Phytochemical Screening and Anti-hyperglycemic Effects of *Crotalaria verrucosa* Leaves

By

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A thesis submitted to the Department of Pharmacy in partial fulfillment of the requirements for the degree of
Bachelor of Pharmacy (Hons.)

Department of Pharmacy
Brac University
September 2019

Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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Approval

The thesis/project titled "Phytochemical Screening and Anti-hyperglycemic Effects *Crotalaria verrucosa* Leaves" submitted by Walid Ibn Amin (15146112) of Spring, 2015 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy (Hons.) on September.

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Ethics Statement

The project titled “Phytochemical Screening and Anti-hyperglycemic Effects *Crotalaria verrucosa* Leaves” required testing on animals. The animals for the in-vivo studies were Long Evan rats and the tests were conducted in icddr,b. The animal models were used with the prior permission of the respective authorities. Most importantly, they were handled with care and taken to processes only required in the protocol, unnecessary hurting of the animals was strictly avoided.

Abstract

Diabetes mellitus in Bangladesh has become a major issue. There are many medications available to treat this disease. However, most of them have side effects and the need for new treatments is required. *Crotalaria verrucosa* is a plant which is native to Bangladesh and it is found in Bandorban, Sundarbans, Khagrachori. Its medicinal properties have been known for a long time and used as a traditional medicine in many parts of the country. This study included phytochemical analysis to identify potential anti-hyperglycemic chemicals, in-vivo and in-vitro experiments to determine anti-hyperglycemic ability of the extract. Phytochemical screening of the plant leaves showed the presence of alkaloids, glycosides, flavonoids etc. Furthermore, this study showed that the ethanolic extract of the plant does not inhibit disaccharidase enzymes in the small intestine and does not change gastrointestinal motility in Long Evan rats. However, in-vitro study showed that the extract has a potential glucose adsorption capacity.

Keywords: anti-hyperglycemic; *Crotalaria verrucosa*; six-segment; gastrointestinal tract; glucose adsorption.

Dedication

Dedicated to My Family & Friends

Acknowledgement

This research could not have been completed without the support of many people who are gratefully acknowledged here.

First and foremost, my special gratitude is due to my supervisor, Easin Uddin Syed, Lecturer, Department of Pharmacy, Brac University. He has been content to let me shape this work as I thought best, yet his criticisms and suggestions have always been available and have always proved stimulating. His constant effort and encouragement towards my research based project allowed me to grow as a research scientist. His skills and experience in the field of pharmacology helped to conduct this study smoothly. He continually and persuasively conveyed a spirit of adventure in regard to research and an excitement in regard to teaching. I would like to express my appreciation to our chairperson Professor Dr. Eva Rahman Kabir, Department of Pharmacy, Brac University, who I am deeply thankful for her valuable contribution.

Last but not the least, I would like to give a special gratitude to my parents for their constant invaluable support and prayers which have enabled me to dream bigger and pursue something which can only be realistic after passing hurdles and my friends who have supported me always.

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List of Acronyms

BNH	Bangladesh National Herbarium
CAM	Contemporary & Alternate Medication
CVLE	Ethanolic Extract of *Crotalaria verrucosa* Leaves
DPP-4	Dipeptidyl Peptidase-4
GAD	Generalized Anxiety Disorder
GDM	Gestational Diabetes Mellitus
GI	Gastrointestinal
GLP-1	Glucagon Like Peptide-1
GLUT2	Glucose Transporter 2
GLUT4	Glucose Transporter 4
GLUT5	Glucose Transporter 5
GOD-PAP	Glucose Oxidase-Phenol Amino Phenazone
HLA	Human Leukocyte Antigen
HNF-1α	Hepatocyte Nuclear Factor 1 Alpha
IRS1	Insulin Receptor Substrate 1
NPH	Neutral Protamine Hagedorn
PDK1	3-Phosphoinositide Dependent Kinase-1
PI	Protease Inhibitor

Pkb	Protein Kinase B
P13K	Phosphoinositide 3-kinase
PI(3)P	Phosphatidylinositol 3-Phosphate
ROS	Reactive Oxygen Species
SGLT1	Sodium Dependent Glucose Co-transporter 1
SGLT2	Sodium Dependent Glucose Co- transporter 1
TZD	Thiazolidinedione

Chapter 1

Introduction

1.1 Regulation of blood glucose

Insulin is secreted by the pancreatic β-cells. High blood glucose stimulates the release of insulin. Insulin secretion changes with the change of blood glucose level.

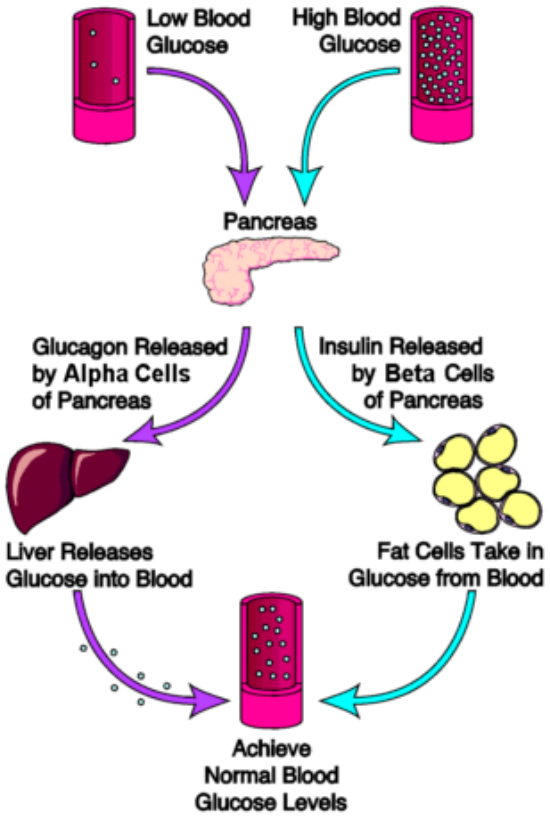

Figure 1: Regulation of glucose level by insulin and glucagon (Norman, 2014).

As can be seen in figure 1 insulin has an effect on a number of cells, including muscle, red blood cells, and fat cells. In response to insulin, these cells absorb glucose out of the blood lowering the amount of glucose in the blood.

Glucagon similarly to insulin is released from the pancreas. However, the actions of these two hormones are totally opposite. Glucagon is not secreted when blood glucose is high. Rather it is secreted when blood glucose gets low. It effects many cells of the body but the

hepatic cells are the ones which are most affected. Glucagon stimulates gluconeogenesis to break protein molecules and produce glucose which increase glucose level. In the end, it is the balance between insulin and glucagon secretion that maintains a normal blood glucose level (Norman, 2014).

1.2 Digestion and absorption of carbohydrates

The breaking down of carbohydrates begins in the mouth. The food is chewed and carbohydrates are broken down by enzymes released by the salivary gland. The carbohydrate is coated with saliva and form a uniform chyme. The chyme doesn't undergo any further degradation in the stomach.

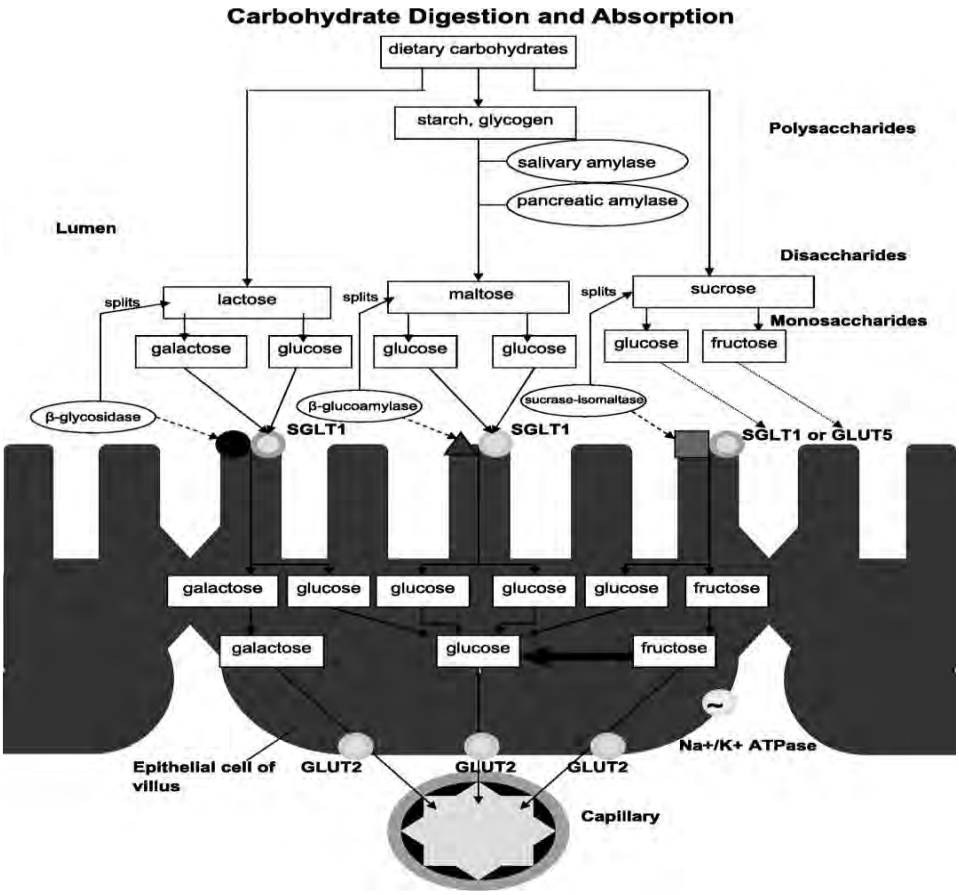

Figure 2: Absorption of monosaccharides in the small intestine (Goodman, 2010).

