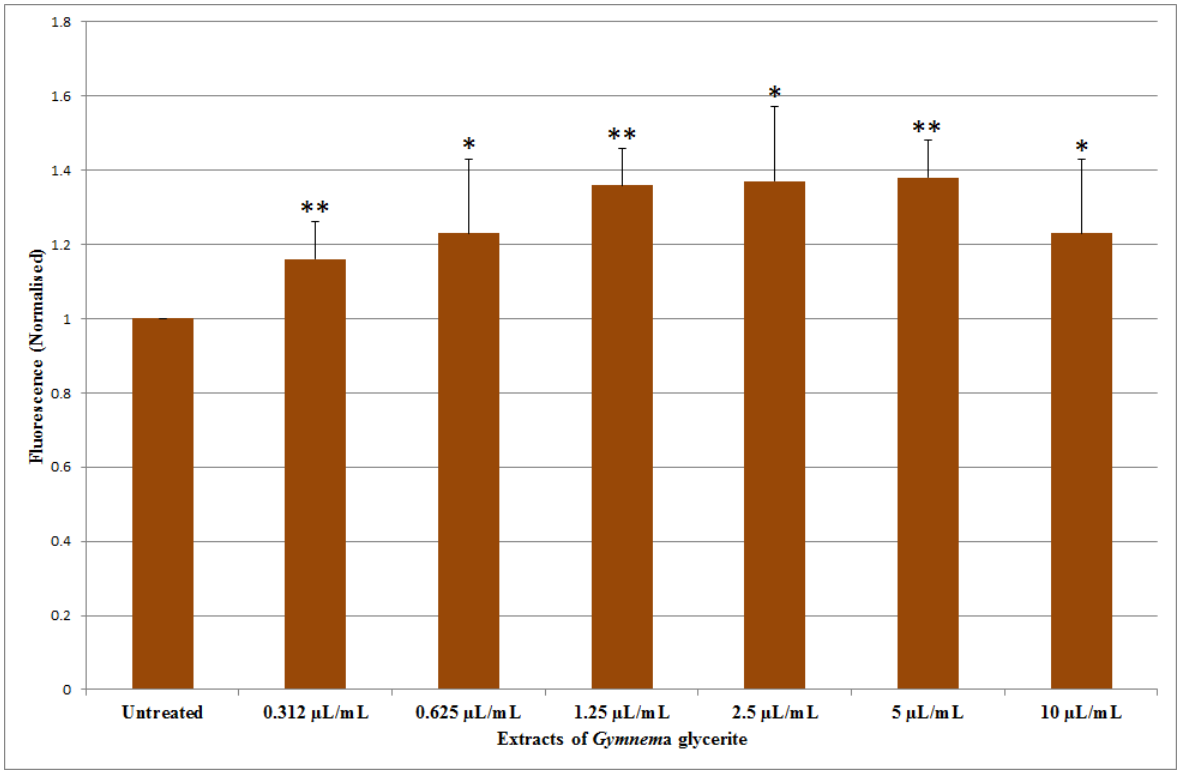


Figure 66: Effect of a short-term treatment of *Gymnema* glycerite extracts on uptake of 2-NBDG by confluent HepG2 cells. Data are changes in fluorescence normalised for protein content, and normalised to untreated control, and are the mean + SD from three experiments with goldenseal glycerite extract and five experiments with goldenseal ethanolic extracts. The treatments in each experiment were conducted in triplicate wells. Significant difference from untreated control to target treatments were assessed by employing one-way ANOVA with Bonferroni's multiple comparison post hoc test, *$p< 0.05$, and **$p<0.01$.

As shown in Figure 66, in confluent HepG2 cells a 1 hr treatment with different concentrations of *Gymnema* glycerites stimulated glucose uptake slightly, i.e., at the top concentration (10 µL/mL), by 1.3 times ($p<0.05$), and 1.4 times at concentrations 1.25 µL/mL ($p<0.01$), 2.5 µL/mL ($p<0.05$), and 5 µL/mL ($p<0.01$) compared with untreated cells.

3.2.1.2.3. Effect of *Stevia* aqueous extracts on 2-NBDG uptake in HepG2 cells after short-term treatment

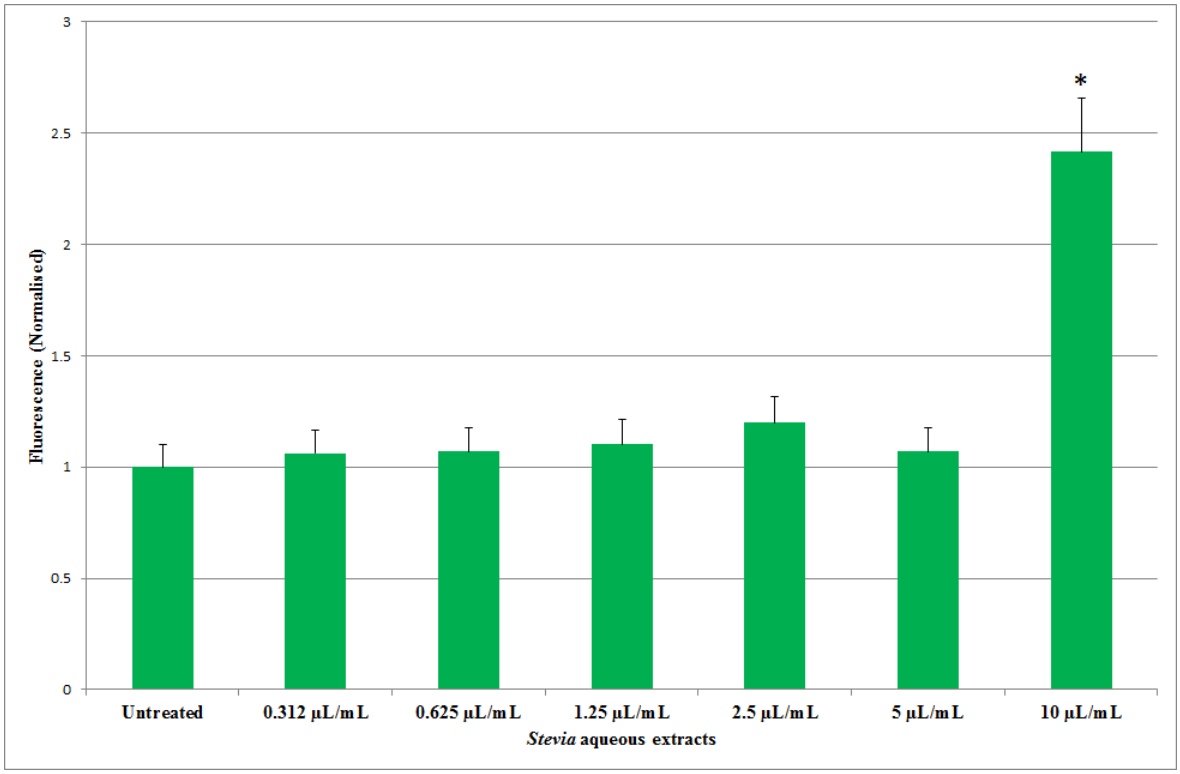


Figure 67: Effect of a short-term treatment of *Stevia* aqueous extracts on uptake of 2-NBDG by confluent HepG2 cells. Data are changes in fluorescence normalised for protein content, and normalised to untreated cells, and are the mean +SD from five experiments with *Stevia* aqueous extracts, and the treatments in each experiment were conducted in triplicate wells. Significant difference from untreated control to target treatments were assessed by employing one-way ANOVA with Bonferroni's multiple comparison post hoc test, $*p<0.05$.

As shown in Figure 67, in confluent HepG2 cells a 1 hr treatment with different concentrations of *Stevia* aqueous extracts, the top concentration (10 µL/mL) stimulated glucose uptake 2.4 times ($p>0.05$), compared with the untreated cells, whereas the other concentrations had little effect on glucose uptake.

3.2.1.3. Effect of goldenseal ethanolic extracts, *Gymnema* ethanolic extracts, goat's rue ethanolic extracts and *Stevia* ethanolic extracts and rebaudioside - A on 2-NBDG uptake in HepG2 cells after long-term treatment (48 hr)

3.2.1.3.1. Effect of goldenseal ethanolic extracts on 2-NBDG uptake in HepG2 cells after long-term treatment

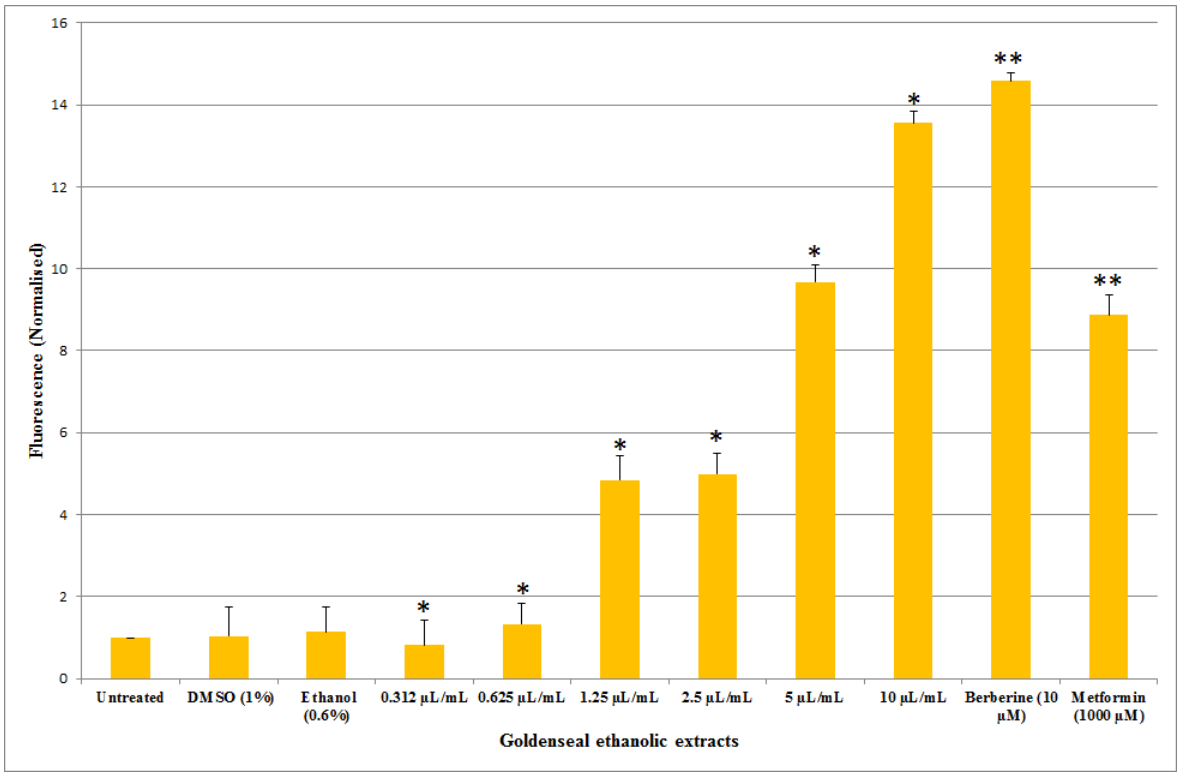


Figure 68: Effect of long-term treatment of goldenseal ethanolic extracts on uptake of 2-NBDG by confluent HepG2 cells. Data are changes in fluorescence normalised for protein content, and normalised to untreated cells, and are the mean + SD from five experiments with goldenseal ethanolic extracts, and the treatments in each experiment were conducted in triplicate wells. Significant difference from untreated control to target treatments were assessed by employing one-way ANOVA with Bonferroni's multiple comparison post hoc test, $*p<0.05$ and $**p<0.01$.

As shown in Figure 68, in confluent HepG2 cells, 48 hr treatment with different concentrations of goldenseal ethanolic extracts, the top concentration (10 µL/mL) stimulated glucose uptake 13.5 times ($p<0.05$), compared with the untreated control. The negative control 1% DMSO and the solvent control (0.6% ethanol) are not much effective compared with the untreated control ($p>0.05$), and the positive control metformin stimulating 9 times more glucose uptake ($p<0.01$), compared with untreated cells, whereas

berberine stimulating 14.2 times more glucose uptake (*p<0.01*), compared with the untreated control.

In Figure 68, there is >10 times glucose uptake by berberine after 48h than after 24h (Figure 64), which is contrary to the effect of 24h versus 1h (Figures 61&62), this is because as these studies were performed in 2017-2019, protein estimation studies were performed, and the glucose uptake outcome data was normalised with protein content to know the exact effect.

3.2.1.3.2. Effect of *Gymnema* ethanolic extracts on 2-NBDG uptake in HepG2 cells after long-term treatment

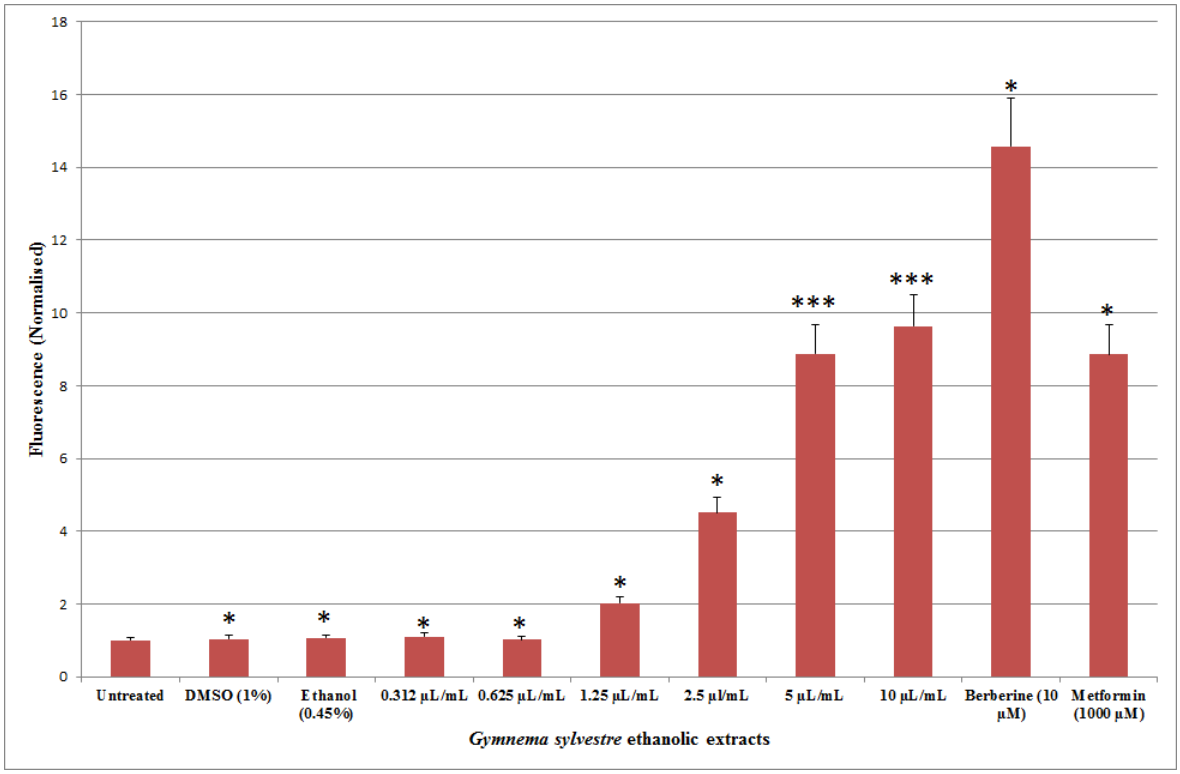


Figure 69: Effect of long-term treatment of *Gymnema* ethanolic extracts on 2-NBDG uptake by confluent HepG2 cells. Data are changes in fluorescence normalised for protein content, and normalised to untreated cells, and are the mean +SD from five experiments with *Gymnema* ethanolic extracts, and the treatments in each experiment were conducted in triplicate wells. Significant difference from untreated control to target treatments were assessed by employing one-way ANOVA with Bonferroni's multiple comparison post hoc test, **p<0.05*, and ****p<0.001*.

As shown in Figure 69, the long-term treatment (48 hr) with different concentrations of *Gymnema* ethanolic extracts, the top concentration (10 µL/mL) stimulated glucose uptake

9.5 times (*p<0.001*), compared with the untreated control. The negative control DMSO (1%) and the solvent control (0.6% ethanol) are not much effective compared with the untreated control (*p<0.05*), and the positive control metformin stimulating 9 times more glucose uptake (*p< 0.05*), compared with untreated cells, whereas berberine stimulating 14.2 times more glucose uptake (**p<0.05*), compared with the untreated control.

3.2.1.3.3. Effect of goat's rue ethanolic extracts on 2-NBDG uptake in HepG2 cells after long-term treatment

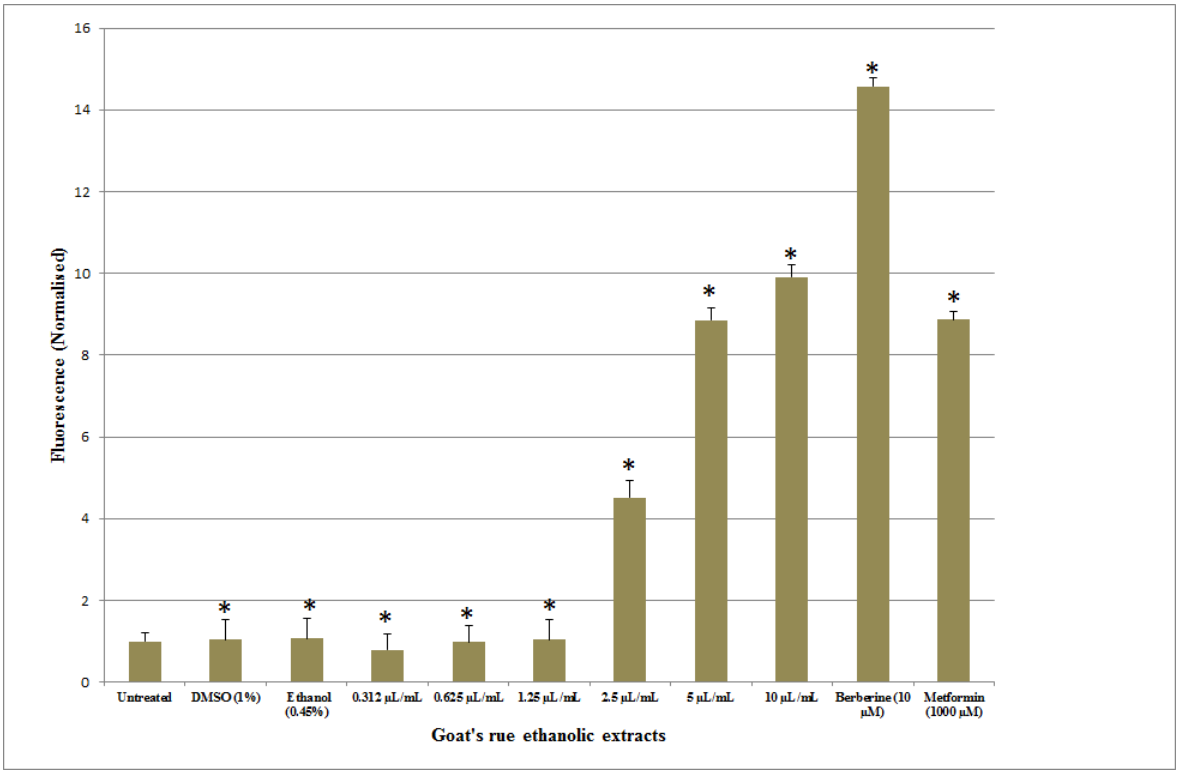


Figure 70: Effect of long-term treatment of goat's rue ethanolic extracts on 2-NBDG uptake by confluent HepG2 cells. Data are changes in fluorescence normalised for protein content, and normalised to untreated cells, and are the mean +SD from five experiments with goat's rue ethanolic extracts, and the treatments in each experiment were conducted in triplicate wells. Significant difference from untreated control to target treatments were assessed by employing one-way ANOVA with Bonferroni's multiple comparison post hoc test, **p<0.05*.

As shown in Figure 70, the 48-hr treatment with different concentrations of goat's rue ethanolic extracts, the top concentration (10 µL/mL) stimulated glucose uptake 10 times (*p<0.05*), compared with the untreated control. The negative control 1% DMSO and the solvent control (0.6 % ethanol) are not much effective compared with the untreated control

($p<0.05$), and the positive control metformin stimulating 9 times more glucose uptake ($p<0.05$), compared with untreated cells, whereas berberine stimulating 14.2 times more glucose uptake ($p<0.05$), compared with the untreated control.

