PHYTOCHEMICAL AND ANTIDIABETIC STUDIES OF *PERESKIA BLEO* EXTRACTS

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2017

PHYTOCHEMICAL AND ANTIDIABETIC STUDIES OF *PERESKIA BLEO* EXTRACTS

by

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Thesis submitted in fulfilment f the requirements
for the degree of
Master of Science

July 2017

ACKNOWLEDGEMENT

All praises is to Allah, The Greatest, with His Guidance and Love, I may complete this research.

First of all, I would like to offer my gratitude to my supervisor, Associate Prof. Dr. Roziahanim Mahmud, and my co-supervisor, Prof. Dr. Mohd Zaini bin Asmawi who had always gave me their time, supported me and contributed me their deep knowledge throughout this research.

I would also like to address my sincerely thanks to all staff in School of Pharmaceutical Sciences, especially Mr. Roseli Hassan, Mr Anuar Apandi Ahmad, Mr Fisal Jamaludin, Mr Mohd Jasmie Ikhram Ab Rahaman, Mrs. Azlina Amil and others for their technical and administration assistance, which help me a lot in this research.

I am also very grateful to my colleagues who are always my real friends, especially Norliyana Amran, Syarifah Nor Syakira, Radiah Ahmad and Emad Al-Samarrai for their support and true friendship. One simply could not wish for better friends.

Lastly, special thanks to my parents, Abdul Rani Chik and Fatimah Sam Ghazali, and my family members as without them, I would not be who I am now. Their prayer, support, understanding and endless love had always guide me to finish this research.

And for the rest I could not mention, believe me you are not forgotten.

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ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
b.w	Body weight
DPPH	2,2-diphenyl-1-picrylhydrazyl
FTIR	Fourier Transform Infra-red
FRAP	Ferric Reducing Power
HPLC	High Performance Liquid Chromatography
IC	Inhibition concentration
IDDM	Insulin-Dependent Diabetes Mellitus
IPGTT	Intra Peritoneal Glucose Tolerance Test
LCMS	Liquid Chromatography Mass Spectrometry
LD	Lethality Dose
NIDDM	Insulin-Dependent Diabetes Mellitus
SEM	Standard Error of Mean
STZ	Streptozotocin

LIST OF SYMBOLS

%	Percentage
°C	Degree celcius
A	Alpha
B	Beta

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KAJIAN FITOKIMIA DAN ANTIDIABETIK EKSTRAK

PERESKIA BLEO

ABSTRAK

Kajian ini telah dijalankan bagi mengkaji kesan antidiabetik berserta mekanisma tindakan bagi ekstrak daun *Pereskia bleo*. *Pereskia bleo* yang berasal dari family *Cactaceae* merupakan salah satu remedi yang sering digunapakai dalam perubatan tradisional di Malaysia bagi mengubati beberapa jenis penyakit seperti kencing manis, kanser, tekanan darah tinggi, dan beberapa penyakit berkaitan dengan penyakit sendi dan keradangan. Bagaimanapun, kajian saintifik masih belum mencukupi bagi membuktikan kenyataan ini, terutamanya yang berkaitan dengan penyakit kencing manis. Empat jenis ekstrak telah dihasilkan menggunakan cecair kimia yang berbeza kepolaran, iaitu petroleum-eter, kloroform, methanol dan air. Dalam kajian hipoglisemik dan ujian toleransi glukosa intra-peritoneal (IPGTT), kesemua ekstrak didapati tidak memberikan sebarang kesan secara signifikan dalam menurunkan aras glukosa darah tikus normal. Manakala, dalam kajian lain mengenai kesan antihiperglisemik yang mana tikus yang digunakapakai telah diaruh menjadi diabetik dengan memberikan suntikan streptozotocin sebanyak 50 mg/ kg berat tikus. Pengambilan ekstrak air *P. bleo* telah didapati memberikan kesan signifikan dalam menurunkan aras glukosa darah tikus aruhan diabetik tersebut seawal 6 hari selepas rawatan bermula. Oleh itu, ekstrak air *P. bleo* telah dipilih sebagai ekstrak paling aktif dan difraksikan menggunakan teknik pelarut-pelarutmenjadi fraksi etil asetat, n-butanol dan air. Kesan antihiperglisemik fraksi-fraksi ini telah diuji pada tikus aruhan diabetik yang mana pengambilan fraksi air *P. bleo* telah didapati memberikan