The chyme is progressively driven into the upper portion of the small intestine. Once the chyme has entered the small intestine, pancreatic fluid is released via a duct. This pancreatic fluid contains pancreas amylase enzyme which again breaks carbohydrates into smaller and smaller strands. The intestinal cells which border the villi also secrete enzymes which are sucrase, maltase and lactase. They are jointly regarded as disaccharidases also known as brush border enzymes. Sucrase splits sucrose into the molecules of glucose and fructose. Maltase breaks the connection between the two glucose units in maltose and lactase cuts the connection in lactose to form galactose and glucose.

It can be seen from figure 2 that, small intestinal cells have membranes containing many transport proteins mainly Sodium-dependent glucose cotransporters 1 to get the monosaccharides and other nutrients into cells. GLUT5 is solely responsible for the absorption of fructose from the intestine. These monosaccharides (galactose, glucose and fructose) are then transported to the blood and spread to the rest of the body by GLUT2 transport proteins. Facilitated diffusion absorbs fructose while active transport is responsible for the absorption of glucose and galactose. A small amount of carbohydrates which are not absorbed in the small intestine are broken down by bacterial enzymes in colon (Goodman, 2010).

1.3 Definition of diabetes mellitus

Diabetes mellitus refers to a metabolic disorder having several etiology characterized by chronic hyperglycemia and associated with carbohydrate, lipid and protein metabolism disturbance caused by defected insulin secretion, insulin action or both (Amereican diabetes association, 2004). In every country in the world this disease has reached epidemic condition. According to World Health Organization 200 million people of the world are currently suffering from diabetes and this figure is expected to reach 300 million by 2025. Patient

suffering from diabetes are very susceptible to neurological, cardiovascular, retinal and renal complications which can result in untimely death often caused by diabetes induced oxidative stress (Rahmatullah et al., 2012).

1.4 Mechanism of insulin

Upon binding to the receptor tyrosine kinase, insulin is phosphorylated and activated. Followed by this, several other proteins also undergo a cascade of activation and phosphorylation by the kinase enzyme as they bind to it. One of these proteins is responsible for gene transcription. As can be seen from figure 3, another protein called Insulin Receptor Substrate 1 (IRS1) causes the transport of GLUT4 protein across the cell surface. IRS1 is also called a scaffolding protein. After the activated insulin receptor protein tyrosine kinase phosphorylates IRS1, it binds phosphatidylinositol 3-kinase (P13K) that causes the phosphorylation of the 3'OH on phosphatidyl inositol (PI) in the inner portion of the membrane to form Phosphatidylinositol 3-Phosphate [PI(3)P]. P13K is a kinase enzyme that can phosphorylate PI. In human cancers, it can be seen that this particular cellular metabolic pathway mechanism is mutated. PI(3)P can be seen to include other inactive kinases such as phosphoinositide-dependent kinase 1 (PDK1) and Akt.

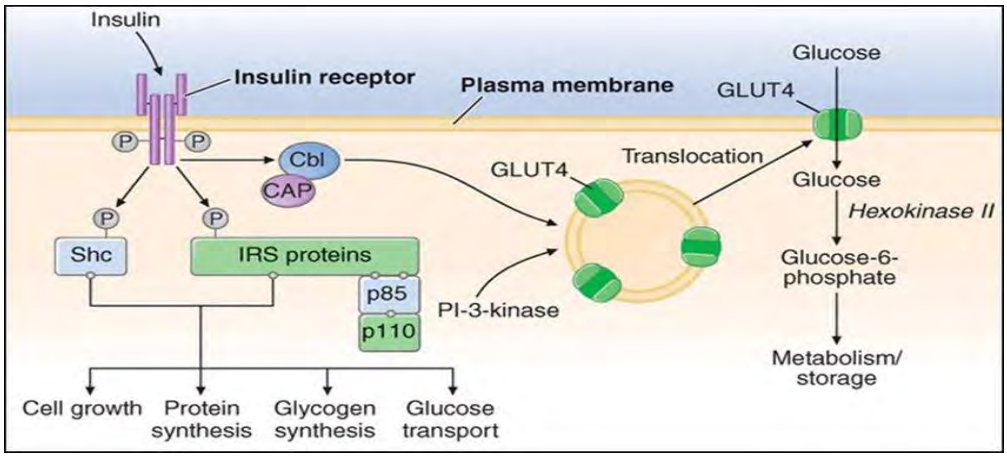

Figure 3: Mechanism of action of insulin (Giammona, Mauro, & Scialabba, 2016).

Many proteins which are responsible for cell activities such as regulation of glucose transport, cell proliferation and cell death can be regulated by the family of three Act kinases. These are major Ser/Thr protein kinases that phosphorylate such major proteins. In the insulin signalling pathway, phosphorylated Act leads to the transport of the GLUT4 protein from intracellular endosomal vesicles to the surface of the cell. As a result, this provides a faster pathway of glucose import into the cell (Jakubowski, 2013).

1.5 Classification of diabetes mellitus

1.5.1 Type 1 Diabetes

Type 1 diabetes most commonly afflicts children, adolescents, or young adults, but some latent forms occur later in life. As figure 4 suggests Type 1 diabetes refers to an utter insufficiency of insulin resulting from destruction of β-cells. β-cells may lose its function due to autoimmune-mediated processes that can be activated by viruses or environmental toxic substances. In this situation the pancreas is unable to respond to the glucose levels. A person with Type 1 diabetes suffers from polyphagia, polyuria, polydipsia and weight loss. This condition will require exogenous insulin to avoid serious hyperglycemia and the catabolic state of ketoacidosis which can be life threatening.

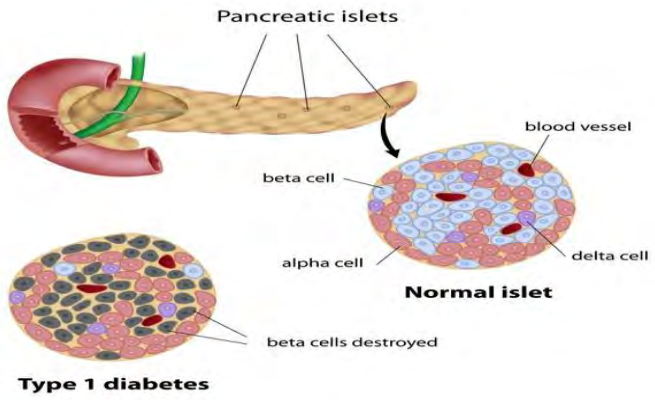

Figure 4: Difference of β-cells in Type 1 diabetes pancreas and in normal pancreas (Baker & Steck, 2011).

During post absorptive state, β-cells maintains low concentration of insulin in the blood which suppresses glycogenolysis, lipolysis and proteolysis. Insulin concentration increases after having meal which maintains the blood glucose level. However, without β-cells functioning properly the circulation glucose level is not maintained (Finkal Richard, cubeddu Luigi X, 2009).

1.5.2 Type 2 diabetes

Type 2 diabetes is attributed by the pancreas having some functional β-cells as illustrated in figure 5. Type 2 diabetes is more common than Type 1 and occurs about 9 in 10 cases of diabetes compared to Type 1 diabetes. Unlike Type 1 diabetes Type 2 diabetes is not an autoimmune disease where cells are destroyed. Factors that influence Type 2 diabetes are genetic makeup, aging, obesity, and peripheral insulin resistance rather than autoimmune processes. The metabolic changes are less severe in Type 2 diabetes. However, the long-term clinical outcomes are not dissimilar. Overtime the number of β-cells slowly dwindles and usually in most cases of Type 2 diabetes the patient is obese (Finkal Richard, cubeddu Luigi X, 2009).