3.2.1.3.4. Effect of *Stevia* ethanolic extracts and rebaudioside- A on 2-NBDG uptake in HepG2 cells after long-term treatment

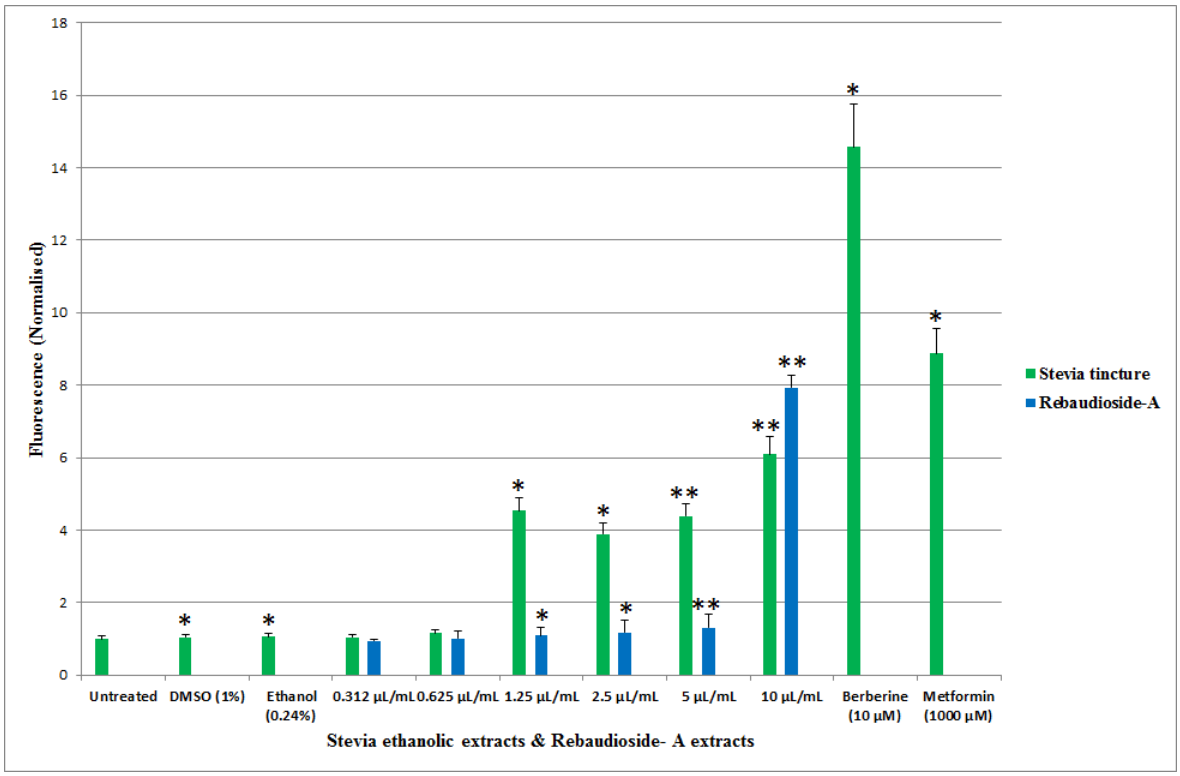


Figure 71: Effect of long-term treatment of *Stevia* ethanolic extracts and rebaudioside-A on 2-NBDG uptake by confluent HepG2 cells. Data are changes in fluorescence normalised for protein content, and normalised to untreated cells, and are the mean +SD from five sets of experiments with *Stevia* ethanolic extracts and five sets of experiments with rebaudioside-A, and the treatments in each experiment were conducted in triplicate wells. Significant difference from untreated control to target treatments were assessed by employing one-way ANOVA with Bonferroni's multiple comparison post hoc test, *$p<0.05$, and **$p<0.01$.

As shown in Figure 71, the long-term application (48 hr) of different concentrations of *Stevia* ethanolic extracts and rebaudioside-A, the top concentration (10 µL/mL) of *Stevia* application, stimulated glucose uptake 6 times ($p<0.01$), and the top concentration of rebaudioside- A stimulated 8 times ($p<0.01$) more glucose uptake compared with the untreated control. The negative control 1% DMSO and the solvent control (0.24% ethanol)

are not much different compared with the untreated control ($p<0.05$), and the lower concentrations 0.312 µL/mL and 0.625 µL/mL are showing a little uptake compared to the untreated control ($p>0.05$). The positive control metformin stimulating 9 times more glucose uptake ($p<0.05$), compared with untreated cells, whereas berberine stimulating 14.2 times more glucose uptake ($p<0.05$), compared with the untreated control.

3.3. The effect of herbal extracts on glucose consumption

After 48 hr treatment of confluent HepG2 cells with target phyto extracts, the media was collected, tested, and measured for the decrease of glucose content in the cell culture medium, as described in Section 2.5.

3.3.1. Effect of goldenseal ethanolic extracts on glucose consumption in HepG2 cells after long-term treatment

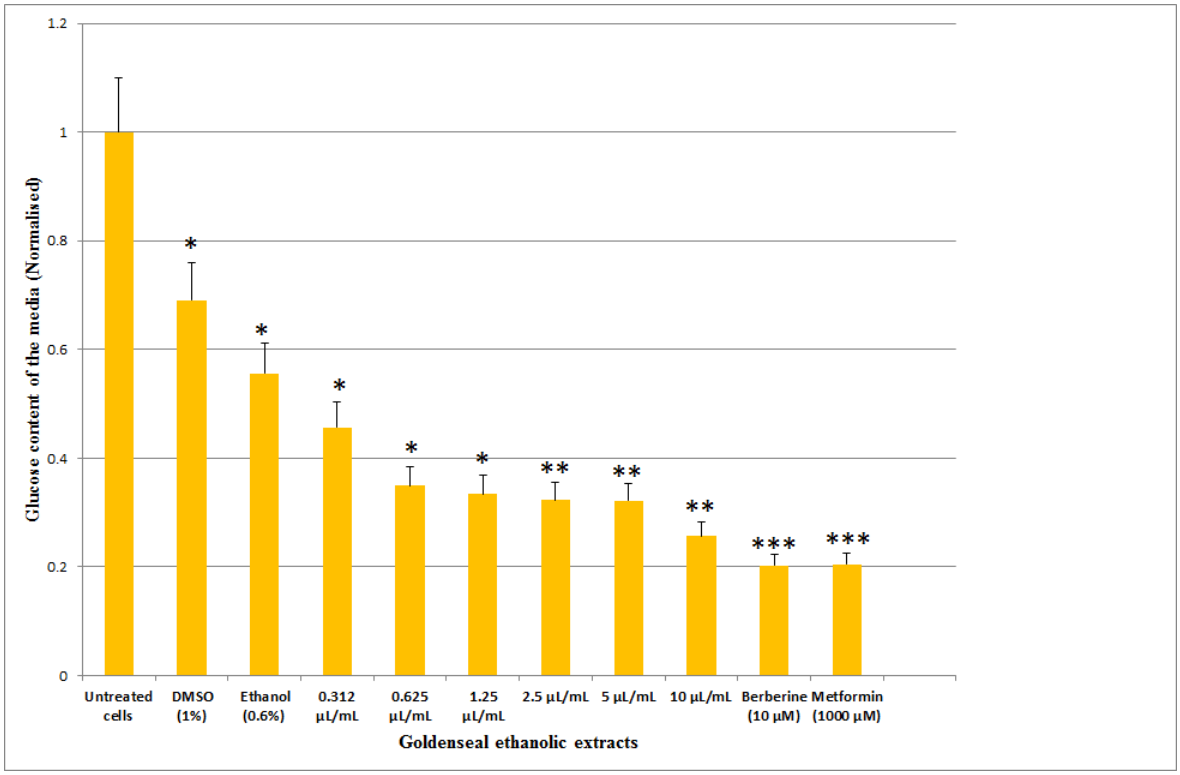


Figure 72: Effect of long-term treatment of goldenseal ethanolic extracts on glucose consumption by confluent HepG2 cells. Data are changes in glucose levels in treated cell culture media normalised for cell protein content and normalised to untreated cells. Data are the mean +SD from seven independent experiments, and the treatments in each experiment were conducted in triplicate wells. Here, the mean value of the triplicate determination of glucose levels of RPMI-1640 media with no cells was 9.65 mM. Significant difference from untreated control to target treatments were assessed by

employing one-way ANOVA with Bonferroni's multiple comparison post hoc test, *$p< 0.05$, **$p<0.01$, and ***$p<0.001$.

As shown in Figure 72, in confluent HepG2 cells, after 48-hr treatment with different concentrations of goldenseal ethanolic extracts, at the top concentration (10 µL/mL) glucose content in the medium is reduced by 75% ($p<0.01$), compared with the untreated control (100%), and by 80% by berberine ($p<0.001$), compared with untreated cells. The glucose content of the medium was 9.65 mM.

3.3.2. Effect of *Gymnema* ethanolic extracts on glucose consumption in confluent HepG2 cells after long-term treatment

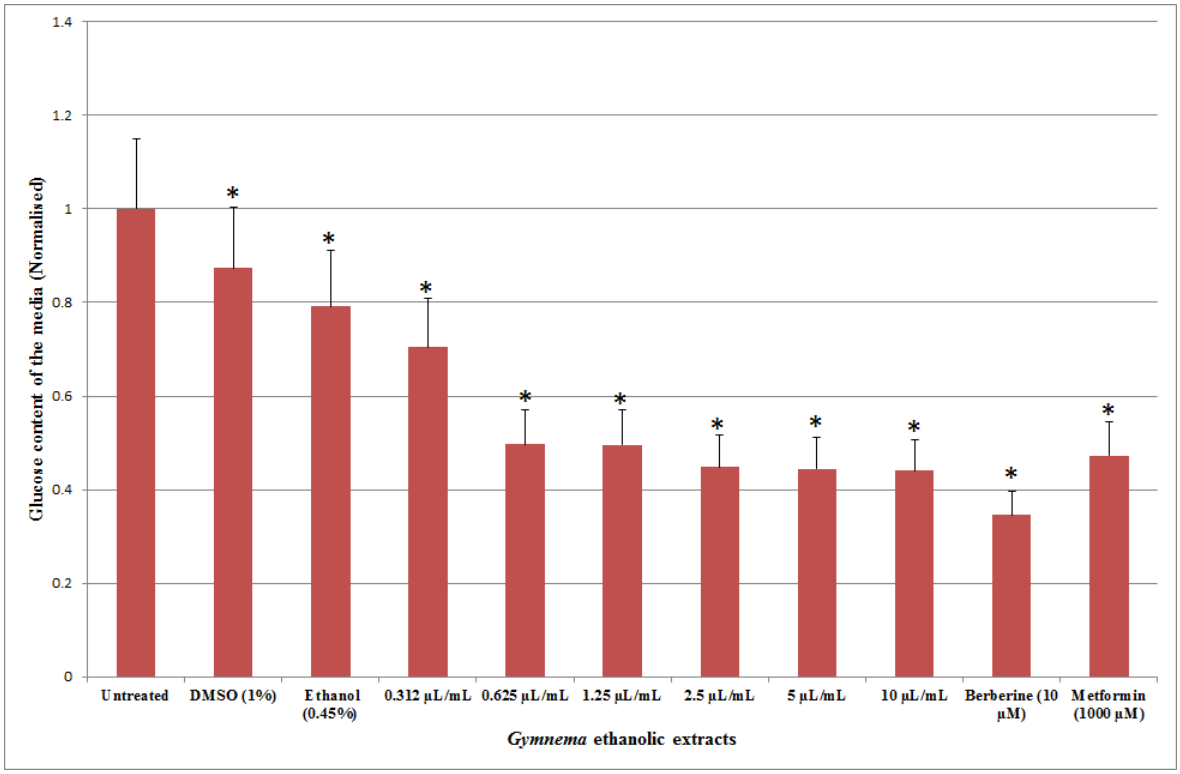


Figure 73: Effect of long-term treatment of *Gymnema* ethanolic extracts on glucose consumption by confluent HepG2 cells. Data are changes in glucose levels normalised for protein content, and normalised to untreated cells, and are the mean +SD from seven independent experiments. The treatments in each experiment were conducted in triplicate wells. Here, mean value of the triplicate determination of glucose levels of RPMI-1640 media with no cells was 10 mM. Significant difference from untreated control to target treatments were assessed by employing one-way ANOVA with Bonferroni's multiple comparison post hoc test, *$p<0.05$.

As shown in Figure 73, in confluent HepG2 cells, long term treatment (48 hr) with *Gymnema* ethanolic extracts, at the top concentration (10 µL/mL) glucose content is reduced by almost 60% ($p<0.05$), compared with the medium of untreated controls. Metformin and berberine reduced the glucose content by about 50 and 70% respectively ($p<0.05$), compared with untreated cells. The glucose content of the medium was 10 mM.

3.3.3. Effect of goat's rue ethanolic extracts on glucose consumption in confluent HepG2 cells after long-term treatment

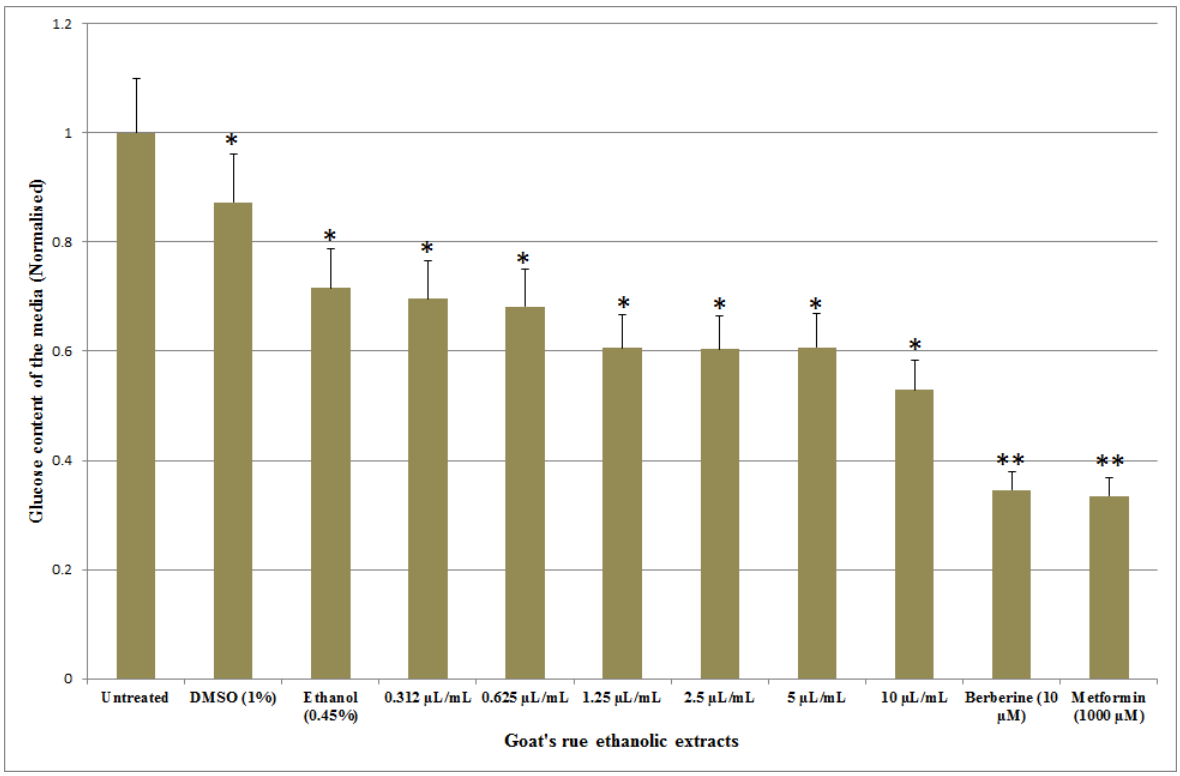


Figure 74: Effect of long-term treatment of goat's rue ethanolic extracts on glucose consumption by confluent HepG2 cells. Data are changes in glucose levels in treated cell culture media normalised for cell protein content, and normalised to untreated cells, and are the mean +SD from seven independent experiments, and the treatments in each experiment were conducted in triplicates. Also, mean value of the triplicate determination of glucose levels of RPMI-1640 media with no cells was 10 mM. Significant difference from untreated control to target treatments were assessed by employing one-way ANOVA with Bonferroni's multiple comparison post hoc test, $*p<0.05$, and $**p<0.01$.

As shown in Figure 74, in confluent HepG2 cells, 48 hr treatment with goat's rue ethanolic extracts, at the top concentration (10 µL/mL) glucose content is 50% ($p<0.05$), compared with the untreated control (100%), and glucose content at positive control metformin and

berberine is 35% (*p<0.01*), compared with untreated cells. The RPMI-1640 with no cells had a glucose content of 10 mM.

3.3.4. Effect of *Stevia* ethanolic extracts and rebaudioside-A on glucose consumption in confluent HepG2 cells after long-term treatment

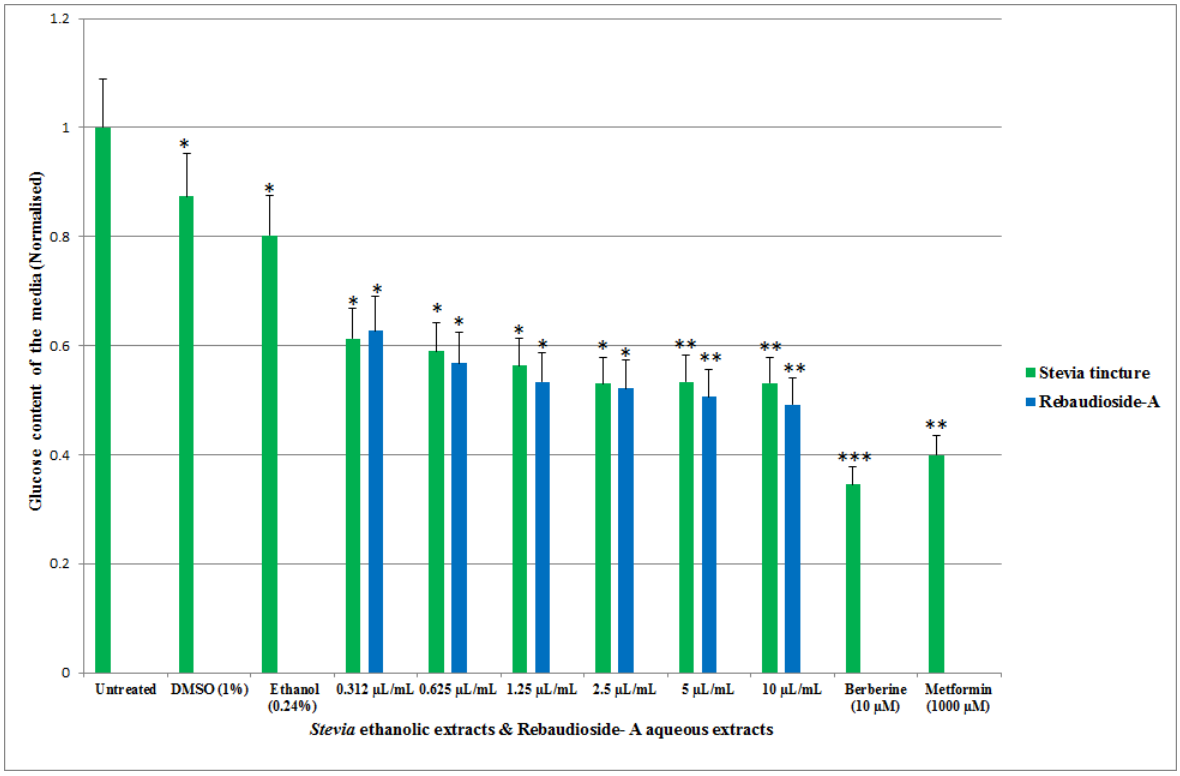


Figure 75: Effect of long-term treatment of *Stevia* ethanolic extracts and rebaudioside-A on glucose consumption by confluent HepG2 cells. Data are changes in glucose levels in treated cell culture media normalised for cell protein content, and normalised to untreated cells, and are the mean +SD from seven independent experiments, and the treatments in each experiment were conducted in triplicates. Media (RPMI-1640) with no cells displaying 10 mM of glucose content, compared with untreated control. Significant difference from untreated control to target treatments were assessed by employing one-way ANOVA with Bonferroni's multiple comparison post hoc test, *$p< 0.05$, **$p<0.01$, and ***$p<0.001$.

As shown in Figure 75, in confluent HepG2 cells, 48 hr treatment with *Stevia* ethanolic extracts and rebaudioside-A, at the top concentration (10 µL/mL) glucose content in the media is 50% (*p<0.01*) and 45% (*p<0.01*) compared with the untreated control (100%), and glucose content in media of cells treated with metformin and berberine is about 40% and 30% (*p<0.01*) respectively, compared with untreated cells. The glucose content of the medium was 10 mM.

3.3.5. Discussion

In the first phase of the project, screening tests were carried out to detect the potential glucose uptake and consumption properties of the target herbal compounds and are used to identify the key compounds and optimal treatments to carry out additional testing in the second phase (Chapter 4), to determine the additional metabolic properties of the selected compounds.

The intended application of *Stevia* herb in this research was to evaluate its effect on glucose uptake and consumption activity in HepG2 cells. Chromatographic analysis of the shade dried *Stevia* leaf, glucose uptake and consumption, and protein estimation studies were carried out with target herbal compounds and extracts, i.e., pure compounds, phloretin, ouabain, berberine, metformin, and rebaudioside-A, and extracts of *Stevia*, *Gymnema*, goat's rue and goldenseal. And the herbs such as goat's rue (*Galega officinalis*) is the founder herb of metformin, goldenseal (*Hydrastis canadensis*) for berberine, *Stevia* (*Stevia rebaudiana Bertoni*) for rebaudioside-A. Also, *Gymnema* is included in this research for its known anti-diabetic activity. Therefore, the influence of the extracts on glucose uptake and consumption was tested, and the efficacy was compared with pure compounds.

The concentration of stevioside in the leaves of *Stevia* varies substantially (2-20%) based on the cultivation, geographical and climatic conditions such as temperature, rain fall and soil (De Oliveira *etal.,* 2004; Yao *etal.,* 1999). And the methods of extraction, harvesting, and preparation of the samples and analysis causes the variation in glycoside composition in *Stevia* leaves (Midmore and Rank, 2002). Also, nutrition plays a vital role in glycoside formation, which is strongly governed by the quality of the soil bed and climatic conditions. For instance, a study suggested, in northern India during winter, which has similar climatic conditions of UK in summer, due to the interactive effects of crop ecology and nutrition on the crop yield and secondary metabolites of *Stevia,* promoted accumulation of stevioside; however, the accumulation of rebaudioside-A is not much influenced by these two factors. Therefore, leaf yield and steviol glycoside profiles can be improved through the selection of suitable growing locations and appropriate nutrient management (Pal *etal.,* 2015). In general plants grown in summer in the UK express elevated levels of stevioside in their leaves due to temperate climate, while plants grown in spring express higher levels of rebaudioside-A (*Stevia*.net, 2015; *Stevia* info,2015; Saifi *etal.,* 2014), therefore, as these plants were grown in summer in northwest of UK, the

analysis of the shade dried leaves has expressed stevioside as the most prominent glycoside (about 8%; Figure 60), and rebaudioside-A as the second leading compound (about 3.5%; Figure 60).