kesan signifikan bagi menurunkan aras glukosa darah. Kajian ini juga mendapati bahawa ekstrak air dan fraksi air *P. bleo* juga telah memberikan kesan signifikan dalam menurunkan aras glukosa darah, lipid berketumpatan rendah (LDL), trigliserida (TG) dan jumlah kolestrol (TC), mengurangkan kesan penurunan berat badan dan juga memberikan kesan signifikan dalam meningkatkan kadar insulin dalam darah. Mekanisma berkaitan kesan tersebut juga telah diuji dan didapati bahawa fraksi air *P. bleo* menujukkan kesan yang lebih menonjol berbanding ekstrak air *P. bleo* dimana ia mampu merencatkan kesan enzim α-amylase secara sederhana dan merencatkan kesan enzim α-glucosidase secara aktif. Dalam ujian antioksidan secara in vitro, ekstrak air dan fraksi air P.bleo menunjukkan kesan antioksidan yang sangat lemah dengan kesan lebih menonjol ke arah ujian β-karotene. Selanjutnya, analisis sektrum LCMS (Kromatografi Cecair Berprestasi Tinggi Spektroskopi Jisim) bagi fraksi air *P. bleo* telah mendapati tiga puncak utama, iaitu N, N-diethylglycine, N-stearoylvaline dan paraxanthine. Secara keseluruhannya, *P. bleo* memberikan kesan antihyperglysemik dengan merangsang perembesan insulin dan dengan merencatkan kesan enzim α-amylase dan α-glucosidase. Berdasarkan kesemua hasil kajian ini menunjukkan bahawa pokok ini berpotensi untuk dijadikan bahan kajian dengan lebih mendalam bagi mengetahui mekanisma yang terbabit bagi kesan antihyperglysemik tersebut.

PHYTOCHEMICAL AND ANTIDIABETIC STUDIES OF *PERESKIA BLEO* EXTRACTS

ABSTRACT

This present study was done to evaluate the antidiabetic activities and the underlying mechanism associated to the observed bioactivity of *Pereskia bleo* leaf extracts. *Pereskia bleo* which belongs to the family *Cactacae* is a well-known traditional remedy in Malaysia for the treatment of diabetes, cancer, high blood pressure and diseases associated with rheumatism and inflammation. However, the scientific information on the usefulness in the medicinal importance of this plant extracts is still lacking, especially on the antidiabetic properties. Four different extracts of differing degrees of polarity of *P. bleo* leaf are selected namely petroleum-ether extract, chloroform extract, methanol extract and aqueous extract respectively. In the hypoglycaemic test and intraperitoneal glucose tolerance test (IPGTT), all extracts were found to be insignificant in lowering glucose level in blood compared to the control group. In antihyperglycaemic test, the diabetic type 2 was induced by injecting 50 mg/kg of STZ in Sprague Dawley rats. The administration of *P. bleo* aqueous extract was found to be significant ($p<0.05$) in lowering glucose level as early as day 6 compared to negative control. Hence, *P. bleo* aqueous extract was chosen as the most active extract and was further fractionated using solvent-solvent extraction into ethyl acetate, butanol and aqueous fractions. These fractions were then being determined for the antihyperglycaemic properties in STZ-induced diabetic rats and the administration of *P. bleo* aqueous fraction was found to be the most potent fraction in lowering the blood glucose level.