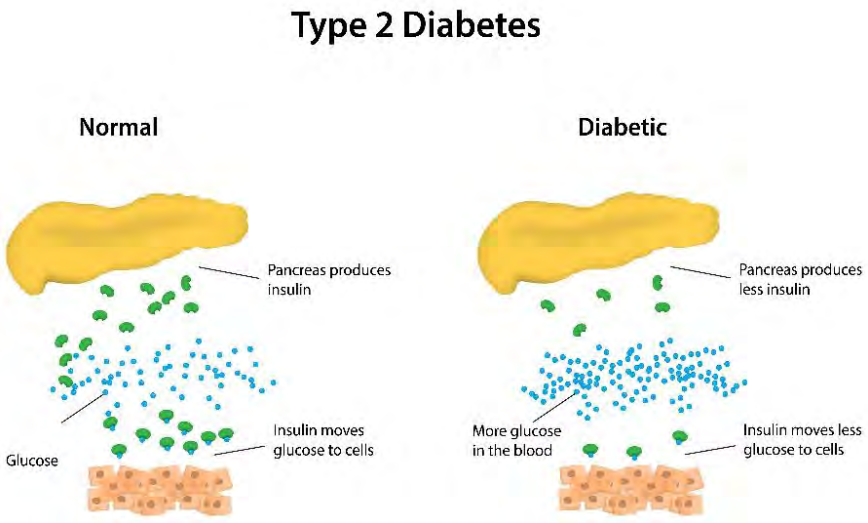

Figure 5: Pancreas producing less amount of insulin in Type 2 diabetes (Anderson et al., 2014).

1.5.3 Uncommon forms of immune-mediated diabetes

There are many conditions in this category. However, two conditions are more likely to occur.

Firstly, there are antibodies called anti-insulin receptor antibodies that blocks the binding of insulin to its receptor in the tissue. They do this by binding with the insulin receptors resulting in antagonistic effect. However, in some cases after binding to the receptors they can cause low blood glucose level. These antibodies are sometimes found in several autoimmune conditions like systemic lupus erythematosus. In addition, patients having acanthosis can suffer from severe insulin resistance.

Secondly, uncommon diabetes occurs during the stiff-man syndrome which is an autoimmune condition occurring in the central nervous system. This disorder is usually associated with high titers of the GAD autoantibodies, and roughly one-third develop diabetes.

1.5.4 Gestational diabetes mellitus (GDM)

Any level of glucose intolerance during pregnancy is referred to gestational diabetes mellitus. GDM is defined as any degree of glucose intolerance with onset or first recognition during pregnancy. Insulin or diet modification can be used for the treatment of GDM. In many cases this condition persists after pregnancy. The occurrence of GDM ranges from 1 to 14% of all pregnancies varying in different populations. In addition, nearly 90% of diabetic complications during pregnancy are caused by GDM (Amereican diabetes association, 2004).

1.6 Causes of diabetes

1.6.1 Genetic defects of the β-cell

Monogenetic defects in β-cells accounts for several forms of diabetes mellitus. These hyperglycemic conditions usually occur before the age 25. In this condition impaired glucose

secretion is observed. However, glucose action is slightly impaired or not impaired at all. Genetic alterations in six different genetic loci in different chromosomes have been identified. Mutations on chromosome 12 in a hepatic transcription factor known as hepatic nuclear factor (HNF)- 1α are most common.

1.6.2 Genetic defects in insulin action

These defects are not as common as defects in β-cells. Abnormalities in insulin actions are observed in these cases. The alterations in insulin receptor may cause hyperinsulinemia, modest hyperglycemia to severe diabetes. It is assumed that the abnormalities are located in the postreceptor signal transduction pathways.

1.6.3 Diseases of the exocrine pancreas

Diabetes can result from damages in the pancreas. Damages can be caused by infections, trauma, pancreatectomy and pancreatic carcinoma. Injury of the pancreas needs to be severe to cause diabetes except pancreatic carcinoma.

1.6.4 Endocrinopathies

Several hormones produced excessively in the body can inhibit insulin action and lead to diabetes. These include growth hormone, cortisol, glucagon and epinephrine.

1.6.5 Drug or chemical-induced diabetes

Many drugs can cause abnormalities in insulin secretion. These drugs may not cause diabetes itself but the resulting impaired insulin secretion can eventually lead to diabetes. Examples include, certain toxins (vacor), intravenous pentamidine, nicotinic acid and glucocorticoids.

1.6.6 Infections

There are specific viruses which have been linked with β-cell destruction. For example, adenovirus, cytomegalovirus, and mumps have been associated with diabetes. In addition, in

many cases patients suffering from congenital rubella develop diabetes. However, most of these cases are associated with HLA and immune markers characteristic of Type 1 diabetes (Amereican diabetes association, 2004).

1.7 Prevalence diabetes in Bangladesh

In a nationally representative study conducted by WHO, it has been found that diabetes has become epidemic in adult population of Bangladesh. Similar conditions have been observed in China, India, Pakistan, and Iran. However, prevalence in Vietnam was significantly lower due to different climactic condition and food habits (Akter, Rahman, Abe, & Sultana, 2014).

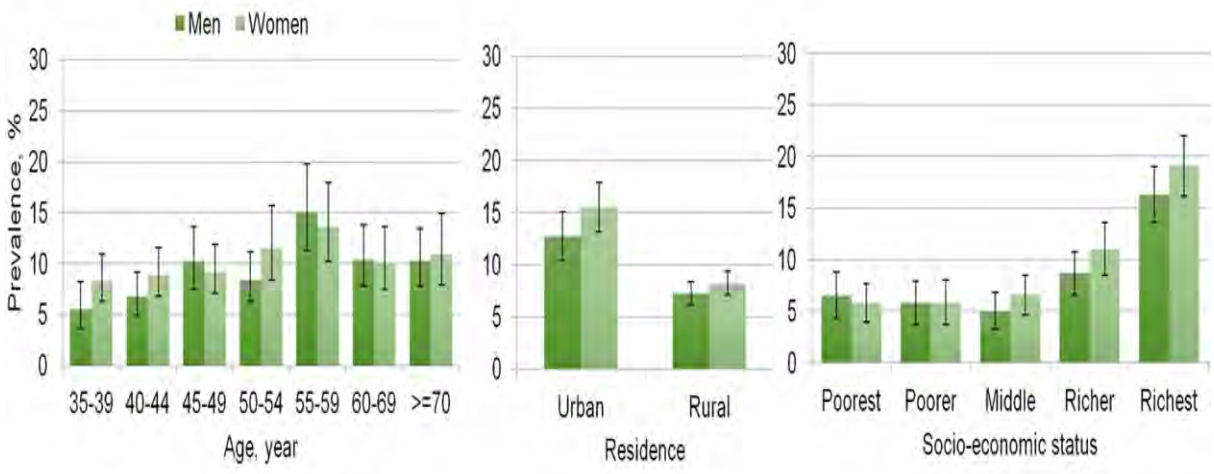

Figure 6: Prevalence of diabetes in different groups (Rahman et al., 2015).

The graphs in figure 6 refers that, diabetes was more frequent in elderly persons (aged 55-59). The experiment was based on age-standardization and sex. It has been found that diabetes increased with the increase of socio-economic status. The increase of diabetes in rural areas can also be seen (Rahman et al., 2015).

1.8 Treatment of Type 1 diabetes

Diabetes cannot be cured so the treatments are designed to maintain the blood glucose levels as normal and control symptoms, to avoid health difficulties that arise later in life. The pancreas does not generate any insulin due to Type 1 diabetes. In order to maintain ordinary

glucose concentrations, regular insulin therapy is needed ("Type 1 diabetes - About insulin - NHS," n.d.).

There are different sorts of insulin preparations is use. Each of them works slightly differently as illustrated in figure 7. For example, some last up to a whole day, some last up to eight hours and some work quickly but don't last very long. These are rapid acting, short acting, intermediate acting and long acting insulin preparations. Depending upon the condition and needs of the patient the doctor prescribes different sorts of preparations. For example, NPH insulin should be given only subcutaneously (never IV), and it should not be used when rapid glucose lowering is needed (for example, diabetic ketoacidosis) (Aitchison, Gilchrist, & Bawden, 2000).