As steviol glycoside content and composition are the main quality criteria of *Stevia* extracts, initially experiments were performed with crude *Stevia* extracts i.e., aqueous extracts were used in the short-term treatment to check the working potential of the crude extracts and due to the increased bioavailability, ethanolic extracts (The Pharmacopoeia of the United States, 1850) were used in the long-term treatment. Also, effects of the extracts of whole herb were tested, along with other phytocompounds (i.e., goldenseal extracts, *Gymnema* extracts, goat's rue extracts, rebaudioside-A and berberine), in comparison to the standard drug metformin.

The HPLC/MS analysis was performed to establish the percentage of prevailing glycosides in *Stevia* crude extracts, and further, glucose uptake and consumption experiments were performed with the individual principal glycoside rebaudioside-A, which promoted glucose uptake (Figure 71) and consumption (Figure 75) in HepG2 cells in the long-term treatment. Although, *Stevia* is reported to promote glucose transport (Rizzo *etal.,* 2013), there is no evidence found using it in any herbal therapies worldwide. Instead, it has been in usage recently as a natural sweetener due to its zero calorific value and as a general approach to prevent dental caries, and obesity associated diseases such as diabetes and hyperlipidemia (Samuel *etal.,* 2018). It has also been consumed routinely by the natives of Paraguay, Brazil and Central America to sweeten beverages or chewed them for their sweet taste (Ashwell, 2015).

Chromatographic analysis of *Stevia* leaf has expressed about 8% stevioside, and 3.5% rebaudioside- A (Figure 60), the glycoside profiles of the leaves depend on interactive herb ecology (De Oliveira *etal.,* 2004; Yao *etal.,* 1999), and can be changed by plant ecology management (Pal *etal.,* 2015). After the short-term treatment, aqueous extracts of *Stevia* have displayed 2.4 times more glucose uptake at the optimal concentration (10 µL/mL) compared to the untreated control (Figure 67). After the long-term treatment *Stevia* ethanolic extracts and rebaudioside-A stimulated glucose uptake 6 times, and 8 times respectively (Figure 71) at the optimal concentration 10 µL/mL. The positive control metformin stimulated 9 times more glucose uptake, and berberine stimulated 14.2 times more glucose uptake, compared with the untreated cells (Figure 71). In this case, rebaudioside-A stimulated more uptake than the *Stevia* extract, and berberine stimulated

greater uptake than rebaudioside-A and *Stevia* extract. After the long-term treatment, the optimal concentrations of *Stevia* ethanolic extracts and rebaudioside-A stimulated glucose consumption, and the glucose content in the treated cell culture media was 50% and 45% respectively (Figure 75). In comparison metformin and berberine stimulated a greater consumption of glucose, 60% and 70% respectively. These data suggest that *Stevia* and its component compounds have an ability to stimulate glucose consumption and have potential anti-diabetic *in vivo*.

After one hour treatment the pure compounds phloretin, ouabain, berberine and metformin stimulated glucose uptake in growing and confluent HepG2 cells. In growing cells a short-term treatment with berberine had a prominent stimulation of glucose uptake (110%), compared with DMSO, and 20% by phloretin and 0.4% by ouabain, and metformin stimulated a 40% upregulation (Figure 61), and after the 24-hour treatment berberine has stimulated upregulation of the glucose by 78%, followed by metformin (60%) , phloretin (53%) and ouabain (20%), compared with the control cells (Figure 62). Whereas, in confluent cells after the one-hour treatment, berberine has stimulated glucose uptake by 117%, phloretin (28%), and ouabain has no effect and metformin has stimulated 32% of the upregulation compared with control (Figure 63), and after 24-hour treatment, berberine has stimulated glucose uptake by 50% compared to the control, and ouabain (.07 %), phloretin (20%) and metformin (31%) (Figure 64). In all four cases, berberine has the highest stimulation of glucose uptake in comparison to phloretin, ouabain and metformin. Therefore, berberine was selected for comparison with herbal extracts from goldenseal, goat's rue, *Gymnema*, and *Stevia* and its component rebaudioside-A.

In confluent cells, a short-term (i.e.,1 hour) treatment with goldenseal glycerites and ethanolic extracts stimulated glucose uptake at the optimal concentration (10 µL/mL), by 6.5 times and 5.4 times respectively, compared with untreated cells (Figure 65). And ethanolic extracts, at the optimal concentration stimulated glucose uptake 13.5 times compared with the untreated control, positive control metformin stimulated 9 times, berberine stimulated 14.2 times more glucose uptake, compared with the untreated control (Figure 68). Also, when it comes to glucose consumption rate, after long-term treatment, at the optimal concentration, glucose content was 25%, and at positive control metformin and berberine is 20%, compared with untreated cells (100%) (Figure 72). In both cases, at the optimal concentration of goldenseal ethanolic extract, and berberine has stimulated prominent glucose uptake 13.5 times and 14.2 times respectively, and prominent glucose consumption 75% and 80% respectively.

In confluent cells, the long-term treatment with goat's rue ethanolic extracts, the optimal concentration (10 µL/mL) upregulated glucose uptake 10 times, and the positive control metformin stimulated 9 times more augumentation, whereas berberine augumentated 14.2 times more glucose uptake, compared with the untreated control (Figure 70). Also, at the top concentration (10 µL/mL) glucose consumption rate was 50%, and glucose consumption rate of positive control metformin and berberine was 65%, compared with untreated cells (Figure 74). In both cases, at the optimal concentration of goat's rue extract and berberine has stimulated prominent glucose uptake 10 times and 14.2 times respectively, and prominent glucose consumption 50% and 65% respectively.

After short term treatment, *Gymnema* glycerites improved glucose uptake slightly at the optimal concentration, by 1.3 times, and 1.4 times at concentrations 1.25 µL/mL, 2.5 µL/mL and 5 µL/mL compared with untreated cells (Figure 66), and after long term treatment with *Gymnema* ethanolic extracts, the premier concentration stimulated glucose uptake 9.5 times, and the positive control metformin stimulated 9 times, whereas berberine stimulated 14.2 times more glucose uptake, compared to the untreated control (Figure 69). When it comes to the glucose consumption rate, at the optimal concentration (10 µL/mL) glucose consumption rate was 55%, and at positive control metformin and berberine, it was 50% and 65 %, compared with untreated cells (Figure 73). In both cases, at the optimal concentration of *Gymnema* extract and berberine has stimulated prominent glucose uptake 9.5 times and 14.2 times respectively, and prominent glucose consumption 55% and 65 % respectively.

The above observations serve as the basis for estimating the key compounds, and conclusions can be drawn from the tendency of the compound action, as berbeine and goldenseal (10 µL/mL) are consistently expressed prominent glucose uptake and consumption throughout the study than their counterparts, therefore the experimental data revealed that goldenseal and berberine have significant glucose uptake and consumption activity in HepG2 cellular models compared to the standard drug metformin. The goldenseal extracts and berberine seem promising for the development of oral anti-diabetic agents, therefore these extracts and compounds i.e., Goldenseal ethanolic extracts (10µL/mL), and berberine (10µM) have been identified as key compounds of the research to focus and explore the next series of experiments, i.e., cytotoxicity assays, flow cytometry based glucose uptake assays, western blotting GLUT-1 analysis, and estimation of the glycogen content and glucose release, and the seahorse metabolic analysis of the treated HepG2 cells (Chapter 4). As there is a separate chapter for the comprehensive

discussion (Chapter 5), in this section, the outcome of the screening experiments has been discussed as a summary.

Chapter 4: Metabolic properties of the key herbal compounds and extracts

Following the initial screening of candidate extracts and compounds for potential anti-diabetic activity, as indicated by stimulation of glucose uptake by HepG2 cells described in Chapter 3, goldenseal ethanolic extracts (10µL/mL), berberine (10µM) and metformin (1000 µM) were identified for further analysis. The experiments described in this chapter were designed to investigate further the effect of these treatments on key aspects of glucose metabolism in HepG2 cells, to confirm, or not, the anti-diabetic potential of the treatments. Cytotoxicity assays, flow cytometry-based glucose uptake, western blotting GLUT-1 analysis, and estimation of the glycogen content, glucose release, and the Seahorse metabolic analysis of the long term treated HepG2 cells were carried out.

4.1. Cytotoxicity assays (MTT assays)

Relative live cells were firstly checked to ensure that the selected treatments had no adverse effects under the conditions to be used for the experiments described in this chapter, normally cells grown to 70-80% confluency before long-term treatment. Goldenseal (10 µL/mL), berberine (10 µM), and the respective solvent controls ethanol (0.6%), and DMSO (1%), and a positive control metformin (1000 µM), and an untreated control were assessed. The reduction of tetrazolium salts reflects relative live cells in a cell population, the yellow tetrazolium MTT was reduced by live and active cells by dehydrogenase enzymes and generate reducing compounds NADH and NADPH, and the resultant purple formazan was quantified using a plate reader (Chapter 2; Section 2.4.B).

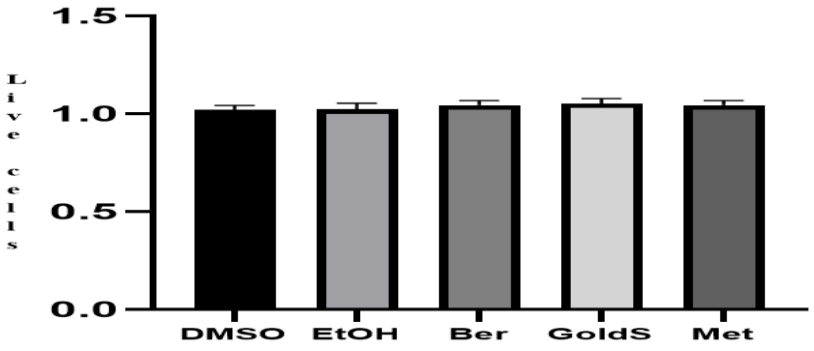


Figure 76: Effect of the target herbal compounds on cell viability after long-term treatment of the confluent HepG2 cells. Data are changes in absorbance and are the mean +SD from three experiments, and the treatments in each experiment were conducted in triplicate wells. Significant differences between the control and target treatments were assessed by

employing Graph Pad Prism 9, with a student t-test, and between the treatments and the control $p<0.05$ for Ber and Met and for Gold S $p<0.01$.

As shown in the Figure 76, in confluent HepG2 cells after 48 hours treatment, there were no signs of toxicity.Following this viability assay, it was confirmed the identified key compounds were nontoxic at the concentrations used, and the research was further explored by assay of glucose uptake with the selected key compounds, and by flow-cytometry to complement the glucose uptake assays performed by the plate reader method in Chapter 3, Section 3.2.

4.2. Flow cytometric detection of 2-NBDG uptake in HepG2 cells stimulated by berberine and goldenseal

The results of the glucose uptake experiments described in Chapter 3 showed that berberine and goldenseal induced a marked increase in glucose uptake by HepG2 cells, and it was interesting to supplement these results with data from a different technique, flow cytometry. This technique allows for a direct measurement of each cell's fluorescence and allows for a quantitative comparison of cells treated with test compounds, controls, and untreated non-fluorescent negative control cells. The 2-NBDG uptake assay is limited because it is sensitive to light and is used by cells. So, it's important to limit the number of samples so that the last one can be evaluated within 30 minutes of the first one.

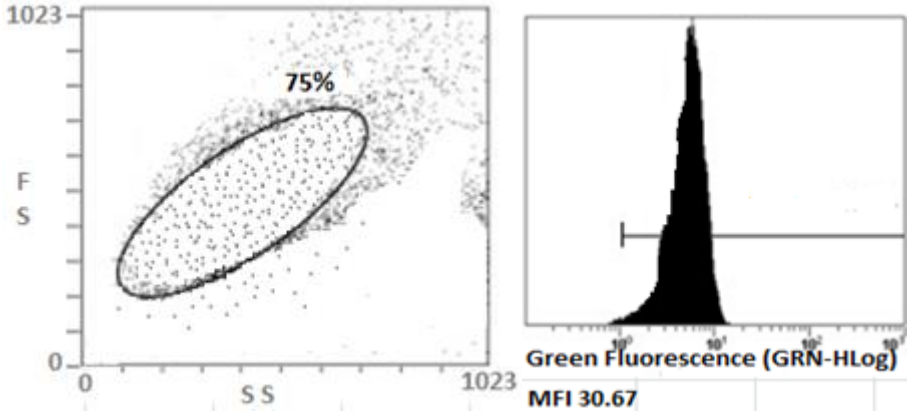
For these investigations, confluent HepG2 cells were treated for 48 hours with test compounds goldenseal ethanolic extracts (10μL/mL), berberine (10 μM), and positive control metformin (1000 μM), solvent controls DMSO (1%) and 0.6 % (v/v) ethanol. Additionally, to allow the setup for analysis to be accurately understood, untreated non-fluorescent negative control cells (Chapter 2; Section 2.2.2.1.10) were present, and a gating method was used to separate the populations of fluorescent cells. An active gate was drawn based on the cell size and granularity, to avoid the heavily granulated cells which are generally dead cells, and often found at the bottom of the plot, and this gate excludes debris. Also, the auto-fluorescence of the unstained gated cells in the dot plot (Plot-1A) was plotted in the histograms (Plot-1B).

The gated cells' fluorescence was plotted separately in the histograms (Plot-B), which shows the green fluorescent profile of the gated population. On the X-axis of Plot-B, cells with green fluorescence greater than 100 were looked at, and the mean intensity of the green fluorescence was used to measure how much 2-NBDG was taken up.

The results of individual test compounds and their respective solvent control and an untreated negative control were obtained based on the mean fluorescence intensity of the green fluorescence values of the Plot-B (Figure 77 plots-1B to 6B). Due to the large amount of data obtained in these four experiments, a single set of data (Figure 77 and Table 5) is presented here, and to distinguish the population of fluorescent cells from the negative control cells for goldenseal and berberine only presented below to understand the results of the experiments and the similar pattern observed for the remaining samples.

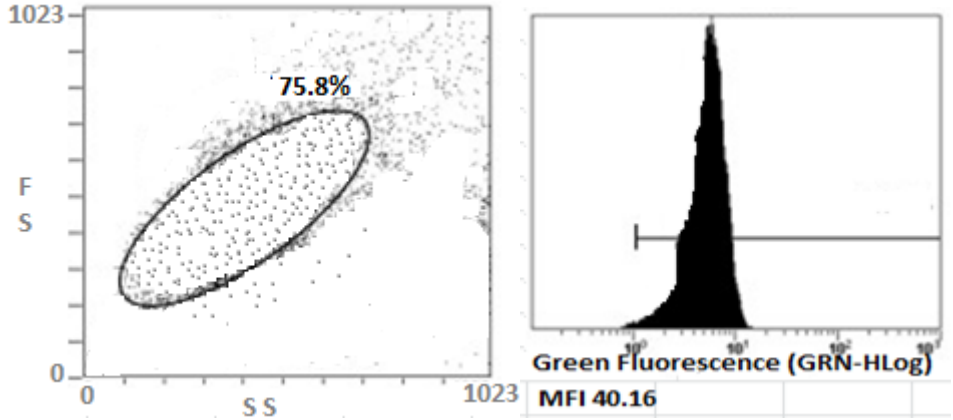
1) Untreated non-fluorescent negative control cells

Plot-1A (Dot plot) **Plot-1B (Auto-fluorescent profile)**



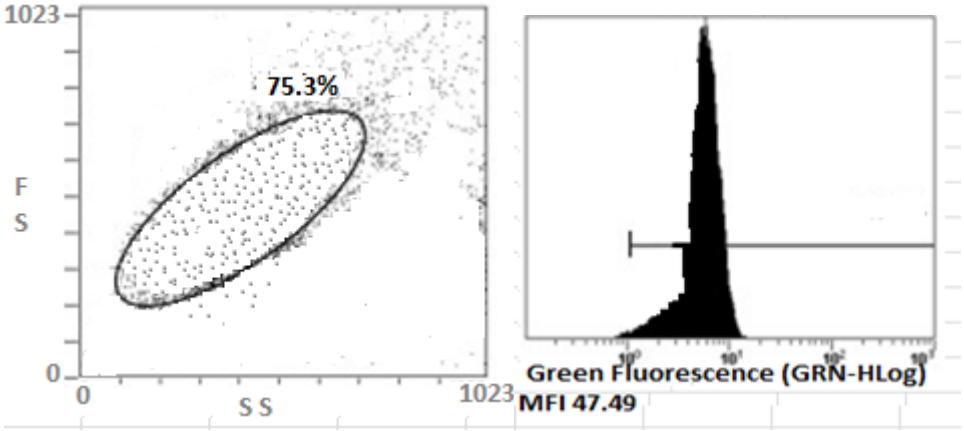
2) DMSO (1%) treated cells

Plot-2A (Dot plot) **Plot-2B (Fluorescent profile)**



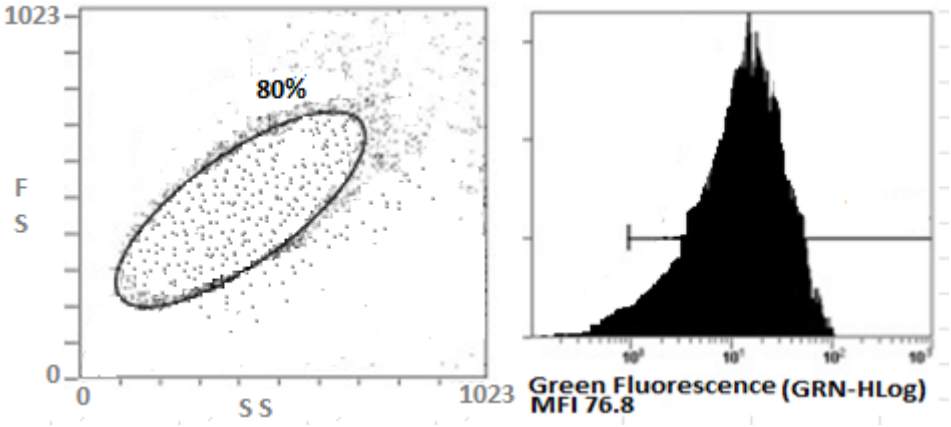
3) Ethanol (0.6%)

Plot-3A (Dot plot) **Plot-3B (Fluorescent profile)**

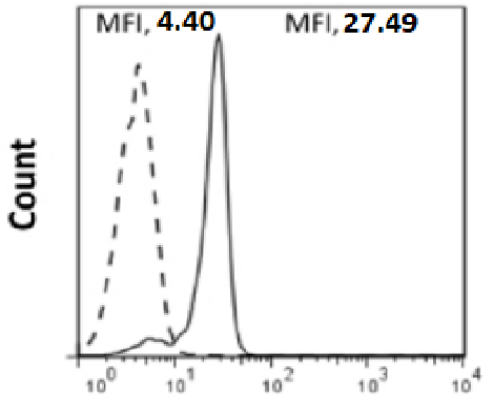


4) Goldenseal (10 µL/mL)

Plot-4A (Dot plot) **Plot-4B (Fluorescent profile)**



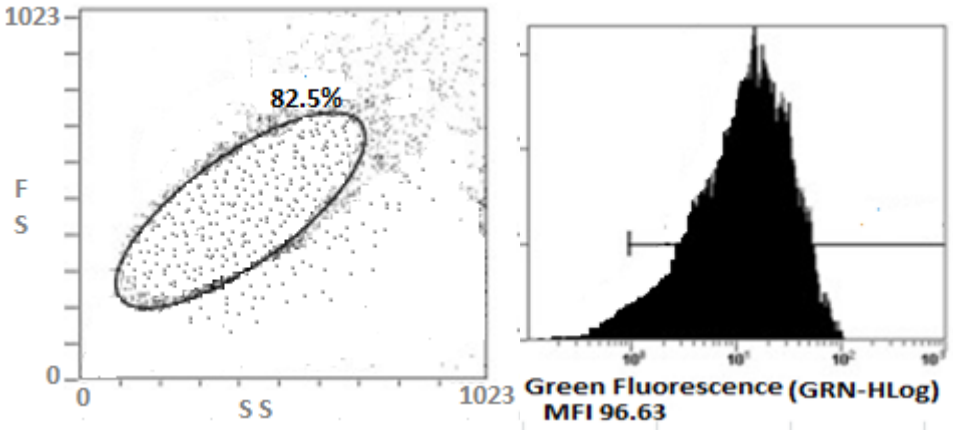
Plot-4C (Negative control and Goldenseal sample)



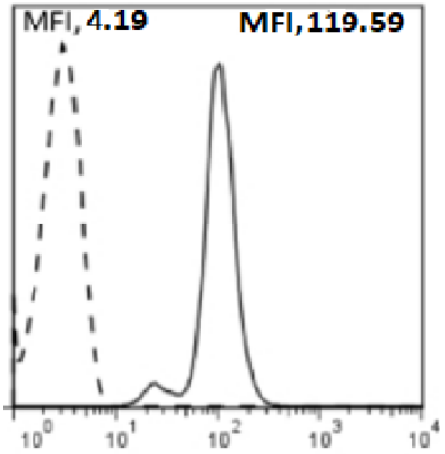
Plot-4C shows the negative control (- - - -), and the Goldenseal with 2- NBDG treated sample (—). The negative control's MFI is 4.40, whereas the Goldenseal sample's MFI is 27.49.

5) Berberine (10 µM)

Plot-5A (Dot plot) **Plot-5B (Fluorescent profile)**



Plot-5C (Negative control and Berberine sample)



Plot-5C shows the negative control (- - - -), and the Berberine with 2-NBDG treated sample (—). The negative control's MFI is 4.19, whereas the Berberine sample's MFI is 119.59.