Our finding also reveals that *P. bleo* aqueous extract and its aqueous fraction also exhibited significant decrease in the glucose level, low density lipid (LDL), triglyceride (TG) and total cholesterol (TC), significant lowering effect in the decreasing percentage of the body weight and significant increase in plasma insulin level compared to diabetic control. The mechanism underlying observed antidiabetic activity was carried out in vitro and the result indicates that *P. bleo* aqueous fraction had demonstrated a more potent inhibitory agent compared to aqueous extract as it possessed a mild α-amylase inhibitory and strong α-glucosidase inhibitory properties respectively. In in vitro antioxidant studies, *P. bleo* aqueous extract and aqueous fraction exhibited weak antioxidant activity with the preferences towards β-carotene bleaching activity. Further LCMS analysis showed that the major peaks identified in most antidiabetic bioactive fraction, *P. bleo* aqueous fraction were N, N-diethylglycine, N-stearoylvaline and paraxanthine. Overall, *P. bleo* extracts exhibited potent antihyperglycaemic property by stimulating the insulin secretion and inhibiting α-amylase and α-glucosidase activities. All these findings showed the plant extract potentials for further in-depth mechanism study associated with observed antidiabetic properties.

CHAPTER 1: INTRODUCTION

1.1 Introduction

1.1.1 Diabetes

Diabetes mellitus which is a hyperglycaemic condition characterized by high glucose level is a chronic endocrine syndrome associated with abnormality of glucose metabolism, leading to the high triglyceride and lipoprotein levels (Erciyas et al., 2004). This disease is caused by a loss of glucose homeostasis mainly either due to the defects of insulin secretion or the insensitiveness of insulin towards target organs, or both (Maiti et al., 2004; Alarcon-Aguilara et al., 1998). In chronic condition it leads to a long term dysfunction, damage or even failure of several organs in patients, mainly the eyes, nerves, kidney, heart and blood vessels (Poongothai et al., 2011). However this endemic condition has always been underestimated as the underlying cause of death and no proper attention has been given (Fuller et al, 1983).

1.1.2 Diabetes statistics

Diabetes mellitus is now being considered as one of the world's five leading causes of death. In 2010, there were about 285 million people around the world suffering from diabetes and it is increasing throughout the years especially in rural population (Shaw et al., 2010). Even Malaysia today had been known as diabetic capital, where more than 8.2% of its population as diabetic sufferer (Rugayah, 1997). There is an estimated statistics by World Health Organization (WHO) that by 2035, about 592 million of world's population will suffer from this deadly disease especially from low and middle income class populations (International Diabetes Federation Atlas, 2013).

1.1.3 Type of diabetes

The National Diabetes Data Group of the National Institute of Health of Bethesda, Maryland, USA had classified diabetes into 5 types (National Diabetes Data Group, 1979). Type 1 diabetes is usually known as insulin-dependent diabetes mellitus (IDDM). This type of diabetes is associated with the increase and decrease frequency of histocompatibility antigens (HLA). This type of diabetes usually can occur at any age, including juvenile. Treatment for this type of diabetic including the administration of insulin which usually be taken by mean of injection or inhalation (Widmaier, 2013).

Type 2 diabetes is the noninsulin-dependent diabetes mellitus (NIDDM). This type of diabetes is very common worldwide cover up at least 90% of the diabetic statistics (Widmaier, 2013). This type of diabetes can be divided into two subclasses, the obese NIDDM and the nonobese NIDDM. Type 2 diabetes usually occurs in elderly stage of life. The therapy for this type of diabetes usually involved some combination of insulin or drug which can increase the insulin sensitiveness (Widmaier, 2013).

Other types of diabetes are type 3, type 4 and type 5 which are rare in population. The diabetes caused by other conditions is being classified as type 3 diabetes mellitus. It is diabetes associated with certain condition and syndromes which can be divided to known or suspected relationships. Type 4 diabetes is the gestational diabetes which is restricted to pregnant women with glucose intolerance developed or discovered during the pregnancy. The last type of diabetes is type 5, where the

individuals have plasma glucose levels that are at intermediate level between being diabetic and normal. This condition is being referred as impaired glucose tolerance.