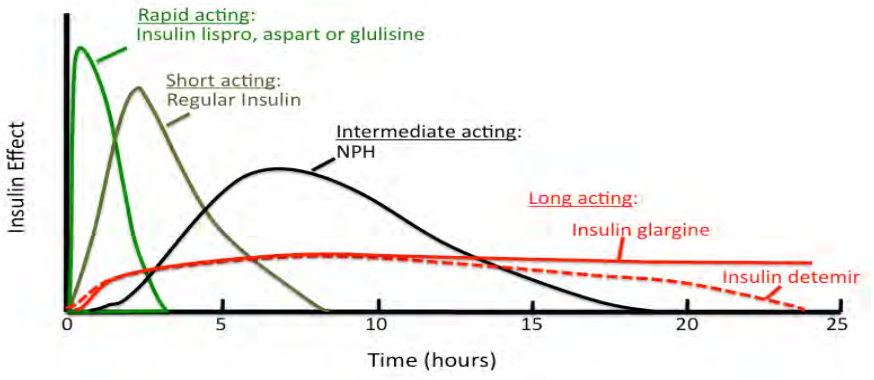

Figure 7: Effects of different insulin preparations (Kendall, Cuddihy, & Bergenstal, 2009).

1.9 Treatment of Type 2 Diabetes

In the course of time, Type 2 diabetes generally gets worse. Changes in lifestyle, such as changing the diet and more exercise, may assist patients initially regulate their blood glucose concentrations, but they may not be sufficient in the long run. Ultimately, they may need to take medication to help control blood glucose levels. This will generally be in tablet form at the beginning and can sometimes be a mixture of more than one tablet type. Insulin injections may also be required ("Type 2 diabetes - Understanding medication - NHS," n.d.).

1.9.1 Drugs used to treat Type 2 diabetes

Metformin: Typically, it is the first treatment for Type 2 diabetes. It operates by decreasing the quantity of glucose by reducing the amount of glucose liver releases into the bloodstream. It also enhances the reaction of the body cells to insulin. Metformin is recommended for adults with a high risk of developing Type 2 diabetes. Additionally, it is used in cases where despite lifestyle modifications that are essential, blood glucose continues to progress towards Type 2 diabetes. Metformin does not cause an extra weight gain than other drugs used to treat Type 2 diabetes. Examples include: Glucophage**,** Glumetza.

Sulphonylureas: Sulfonylureas are a group of medications that operate by enhancing the insulin content. Examples include, glibenclamide, glimepiride, glipizide, gliquidone. These medications are prescribed for patients who are unable to take metformin or not overweight. Patients who don't have control of blood glucose alone by metformin may be prescribed sulphonylurea. Sulfonylureas can increase the danger of hypoglycaemia by increasing the quantity of insulin in the body of the patient. Sometimes they can also trigger side effects such as weight gain, nausea, and diarrhoea.

Pioglitazone: Pioglitazone is the sort if thiazolidinedion (TZD) drug, which increases the sensitivity of the body cells to insulin so that more of the blood glucose is taken. It is usually used with metformin or sulfonylureas or both. It can lead to weight gain and inflammation of the knee.

Gliptins (DPP-4 inhibitors): Gliptins operate to prevent a natural hormone called GLP-1 from breaking up. In reaction to high blood glucose concentrations, GLP-1 enables the body create insulin, but quickly breaks down. Gliptins (linagliptin, Saxagliptin, Sitagliptin and Vildagliptin) prevent elevated blood glucose but do not produce episodes of hypoglycaemia by stopping such breakdown. Gliptin may be prescribed if a patient cannot take sulphonylurea or in conjunction with sulphonylurea or glitazones.

SGLT2 inhibitors: Inhibitors of SGLT2 work by increasing the amount of glucose excreted in urine. It may be considered if metformin and DPP-4 inhibitors are not appropriate. The three SGLT2 inhibitors that may be prescribed include: dapagliflozin, canagliflozin and empagliflozin. A higher risk of genital and urinary tract infections is the main side effect.

GLP-1 agonists: Similar to the GLP-1 natural hormone, GLP-1 agonists work increasing the insulin production.

Acarbose: Acarbose helps avoid an increase in blood sugar after a meal. It slows down the rate of carbohydrate division into glucose in the digestive system. Type 2 diabetes is often not treated with acarbose as it generally produces adverse effects such as bloat and diarrhoea. However, if a patient cannot take other medicines for Type 2 diabetes it can be prescribed.

Nateglinide and repaglinide: Nateglinide and repaglinide boost pancreatic production of insulin. Their effects don't last very long hence they are not frequently used. However, if taken shortly before a dinner, they are efficient. It can lead to adverse effects like weight gain and hypoglycemia.

Insulin Treatment: If glucose-reduction pills do not control blood glucoses, insulin therapy might be necessary. Depending on the amount and the manner the person gets it, this can be done instead of or alternatively with the pills (York et al., 2015).

1.10 Complementary and alternative treatment

A number of other types of medicines and techniques has been used to treat diabetes and its effects on the body. Complementary and alternative medicine refers to a wide range of clinical therapies and techniques. The term complementary means the treatment used alongside conventional methods and alternative treatment refers to the treatment used instead of conventional medicine.

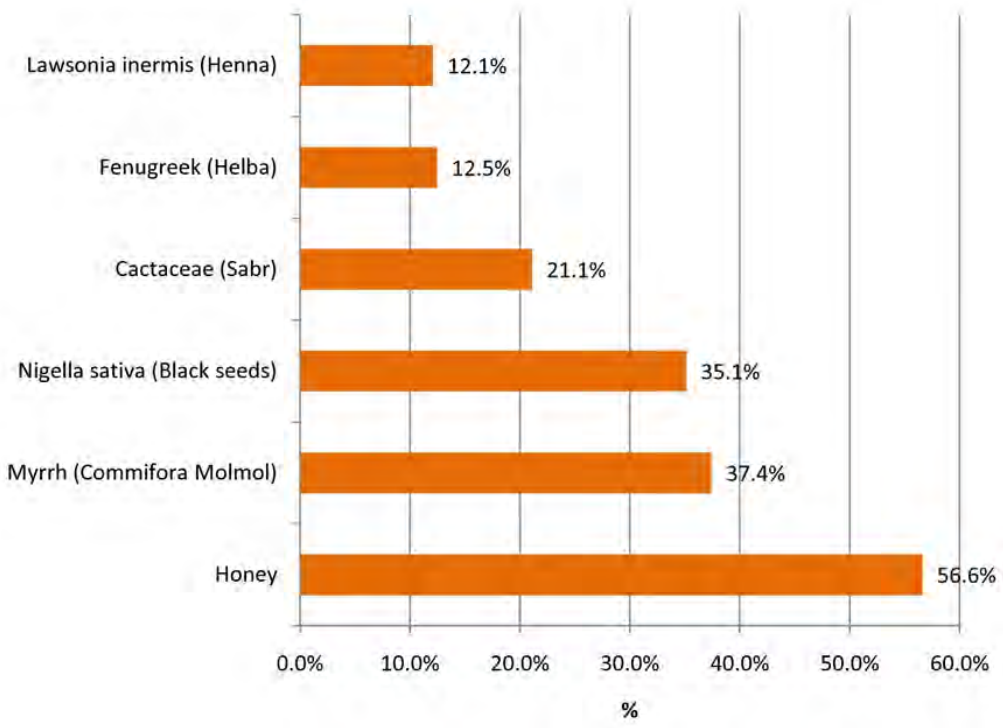

Figure 8: CAM products which are used in foot disorders in Saudi Arabia (Bakhotmah & Alzahrani, 2010).

These techniques can control blood sugar level. However, their mechanism of action and pharmacodynamic parameters are not quite established. There are a wide range of complementary and alternative therapies available which include parts of herbs and plants, mind body medicine technique, acupuncture etc. However, parts of plants and herbs are most common form of CAM across the world (Birdee & Yeh, 2010). For example, figure 8 demonstrates the use of herbal CAM products in treating foot disorders linked with diabetes.

1.11 Use of plants to treat diabetes

In the past three decades, despite significant progress made in the treatment of diabetes, the results of treatment are still far from perfect (Rahmatullah et al., 2010). Allopathic medicine has no known total cure for this disease, but merely alleviates one or more symptoms, which may lead to delay of development of other complicating factors arising out of the disease (Rahmatullah et al., 2012). These medications have certain disadvantages, such as drug resistance, negative impacts and even toxicity. For instance, after six years of therapy in 44

percent of patients, sulfonylurea loses its efficacy. The glucose-lowering medicines are also said to be unable to regulate hyperlipidemia (Rahmatullah et al., 2010). In the science and medical societies, therefore, there is an extensive interest in the development of more effective anti-diabetic medicines. Today many medicines are suggested which include the use of medicinal plants. In most plants carotenoids, flavonoids, terpenoids, alkaloids, glycosides are present and can often be anti-diabetic. Often owing to their capacity to enhance the pancreatic tissue's efficiency, which is achieved with increased insulin secretions or a reduction of intestinal glucose absorption, the anti-hyperglycemic consequences of treatments with plants. It has been observed that many contemporary medicinal products have been found from plants through the reflection of the herbal methods of indigenous populations (Rahmatullah et al., 2012).