6) Metformin (1000 µM)

Plot-6A (Dot plot) **Plot-6B (Fluorescent profile)**

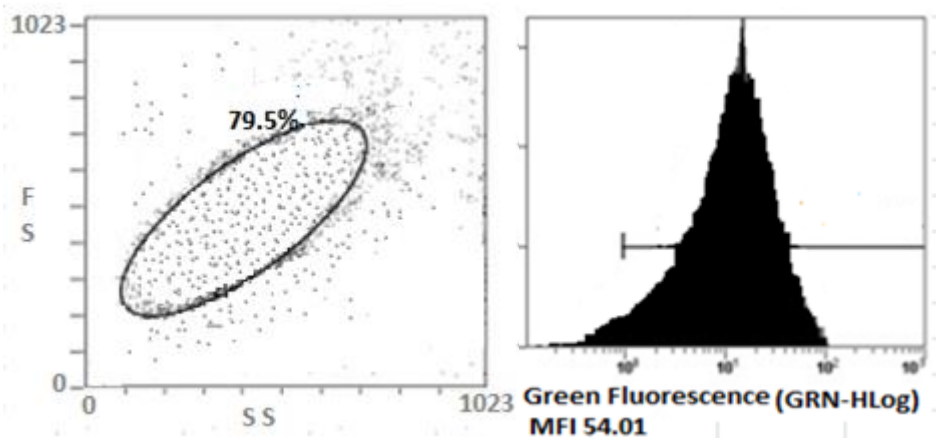


Figure 77: Flow cytometric analysis of 2-NBDG uptake by HepG2 cells. Plots A show dot plots of forward vs side-scatter. The gated cell population chosen for analysis of 2-NBDG uptake, as assessed by green fluorescence, is shown by the oval in each plot. The Plots B displays the green fluorescent profile of the gated population of the corresponding Plot-A. The cells that have green fluorescence greater than 10^0 on the X-axis of the Plot B, and the mean fluorescence intensity of the green fluorescence, was the criteria used to quantify 2-NBDG uptake.

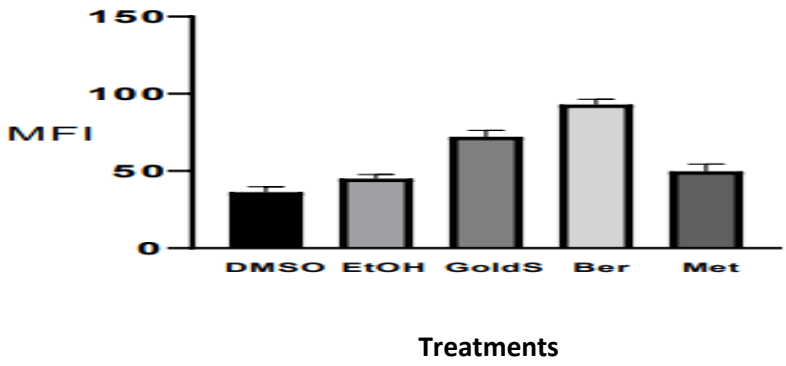


Figure 78: Uptake of 2-NBDG by HepG2 cells assessed by flow cytometry. The treatments in each experiment were carried out in triplicate wells, and the data represent changes in the mean fluorescence intensity of the green fluorescence and are the mean + SD from four trials. With the use of Graph Pad Prism 9, a student t-test was used to determine whether there was a significant difference between the target treatments and the solvent control (DMSO), and $p<0.001$ in all groups. The data can be compared to the findings of the plate reader tested 2-NBDG uptake, which are depicted in Figure 68, Chapter 3.

Table 5: A single set of the data of the mean values of the green fluorescence in plots B of a negative control, DMSO (1%), and ethanol (0.6%), and goldenseal (10 µL/mL), berberine (10 µM), and metformin (1000 µM).

	MFI (Mean fluorescence intensity of the Green fluorescence)					
	Negative control	**DMSO**	**EtOH**	**GolS**	**Ber**	**Met**
	30.67	40.16	47.49	76.8	96.63	54.01
	33.29	40.5	42.33	77.96	95.01	55.05
	30.29	41	45.4	77.7	96.5	54.97
Average	31.41	40.55	45.07	76.82	96.04	54.67
S.D.	1.63	0.42	2.59	0.87	0.9	0.57

As shown in the Figure 78, the outcome of the experiments shows that berberine stimulates 2.5 times more glucose uptake ($p<0.001$), than the solvent control (DMSO), and goldenseal is the second most active compound with 1.98 times more uptake ($p<0.001$), and metformin stimulates 1.37 times uptake ($p<0.001$), compared to the DMSO. Following the glucose uptake experiments, the research has explored the effect of goldenseal and berberine on stimulation of GLUT-1 in the long term treated HepG2 cells. These data

confirm qualitatively the stimulation of 2-NDBG uptake by berberine, goldenseal and metformin measured using a plate reader (Figure 68).

4.3. Effect of Goldenseal extract and Berberine on expression of GLUT-1 in HepG2 cells

To further explore the mechanisms by which the selected phytochemicals regulate glucose metabolism in HepG2 cells, an analysis of the expression of the glucose transporter-1 (GLUT-1) was carried out by western blotting. Recently the practice of using housekeeping controls, such as β-actin, to normalise expression data from western blots has come under criticism, and instead total staining of blotted proteins is recommended practice by some journals, including the Journal of Biological Chemistry (J Biol Chem, 2018). Accordingly, equivalent amounts of protein per sample were loaded, according to Bradford assay of sample lysates, and after blotting, all blotted proteins were transiently stained and recorded as described in methods (2.6.1.6.B).

In Section 4.7, it was explained why HepG2 cells were used in this study rather than primary hepatocytes. Confluent HepG2 cells were treated with goldenseal extract (10 µL/mL), and berberine (10µM), and the respective solvent controls ethanol (0.6%), and DMSO (1%) for 48 hours. GLUT-1 protein was detected by using a rabbit primary anti GLUT-1 antibody, and a goat anti-rabbit IgG labelled with horse-radish peroxidase secondary antibody. GLUT-1 was identified as a band of about 55kDa (Figure 79), in accordance with published values **(**Mueckler *et al.*,1985). Treatments of HepG2 cells with goldenseal (10 µL/mL), and berberine (10 µM) induced a noticeable increase in expression of GLUT-1 compared with the solvent controls ethanol (0.6%) and DMSO (1%).

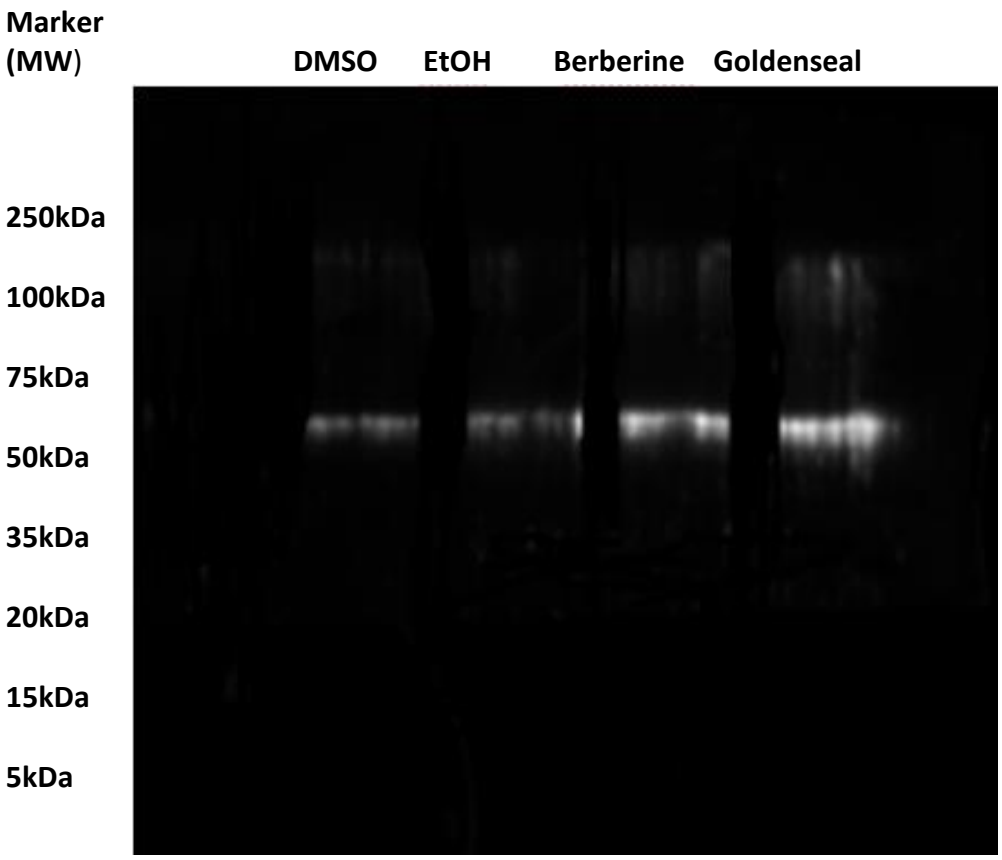


Figure 79: GLUT-1 expression in treated HepG2 cell lysates. A representative western blot showing changes in expression of GLUT-1 in HepG2 lysates induced by goldenseal (10µL/mL), and berberine (10 µM), compared with control treatments ethanol (0.6%), and DMSO (1%).

The band intensities on gels were quantified using Image J software to compare the expression of GLUT-1 in each sample. A rectangular region was chosen around each band, and the intensity of each sample was quantified. Intensities were normalised to intensities of protein blots of the same sample. The data, shown in Figure 80, suggest that extracts from goldenseal (10 µL/mL), and berberine (10 µM), could stimulate an increased expression of GLUT-1 protein in HepG2 cells.

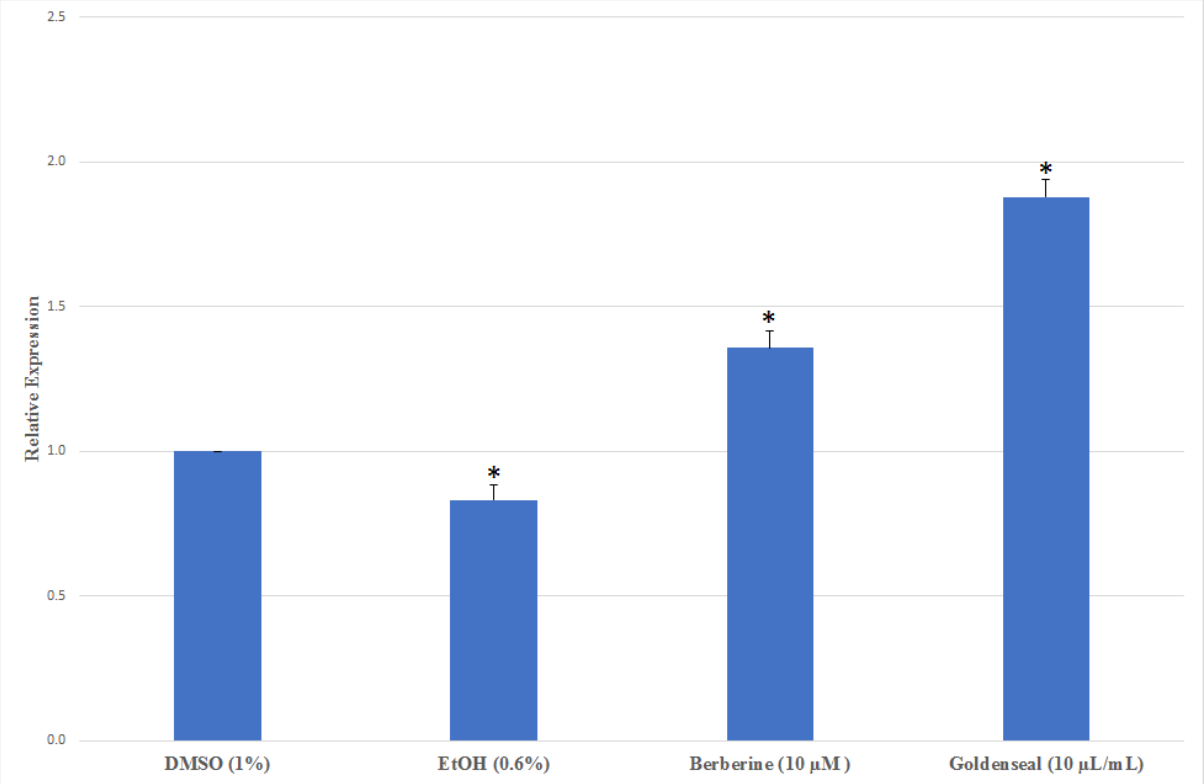


Figure 80: Quantification of the expression of GLUT-1 protein detected by western blotting in HepG2 cells. A plot of the average relative intensities of the GLUT-1 bands from the image of four blots, quantified using ImageJ. Data are changes in the intensity normalised to the DMSO control and are the mean +SD from four experiments. Significant difference from the control to target treatments were assessed by employing one-way ANOVA with Bonferroni's multiple comparison post hoc test, *$*p<0.05$*.

As shown in the Figure 80, goldenseal stimulates the expression of GLUT-1, 1.7 times ($p<0.05$), and berberine stimulates 1.4 times ($p<0.05$), compared to the control. These data provide an explanation for the enhanced uptake of 2-NBDG induced by goldenseal and berberine. The next experiments were designed to explore the effect of berberine and goldenseal on the fate of glucose once taken up by HepG2 cells.

4.4. Effect of Goldenseal extract, Berberine, and Metformin on Glycogen content in HepG2 cells

Glycogen is the storage form of glucose, mainly found in the liver and the skeletal muscles of the body and is a multi-branched polysaccharide. Generally, insulin stimulates glycogen synthesis by activating the hexokinase enzyme, which phosphorylates additional glucose, and deposits it within the liver cell (Sonksen and Sonksen, 2000). As the target herbal compounds demonstrated stimulation of glucose uptake and consumption efficacy in HepG2 cells (Chapter 3), it is of the interest to investigate the efficacy of the berberine, goldenseal and metformin in stimulating glycogen synthesis in the long-term treated confluent HepG2 cells (Chapter 2; Section 2.7).

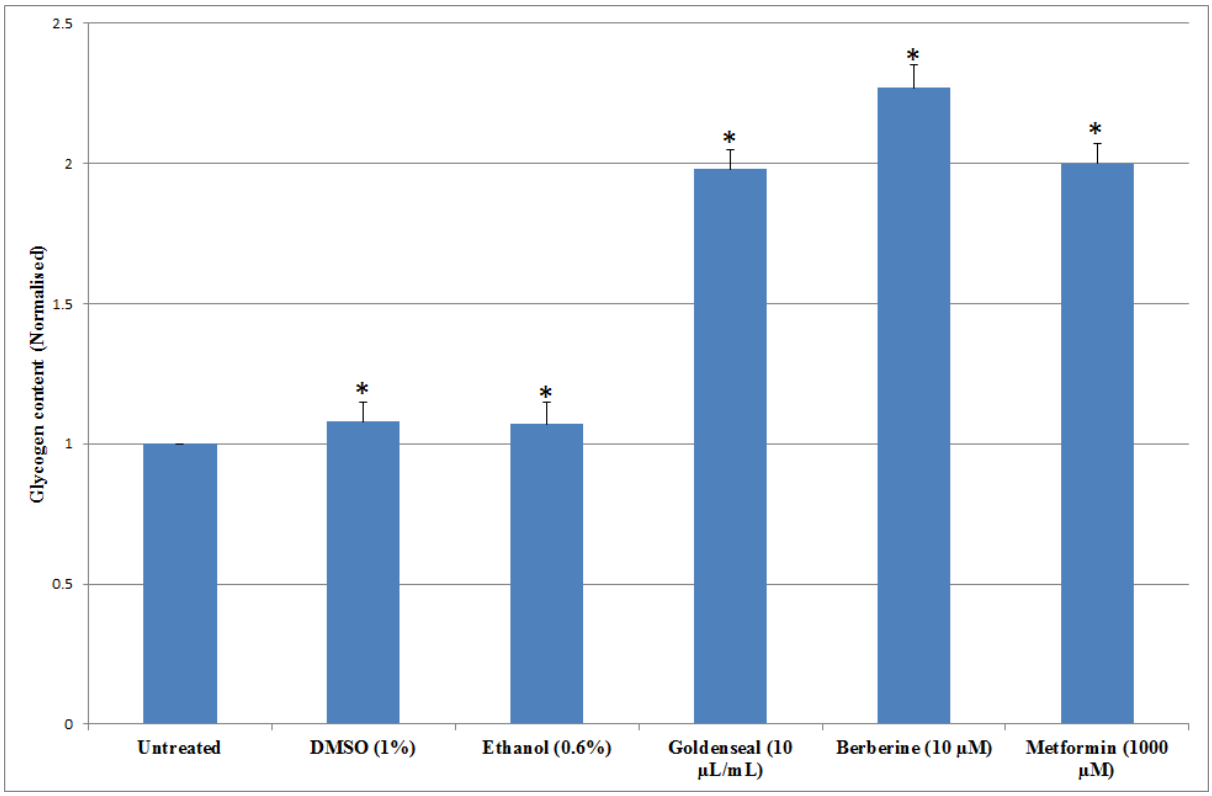


Figure 81: Effect of the target herbal compounds on glycogen content after long-term treatment of the confluent HepG2 cells. Data are changes in glucose normalised by the protein content of the treated cells (Figure 84), to an untreated control and are the mean +SD from four experiments, and the treatments in each experiment were conducted in triplicate wells. Significant difference from the untreated control to target treatments were assessed by employing one-way ANOVA with Bonferroni's multiple comparison post hoc test, *$p<0.05$.

As Figure 81 shows, after 48 hr treatment, berberine promotes an increase in glycogen content 2.4 times, metformin 2 times, goldenseal around 2 times compared to the untreated control ($p<0.05$). As stated in the legend, the values of the glycogen content were normalised with the protein content of the treated cells. The values of the protein content from the Bradford assay were calculated by the formula of the standard curve.

The details of the standard curve and the protein estimation chart have been displayed below (Figures 82 & 83), and the same standard curve was used to assess the estimation of protein in Figure 86, Section 4.5. Also, as glycogen stores in polysaccharide form in the cytosol, the treated cells were broken by exposing the temperatures at 95-100 °C for 5 minutes. To ensure complete homogenisation of the pellet, mixtures were vortexed thoroughly and incubation at 95-100 °C continued for a further 55 minutes. Levels of glucose were determined through the amount of NADPH generated by the combined action of hexokinase and glucose-6-phosphate dehydrogenase, measured by fluorescence (Chapter 2; Section 2.7). Glucose standards of 0 to 50 µM were used to calibrate the assay and the values of the glucose content from the broken glycogen, which was released into the supernatant was calculated by the formula of the glucose standard curve, and the same standard curve was used for glucose release assay data in Figure 85, Section 4.5. The details of the standard curve are displayed in the Figure 82.

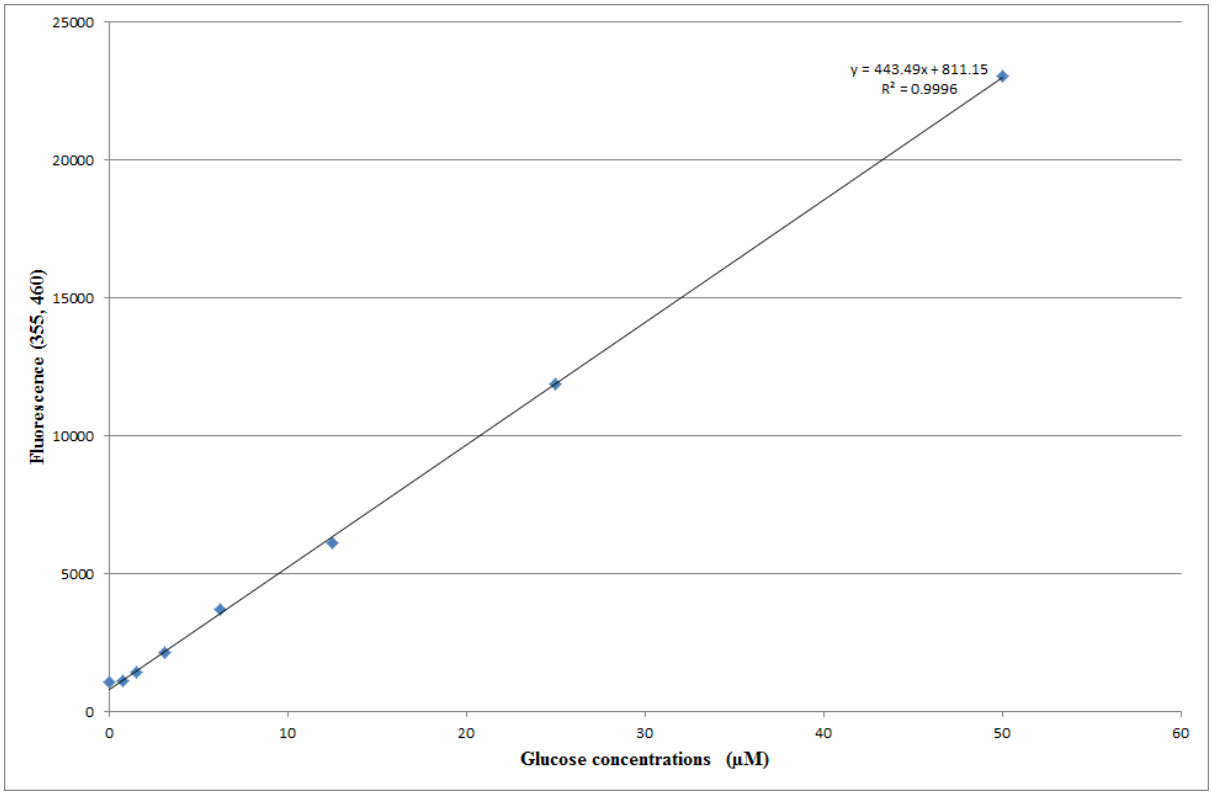


Figure 82: Standard curve for the glucose estimation. A typical standard curve with a range of 0 to 50 µM. Glucose standards were used to determine the sensitivity of the assay, and to measure the glucose concentrations expressed by treated samples by using the equation. Curve fitting shows the equation (Y=443.49x+811.15), where X is the glucose concentration and Y is the measured fluorescence value of the individual treatments. The regression value is 0.9996 suggesting accuracy of the curve fitting.