1.1.4 Causes of diabetes

The main factor leading to diabetes is based on the insulin, whether insulin resistance, insulin action or both (Maiti et al., 2004; Alarcon-Aguilara et al., 1998). In type 1 diabetes mellitus, it is due to the insulin deficiency which happen because of the total or near-total destruction of pancreatic β-cells, occurs as the auto-immune response of body's white blood cells (Widmaier, 2013). The therapy involved insulin intake via parenteral injection as the body failed to produce by its own. Meanwhile, in type 2 diabetes mellitus, the diabetic condition occurs as the inadequate secretion of insulin which may be due to the defects or compromised function of β-cells, and thus failed to normalise the plasma glucose concentration (American Diabetes Association, 2010). Besides, the other factor leading to the type 2 diabetes mellitus is due to the target organs or cells which become hyporesponsive towards the insulin, or being known as insulin resistance (Widmaier, 2013). It is defined as the impaired ability of the insulin to control the glucose in the target organs (Oberley, 1988). These conditions, the insulin secretion impaired and insulin action defects usually coexist in one person (American Diabetes Association, 2010).

1.1.5 Diabetic induced in laboratory

Although there are about 5 types of diabetes occur, only type 1 and type 2 diabetes (IDDM and NIDDM respectively) were common in scientific research on natural products. Experimental diabetes had been induced in laboratory research on

animal model through chemicals which selectively destroy the β-cells in pancreas, thus reducing the total insulin secretion. Alloxan and streptozotocin are the most common chemicals used to evoke such diabetes condition.

1.1.5(a) Mechanism of action of alloxan

Alloxan or 2,4,5,6-tetraoxypyrimidine was known to exhibit the diabetogenic properties and commonly used in laboratory research to induce type 1 diabetes mellitus or insulin de pendent diabetes mellitus (IDDM) in animal model (Dunn et al., 1943). Alloxan were administered to animal through parenterally either by intravenous, subcutaneous or intraperitoneal injection (Szkudelski, 2001). The alloxan were taken up by insulin-secreting cell rapidly and formed a reactive oxygen species in β-cells (Heikkila et al., 1976). The alloxan will get reduced prior to the reactive oxygen species formation and leading to the formation of disulphide bond and inactivation of enzymes (Szkudelski, 2001). The diabetogenic action of alloxan on β-cells in pancreas were either by oxidation of essential -SH groups, free radical formation, glucokinase inhibition or intracellular calcium homeostasis disturbances (Szkudelski, 2001). However, the alloxan action is not so selective and the range of diabetogenic dose is quite narrow making alloxan use to induce diabetic in animal model is a difficult dosage optimization task.

1.1.5(b) Mechanism of action of streptozotocin

Streptozotocin (STZ) or 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose is synthesised by *Streptomycetes achromogenes*. STZ administration dose for diabetogenic was not as narrow as alloxan and was widely used in laboratory research to induced both type 1 (insulin-dependent) and type 2 (non-insulin-dependent) diabetes mellitus (IDDM and NIDDM respectively) in animal

model (Szkudelski, 2001). STZ will destroy the β-cells and inhibits its insulin secretion through its alkylation potency of DNA (Lenzen, 2008; Delaney et al., 1995; Elsner et al., 2000). The STZ is more selective on the β-cells specificity compared to alloxan, making it as the primary chemical-inducing diabetic agent in animal model (Lenzen, 2008).

1.1.6 Treatment of diabetes

1.1.6(a) Oral hypoglycaemic drug

There are several classes of oral antidiabetic drug, which had been classified based on their effects, mechanism of action and adverse reaction on diabetes condition which are sulfonylureas, biguanides, alpha-glucosidase inhibitors, meglitinides, thiazolidinediones and many more. The main goals of clinical therapy on diabetic patients are to achieve better glucose level and to prevent the long term diabetic complication (Turner et al., 1999). In comparing to the newer agents such as alpha-glucosidase inhibitors, meglitinides, and thiazolidinediones, the older agents such as sulfonylureas and biguanides are cheaper and exhibited the same therapeutic values in managing the glycaemic level (Bolen et al., 2007).These clinical drugs were usually being prescribed alone as monotherapy or by combination of two or more depending on the diabetes condition (Emilien et al., 1999). Majority of the diabetic patients needs multiple therapies to maintain their glycaemic levels for long term (Turner et al., 1999).