Bangladesh has a number of indigenous communities or tribes who still rely on their traditional medicinal practices for treatment of a diverse variety of diseases. Medicinal plants have throughout centuries formed the mainstay of these tribal practitioners for treatment. Since diabetes affects also the tribal peoples and is recognized as a disease by their practitioners, they use a variety of medicinal plants for treatment of this disease, and claim to completely cure or at least alleviate major symptoms of the disease through administration of medicinal plants (Rahmatullah et al., 2012). In Rangpur and Dinajpur santal communities use *Carica papaya, Cassia occidentalis, Tamarindus indica, Psidium guajava, Syzygium cumini, or Aegle marmelos* plants to treat diabetes (Rahmatullah et al., 2010). In addition, in the northern and central forested regions of the country the Garos use *Lannea coromandelica, Alstonia scholaris, Catharanthus roseus, Enhydra fluctuans, Terminalia chebula, Coccinia grandis, Momordica charantia, Cuscuta reflexa, Phyllanthus emblica, Syzygium aqueum, Drynaria quercifolia* and *Clerodendrum viscosum* to treat diabetes (Rahmatullah et al., 2012).

Antidiabetic study of plants have been extensively conducted in laboratories across the country. Study conducted on animal models found anti-diabetic effect in a number of plants. Such as, *Acacia Arabica, Achyranthes aspera, Acosmium panamense, Aegle marmelose, Allium sativum, Aloe barbadensis Miller, Andrographis paniculata, Annona squamosal, Argyreia nervosa, Artemisia herba, Averrhova bilimbi, Azadirachta indica, Barleria prionitis, Biophytum sensitivum, Brassica nigra, Bryonia alba, Caesalpinia bonducella, Cajanus cajan, Carum carvi, Casearia esculenta, Chamaemelum nobile, Cichorium intybus, Citrulus colocynthis, Coriandrum sativum, Dorema aucheri, Eclipta alba, Fraxinus excersior, Helicteres isora, Hypoxis hemerocallidea, Lepidium sativum, Mangifera indica, Myrcia bella, Nigella sativa, Ocimum sanctum, Origanium vulgare, Phyllanthus amarus, Prangos ferulacea (L.) lindl, Rhus coriaria, Salacia reticulate, Securinegra virosa* (Kooti, Farokhipour, Asadzadeh, Ashtary-Larky, & Asadi-Samani, 2016).

1.12 Plant background information

A member of the Leguminosae, *Crotalaria verrucosa Linnaeus* is a quite branched herbaceous plant. Leguminosae is often referred to as legume, pea or bean family. Currently it the most economically essential family of flower plants. Furthermore, in terms of number of species it is the third largest in plant kingdom being only behind orchidaceae and asteraceae. *Crotalaria verrucosa L*. is also known as “Blueflower”, “Rattlepod” and “Jhanjhania” or “Bansan” in Bangladesh is an important medicinal plant in this family. The appearance of the plant can be seen in figure 9. Their abundantly ramified stumps with bent spines and the ovate elliptic formed leaves can better identify them. When it is young, the plant generates usually flowers and once it has a height of 1 to 1.5 meters, it bears fruit, as well as blossoms. *Crotalaria verrucosa* is the only perennial shrub to have a unique medicinal value in comparison to other species of Crotalaria (Ahmed, 2016).

Figure 9: Crotalaria verrucosa ("Crotalaria verrucosa / Blue Rattle Snake," n.d.).

It is known that this ethno- botanical herb has medicinal properties (Hussain, Chandrasekhar, & Gopal, 2008). In traditional medicine, leaf extract is used against scabies, impetigo, dyspepsia, diarrhea, dysentery and leprosy. This plant is native to Bangladesh's Chittagong, Khulna, Rajshahi and Sylhet. Preliminary phytochemical screening of *Crotalaria verrucosa* extract from leaves has shown that flavonoids, phenolic compounds, alkaloids, tannins, steroids and glycosides are present (Ahmed, 2016).

1.13 Aim of the study

A study conducted previously described the anti-hyperglycemic effects of *Crotalaria verrucosa* leaves in alloxan induced diabetic rats. The study however did not indicate the mechanism of the plant leaves reducing the blood glucose level. Our aim of the study is to

1. Identify potential anti-diabetic phytochemicals
2. Determine the anti-hyperglycemic effect of the leaves by:
    - Inhibiting disaccharidase enzyme which reduces absorption of sucrose from the gastrointestinal tract.

- Observing glucose adsorption capacity of the plant extract.

Chapter 2

Methodology

2.1 Collection and preparation of plant material

Figure 10 outlines the collection and extraction process of the plant. The entire plant of *Crotalaria verrucosa* was collected from the hilly region of Bandorban on February 2019. As shown in figure 11(a) a sample was sent to Bangladesh National Herbarium (BNH) which can be used for further references (Accession number: DACB 46913).

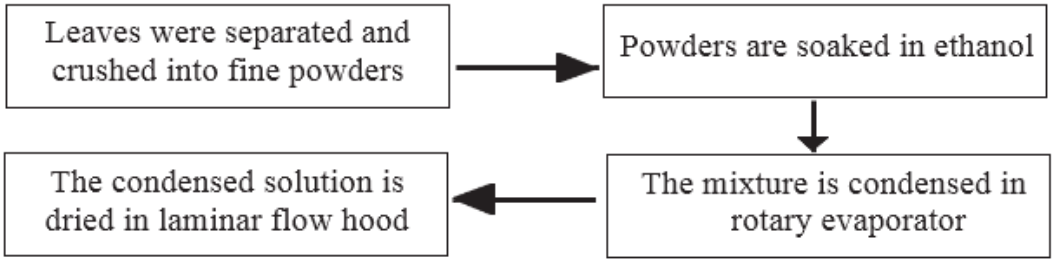

Figure10: Extraction process of the plant leaves

The leaves were separated from the rest of the plant and washed thoroughly. Then they were dried under ceiling fan. Afterwards, the leaves were crushed into powders and sieved to get a uniform particle size. 250 g of the leaves were soaked in 500 mL ethanol for 3 days in a closed container as shown in figure 11(b). The mixture was stirred 3 times every day. Afterwards, the mixture was filtered using Büchner funnel. The filtrate was condensed using a rotary evaporator at 45 °C which can be seen in figure 12(a).

a b

Figure 11: (a) Verification by Bangladesh National Herbarium, (b) soaking of leaf powder in ethanol

Finally, the mixture was dried inside a laminar flow hood [shown in figure 12(b) to obtain viscous ethanolic extract (20 gm approx.)]. After extraction the extract was kept in a petri dish and stored in freezer (Nawrin et al., 2015).

a b

Figure 12: (a) Mixture being condensed in rotary evaporator; (b) crude extract stored in petri dish

2.2 Phytochemical screening

Phytochemical analysis is required in order to access the extract's qualitative chemical compositions mainly alkaloids, carbohydrates, tannin, flavonoids, glycosides, etc. It was performed by dissolving crude plant extract to distilled water to create an aqueous solution.

The aqueous solutions were assessed by using various standard methods. The characteristic colored solutions and precipitates are presented in figure 13.