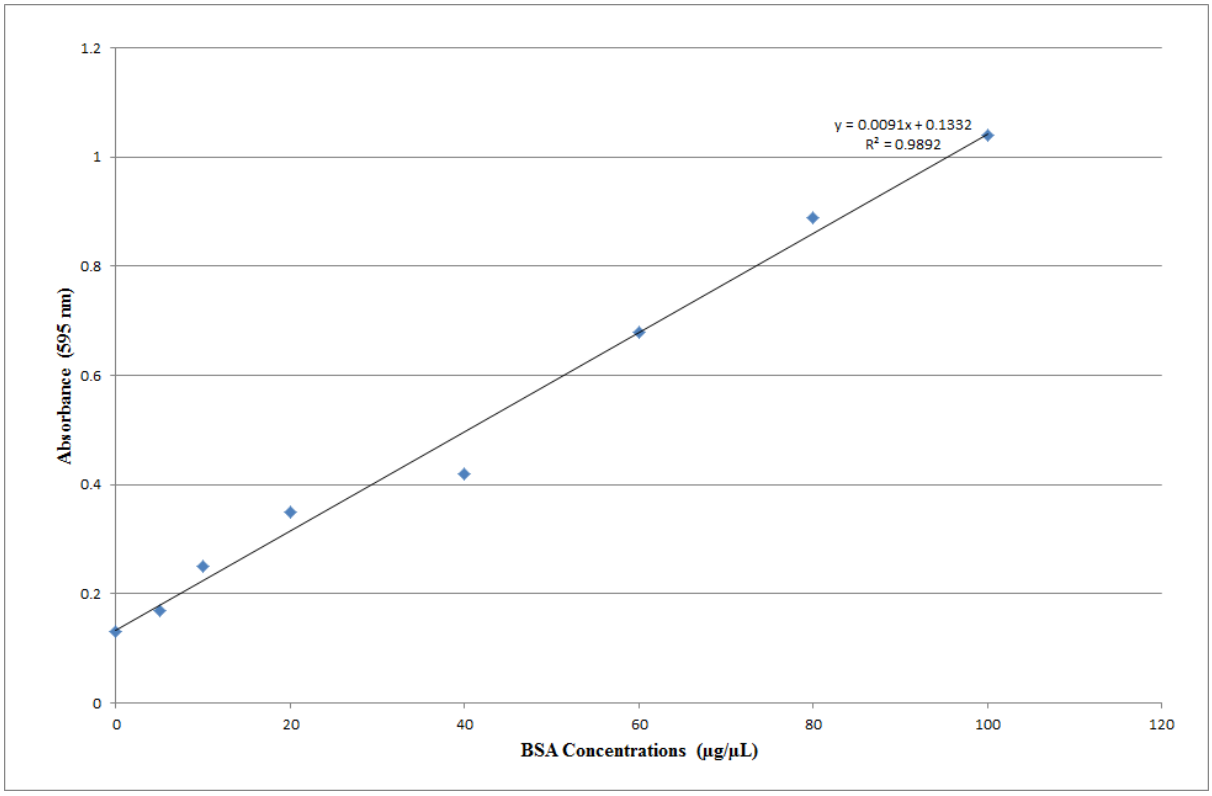


Figure 83: Standard curve for the protein estimation. A typical standard curve with a range of 0 to 100 µg/µl. BSA standards were used to determine the sensitivity of the assay, and to measure the protein concentrations expressed by treated samples by using the equation. Curve fitting shows the equation (Y=0.0091X+0.1332), where X is the protein concentration and Y is the measured absorbance value of the individual treatments. The regression value is 0.9892 suggesting accuracy of the curve fitting**.**

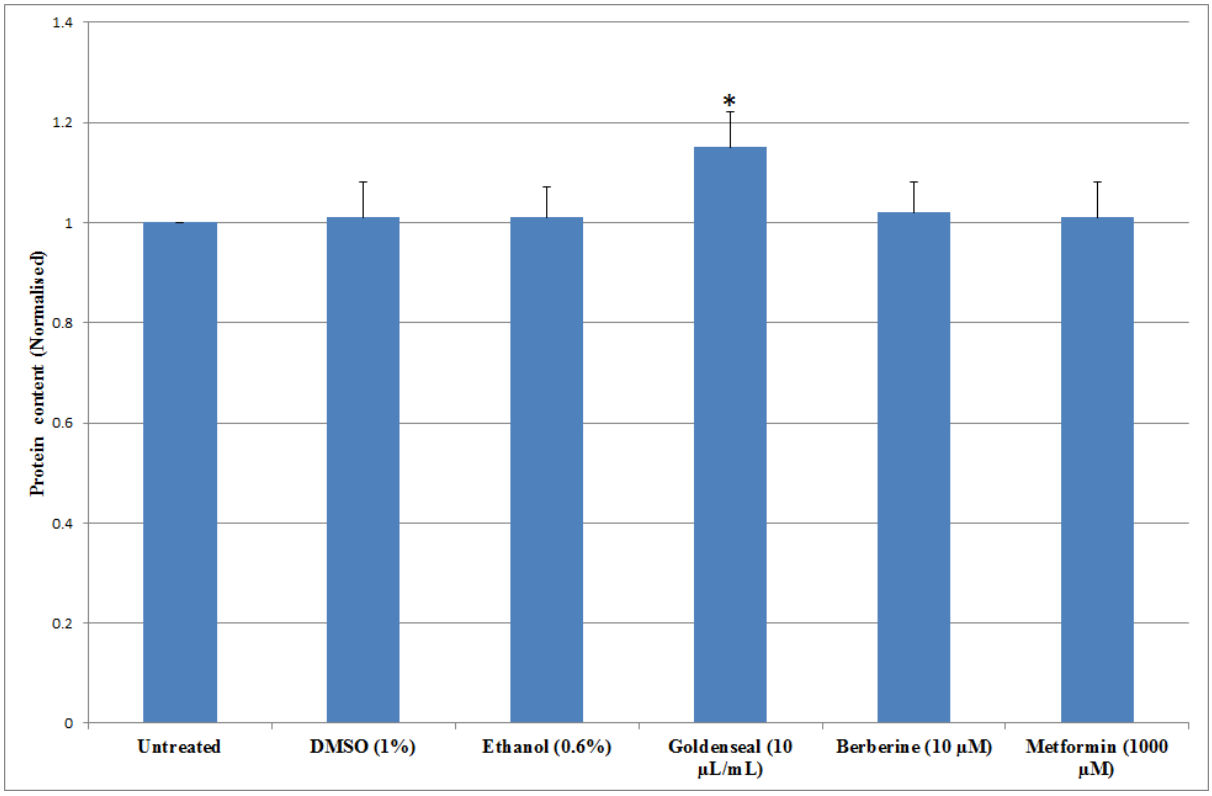


Figure 84: Protein concentrations in confluent HepG2 cells after long-term (48-hour) treatments. Data are protein concentration levels in treated cells normalised to protein concentrations of untreated cells and are the mean +SD from four independent experiments. The treatments in each experiment were conducted in triplicates. Significant difference from untreated control to target treatments were assessed by employing one-way ANOVA with Bonferroni's multiple comparison post hoc test, $*p<0.05$. The protein concentrations of berberine and metformin are the same as untreated control ($p>0.05$), and as noted before, treatments had little effect on cell biomass, except for goldenseal ($p<0.05$).

4.5. Effect of Goldenseal extract, Berberine, and Metformin on Glucose release from HepG2 cells

Another indication of potential anti-diabetic activity would be the modulation of glucose homeostasis through control of glucose release from glycogen stores. Treatments that lower blood glucose could do so by inhibiting release of glucose from stores such as glycogen in liver. Accordingly, HepG2 cells were grown to confluency and treated for 48 hours in normal medium containing 22 mM glucose. Under these conditions it has already been shown that goldenseal and berberine promote glucose uptake (Sections 3.2 and 4.2) and increase levels of glycogen (Section 4.4). Normal cell culture media was replaced with glucose and phenol-free medium (Section 2.8), and the amount of glucose released from cells during a four-hour period was measured.

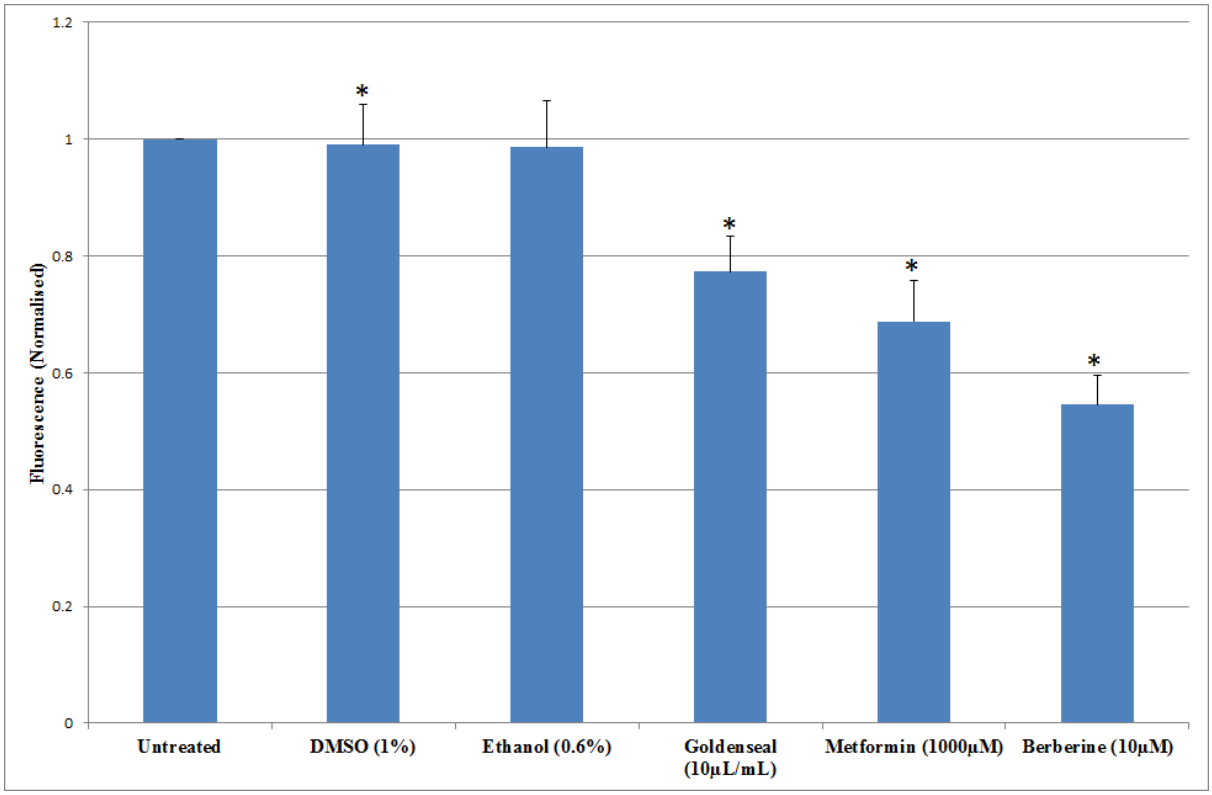


Figure 85: Effect of the target herbal compounds on release of glucose from HepG2 cells. Data are changes in fluorescence normalised by the protein content of the treated cells (Figure 86), to an untreated control and are the mean +SD from four experiments, and the treatments in each experiment were conducted in triplicate wells. Significant difference from the untreated control to target treatments were assessed by employing one-way ANOVA with Bonferroni's multiple comparison post hoc test, *$p<0.05$.

As Figure 85 indicates, when compared to the untreated control HepG2 cells, cells treated with the solvent controls DMSO, and ethanol released almost the same amount of glucose. In contrast pre-treatment with the test reagents significantly inhibited release of glucose - berberine, metformin, and goldenseal inhibited release by 50%, 30%, and 25% ($p<0.05$) respectively. The protein content of each sample was almost the same (Figure 86), except after treatment with goldenseal, which increased cell protein by 15%. Even after accounting for this, the goldenseal treatment considerably reduced glucose release by 25% (Figure 85).

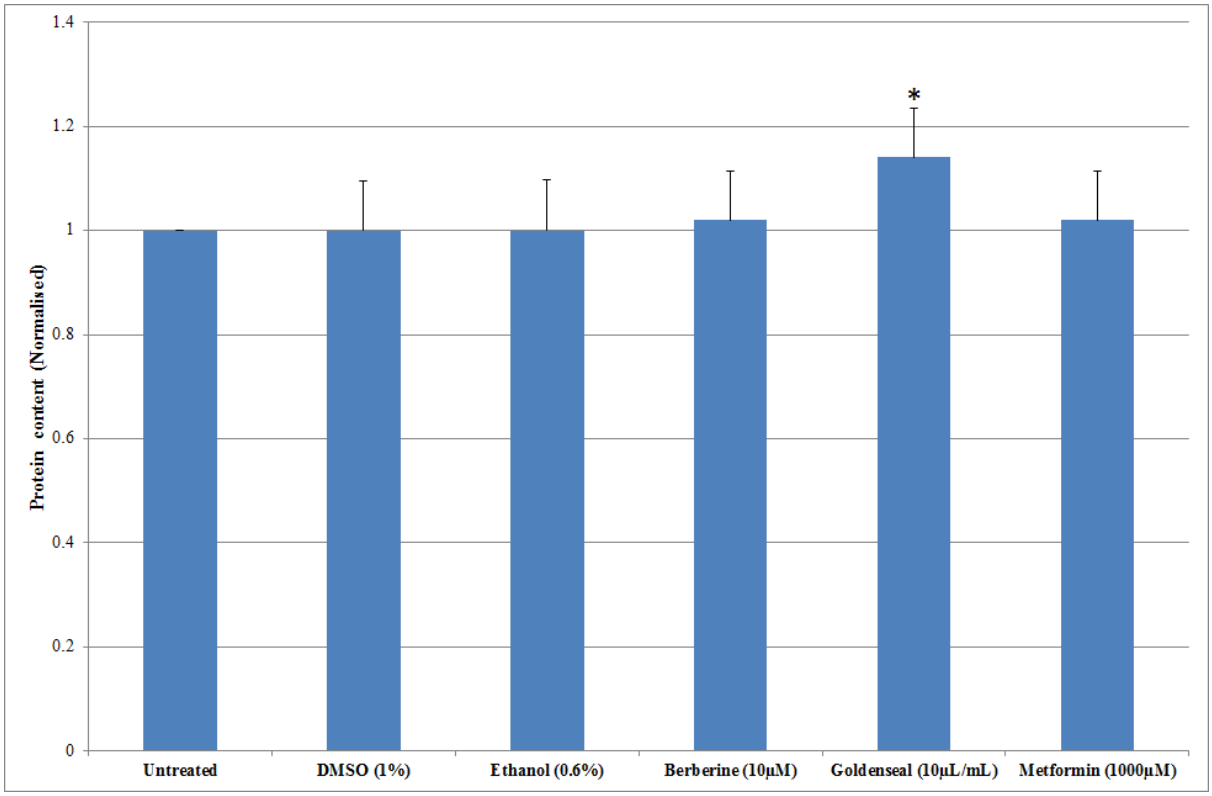


Figure 86: Protein concentrations in confluent HepG2 cells after long-term (48-hour) treatment with goldenseal ethanolic extract, berberine, and metformin, and their respective solvent controls ethanol, DMSO, and an untreated control. Data are protein concentration levels in treated cells, which is normalised to protein concentrations of untreated cells, and are the mean +SD from four independent experiments. The treatments in each experiment were conducted in triplicates. Significant difference from untreated control to target treatments were assessed by employing one-way ANOVA with Bonferroni's multiple comparison post hoc test, $*p<0.05$. The treatments had little effect on protein concentrations, except for goldenseal ($p<0.05$).

Following the outcome of these studies, the research further investigated the efficacy of the key compounds in Seahorse metabolic analysis of the long term treated cells (Section 4.6).

4.6. Effect of Goldenseal extract, Berberine, and Metformin on Seahorse metabolic analysis of the treated HepG2 cells.

The experiments described so far indicate that goldenseal and berberine have marked effects on glucose metabolism in HepG2 cells, consistent with an anti-diabetic effect of stimulating glucose uptake into cells, and inhibiting, partially, release of glucose from cell stores of glycogen. Both compounds increase glycogen content of cells, which fits well with the enhanced uptake of glucose, mediated via an increase in GLUT-1 glucose transporters. A further question arises whether it is possible that berberine or goldenseal could affect the rate of glucose metabolism, to examine the possibility that these compounds may, mildly, uncouple oxidative phosphorylation. By acting in this way, the compounds could stimulate metabolic breakdown of glucose without producing energy. To address this question some analysis of the effect of berberine and goldenseal, in comparison with metformin, on glucose metabolism was undertaken using the Seahorse system.

The Seahorse instrument uses fluorescence probes to continually monitor rates of oxygen consumption and production of extracellular acidification, assumed to be due to lactate formation. By use of selective inhibitors of different points of the electron transport chain it is possible to infer the contribution of mitochondria, as opposed to other cellular processes, to the consumption of oxygen. To help interpret the data, the diagram from Figure 54 in Chapter 2 is reproduced in Figure 88 along with the corresponding profile of oxygen consumption of HepG2 cells subjected to the treatments. Three separate experiments were performed, following the procedure outlined in Section 2.9, and the outcome of the individual treatments were normalised with the viable cell count (Figure 87), which was performed by Crystal violet staining assay (Section 2.9.4), and the results were analysed using the Agilent Seahorse XF Cell Mito Stress Test software (XF Cell Mito Stress Test Report Generator, 2018), and graphs from the output are presented in Figure 89.

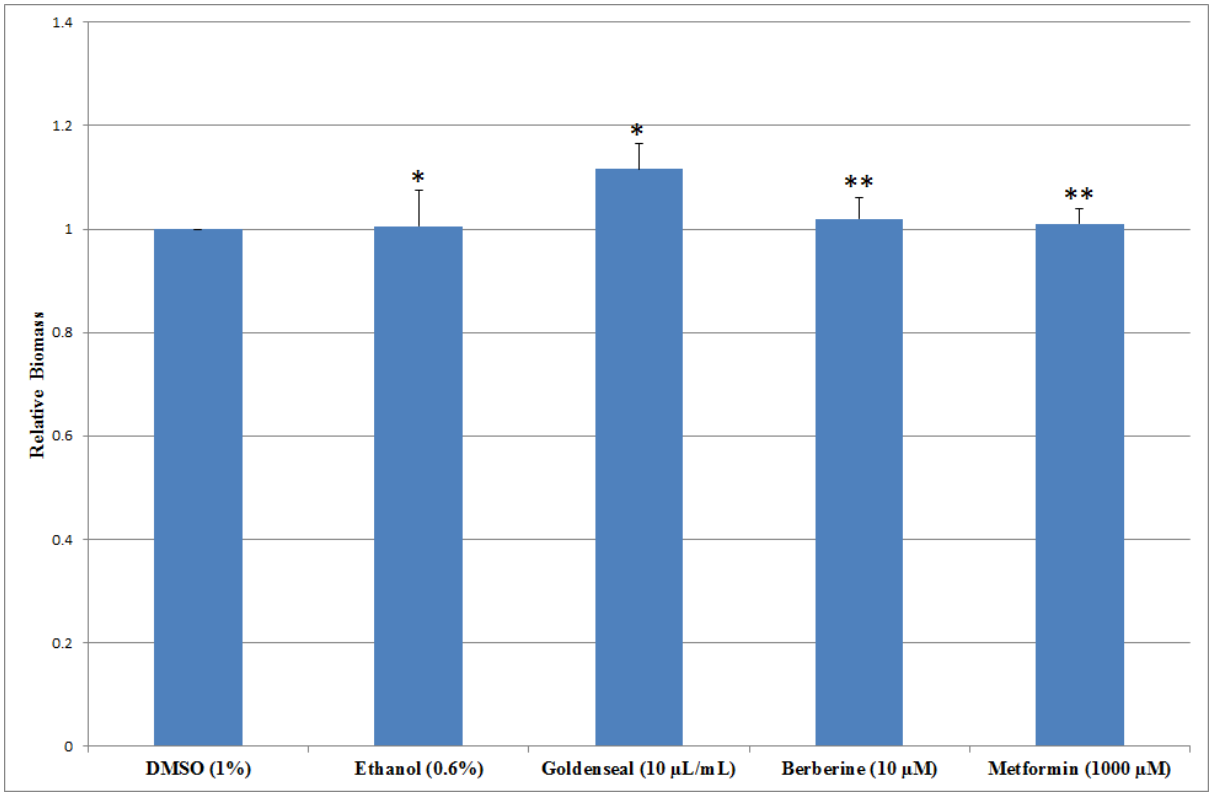


Figure 87: Relative biomass of HepG2 cells after long-term (48-hour) treatments. Cells were treated with goldenseal ethanolic extract, berberine, and metformin, and solvent controls ethanol, and DMSO. Data are relative cell mass, which is normalised to DMSO control, and are the mean +SD from three independent experiments. The treatments in each experiment were conducted in triplicates. Significant difference from control to target treatments were assessed by employing one-way ANOVA with Bonferroni's multiple comparison post hoc test, $*p<0.05$, and $**p<0.01$. The treatments had little effect on cell population.