The sulfonylureas improves the glucose management by stimulating the insulin secretion (Pfeifer et al., 1980). Glibenclamide is a potent second generation sulfonylurea drug that improves glucose control by stimulating the insulin secretion

and action (Luzi & Pozza, 1997). It is also one of commercial drugs used to treat diabetes. The principle of action in glibenclamide is proven to be on the β-cells of the islets of Langerhans in pancreas, stimulating insulin secretion and action and thus reducing the plasma glucose concentration. Adverse reaction of clinical treatment of this drug and other sulphonylurea derivatives however showed cardiovascular events in patients with non-insulin dependent diabetes mellitus (Smits, et al., 1995).

Metformin (1.1-dimethylbiguanide hydrochloride), a commercial hypoglycaemic drug for the treatment of diabetes is the only drug that belongs to the biguanide class, which is the guanidine derivatives. Metformin was first extracted from *Galega officinalis* and it is the first-line oral antidiabetic drug for the treatment of type 2 diabetes. Its mechanism of action is through promoting glucose uptake by tissues (via glucose transporters), especially those of skeletal muscles and inhibits hepatic gluconeogenesis. Activation of AMPK (AMP-activated protein kinase) appears to be the main mechanism of its antihyperglycaemic action, but metformin also acts on small-intestine glucose metabolism (Bouchoucha et al, 2011).

The other class of oral antidiabetic drug is the α-glucosidase inhibitors. This class of drug functions directly by inhibiting those enzymes responsible for the glucose metabolism and thus delay the digestion of carbohydrate, prolong the time needed for the digestion and thus will reduce the glucose absorption rate that will aid in preventing the postprandial glucose rise in blood (Rhabasa-Lhoret & Chiason, 2004). The drugs which fall under this class include the acarbose, miglitol and voglibose. The long term and continuous intake of this class of drug should be controlled and limited as it produce adverse reaction such as abdominal cramps, diarrhoea,

flatulence, vomiting and even some serious incidence like acute hepatitis and renal tumors (Hanefeld, 1998; Diaz-Gutierrez et al., 1998). This adverse reaction is the result of the bacteria fermentation of undigested carbohydrate in colon (Bischoff, 1994).

1.1.6(b) Insulin

Insulin is a key regulating hormones that control the blood glucose concentration in body. In normal condition, β-cells in pancreas will secrete the insulin. However, in certain condition, as diabetes, the body failed to secrete the insulin, or secrete inadequately to lower the glucose level to normal level. Hence, for some type of diabetes, especially for type 1 diabetes mellitus, the insulin injection is needed in managing the diabetes condition (Widmaier, 2013). Insulin secretion and action are based on the plasma glucose concentration (Widmaier, 2013).Insulin acts in body by enhancing the glucose transport through the cell membrane and facilitating the glucose metabolism, and thus will lower the glucose level in blood (Dimitriadis et al., 2011).This hormone affects majorly on muscle and adipose tissue by aiding in carbohydrate metabolism, lipid metabolism and protein metabolism. In carbohydrate metabolism, the insulin increases the transportation rate of glucose through the cell membrane, increases the glycolysis (glucose breakdown to pyruvate or lactate) rate by stimulating the hexokinase and 6-phosphofructokinase activity, increases the glycogen synthesis rate and inhibits the glycogenolysis (glycogen breakdown to glucose) and gluconeogenesis (formation of glucose from pyruvate, lactate, glycerol or amino acids) rate (Dimitriadis et al., 2011). Upon lipid intake, the insulin acts by decreasing the lipolysis and fatty acid oxidation rate, stimulates the synthesis of fatty acid and triacylgylcerol and increases triacylglycerides uptake by

adipose tissue and muscle (Dimitriadis et al., 2011). Insulin which also acts on protein metabolism also increases the amino acids transport rate, increases the protein synthesis rate and decrease the protein degradation rate in muscle (Dimitriadis et al., 2011). The overall insulin mechanism is an effective therapy in managing the glucose level in blood especially after carbohydrate consumption, since the increasing postprandial blood glucose holds more risk to cardiovascular disease as compared to fasting glucose level (Narkhede et al., 2011).