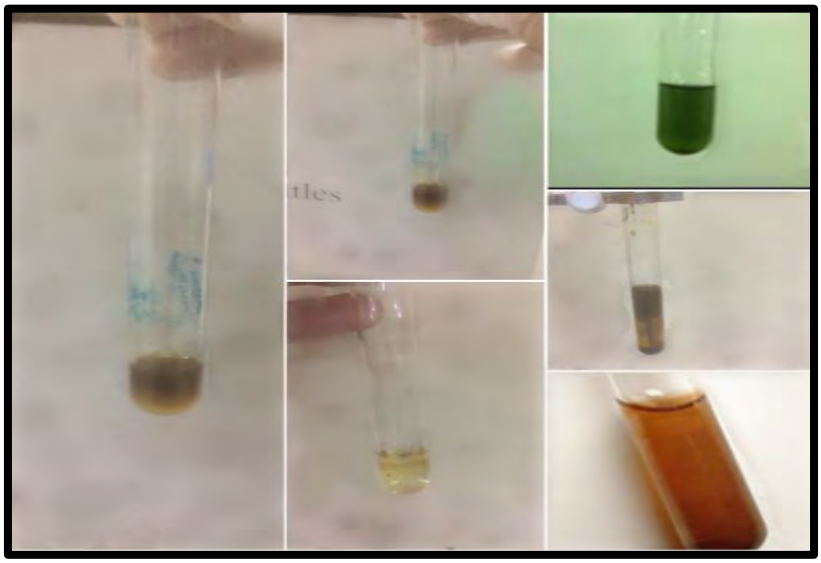

Figure 13: Phytochemical analysis indicating the presence and absence of various compounds

2.2.1 Detection of alkaloids

Mayer's Reagent was prepared by dissolving 0.1358 g of mercuric (II) chloride and 0.5 g of potassium iodide in 10 mL distilled water. Afterwards, few drops of Mayer's reagent was added to 2 mL of filtered plant extract solution. Formation of a white precipitate confirmed the presence of alkaloids (Ahmed, 2016).

2.2.2 Detection of glycosides

1 drop of 2.0 % $FeCl_3$ was mixed with 4 mL glacial acetic acid. Then 1 mL of concentrated H_2SO_4 was added with 10 mL of aqueous plant solution. The two solutions were mixed and appearance of a brown ring between the layers of the two solution indicated the presence of cardiac glycosides (Gul, Jan, Faridullah, Sherani, & Jahan, 2017).

2.2.3 Detection of phenols

This experiment was carried out by measuring 2 mL of aqueous extract in a test tube followed by adding 3-4 drops of 15 % (w/v) ferric chloride solution. The formation of a bluish-black precipitate indicated the existence of phenols.

2.2.4 Detection of tannins

5 % ferric chloride solution was prepared by dissolving 0.5 g of ferric chloride in 10 mL distilled water. 5 mL of aqueous solution was been dissolved in 1 mL of 5 % ferric chloride solution and the emergence of greenish black precipitate suggests the existence of tannins (Ahmed, 2016).

2.2.5 Detection of flavonoids

2 mL of 2 % NaOH was mixed with aqueous crude plant extract. Focused yellow color was generated which became colorless when 2 drops of diluted acid were added to the blend. This outcome showed that flavonoids were present (Gul et al., 2017).

2.2.6 Detection of diterpenes

Aqueous plant extract was treated with copper acetate solution. Green color emergence showed the presence of diterpenes (Pandey, Tripathi, & Pandey, 2014).

2.2.7 Detection of steroids

1 mL of aqueous plant extract was treated with 2 mL of chloroform and 1 mL of H_2SO_4. The formation of red color indicated the presence of steroids (Ahmed, 2016).

2.2.8 Detection of terpenoids

With 5 mL aqueous plant extract, 2 mL of chloroform was added and evaporated in the water bath and then heated with 3 mL of concentrated H_2SO_4. A gray color formed showing the presence of terpenoids (Gul et al., 2017).

2.2.9 Detection of saponins

Five milliliters the aqueous plant extract were put in a test tube. For two minutes, the blend was shaken forcefully. The presence of saponins can be verified by persistent appearance of foam lasting at least fifteen minutes or the formation of an emulsion we when added olive oil (Kareru, Keriko, Gachanja, & Kenji, 2008).

2.3 Glucose adsorption capacity of *Crotalaria verrucosa*

9 mg of glucose was added to 50 mL of water to make a glucose solution. Different amounts (0.25 g, 0.5 g, 0.75 g, 1g, 1.25 g) of plant extract were added to the solution and heated for 6 hours in water bath. Then the solution was centrifuged at 3000 rpm for 20 minutes. Glucose concentration of the supernatant was determined using GOD-PAP method (Kabir et al., 2014a).

2.4 Effects of *Crotalaria verrucosa* on sucrose absorption from the gastrointestinal tract

The sucrose absorption from the gastrointestinal tract depends on three factors. The adsorption ability of the plant extract, gut motility and disaccharidase enzyme activity.

The gut motility experiment was conducted in the animal resources facility of icddr,b. The animals were weighed and divided into 2 groups. Each group of animals contained three animals. Control group who received $BaSO_4$ milk prepared earlier inserted by gavage and treated group. They received CVLE 500 mg/kg solution by gavage and after 60 minutes they received $BaSO_4$ milk inserted by gavage.

15 minutes after the administration of $BaSO_4$ milk, the rats were sacrificed and gastrointestinal tract was excised. The distance the milk travelled was observed (shown in figure 14).

Figure 14: Gut motility test of the gastrointestinal tract

The distance traversed by $BaSO_4$ milk was measured and expressed as a percentage of the total length of small intestine (Hannan et al., 2006).

In oreder to determine disaccharidase enzyme activity alteration the unabsorbed sucrose content of the rats was measured. Six-segment assay was conducted for this purpose. The study was conducted in the animal resources facility of icddr,b. Six rats were kept for one week in the laboratory to acclimatize to the conditions. By doing this, the stress levels of the rats were reduced. The animals were fasted for 20 hours. Afterwards, the animals were weighed and divided into 2 groups. Each group of animals contained three animals. Control group who received the 2.5 g/kg sucrose solution at the specified time by gavage and the treated group. They received the 2.5 g/kg sucrose solution, CVLE 500 mg/kg at the specified time by gavage.

500 mg of CVLE was inserted into rats by using a syringe which had a smooth curved end, which can lead the extract directly to the stomach. The tail vein was anesthetized by using mild diethyl ether. Blood samples were obtained from the tail vein of all the rats 30 minutes before and 30, 60, 120 minutes after sucrose load for the determination of glucose using blood glucose meter.

Different surgical procedures are shown in figure 15. After 10 minutes of the sucrose administration the rats were euthanized to determine of unabsorbed sucrose contents of the gastrointestinal tract.

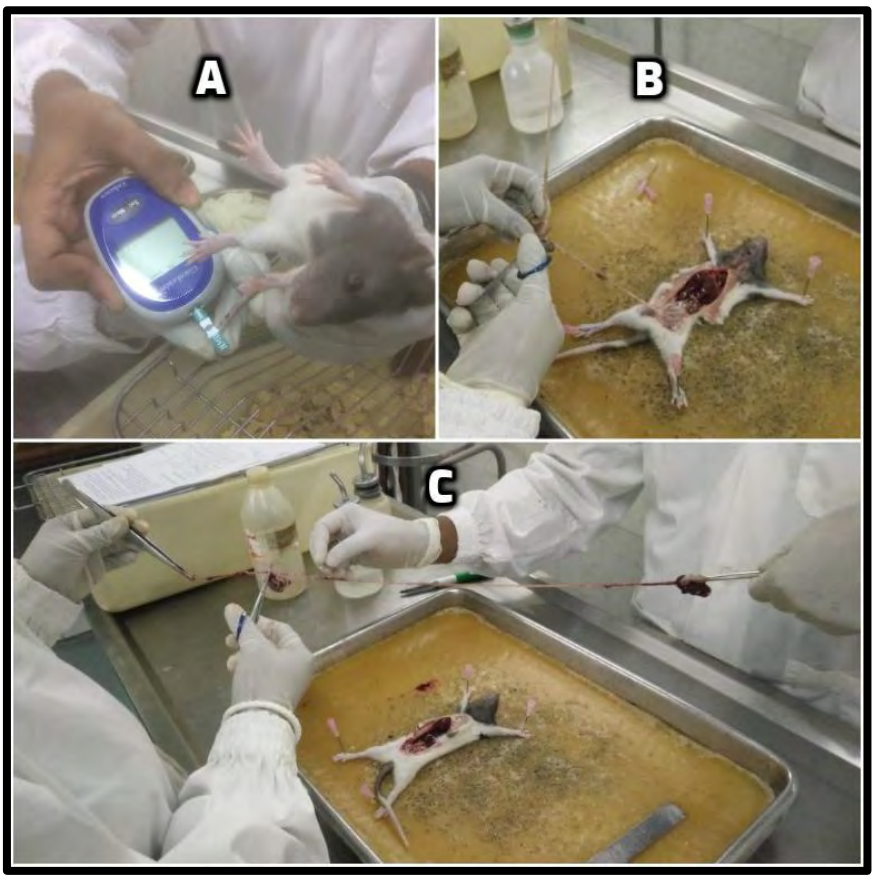

Figure 15: (A) Blood glucose being measured; (B) parts of the GI tract being surgically removed; (C) parts of the GI tract being cut into segments.