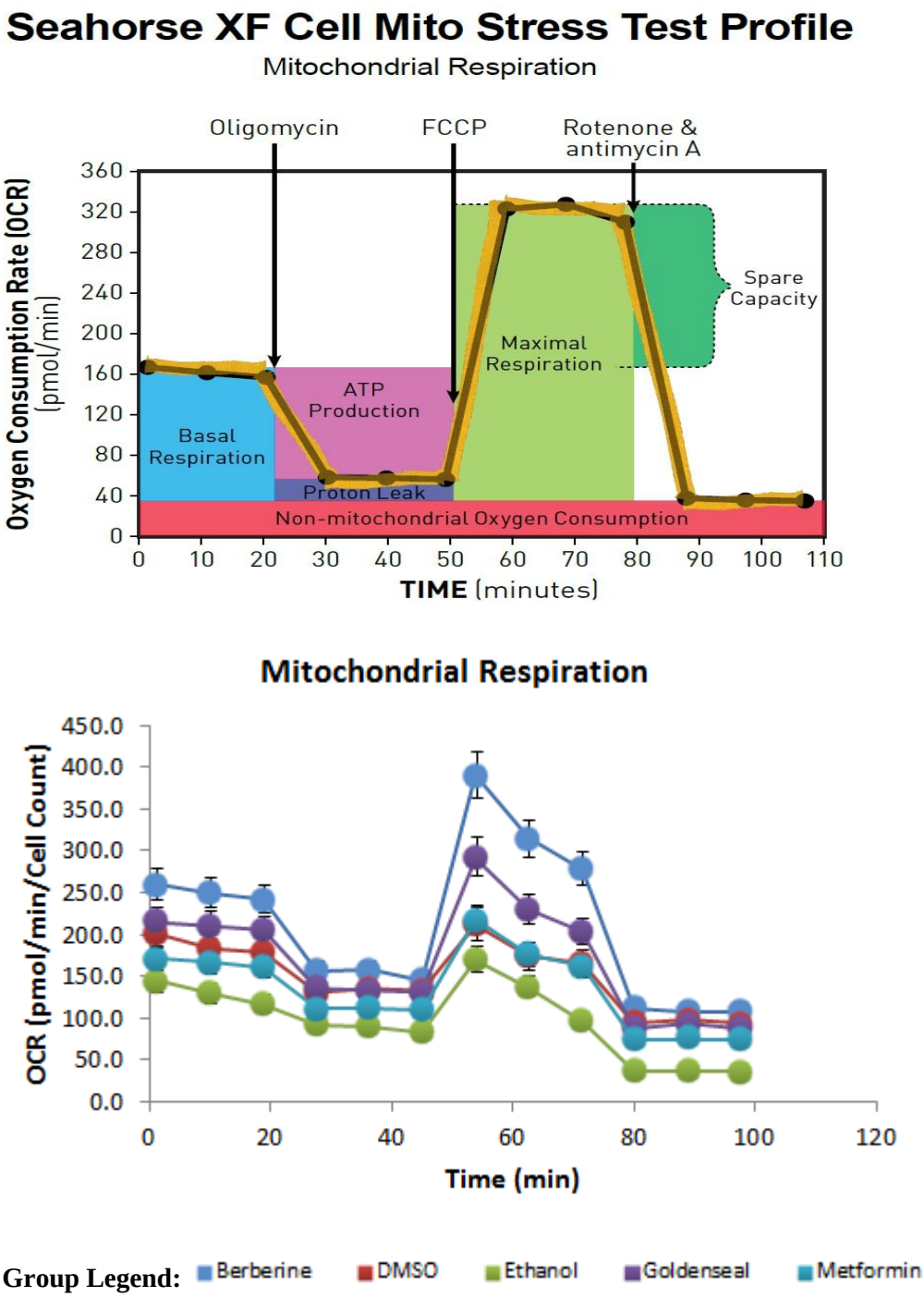


Figure 88: Effect of treatments on the mitochondrial respiration profiles of HepG2 cells. A representative output of three independent experiments presented here. Oxygen consumption rate measurement of mitochondrial respiration (lower chart) of long-term treated HepG2 cells, the data are the mean ±SD from three measurements (student's t-test $p<0.05$).

This image was created using the MitoStress test report generator for Excel and by exporting the data from the Wave software; because it is auto generated, it is not possible

to change the colour of one of the compounds, i.e., berberine or metformin, to distinguish it; however, the colour difference between these two compounds is distinguishable in subsequent images (Figures 89,90 and 91).

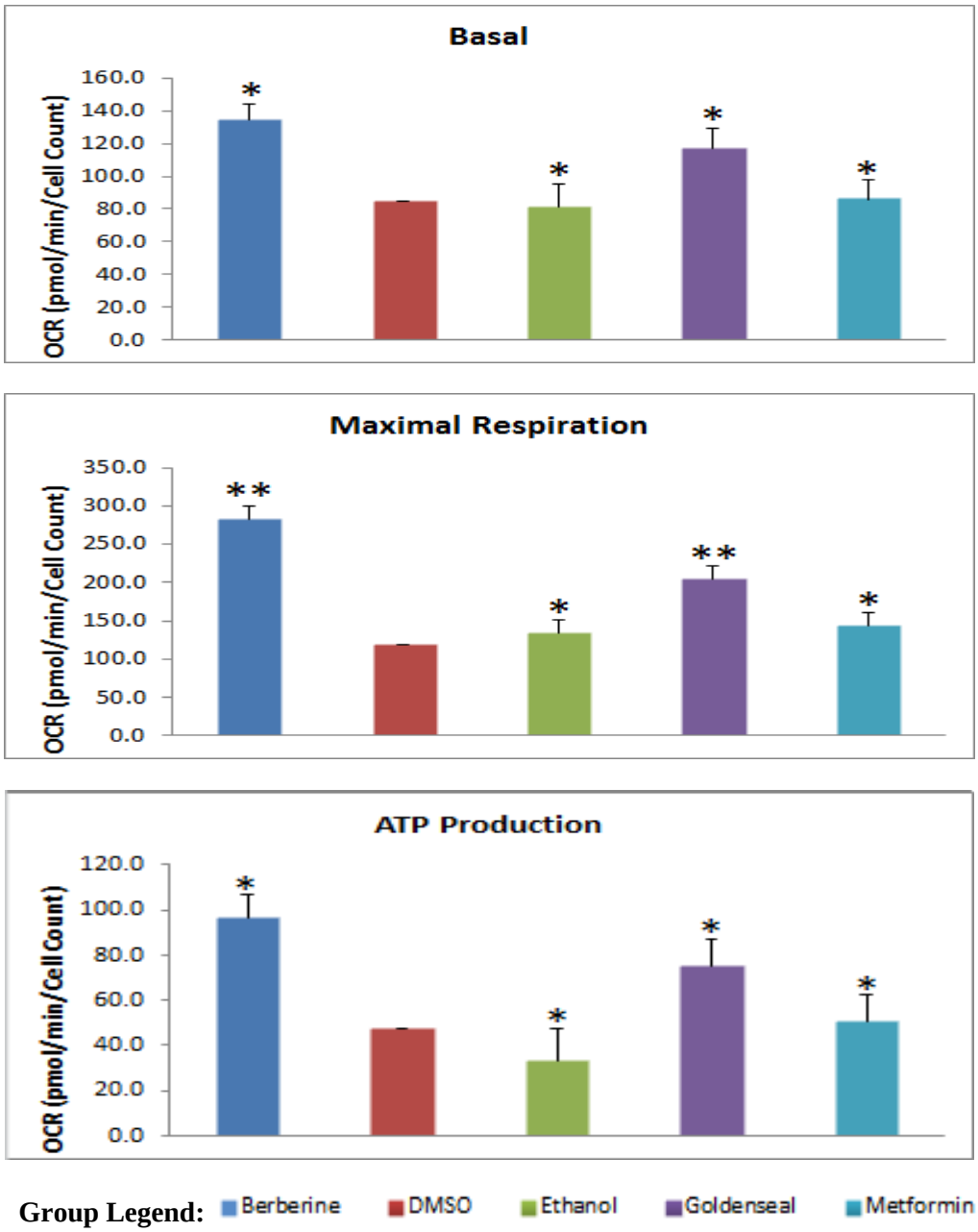


Figure 89: Basic Metabolic parameters. Oxygen consumption rate measurement of basal respiration (the top chart), maximal respiration (the middle chart), and ATP production (the bottom chart), of long term treated HepG2 cells, the data are the mean +SD from three measurements (student's t-test *$p<0.05$, and **$p<0.01$).

The data in Figure 89 indicate that berberine and goldenseal both stimulate the rate of mitochondrial metabolism as shown by increased production of ATP and enhanced

maximal respiration, compared with solvent controls. Treatment with metformin shows a similar, but less marked, effect.

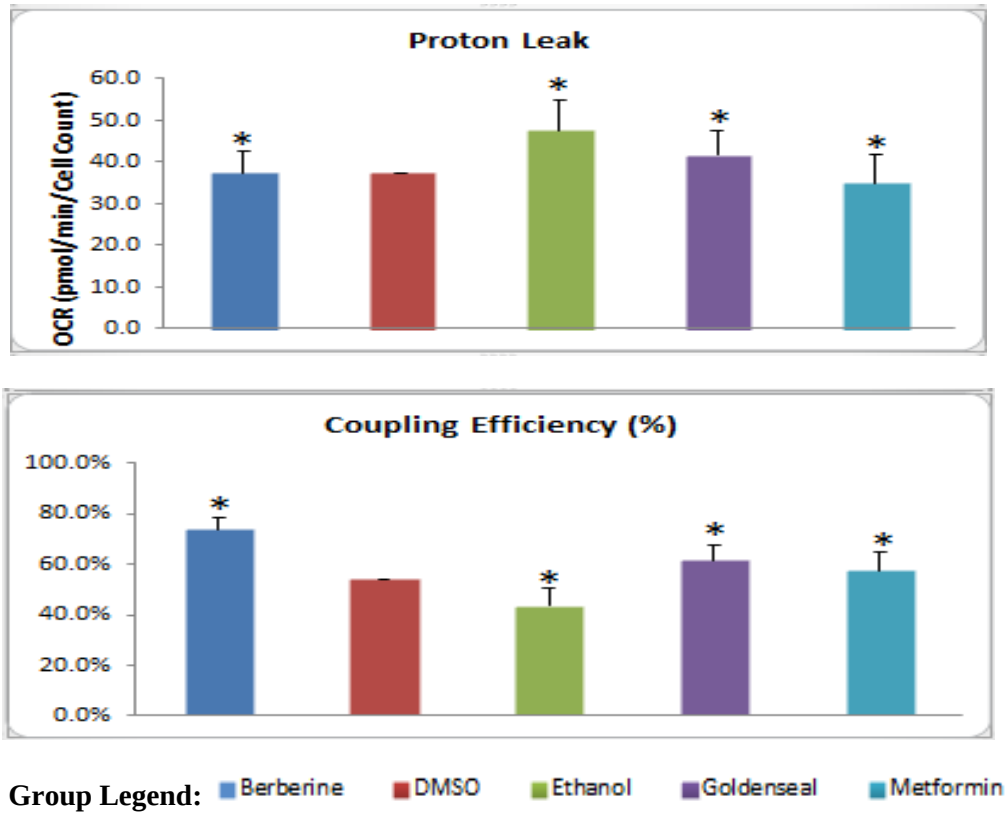
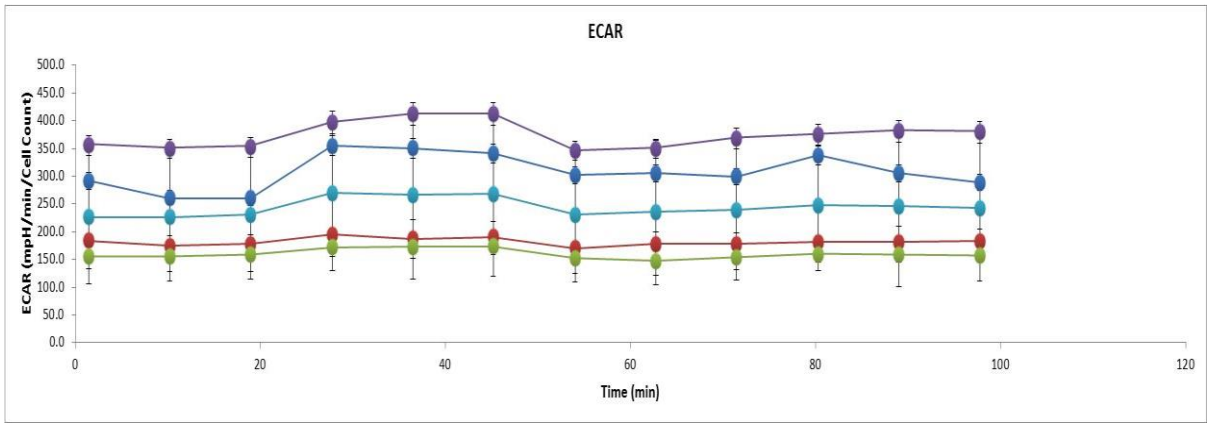


Figure 90: Assessment of Uncoupling. Oxygen consumption rate measurement of the proton leak (the top chart), and coupling efficiency (the bottom chart), of long-term treated HepG2 cells, the data are the mean +SD from three measurements (student's t-test *$p<0.05$).

The data in Figure 90 indicate the effect of treatments on coupling between the electron transport chain and ATP production. If there is enhanced leakage of protons back across the inner membrane of the mitochondria, this would indicate an uncoupling, and would be reflected in a lower coupling efficiency. The data indicate that berberine and goldenseal change proton leakage and improve coupling efficiency.

Finally, the Seahorse analysis provides data on the rate of glycolysis, shown in Figure 91. There is a difference within the treatments, all of which stimulate glycolysis compared with control cells. Goldenseal and berberine stimulate glycolysis to rates 100-125% higher than controls and glycolysis in cells treated with metformin is about 50% than controls (Figure 91). These data are consistent with a higher flux of glucose through cells treated with goldenseal, berberine or metformin.



Group Legend: Berberine DMSO Ethanol Goldenseal Metformin

Figure 91: Effect of treatments on the rate of glycolysis in HepG2 cells. Measurement of the extracellular acidification rate of the long term treated cells with target herbal compounds, the data are the mean ±SD from three measurements (student's t-test $p<0.01$).

4.7. Discussion

About the application of HepG2 cells in anti-hyperglycemic herbal studies:

Cell lines are frequently used as a model for healthy or diseased tissue in molecular pharmacology studies because they provide an easy, inexpensive, and reliable platform. Sometimes, though, they don't quite capture the effects in real time. However, another approach for simulating complex physiological behaviour may be human primary cells. Primary cells, on the other hand, do not endure forever. They go through senescence and have limited capacity for self-differentiation and rejuvenation. They should be employed in early stages since they experience morphological and functional changes as they get older. Because donors' cells can respond differently under the same culture circumstances, genetic features and age of donors are also significant. Using human basic cells has the benefit of eliminating the need for animal models. By doing so, you can prevent inter-species variations in anatomy, molecular mechanisms, and metabolism that may impact the toxicity and mode of action of an employed drug.

Either healthy or cancerous cells can be used to start primary cell cultures of humans. The supply of human primary cells is constrained since they are derived from healthy donors, organ donors, surgical specimens, foetal tissues, and post-mortem donors. This implies that you might not be able to get more content from the same donor. Additionally, if you wish to start more homogeneous cultures, you might need to filter cell types because primary cultures made from tissue explants contain a mixture of different cells at various stages.

Because primary cells are more sensitive than cell lines, they require more nutrition and growth hormones. Additionally, because different primary cell types require various media to develop and survive, the circumstances of each cell type's culture must be optimised. Cell line serum concentrations must be quite high. But because serum is not standardised, the many distinct compounds it contains can vary greatly between different lots.

The research findings may be significantly impacted by this, and they may even become inconsistent. Primary cells are frequently at risk from contamination due to their sensitivity. In addition to causing issues for bacteria, fungus, yeast, and myco-plasma, endotoxins also affect cellular development and function (Gould,1984). Furthermore, the viability and proliferation of human primary cells are decreased by high antibiotic doses. The researcher must assess the characteristics of the cells as they go through the culture stages and note any morphological or func-tional changes (Pamies *et al.*,2018).

Working with primary cells is founded on decades of cell line research, whereas cell lines constitute a crucial part of biological study. Immortalized cell lines are either cancerous cells that never stop dividing or cells that have been genetically altered to continuously divide indefinitely so they can be grown for many generations. Since the early 20th century, cell lines have given researchers insightful knowledge about the biological workings of human cells.

The development of immortal cell lines has made them an effective tool for a variety of tasks, such as studying the cytotoxicity and metabolism of drugs, researching the function of genes, and creating vaccines, antibodies, and other biological substances. The sort of cell culture used depends on the research goal, as was already mentioned. Researchers that want to examine fundamental biological processes, change cellular activities, create novel techniques, or carry out drug screenings and metabolism frequently use immortalised cell lines.

Why? because they are inexpensive, simple to use, and can be maintained in culture for a long time. Additionally, cell lines are simple to develop and alter, and having an unending supply of material is always advantageous (Kaur and Dufour,2017). Additionally, immortalised cells are extremely well characterised because they are standard lines that are employed by many different labs. Immortalized cells are easier to cultivate than primary cultures because they grow more robustly and do not need to be taken from a living animal. Additionally, due to their ongoing and rapid growth, enormous amounts of proteins can be extracted for biochemical assays (Carter and Shieh, 2015).

HepG2 cells were employed extensively in this study and are routinely used in toxicological and pharmacological studies due to their substantial research and wide use in high-throughput platforms for cytotoxicity and other molecular endpoints (Van Delft and Price, 2014). In addition, substances that require metabolic activation can be detected using a number of assays, as shown by a toxicogenomics study from 2012, HepG2 cells can be used to correctly assess genotoxicity (Van Delft and Price, 2014). A practical and ethical replacement for primary hepatocyte cell cultures is the creation of hepatoma derived cell lines that express hepatocyte markers and display typical morphological properties of hepatocytes.

In an article published in the journal pLoS One in 19 July, 2018,"Potential activities and mechanisms of extracellular polysaccharopeptides from fermented Trametes versicolor on regulating glucose homeostasis in insulin-resistant HepG2 cells", the researchers Teng *et*

al.,(2018) employed HepG2 cells, and stated that, for screening anti-hyperglycemic agents in *in vitro* studies using hepatocytes is advantageous, since HepG2 cells regulates glucose metabolism, and maintains liver cell morphology and function and mimic the different diabetic conditions, such as type 1 and type 2 diabetes,therefore they are crucial in dealing with large number of preliminary products (Teng *et al.*,2018).

HepG2 cells are widely employed as a substitute for primary human hepatocytes. The longevity, phenotypic stability, accessibility, and manageability are exceptional. Their main shortcoming, however, is that they display less metabolic activities than hepatocytes do. The HepG2 human hepatoma cell line is widely utilised for research into drug metabolism and hepatotoxicity. Non-tumorigenic, highly proliferative, and having an epithelial-like shape, HepG2 cells are responsible for a wide variety of differentiated hepatic functions. Lipid accumulation, glycogen storage, urea synthesis, and the ability to undergo phases I and II of drug metabolism studies are all measured in HepG2 cells (Donato *et al.*, 2015).

On the other hand, preparing primary hepatocytes is more labor- and technically intensive than preparing immortalised cell lines and necessitates the use of animals. Furthermore, the availability of human-originated hepatoma cell lines like HepG2 could be seen as a major benefit given the dearth and expensive cost of human primary hepatocytes. It follows that the use of hepatoma cell lines, most frequently HepG2 cells, to simulate hepatocytes in metabolic research has become widespread (Molinaro, Becattini, and Solinas, 2020).

In another study, two lines of immortalised hepatocytes were compared to HepG2 cells for insulin action and hepatokine gene expression. According to the findings of this study (Sefried *et al.*, 2018), HepG2 cells provide a reliable model for studying insulin signalling and show gluconeogenic and hepatokine gene expression patterns comparable to those reported *in vivo*.

Researchers proved the effectiveness of high throughput RNAi screens and discovered that GLUT-1 and GLUT-9 are the primary drivers of glucose uptake in HepG2 cells using innovative FRET sensors. Additionally, they demonstrated that GLUT-1 is the primary glucose transporter in HepG2 cells, and that GLUT-9 also participates, albeit to a lower degree, using siRNA to suppress members of the GLUT family (Takanaga, Chaudhuri, and Frommer, 2008).

Though primary hepatocytes may more accurately represent what happens in liver tissue, it is also acceptable to investigate how testing compounds affect HepG2 cells as long as the results are not extrapolated too far, and HepG2 cells are frequently used to investigate glucose uptake and metabolism (Kowalczuk *et al.,* 2021). Several studies have been conducted using HepG2 cells to investigate the effect of various compounds on glucose uptake. For example, Kowalczuk *et al.,* 2021, discovered that nortropane alkaloids from *Hyoscyamus albus* can regulate glucose metabolism in HepG2 cells by stimulating glucose uptake and modulating the SIRT1/Foxo1/G6PC/mTOR pathway. Another study discovered that combining galangin, kaempferol, and quercetin can boost glucose uptake in HepG2 cells (Kerimi, Jailani, and Williamson, 2015).

This study looked at the effects of herbal compounds on HepG2 cellular models' glucose uptake, consumption, glycogen synthesis, glucose release, and metabolic parameters. The study's main objective was to compare the effects of botanical compounds and herb extracts on glucose uptake, consumption, glycogen synthesis, glucose release, and other metabolic processes only in HepG2 cells with that of the drug metformin, which belongs to the most widely used biguanide class currently.

Based on the outcome of the screening experiments, berberine (10 μM), goldenseal (10μL/mL), and the positive control metformin (1000 μM) were considered as key compounds. To check the toxic effects of the key compounds on cell viability, MTT assay was performed (Figure 76), in confluent cells, after long-term application, the cells were well tolerated with the applied doses of the target compounds, but the cells treated with goldenseal (10 μL) were displayed a little bit of increase i.e., around 20% in the proliferation, in this case the treated cells could be more metabolic active and trying to deal with the multiple compounds in the extract, or the wholesome effect of the multiple compounds could be potentiating the cells. Interestingly, a relative low concentration of berberine (10 μM) didn’t affect the cell viability (Figure 76). Also, the positive control metformin had no toxic effects on cell viability (Figure 76). Therefore, it was evident that, the chosen compounds with respective doses, are nontoxic to HepG2 cells. Therefore, based on the outcome of the cytotoxicity assays the identified key compounds were finalised to carry out further research on flow cytometry-based glucose uptake, GLUT-1 assay, glycogen synthesis, glucose release and the Seahorse metabolic analysis.