1.1.6(c) Diet control

Beside the antidiabetic drugs, the diabetic patients are highly recommended to control their diet in order to achieve a well maintained glycaemic index to prevent the associated risk. Some of the diabetic sufferer can maintain their blood glucose level just by controlling the diet alone as a short term therapy. The intensive diet program as early as the diabetes is diagnosed is likely to improve glycaemic control and thus manage the blood glucose level (Andrews et al., 2011). The diet controlled program which includes eating a balanced diet, taking appropriate snack to prevent hypoglycaemia, avoiding high glucose intake, maintaining food portion and eating time, avoiding high saturated fat food intake and supplementation with appropriate dietary fibres may be beneficial in aiding the diabetic management (Chase, 1979).

1.1.6(d) Exercise

Exercise has the key important role in managing diabetes. Together with the clinical drug, a controlled diet and an appropriate physical activity program can be a better therapy to improve glycaemic control and reduce the cardiovascular risk associated with diabetic (American Diabetes Association, 2002). This recommendation is proposed as the benefits of exercise may underweight the

associated risk. The physical activity may improves body fitness and thus affect the metabolic response. Upon frequent endurance exercise, the insulin sensitiveness may increase as it increases the total number of plasma membrane glucose transporter and hence aids in diabetic management (Widmaier, 2013). Therefore, self-monitoring of blood glucose level is highly recommended to diabetes patients who embark on this exercise program as to prevent the hypoglycaemic condition which may be fatal (American Diabetes Association, 2002).

1.1.7 Natural products as the alternative source for human diseases treatment

Natural products have been playing a dominant role in the discovery of leads for the development of drugs for the treatment of human diseases. An encouraging report from world ethnobotanical information about medicinal plants states that as many as 800 plants are being used in the control of diabetes mellitus to date (Alarcon-Aguilara et al., 1998). Many of the currently available drugs have been derived directly or indirectly from plant. Indeed, the vast majority of the existing antidiabetic drugs are based on natural products such as metformin which originates from *Galega officinalis*, and this fact anticipates that new more effective leads may certainly emerge from the tropical plant sources, since biological chemodiversity continues to be an important source of molecular templates in the search for new antidiabetic agents. Natural products are a primary source to obtain cheap and alternative antidiabetic drug. Moreover, the need for new safe template is needed as existing clinical drugs for the treatment of diabetes can produce a number of adverse reactions among diabetes patients.

1.1.8 *Pereskia bleo*

Pereskia which belongs to the family *Cactaceae* is the only genus in this family that is leafy and not a desert-adapted cactus. This genus was named as an honour to a French botanist in 16th century, Nicolas Fabre de Peiresc (Hassanbaglou et al., 2012). There are 17 species that are fall under the *Pereskia* genus with two sub groups. Most of them were economically unimportant and some were considered as troublesome to the biodiversity (Campbell, 1988). However, there are three species that are traditionally popular for their medicinal properties which are *Pereskia aculeata* (Miller), *Pereskia grandifolia* (Haw) and *Pereskia bleo* (Kunth). In Malaysia, *Pereskia grandifolia* and *Pereskia bleo* were locally known as Jarum Tujuh Bilah and usually cultivated as an ornamental plant. *P. grandifolia* and *P. bleo* can visually distinguished by the colour of their flowers which are purple coloured in *P. grandifolia* and orange-red coloured in *P. bleo*.

1.1.9 Morphological decription of *Pereskia bleo*

Pereskia bleo is a spiny shrub with lot of oval-in-shape, glossy, green in colour leaf (Figure 1.1 and Figure 1.2). Locally known as Jarum Tujuh Bilah (seven needles), this plant has a lot of spines that are usually seven in average for one spot. It produces visible fruits with waxy hemispherical in shape, which is green in colour and will turn to yellow upon ripening. The medium size orange-red coloured flower will bloom during late afternoon and only lasts for one day (Sim et al., 2010). This species prefer dry and full sun condition and can be cultivated by their stem cutting. The complete taxonomy classification of this species is as tabulated in Table 1.1. Traditionally, it was claimed to be useful in treating several diseases such as diabetes, rheumatism, inflammation, ulcer, gastric pain, tumor, cancer, hypertension,

anti-snake bite and revitalizing the body (Wahab et al., 2009; Sharf et al., 2013). The local folks usually consumed it as a brewed concoction or as raw leaf salad. There was a preliminary survey done in Kelantan that reported *P. bleo* leaf wide use by local diabetic patient for lowering the blood glucose level (Khor et al, 2013).