The GI tract was excised and divided into six segments: the stomach, the upper 20 cm, middle and lower 20 cm of the small intestine, the caecum and the large intestine. Each segment was washed out with ice-cold saline. Afterwards, each segment was acidified with 2 mL of 2 N of sulfuric acid. Then the segments were centrifuged at 1000 G for 10 minutes. The resulting supernatant was boiled for 2 hours to hydrolyze the sucrose following neutralization of the solution with 1 N NaOH. Amount of glucose liberated from residual sucrose was measured by glucose oxidase (GOD-PAP) method (Goto et al., 1995).

2.5 Statistical analysis

Statistical tests were conducted using Statistical Package for Social Science Software (SPSS) version 20 (IBM, Inc., Chicago, IL, USA). Results are presented as means and standard deviation. Data from experimental groups were compared using independent variable t test. A two-tailed P value less than 0.05 was considered statistically significant and a two- tailed P value higher than 0.05 was considered not significant.

Chapter 3

Results

3.1 Phytochemical screening

The project began with a total of 250 g of powdered plant material, which after extracting with ethanol, a percentage yield of 8.21 % of dried extract of *Crotalaria verrucosa* was obtained. This 20.3 g plant material was later used for phytochemical screening and investigating its hypoglycemic activity in the gastrointestinal tract.

Ethanolic extract of the *Crotalaria verrucosa* leaves were dissolved in water. Preliminary phytochemical analysis was performed and showed the presence of alkaloids, carbohydrate, glycosides, phenols, tannins, diterpenes, terpenoids which are listed in table 1.

Table 1: Phytochemical analysis of Crotalaria verrucosa leaves.

Phytochemical test		**Result**
Alkaloids	Mayer's test	++
Glycosides	Keller-kiliani test	++
Phenols	Ferric chloride test	++
Tannins	Ferric chloride test	++
Flavonoids	Alkaline reagent test	+
Diterpenes	Copper acetate test	+
Steroid	Chloroform test	++
Terpenoids	Sulfuric acid test	-
Saponins	Olive oil test	-

*Key: + indicates presence; - indicates absence. ++ >+: indicates the intensity of the characteristic color or precipitate.

3.2 Glucose adsorption capacity of *Crotalaria verrucosa*

The experiment revealed a clear glucose adsorption ability of the plant extract. The test was conducted by adding different amount of plant extract in glucose test solutions. Using UV-visible spectroscopy to measure the glucose concentrations it was evident that if the amount of plant extract was increased the amount of glucose decreased.

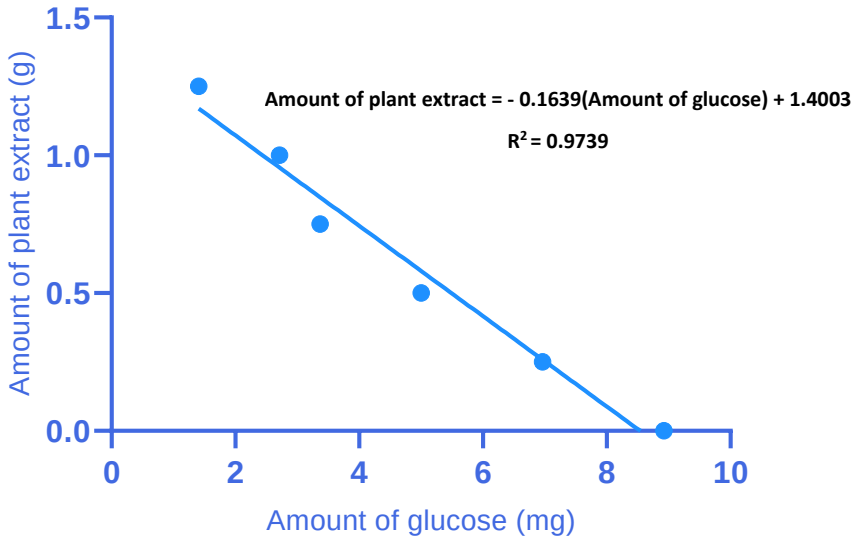

Figure 16: Effect of plant extract on glucose adsorption

Figure 16 was constructed by plotting amount of plant extract versus amount of glucose in the test solutions. Highest amount of glucose (8.92 g) was observed when there was no plant extract in the solution. The glucose amount gradually decreased and eventually reached its lowest (1.40 g) when the plant amount was 1.25 g. These variables had a negative gradient and the straight line had a strong R squared value.

3.3 Effects of *Crotalaria verrucosa* leaves on sucrose absorption from the gastrointestinal tract

The gut motility study makes it evident that the ethanolic plant extract had no effect on the gut motility of the rats. The relative distance travelled by $BaSO_4$ milk (92.68% in control versus 84.04% in treated) was not significantly different ($P > 0.05$).

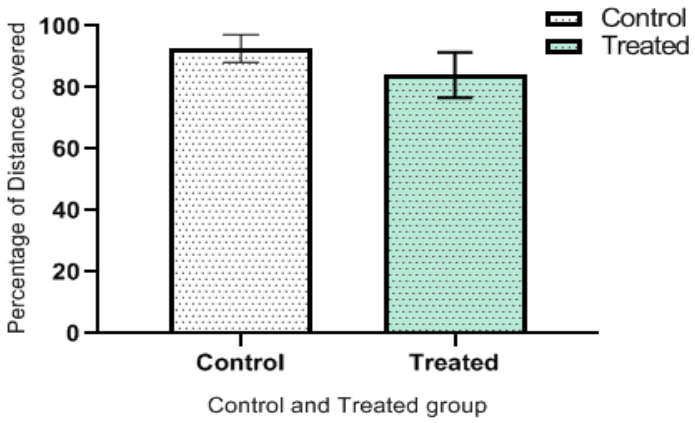

Figure 17: Effect of Crotalaria verrucosa on gut motility

It can be seen from figure 17, the mean percentage of distance covered in control and treated group is very similar. The horizontal lines represent mean values and the vertical bars show the standard deviation (n=3).

The six-segment test revealed that the sucrose contents in the three parts of the small intestine and caecum between control and treated group were similar. However, the sucrose content in the stomach was significantly higher in the treated group (10.43 mg versus 3.06 mg in the control; P = .022) and in the large intestine where it was higher in the controlled group (3.65 mg versus 1.12 mg in the treated group; P = .036).

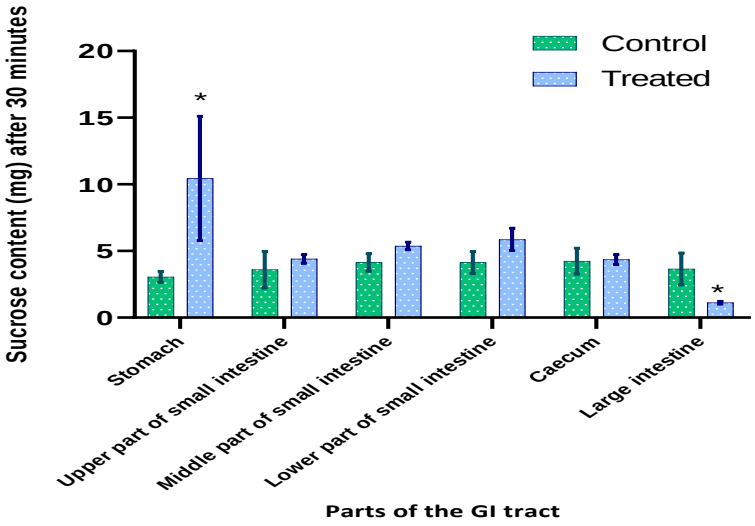

Figure 18: Effects of Crotalaria verrucosa on gastrointestinal sucrose content

Figure 18 outlines the amount of sucrose found by glucose-oxidase method in different parts of the small intestine in control and treated group. Values are means and standard deviations represented by vertical bars (n=3). Mean values marked with an asterisk (*) were significantly different from those of control rats ($p<0.05$). The graph clearly points out that in the stomach and in the large intestine there was a significant difference in the amount. Other than that, the amounts were similar.

Chapter 4

Discussion

We conducted this research to find potential anti-hyperglycemic ability of *Crotalaria verrucosa* leaves. It revealed different phytochemicals which can contribute to the anti-hyperglycemic activity of the extract. In addition, during in-vitro studies the extract showed potential glucose adsorption ability. However, in-vivo studies revealed no anti-hyperglycemic potential of this plant extract.