Flow cytometry-based glucose uptake assays with key compounds were carried out to complement the plate reader-based assays (Chapter 3). In these studies, berberine has proved as the champion among its counterparts goldenseal, and metformin, it stimulates 3.4 times more glucose uptake, goldenseal stimulates 2 times, and metformin stimulates 1.8 times compare to the untreated cells (Figure 78). In this case it is evident that the pure compound berberine stimulates more uptake than its founder crude extracts of goldenseal. Likely the crude extract contain components that reduce the effect of berberine, or the pure compound generates a more intensified action than the crude extracts, for instance, in HepG2 hepatocytes and C2C12 myotubes, berberine stimulates not only GLUT-1, but also GLUT-4 and retinol-binding protein-4 in favour of glucose uptake, activates glycolysis by AMPK pathway, and also influences the peroxisome proliferator-activated receptor γ molecular targets of the phosphorylation of insulin receptor substrate-1, and eventually reduces insulin resistance (Xu *et al.*,2014). Perhaps the crude extracts with the right dose could be beneficial in other ways also, such as the nutrients, alkaloids and flavonoids in the extracts could act as buffering agents and antioxidants and protect from the free-radicals and autophagy, thereby promoting cell viability (Figure 76) (Leyte-Lugo *et al.*,2017). Also, goldenseal seems to have antibacterial activity compared with berberine, probably because of the efflux pump inhibitory activity of bacteria (Ettefagh *et al.*,2011).

Insulin-mediated glucose uptake and non-insulin-mediated glucose uptake are the two primary mechanisms by which glucose is taken in by living cells. Only insulin-sensitive tissues, such as skeletal muscle, adipose tissue, and liver tissue, can absorb glucose via an insulin-mediated process. However, even though both insulin-sensitive and non-insulin-sensitive tissues can absorb glucose without being insulin-mediated, the non-insulin mediated glucose uptake pathway accounted for most of the glucose absorption in insulin resistance situations. Furthermore, while type 2 diabetes is distinguished by a significant impairment in insulin-stimulated glucose uptake, the status of non-insulin-mediated glucose uptake mechanisms in type 2 diabetes is unknown. As a result, the purpose of this study was to directly measure non-insulin-mediated glucose uptake in HepG2 cells using the glucose analogue 2-NBDG and the targeted herbal compounds. In addition, there have been few studies on the acute effects of the targeted herbal components on the function of the insulin-insensitive glucose transporter-1 (GLUT-1). Because of this, we looked into the acute effects that the targeted herbal substances have on the non-insulin-mediated glucose uptake in HepG2 cells, which express the ubiquitous glucose transporter-1 (GLUT-1). In addition, Section 3.2.1.1 highlighted the rationale for not using insulin in this trial.

In the GLUT-1 expression assays (Figure 79 & 80), both compounds goldenseal, and berberine stimulated 1.7 times and 1.4 times respectively compare to the control, in this case the alkaloids in the goldenseal extracts i.e., berberine, and hydrastine etc. (Weber *et al.*,2003), could be potentiating a slight increase of the GLUT-1 expression, than the pure compound berberine. According to the European Pharmacopoeia, it is a mandatory to sell goldenseal as a food supplement only if concentrations of alkaloids, berberine are at least 3%, and hydrastine at least 2.5% (Douglas *et al.*,2010). Therefore, the goldenseal extract used in this research had a minimum of 3% berberine (890 µM), which is higher concentration than the active compound berberine. Suggests that active compound berberine is more effective than the crude extract.

In the glycogen synthesis assays, after long-term treatment berberine displayed increased glycogen synthesis by 2.4 times, metformin 2 times, goldenseal around 2 times compared to the untreated control, and goldenseal extract has shown a slight increase of protein concentration about 15% (Figure 81), compared to the untreated control. In glucose release assays, berberine, metformin, and goldenseal induced 50%, 30%, and 23% (Figure 85) less glucose release respectively compared to the untreated control. Also, after long-term treatment goldenseal increased protein by 14% (Figure 86). The crude extracts contain multiple compounds and with suitable concentrations acting as antioxidants and protecting cells from the free-radicals and autophagy (Ettefagh *et al.*,2011), thereby increasing cell viability, therefore the resultant slight increase of the protein content was seen. The Seahorse data suggest that augmentation of the glucose uptake by berberine and goldenseal is interconnected to upregulated metabolism which can be linked to an increase in glucose storage, which would require an increase in metabolic flux of ATP generation. Following the outcome of these studies, it is evident that the pure compound berberine has better efficacy than the extract of goldenseal in stimulating glucose uptake, glycogen synthesis and glucose release, and improving metabolism. In this section, the outcomes of the experiments are discussed as a summary and a comprehensive discussion is provided in Chapter 5.

Chapter 5: Discussion and Conclusion

The focus of this work has been the characterisation of potential anti-diabetic activity of phytochemicals and extracts as assessed through effects on glucose metabolism by HepG2 cells, as a model of hepatocytes. The initial experiments focussed on identifying phytochemical agents and preparations which could enhance glucose uptake.

To perform glucose uptake experiments, fluorescence-based monitoring of cellular glucose uptake was identified as a suitable method when compared to the radioisotope-based monitoring system. Thus, a method was adopted for the rapid and direct glucose uptake measurement with a fluorescent D-glucose analogue 2-NBDG. 2-NBDG was originally synthesized and studied by Yoshioka *et al* (Yoshioka *et al.*,1996), and subsequently used in various, mostly anti-cancer, studies, and for discovering cellular metabolic functions associated with GLUT systems (Jung *et al.*,2011). The advantages with 2-NBDG over other glucose tracers (e.g., 2-deoxy-D-glucose or radioisotope ^{18}F-fluodeoxyglucose) includes its cost-effectiveness, its ability for high temporal and spatial resolution at the single cell level, the lack of ionizing radiation, and its non-destructive and non-hazardous nature, supports direct monitoring of glucose transportation in viable cells (Hassanein *et al.*,2011). Uptake of 2-NBDG directly determines the distribution of single cell glucose uptake rates in a population, which can be measured by flow cytometry, and provides a significant advantage over traditional indirect methods of inferring population-averaged rates from time-course data of bulk glucose concentrations (Hassanein *et al.*,2011).

In live *E. coli* cells 2-NBDG uptake was inhibited by D-glucose but not L-glucose. In HepG2 cells, GLUT-1 in the plasma membrane allows uptake of 2-NBDG, which then enters the glycolytic pathway, and will be converted to the 6-phosphate metabolite, which is also a fluorescent derivative of 2-NBDG. The 2-NBDG metabolite will eventually be transformed into non-fluorescent forms. The 2-NBDG 6 phosphate metabolite can also be converted back to 2-NBDG by glucose-6-phosphatase, which can hydrolyze phosphate ester derivatives of glucose at the C-6 position, and simultaneously releases inorganic phosphate (Yoshika *et al.*,1996). Hence 2-NBDG can act as an indicator of cell viability based on glucose uptake activity (Yoshioka *et al.*,1996). Consequently, in this study glucose uptake by Hep G2 cells also indirectly reflects cell viability.

The application of 2-NBDG as a fluorescent indicator is not only limited to screening insulin mimetic compounds, but also provides an insight into the mechanisms of glucose accumulation and metabolism in different cell types such as primary or cancer cell lines.

Thus, the application of 2-NBDG is not only useful to develop antidiabetic drugs, but also for the fundamental research on cancer (Zou, Wang, and Shan, 2005). However, 2-NBDG was identified to have poor photo-physical properties in aqueous solution and did not compete strongly with D-glucose during cellular uptake under physiological conditions in C2C12 myocytes (Lee *et al.*,2011). Since 2-NBDG passes through cell membranes via the same GLUTs as D-glucose, it is evident that competitive inhibition of 2-NBDG occurs by D-glucose in MCF10A mammary epithelial and CA1d breast cancer cells (Hassanein *et al.*,2011). Therefore, in this study in HepG2 cells, glucose free medium was used to prepare 2-NBDG medium and for the solvent control. In order to overcome the limitations of 2-NBDG, (i.e., due to weak fluorescence intensity), high treatment dosage, and non-compatibility in physiological conditions, a novel fluorescent glucose bio probe, Cy3-Glc-a, was developed and demonstrated its efficiency in promoting fluorescence-based glucose uptake in NIH/3T3 fibroblast cells and C2C12 myocytes (Lee *et al.*,2011).

This study focussed on basal glucose uptake through GLUT-1, located on the plasma membrane. GLUT-1 facilitates basal glucose uptake due to its lower Km value (3-7 mM) (Zhao and Keating, 2007). Generally, a lower Km value indicates a high affinity to glucose uptake. In contrast, GLUT-2 has a higher Km value (17 mM), which leads a lower affinity to glucose uptake. The higher Km value enables glucose sensing, and the rate of glucose uptake is proportional to blood glucose levels (Zhao and Keating, 2007). The major glucose transporter in HepG2 cells is GLUT-1 (Takanaga, Chaudhuri and Frommer, 2008), reflecting the original cloning of GLUT-1 as the first glucose transporter to be cloned, from HepG2 cells (Mueckler *et al.*,1985).In this study, the effects of the phytochemical treatments on the expression of GLUT-1 in HepG2 cells, and changes in expression that may affect downstream events of glucose metabolism was assessed.

Two series of screening were carried out. In the first series active phytochemicals were used, and in the second series, carried out much later, several extracts of herbs were assessed and compared with active phytochemicals that had been characterised in the first series. In the first series four conditions were used to assess the effect of the phytochemicals. In the first experiments treatments were applied for one hour. This short-term treatment was designed to identify mechanisms that would involve immediate effects on cellular properties, as opposed to longer term treatments of 24 or 48 hours. Phloretin, ouabain, berberine and metformin stimulated glucose uptake in growing cells after a one-hour treatment. Berberine stimulated uptake 110% ($p<0.05$), phloretin 20% ($p<0.05$), ouabain hardly at all (0.4%; $p>0.05$), and metformin stimulated a 40% upregulation

($p<0.05$) (Figure 61). For the same compounds applied for 24 hours a similar pattern is seen with berberine again the most potent, stimulating an upregulation of the glucose by 78%, and metformin (60%), phloretin (53%) and ouabain (20%), having more pronounced effects with the longer, compared with the control cells ($p<0.05$; Figure 62).

Treatments were repeated on confluent cells to determine whether cell growth made a difference in sensitivity to the test treatments. A similar profile of effects was seen in confluent cells after one- or 24-hour treatments. Again, berberine showed the greatest effect and after a one-hour treatment stimulated glucose uptake by 117%, phloretin by 28%, ouabain had no effect and metformin stimulated 32% upregulation, all after a one-hour treatment compared with control ($p<0.05$; Figure 63). After a 24-hour application, berberine stimulated glucose uptake by 50% ($p<0.05$), ouabain had no effect ($p>0.05$), phloretin by 20% ($p<0.05$) and metformin by 31% ($p<0.05$) (Figure 64).

In all four modes of treatment, berberine stimulated glucose uptake markedly more than phloretin, metformin and ouabain. Phloretin was selected for study because it increased glucose uptake through stimulation of GLUT-4 and GLUT-1 expression on the plasma membrane of prostate cancer cells (Menendez *et al.*,2014). It had limited effect on glucose uptake in HepG2 cells and was not selected for further study. Similarly, there are limited research papers available on the efficacy of ouabain for its effect glucose transport activity, but a study in dogs (Triner *et al.*,1968) showed a significant decrease in plasma glucose levels was revealed, although no cellular studies were available. In this study ouabain demonstrated almost no stimulation of glucose uptake and was also not selected for further study.

Berberine and metformin were found to be the most active of the four phytochemicals in stimulating glucose uptake (Kandunuri and White, 2012). These experiments were carried out before the publication of Xu *et al.,* (2014) which supported these findings – HepG2 cells treated with berberine or metformin, at the concentrations used in the present study, 10 µM and 1.0 mM respectively, showed enhanced consumption of glucose. The focus of the study of Xu *et al.,* was on the biochemical pathway involved and the effects were found to be independent of the key metabolic regulatory pathway involving AMP-activated protein kinase (AMPK) (Xu *et al.*,2014).

In the second series of screening experiments, several herb extracts were compared for effects on glucose uptake and consumption. One of the early target herbs of the project was *Stevia rebaudiana*, and initially aqueous extracts were employed in glucose uptake

experiments of one hour treatment (Figure 67). The top concentration (10 µL/mL) stimulated glucose uptake 2.4 times ($p<0.05$), compared with the untreated cells, whereas all the lower concentrations stimulated glucose uptake only slightly ($p<0.05$). Ethanolic extracts of *Stevia* proved to be much more potent. The efficacy of the whole extracts of *Stevia* was tested along with rebaudioside-A, berberine, and the positive control was the first line therapy drug metformin (Figure 71). After 48 hours treatment with *Stevia* extracts and rebaudioside-A, the top concentrations, 100-fold dilution (10 µL/mL) of *Stevia* extract, and 103 µM rebaudioside-A stimulated glucose uptake 6 times ($p<0.01$), and 8 times ($p<0.01$) respectively more than the untreated HepG2 cells. The negative control DMSO and the solvent control ethanol were not much stimulated compared with the untreated cells ($p<0.05$), and the metformin, and berberine treated cells stimulated 9 times, and 14.2 times respectively more glucose uptake ($p<0.05$), compared with untreated cells (Figure 71). Also, after 48 hours treatment, the highest concentrations of *Stevia* ethanolic extracts and rebaudioside-A stimulated glucose consumption 50% and 55% respectively ($p<0.01$; Figure 75). In comparison metformin and berberine stimulated a greater consumption of glucose, 60% and 70% respectively compared to the untreated cells ($p<0.01$; Figure 75).

Stevia extracts have been tested for effects on glucose transport in human leukaemia (HL-60) and in human neuroblastoma (SH-SY5Y) cells; the extracts stimulated glucose uptake as efficiently as insulin by modulating GLUT-4 translocation through the PI3K/Akt pathway and corroborated the hypothesis that *Stevia* extracts could mimic insulin by modulating the PI3K/Akt pathway (Rizzo *et al.*,2013). In another study, stevioside potentiated insulin sensitivity by elevating insulin stimulated glucose uptake in 3T3-L1 adipocytes, and the treated cells displayed a two-fold increase in glucose uptake compared to rosiglitazone (Radzman and Adam *et al.*,2013). In contrast, this project explored the glucose uptake activity of *Stevia* and rebaudioside-A in HepG2 cells, which predominantly express insulin independent GLUT-1.

These outcomes suggest that *Stevia* and rebaudioside-A can stimulate glucose consumption and have potential anti-diabetic activity *in vivo*, but despite there being various studies on *Stevia in vivo*, little is reported concerning the underpinning beneficial effects on human health and *Stevia* has not been in usage in any herbal therapies worldwide. Instead, it has been used recently as a natural sweetener due to its zero calorific value, as an aid to help prevent dental caries, and obesity associated diseases such as diabetes and hyperlipidemia (Samuel *et al.*,2018). It has also been consumed traditionally by the natives of Paraguay,

Brazil, and Central America to sweeten beverages or chewed for their sweet taste (Ashwell, 2015). Given that goldenseal proved to be more potent than *Stevia*, as discussed below, *Stevia* was not selected for further analysis.

It was of interest to assess the activity of an herbal extract with a well-established history of diabetes treatment, and so an ethanolic extract of goat's rue (*Galega officianalis*) was tested for its effect on glucose uptake and consumption. In confluent cells, a 48-hour application of goat's rue ethanolic extracts at the top concentration (10 µL/mL) augmented glucose uptake 10 times, compared with nine times by metformin and 14 times by berberine, all compared with the untreated cells ($p<0.05$; Figure 70). Glucose consumption was reduced by 50%, compared with a reduction of 65% by metformin and berberine, compared with untreated cells ($p<0.05$; Figure 74). Goat's rue has been used in traditional medicine to treat diabetes since the early 1900's (Yeh *et al.*,2003; Dey *et al.*,2002). The active constituent in Goat's rue extracts is known to be galegine, a bisguanidine, and it was originally sold under the trade name Synthalin.2,3,4,5. It was galegine from which the most sought after and cost-effective drug for the treatment of type 2 diabetes, metformin was derived (Dey *et al.*,2002). Galegine, promotes glucose lowering by lowering the surplus basal rates of hepatic gluconeogenesis (Samuelsen, 1935; Blatherwick, Sahyun, and Hill, 1927; Bodo, and Marks, 1928; Cusi, Consoli, and DeFronzo,1996). Also, it is likely, that galegine hinders the glucose 6-phosphate produced from glycogen breakdown to be transformed to glucose through an inhibition of glucose 6-phosphatase and phosphoenolpyruvate carboxykinase, as seen metformin as studied by Blatherwick *et al.*,1927, Bodo and Mark, 1928, Mithieux *et al.*,2002, and Shaw *et al.*,2005. As stated in Section 1.3, previously published studies using diverse *in vitro* cell lines served as the foundation for the study for this thesis.

Another candidate herb with a history of diabetes treatment was *Gymnema sylvestre*. After an hour treatment, *Gymnema* glycerites stimulated glucose uptake slightly at the top concentration, by 1.3 times ($p<0.05$), and 1.4 times at the lower concentrations 1.25 µL/mL ($p<0.01$), 2.5 µL/mL ($p<0.05$) and 5 µL/mL ($p<0.01$) compared to the untreated control (Figure 66). After 48 hours treatment with *Gymnema* ethanolic extracts, the highest concentration augmented glucose uptake 9.5 times, compared to the untreated cells ($p<0.05$; Figure 69). In glucose consumption experiments, at the highest concentration (10 µL/mL) consumption rate was 55%, compared with untreated cells ($p<0.05$; Figure 73). These are comparable activities to goat's rue extracts. Methanolic extracts of *Gymnema sylvestre* leaves have been found to augment glucose uptake and ameliorate insulin

resistance by upregulating GLUT-4 in 3T3-L1 adipocytes (Kumar *et al.*,2016), but there are no studies available on the glucose uptake and consumption properties of the *Gymnema* using HepG2 cells.

The final extracts to be discussed here are those from goldenseal (*Hydrastis canadensis* L.). In confluent cells, an hour application with goldenseal glycerites and ethanolic extracts stimulated glucose uptake at the concentration 10 µL/mL, by 6.5 times and 5.4 times correspondingly, compared with untreated control ($p<0.05$; Figure 65). Ethanolic extracts, at the highest concentration stimulated glucose uptake 13.5 times, metformin 9 times, berberine 14.2 times more glucose uptake, compared to the untreated control ($p<0.05$; Figure 69). In glucose consumption experiments, after long term application, at the highest concentration, glucose content was 25% ($p<0.01$), and compared with 20% for positive control metformin ($p<0.001$) and 20% for berberine ($p<0.001$) compared with untreated cells (100%) (Figure 72). In both glucose uptake and consumption studies, goldenseal ethanolic extract (10 µL/mL), and berberine stimulated glucose uptake 13.5 times and 14.2 times respectively, and glucose consumption 75% and 80% respectively. The study's novelty may stem from the fact that there have been no previous *in vitro* reports on the goldenseal's glucose uptake and consumption abilities using GLUT-1 in HepG2 cells. However, it is possible that this is the first report generated.

Based on the above the experimental data, goldenseal and berberine have the most marked effects on glucose uptake and consumption on HepG2 cells compared to the first line therapy drug metformin. The goldenseal extracts and berberine appear promising for the development of oral anti-diabetic agents, thus goldenseal ethanolic extracts (10µL/mL), and berberine (10µM) were identified as the key treatments to be the focus of further studies in this research and their properties were explored further with cytotoxicity assays, flow cytometry based glucose uptake assays, western blotting GLUT-1 analysis, and estimation of the glycogen content and glucose release, and the seahorse metabolic analysis of the treated HepG2 cells (Chapter 4).

To confirm that the test treatments were not cytotoxic MTT assays were performed (Figure 76), on confluent cells. After 48 hours treatment, the cells not only tolerated the applied concentrations of the key compounds, but the cells treated with goldenseal showed a slight increase in the cell number, by 20%. Metformin and berberine also induced small increase in cell number, by about 10% (Figure 76). Thus, it was apparent that the selected compounds with corresponding concentrations were safe to use on the HepG2 cells. Thus,

the identified key compounds were finalised, and flow cytometry-based glucose uptake, GLUT-1 assay, glycogen synthesis, glucose release and the Seahorse metabolic analysis assays were carried out.

Flow cytometry-based glucose uptake assays with test treatments were performed to complement the plate reader-based assays (Chapter 3). Using flow cytometry berberine stimulated 3.4 times more glucose uptake, goldenseal 2 times, and metformin 1.8 times compared to the untreated control (Figure 78). These data confirm qualitatively the stimulation of 2-NDBG uptake by berberine, goldenseal and metformin measured using a plate reader (Figure 68).

In the GLUT-1 expression assays (Figure 79 & 80), goldenseal, and berberine augmented the expression 1.7 times and 1.4 times correspondingly, compared to the control ($p<0.005$). In this case the alkaloids in the goldenseal extract (Weber *et al.*,2003), could be potentiating a small increase of the GLUT-1 expression over the pure compound berberine.

Glycogen is a polymer of glucose stored predominantly in the liver cells and the skeletal muscle cells and provides glucose to the peripheral blood stream for the period of fasting and muscle contraction while on physical workout. Glycogen synthesis is a series of complex reactions, which initiates as the glucose molecules enter the cytosol through glucose transporters, phosphorylation of glucose to glucose 6-phosphate, isomerization to glucose 1-phosphate, and formation of uridine 5′-diphosphate-glucose i.e., it, donates glucose molecules straightaway for glycogen synthesis (Andany *et al.*,2016).