This study found that alkaloids, carbohydrates, phenols, tannins and steroids were present in abundant. However, flavonoids and diterpenes were present poorly. Terpenoids and saponins were absent. Previously conducted phytochemical screening indicated the presence of presence of glycoside, alkaloids, flavonoids, phenols and tannins (Ahmed, 2016). However, in our study flavonoids were present poorly as the alkaline reagent test produced a light-yellow colored solution. This difference can be due to the alkaline reagent being prepared few days before the test. As a result, the integrity of the reagent was hampered which could have caused misleading results. The constituents of the plant can help reduce blood glucose as a number of phytochemicals has been known to regulate glucose levels in blood. These phytochemicals can control the metabolic flux among organs. In rats and other mammalians, the level of wide range nutrient such as glucose, amino acids, fatty acids and related enzymes are sensed by the different types of cells. The phytochemicals reduce and maintain normal blood glucose level by delaying gastric emptying rate, reducing active transport across brush border membrane and inhibiting starch digestion. The GLUT1 facilitated absorption of glucose has been found to be decreased in different polyphenols and saponins. Saponins further prevent glucose transition from the stomach to the small intestine, thus decreasing glucose absorption. Glycosides are also recognized as a possibly hypoglycemic compound. Many, anti-hyperglycemic impacts are found in previous experiments. Examples in this

category include p-sitosterol-D and stigmadine glycoside. These phytochemicals can also help reduce the impact of diabetes in the body. The depletion and contribution of antioxidants to cardiovascular diabetes complications is well documented (Bailey & Day, 1989). Several trials have shown a substantial reduction of anti-oxidants in diabetes causing various problems such as endothelial dysfunction and atherosclerosis. These plasma antioxidants include lutein, zeaxanthin, retinol as well as ascorbic acid. Low levels of plasma antioxidants are even more pronounced in elderly diabetic subjects. Thus, there is a real need of using antioxidants for prevention and treatment of diabetic complication. Flavonoids and terpenoids can be used in these cases. Flavonoids has shown to reduce ROS production by inhibiting several ROS producing enzymes (xanthine oxidase, cyclooxygenase) (Dembinska-Kiec, Mykkänen, Kiec-Wilk, & Mykkänen, 2008). Furthermore, diabetes causes oxidative stress of tissues. These condition can be improved by treating with tannins (Kumari & Jain, 2012). However our study had found the presence of steroids which can often lead to worsen diabetic situation by increasing blood glucose level (UK, 2016).

In a previous study conducted on diabetic induced Wistar rats by alloxan monohydrate showed that the ethanolic extract of *Crotalaria verrucosa* showed significant anti-diabetic activity at 500 mg/kg doses. However, experiments demonstrating the effects of the plant on glucose adsorption and on sucrose absorption from the gastrointestinal tract were not conducted previously (Nawrin et al., 2015).

In-vitro studies involving glucose adsorption capacity of the plant clearly demonstrated glucose being bound by dietary fibers available in *Crotalaria verrucosa* even at low glucose concentrations. It is established that dietary fibers can improve glycemic control, decreases hyperinsulinemia, and lowers plasma lipid concentration. They can also adsorb macronutrients such as glucose, sucrose, amino acids and fatty acids. Glucose is absorbed into the blood stream by transport protein. However, when bound with the dietary fibers of

the plant extract glucose cannot fit in the transport protein. This results in reduced glucose absorption and eventually reduces the blood glucose level (Kabir et al., 2014).

The gastrointestinal sucrose absorption is dependent on three variables. Firstly, there is the adsorption capacity of the extract. The extract had potential adsorption capacity as shown in figure 16. Therefore, alterations in sucrose absorption can be due to the adsorption capacity of the plant. Secondly, on the gut motility which remained unchanged between the two groups. Increased gut motility means the meal gets less time to be absorbed. This can be effective as sucrose is less broken down by sucrase and absorption reduced. On the other hand, if the gut motility is reduced sucrose gets more time to get broken down and absorption is increased (Kabir et al., 2014). Since CVLE had not changed gut motility significantly it did not had impact on the absorption of glucose.

The effect of *Crotalaria verrucosa* on sucrase activity was determined by six-segment assay. Six-segment test revealed that sucrose content was significantly higher in the stomach of the treated group. However, sucrose along with other disaccharides and complex carbohydrates are mainly absorbed in the small intestine. Sucrose itself does not get absorbed due to lack of sucrose carriers in the gastrointestinal tract. Disaccharidase enzymes which break down disaccharides into monosaccharaides are found in the small intestine. There the sucrose is broken down and absorbed. Literally no absorption takes place in the stomach. Therefore, despite having a higher amount of sucrose in the treated group, CVLE had no effect on the absorption. The high of amount of sucrose in the stomach can be due to the adsorption of sucrose by the plant extract itself. We can see from figure 18 that the amount of sucrose is slightly higher in the small intestinal parts as well. The reason cannot be due to alterations in gut motility because there was no significant difference in gut motility established from the gut motility test. However, we have found from the glucose adsorption capacity test that the amount of glucose is decreasing as the amount is plant extract increased. Therefore, we can

interpret that the high amount of sucrose in the stomach and slightly higher amount of sucrose in the gastrointestinal tract are due to the adsorption of sucrose from gastrointestinal tract (Khattak & Khan, 2018).

Sucrase is a disaccharidase enzyme which breaks sucrose into glucose and fructose. Sucrase along with other disaccharidase enzymes is mainly released in the small intestine where glucose absorption takes place. Therefore, the absorption of glucose is mainly determined by these disaccharidase activities and any inhibition of sucrase activity will result in higher amounts of sucrose in the intestine resulting in reduced absorption. This study revealed that there is no significant difference in the amount of sucrose found in the upper, middle and lower parts of small intestine between the control and treated groups which can be interpreted as no change in the sucrase activity. CVLE had shown no effect on the catabolic activity of sucrase which breaks down sucrose. Almost all of the carbohydrates, except for dietary fiber and resistant starches, are efficiently digested and absorbed by the small intestine. Furthermore, amount of sucrose in caecum was also similar in the two groups. Absorption does not take place in caecum and the sucrose content is eventually eliminated in feces. However, some remaining sucrose are broken down by enzymes released by bacteria in the large intestine. The products of bacterial digestion are short-chain fatty acids and some gases. Sodium and water absorption are also stimulated by the unabsorbed sucrose in the colon. These fatty acids are mainly eliminated in the feces or used up by bacteria. Some of these fatty acids are absorbed by the colon and transported to the liver. This absorption does not immediately affect the blood glucose content as the fatty acids are mainly stored in the liver. This assay revealed that there is low amount of sucrose in the large intestine in the treated group which can be interpreted as increased fermentation by bacteria resulting in higher absorption of sucrose as fatty acids. This however does not affect the blood glucose as most of the sucrose is eliminated or used up by bacteria.

This study showed potential ability of glucose adsorption in the plant extract. However, negative in-vivo observations ultimately tell us that the plant extract poses no potential anti-hyperglycemic effect.

This research included certain limitations. First of all, the number of animals in each group during the in-vivo studies was three which is low when we compare it with other similar studies. This low number of samples can often lead to misleading findings. The six-segment assay was not done in different time intervals. Therefore, the effect of the plant was not observed over a course of time. Secondly, the study did not directly observe the concentration of sucrase secreted by the intestinal cells rather we interpreted sucrase activity from the amount of residual sucrose. In addition, the study also did not include the observations of amylase activity which breaks down complex carbohydrates. Furthermore, glucose adsorption capacity of the plant was determined in-vitro. This can lead to misleading understanding of the result as in-vivo conditions are quite different.

Chapter 5

Conclusion

This study conducted on Long Evan rats provides information regarding chemical constituents and effects on the absorption of sucrose from the gastrointestinal tract of a medicinal plant. Our study found new observations of this plant and also confirms previous findings. This study provides information about the chemical constituents which have potential anti-hyperglycemic capacity. Furthermore, it showed promising glucose adsorption capacity. On the other hand, the leaf extract did not produce disaccharidase inhibitory activity or increase gut motility. As a result, the leaf extract had no effect on the sucrose absorption from the gastrointestinal tract. Based on these facts we can conclude that the plant extract was not able to produce the anti-hyperglycemic activities we desired. However, this study provided important insights to the medicinal properties of the plant and hopefully will pave the way for future studies on this plant.

Chapter 6

Future directions

This research revealed a lot of information regarding anti-hyperglycemic potential of *Crotalaria verrucosa*. However, there are still scopes of future studies.

Firstly, the effect of the plant extract on insulin secretion can be determined by isolating pancreatic islets and measuring the amount of insulin released after administration of the extract. Secondly, using in-situ perfusion technique the glucose absorption from the intestine can be measured. Furthermore, the effect of the plant on disaccharidase enzymes can be fully established by measuring the exact amount of disaccharidase enzyme. Similarly, the effects can be observed on α- amylase activity.

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