Glycogenin catalyzes the synthesis of a short polymer of glucose, and the enzyme glycogen synthase continues the reaction. The branching enzymes club the branching points in the glycogen particle at regular interims. The proteins laforin and malin involves in glycogen assembly but their precise role remains unknown (Andany, 2016). Glycogen breakdown (i.e., glycogenolysis) occurs by the enzymes, glycogen phosphorylase, it releases glucose 1-phosphate from the linear chains of glycogen. And glycogen debranching enzyme, it unties the branching points. In the lysosomes, glycogen degradation occurs by enzyme alpha glucosidase. The glucose 6-phosphatase catalyses the de-phosphorylation of glucose 6-phosphate to glucose, which is an essential step to release the free glucose from the cell (Andany, 2016).

In glycogen content experiments, after a 48-hour application berberine treated cells displayed glycogen content 2.4 times, metformin 2 times, goldenseal around 2 times

compared to the untreated control (*p<0.05*; Figure 81). Therefore, it is evident that the key compounds increase glucose transport to the cytosol and store it in glycogen form, and thereby prevent gluco-toxicity and insulin resistance, which is a prominent anti-diabetic activity.

Insulin resistance is the major cause of type 2 diabetes, in which insulin-induced glucose disposal is defective, then, glucose augmentation or hyperglycemia occurs. Chronic hyperglycemia leads to severe complications and further disrupts glucose metabolism such as impairment of insulin-stimulated glycogen synthesis and augmented glucose output. Therefore, the study suggests that inducing glycogen storage is an important means of easing hyperglycemia (Rossetti and Lauglin ,1989; Dimlich, Townsend, and Reilly,1982).

In an *in vivo* study using berberine, liver glycogen content in diabetic mice was reduced compared to control mice by nearly threefold (*p<0.05*), whereas a significant increase by almost two-fold in diabetic mice was noticed after berberine application (Figure 92; Xie *et al.*,2011). Liver glycogen content, the activity of glucokinase and phosphorylated Akt and IRS were severely reduced in diabetic mice, whereas berberine blocked these biochemical changes, and eventually lowered high blood glucose in alloxan-induced diabetic mice (Xie *et al.*,2011).

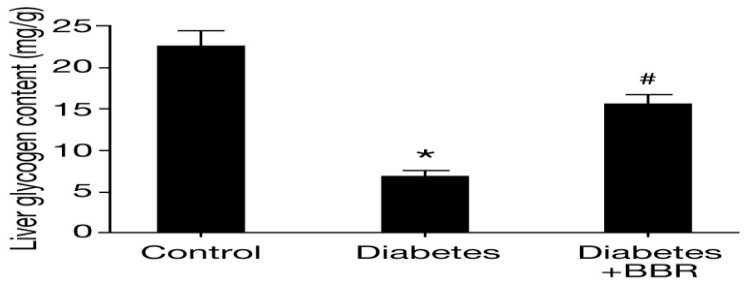


Figure 92: Berberine augumented liver glycogen content in alloxan-induced diabetic C57BL/6 mice. Alloxan-induced diabetic C57BL/6 mice were treated with berberine for 12 weeks, and the liver glycogen content was estimated by an anthrone assay. Data are from Xie *et al*. (2011) and are the mean + SD, n=8. **p<0.05* vs. control group, *#p<0.05* vs. diabetic group by ANOVA.

In glucose release experiments, berberine, metformin, and goldenseal induced release of 50%, 30%, and 23% (Figure 85) less glucose correspondingly compared to the untreated cells. And after long-term application goldenseal treatment induced a 15% increase of the cellular protein content (*p<0.05*; Figure 86). In this case, perhaps the crude extracts contained multiple compounds at suitable concentrations that can act as antioxidants and

protect cells from free-radicals and autophagy (Ettefagh *et al.*,2011), thus increasing cell health and growth, resulting in the subsequent slight increase of the protein content.

These studies indicate that the pure compound berberine has greater efficacy than the extract of goldenseal in upregulating glucose uptake, glycogen synthesis and curbing the glucose release from the cells. As there are no previous reports available on the comparative efficacy of goldenseal and berberine, perhaps, this is the first *in vitro* study performed in this respect.

To probe metabolism of glucose further, measurement of oxygen consumption and of lactate production were carried out using the Seahorse FX analyser, which can measure metabolism of live cells. The data suggest that enhanced stimulation of the glucose uptake by berberine and goldenseal is connected to enhanced metabolism, which can be linked to an increase in glucose storage, which would require an increase in metabolic flux of ATP generation. Berberine and goldenseal both increase the rate of mitochondrial metabolism by increasing production of ATP and enhanced maximal respiration, compared with controls. Metformin has similar properties but is relatively less effective (Figure 89).

It was of interest to note the effect of the treatments on the coupling between the electron transport chain and ATP production, and whether proton leakage across the inner mitochondrial membrane was increased, leading to uncoupling. This could be one way in which the treatments could increase glucose metabolism. However, berberine and goldenseal treatments induced an improved coupling efficiency (Figure 90), therefore the data suggest that berberine and goldenseal do not act by uncoupling oxidative phosphorylation but do enhance metabolic activity. This may be linked to the stimulation of cell growth consistently seen with goldenseal treatment in crystal violet staining assay (Figure 88). Moreover, goldenseal and berberine stimulate glycolysis to rates 100-125% higher than controls, and glycolysis in cells treated with metformin is about 50% higher than controls (Figure 91).

The trio of treatments, berberine, goldenseal extract and metformin showed similar results. Glucose uptake in HepG2 cells stimulated by berberine for concentrations up to 50 µM has been reported by Xu *et al*. (2014), whereas a recent study described the inhibition of HepG2 cells growth and decrease in glucose consumption by 15 - 60 µM berberine (Yan *et al.*,2017). The cause of the discrepancy between the two studies is not clear. The data from the present study suggest that berberine is well tolerated by, or even enhances growth of, HepG2 cells. The mechanism by which berberine increases glucose consumption involves

an increase in glycogen storage as well as decreased glucose release. Metformin has similar effects in the current study, and this is consistent with results reported by Xu *et al.*, (2014) for HepG2 cells.

Studies have reported that metformin inhibit activity of the respiratory chain complex I, in isolated liver mitochondria prepared from 24-h-starved Wistar rats (Owen *et al.*,2000), and a study indicate that dimethylbiguanide decreases oxygen consumption and mitochondrial membrane potential in intact hepatocytes of rats, whereas it has no effect on isolated mitochondria or on permeabilized hepatocytes of rats (El-Mir *et al.*,2000).

In a study, berberine dose dependently inhibited respiration in L6 myotubes and muscle mitochondria of Wistar rats, through a specific effect on respiratory complex I, similar to that observed with metformin and rosiglitazone (Turner *et al.*,2008). The preliminary data presented in Chapter 4 contradict those *in vivo* studies, and this may be, because these are *in vitro* studies mainly dealt with the intact cancer cells (HepG2), or due to the different concentrations and experimental setup, and multiple components of the crude extracts of golden seal and the time course used in the present study. Furthermore, the Searhorse treatments were not optimised due to time constraints, as only three sets of experiments were conducted. A result from the Seahorse XF analysis that is more consistent with the literature is the enhanced rate of lactate production by the three treatments (Xu *et al.*,2014), which would also contribute to increased glucose metabolism.

Finally in conclusion, berberine and the goldenseal extract have fairly comparable activities of enhancing glucose metabolism in HepG2 cells, and it was evident that the pure compound berberine has better efficacy than the crude extract of goldenseal in stimulating glucose uptake, glucose consumption, glycogen synthesis and glucose release and metabolic flux of ATP generation. Therefore, goldenseal extracts and berberine seem promising for the development of alternative oral anti-diabetic agents for the treatment of type 2 diabetes.

5.1. Future directions

Sulfonylureas and other commonly prescribed medications are linked to weight gain and hypoglycemia; consequently, this needs to be considered in future research. When blood sugar levels are normal, some secretagogues, such as repagilinide, do not stimulate the release of insulin (Bell, 1998). Low blood sugar, upper respiratory infections, and back pain are just a few of the undesirable side effects that meglitinides can have (Hu, 2000). Metformin the biguanides class of drugs, which can be damaging to the digestive system and lead to symptoms like nausea, cramping, diarrhoea, lactose intolerance, and, in the long run, a vitamin B-12 deficiency (Bailie, 1992). Hepatic necrosis may develop as a side effect of alpha glucosidase inhibitor use, which works by preventing the digestive enzyme alpha-glucosidase from doing its job and causing gastric distress and intestinal gas (Lebowitz, 2005). The most common side effects of thiazolidinedioes include bloating, weight gain, and anaemia (Olefsky, 2000). Combination drug users, especially those taking metformin and rosiglitazone, have more painful and irregular periods (Venkatesan, 2001).

There is an urgent need to raise awareness of and conduct research on herbal ingredients that may offer an alternative to currently used anti-diabetic medications. Given all of these issues, which are expected to exacerbate a variety of negative health effects (Haq, 2004; Aronson, 2009), as well as the high cost of production and the high cost of existing mainstream pharmaceuticals, it is clear that a change is required.

However, it is widely accepted that the herbal compounds used in this study aid in the reduction of type 2 diabetes symptoms. The purpose of this research was to fill a gap in the existing literature and scientific research on the potential benefits of herbal diabetic treatments. The glucose metabolic activity of selected phytocompounds was studied in order to identify a drug that could compete with the commonly prescribed biguanide drug metformin. Based on a thorough search of the academic literature using reputable search engines such as PubMed and Medline, this study may be the first to use herbal compounds such as Goldenseal, Goat's rue, *Gymnema*, *Stevia* extracts, and Rebaudioside-A to examine glucose uptake and other metabolic parameters in HepG2 cellular models.

In this study, *Stevia* extracts and Rebaudioside-A improved non-insulin mediated glucose uptake and consumption in HepG2 cells, while Stevioside improved insulin sensitivity in 3T3-L1 adipocytes by increasing insulin-stimulated glucose uptake activity (Radzman *et al.*,2013). As a result, it is expected to affect glucose transporters 1 and 4 as well as

activate the AMPK pathway. Consequently, glucose absorption in insulin-sensitive tissues may be enhanced. Those who intend to pursue *in vitro* glucose metabolic research in the future are advised to conduct additional research on this aspect of steviol glycosides, such as Rebaudioside-A; Finding out how the above herbal substances affect the enzymes glucokinase and hexokinase could also lead to new information. The results could also be compared to those of biguanide drugs like metformin that are commonly used.

Glucose uptake studies have revealed that phytocompounds such as Goldenseal extracts and berberine have the potential to replace or supplement metformin in the treatment of type 2 diabetes. Similar studies on different cell lines (i.e., muscle cells and adipose tissue) using prominent herbal compounds such as *Pterocarpus cordifolia* and *Withania somnifera* would be recommended.

Anti-diabetic herbs, which have long been used as a natural source of diabetes treatment, frequently lack thorough characterization of their active constituents. More research is required to determine the mechanism of action of anti-diabetic and insulin-mimetic herbs. Although it is commonly assumed that herbs are generally safe to consume, many herbal formulations are unsafe due to issues such as metal toxicity; thus, a toxicity study should be conducted to standardise for medicinal purposes. As a result, by isolating, purifying, and elucidating the structure of the active compounds, research on hypoglycemic herbs at the cellular, preclinical, and clinical levels may benefit society by providing more affordable and effective alternative drug candidates for the treatment of type 2 diabetes.

Appendix

1.Additional research contributions

1. Presented research work entitled “Potential replacement of Metformin with Berberine in the treatment of type 2 diabetes” in Diabetes spring conference-2012 (diabeticfriend.co.uk/wp-content/uploads/2012/05/DRWF.pdf), organized by Diabetes research and wellness foundation.

2. An abstract entitled “A study on novel phytochemicals for the treatment of type 2 diabetes”, accepted for the poster presentation in World Diabetes Congress-2013, Melbourne, Australia.

3. Professional member of Diabetes UK and attended conferences years-2012 to 2015.

4. Professional member of the Chromatographic Society of UK.

5. Displayed a poster entitled “Hypoglycemic agents for the treatment of type 2 diabetes-A focus on novel phytochemicals”, at PhD student symposium-2012, London Metropolitan University.

6. Displayed a poster entitled “Novel herbal compounds for the treatment of type 2 diabetes” on Industrial day-2013, London Metropolitan University.

7. A review article entitled “An overview on the efficacy of herbs used in ayurvedic formulations for the treatment of type 2 diabetes”, was published in International Journal of Herbal Medicine (2016), 4(5), 116-121.

8. Two potential publications are arising from this thesis.

2. Explanatory note

Figures of glucose uptake, consumption, glycogen content, and glucose release assays show fluorescence fluctuations (i.e., glucose uptake and glucose release studies) or variations in glucose levels (i.e., glucose consumption and glycogen content assays). These results have been normalised for protein content as well as relative to the appropriate control. To aid comprehension, a sample of one set of raw data from each type of study is provided below.

1.Effect of the short-term treatment of Goldenseal ethanolic extracts on uptake of 2-NBDG by confluent HepG2 cells (A single set of data)

1.1. Fluorescence variations before normalisation with protein content.

	LAYOUT				
10µL/mL	5µL/mL	2.5µL/mL	1.25µL/ml	0.625µL/n	0.3125LµL/mL
10µL/mL	5µL/mL	2.5µL/mL	1.25µL/ml	0.625µL/n	0.3125LµL/mL
10µL/mL	5µL/mL	2.5µL/mL	1.25µL/ml	0.625µL/n	0.3125LµL/mL
			0.6%(v/v)I	0.6%(v/v)I	0.6%(v/v)EtOH

	Raw Data (485, 520)					
	1	2	3	4	5	6
A	8112	7980	7225	7115	6550	5995
B	8225	7990	7331	6991	6854	6000
C	8300	7850	7251	6895	7001	6225
D	820	816	814	6441	5445	5225

			Average background	816.666		
Adjusted values with background						
0.6%(v/v)EtC	3125LµL/m	.625µL/mI	1.25µL/mL	2.5µL/mL	5µL/mL	10µL/mL
4408.33	5178.33	5733.33	6298.33	6408.33	7163.33	7295.33
4628.33	5183.33	6037.33	6174.33	6514.33	7173.33	7408.33
5624.33	5408.33	6184.33	6078.33	6434.33	7033.33	7483.33

1.2. Protein concentrations of the different concentrations of Goldenseal ethanolic extracts treated HepG2 cells (Protein content after calculation with STD Curve formula).

	Layout	
GS(ALC)	GS(ALC)	GS(ALC)
10μL/mL	10μL/mL	10μL/mL
5μL/mL	5μL/mL	5μL/mL
2.5μL/mL	2.5μL/mL	2.5μL/mL
1.25μL/ml	1.25μL/ml	1.25μL/mL
0.625μL/n	0.625μL/n	0.625μL/mL
0.3125LμL	0.3125LμL	0.3125LμL/mL
0.6%(v/v)I	0.6%(v/v)I	0.6%(v/v)EtOH
48.6634	42.6684	43.5632
48.7737	48.4474	44.4626
48.5632	47.6053	42.8263
47.8589	43.2426	44.4531
46.7201	44.3526	48.1216
49.0842	47.2947	44.5632
49.8637	40.6158	39.3374

1.3. Divided values of the fluorescence with protein concentration

0.6%(v/v)I	0.3125LμL	0.625μL/n	1.25μL/ml	2.5μL/mL	5μL/mL	10μL/mL
88.407	105.498	122.716	131.602	131.958	146.868	149.914
113.953	109.596	136.121	142.783	136.84	148.064	173.625
142.976	121.363	128.514	136.735	151.643	158.185	171.78

2.Effect of the long-term treatment of Goldenseal ethanolic extracts on glucose consumption by confluent HepG2 cells (A single set of data).

2.1. Glucometer readings before normalisation with protein content.

				LAYOUT							
Media	UT	DMSO	0.6% Etha	0.3125 ul/	0.625 ul/n	1.25 ul/ml	2.5 ul/ml	5 ul/ml	10 ul/ml	Ber	Met
Media	UT	DMSO	0.6% Etha	0.3125 ul/	0.625 ul/n	1.25 ul/ml	2.5 ul/ml	5 ul/ml	10 ul/ml	Ber	Met
Media	UT	DMSO	0.6% Etha	0.3125 ul/	0.625 ul/n	1.25 ul/ml	2.5 ul/ml	5 ul/ml	10 ul/ml	Ber	Met

				Raw data							
15.8	14.99	9.003636	10.60348	5.954804	3.828304	9.487536	2.713188	5.245333	4.807573	2.997647	1.585374
14.5	13.58	13.62	12.57846	2.735057	2.722759	7.524444	9.0333	7.196306	6.674667	3.615111	5.863536
13.8	14.55	15.10207	14.03333	12.73333	10.92408	10.46667	10.86667	10.73333	5.766667	5.666667	8.631515

2.2. Protein concentrations of the different concentrations of Goldenseal (alc) treated HepG2 cells (Protein content after calculation with STD Curve formula).

	Layout	
Met	Met	Met
Ber	Ber	Ber
10µL/mL	10µL/mL	10µL/mL
5µL/mL	5µL/mL	5µL/mL
2.5µL/mL	2.5µL/mL	2.5µL/mL
1.25µL/ml	1.25µL/ml	1.25µL/mL
0.625µL/n	0.625µL/n	0.625µL/mL
0.3125LµL	0.3125LµL	0.3125LµL/mL
0.6%(v/v)	0.6%(v/v)	0.6%(v/v)EtOH
DMSO	DMSO	DMSO
UT	UT	UT

40.111	34.2113	35.7712
39.6654	33.4412	36.111
39.5514	32.2213	35.111
40.3312	42.1111	40.2212
42.3333	45.3333	43.4442
45.3322	43.24263	44.45311
46.72005	48.3322	48.1516
49.1253	47.321	44.435
47.5788	49.1567	48.8892
47.6682	48.2276	49.7782
49.4432	50.3332	49.6675

2.3. Divided values of the Glucometer readings with protein concentration

UT	DMSO	0.6%(v/v)	0.3125LµL	0.625µL/n	1.25µL/ml	2.5µL/mL	5µL/mL	10µL/mL	Ber	Met
0.303	0.188	0.222	0.121	0.081	0.209	0.06	0.13	0.121	0.07	0.03
0.269	0.282	0.255	0.05	0.056	0.173	0.199	0.17	0.207	0.108	0.171
0.292	0.308	0.287	0.286	0.226	0.235	0.25	0.266	0.164	0.156	0.241

3.Effect of the target treatments on the glycogen content after 48 hours treatment of the confluent HepG2 cells.

3.1. Readings before normalisation with protein content.

		LAYOUT		
Ber	GolS	Met	EtOH	DMSO
Ber	GolS	Met	EtOH	DMSO
Ber	GolS	Met	EtOH	DMSO

	Raw Data (355, 460)				
	1	2	3	4	5
A	58790	55856	53256	45405	45555
B	54660	50455	50365	40400	40420
C	50550	52250	51225	42355	42455

		Average background	820.222	
Adjusted values with background				
DMSO	Ethanol	GolS	Ber	Met
44734.78	44584.78	55035.78	57969.78	52435.78
39599.78	39579.78	49634.78	53839.78	49544.78
41634.78	41534.78	51429.78	49729.78	50404.78

3.2. Protein concentrations in treated HepG2 cells

Protein con after calculation with STD Curve formula					
	DMSO	Ethanol	GolS	Ber	Met
	40.47321	43.11161	44.35311	47.95995	43.34263
	43.63001	42.21141	48.32158	46.81005	44.45463
	46.10021	39.26421	44.66316	49.19321	47.39574

3.3. Glycogen content (measured with STD curve formula) before normalization with protein content.

DMSO	Ethanol	GolS	Ber	Met
18.99191	19.10101	24.101	25.67309	23.47209
20.101	20.10111	26.24991	26.53807	26.25309
20.77173	18.99191	24.9488	28.65771	24.9589

3.4. Glycogen content after normalization with protein content.

Divided values of the Glycogen content with protein concentration					
	DMSO	Ethanol	GolS	Ber	Met
	0.469	0.443	0.543	0.535	0.541
	0.413	0.476	0.543	0.566	0.59
	0.45	0.483	0.558	0.582	0.526

4.Effect of the treatments on the release of glucose from HepG2 cells.

4.1. Fluorescence variations before normalisation with protein content.

		LAYOUT			
Ber	GolS	Met	EtOH	DMSO	
Ber	GolS	Met	EtOH	DMSO	
Ber	GolS	Met	EtOH	DMSO	
	Raw Data (355, 460)				
	1	2	3	4	5
A	41224	37120	41118	49000	65192
B	41402	39541	41176	48506	66778
C	42254	41197	42145	50242	68230

		Average background	822.222	
Adjusted values with background				
DMSO	Ethanol	GolS	Ber	Met
64369.78	48177.78	36297.78	40401.78	40295.78
65955.78	47683.78	38718.78	40579.78	40353.78
67407.78	49419.78	40374.78	41431.78	41322.78

4.2. Glucose concentrations of the treated HepG2 cells (Glucose content after calculation with STD Curve formula).

calculation with STD Curve formula				(Glucose output)
DMSO	Ethanol	Gols	Ber	Met
145.168	108.658	81.87	91.124	90.885
148.744	107.544	87.329	91.525	91.016
152.018	111.458	91.063	93.447	93.201

4.3. Protein concentrations of the treated HepG2 cells (Protein content after calculation with STD Curve formula).

Protein con after calculation with STD Curve formula					
	DMSO	Ethanol	GolS	Ber	Met
	42.36321	43.11521	40.25311	46.85895	42.44263
	45.45001	45.65412	48.22458	43.71105	43.44463
	46.11221	40.54371	45.65316	47.29521	46.47474

4.4. Normalised values of the glucose output (after dividing with protein content)

Normalised values of the glucose output (with protein content)					
DMSO	Ethanol	GolS	Ber	Met	
3.456	2.52	2.03	1.94	2	
3.27	2.355	1.81	2.09	2.09	
3.29	2.749	1.99	1.99	2	

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