Table 1.1: Taxonomic classification of *Pereskia bleo*

Taxonomic classification	
Kingdom	*Plantae*
Phylum	*Tracheophyta*
Class	*Magnoliopsida*
Order	*Caryophyllales*
Family	*Cactaceae*
Genus	*Pereskia*
Species	*Pereskia bleo*

Figure 1.1: *Pereskia bleo* tree.

Figure 1.2: a) The leaf and flower of *Pereskia bleo*; b) the ripe fruit of *Pereskia bleo*; c) the spine of *Pereskia bleo* stem.

1.1.10 Pharmacological review on *Pereskia bleo*

Recently, several scientific research have been done to evaluate the potent bioactivity of the plant extract to justify its traditional claims. Ahmed et al. (2014) demonstrated that after single oral administration of 250 mg/kg of body weight aqueous and ethyl acetate extracts of *P. bleo* significantly lowered the blood glucose level of alloxan induced diabetic rats approximately 24 hours following treatment. The study also showed that both extracts inhibited α-amylase and α-glucosidase activities *in vitro*. In an acute oral toxicity study, Sim et al. (2010a) reported that *P. bleo* could be regarded as non toxic in experimental mice with lethality dose, LD_{50}>2500mg/kg.

P. bleo methanol extract was found to be a strong cytotoxic agent against human mammary carcinoma cell, T-47D via apoptosis mechanism by the activation of

caspase-3 ans c-myc pathways (Tan et al., 2005). Other studies by Malek et al., (2008 and 2009) also found that *P. bleo* ethyl acetate and methanol extracts showed significant effect of cytotoxicity in human nasopharyngeal epidermoid carcinoma cell line (KB).

P. bleo also exhibited potent antioxidant activity. In less polar solvent extraction methods, several studies also found that the *P. bleo* hexane extract exhibited strong 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenger capability and significantly reduced the ferric chloride in Ferric Reducing Power (FRAP) assay, while ethyl acetate extract of *P. bleo* showed significant effect in DPPH scavenging activity, in β-carotene bleaching assay, and in 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay (Sim et al., 2010 (a); Sim et al., 2010 (b); Hassanbaglou et al., 2012; Wahab et al., 2009; Bakhari et al., 2010). This plant butanol extract also was reported to possessed good antioxidant properties in ABTS assay (Lee et al., 2009).

P. bleo also exhibited some antimicrobial and anti-noniceptive activities as reported by Philip et al. (2009) which found that *P. bleo* extracts possessed moderate inhibition activity against *Pseudomonas aeruginosa* and some gram-negative bacteria. In another study, *P. bleo* hexane extract also showed significant effect in anti-noniceptive activities as in formalin-induced test, plant ethyl acetate extract showed lowest number of contortions in acetic acid-induced abdominal writhing test while butanol extract exerted anti-noniceptive activity in the hot plate test (Wahab et al., 2012).

1.1.11 Phytochemical review of *Pereskia bleo*

The chemical constituents in *P. bleo* had been evaluated by several studies associated with certain bioactivities. Earlier, Doetsch et al. (1980), showed four constituents of alkaloids were isolated from *Pereskia* plant namely 3,4-dimethyl-β-phenethylamine, mescaline, 3-methoxytyramine and tyramine respectively. Malek et al. (2008; 2009) then isolated several cytotoxic components in *P. bleo* ethyl acetate fraction through bioactivity guided fractionation namely dihydroactinidiolide, mixture of sterol (compasterol, stigmasterol and β-sitosterol), 2,4-di-tert-butylphenol, α-tocopherol and phytol. In another research, Wahab et al. (2012) was then successfully isolated three compounds namely vitexin, β-sitosterol glucoside and β-sitosterol from *P. bleo* ethyl acetate, dichloromethane and hexane extracts respectively and were further tested on anti-noniceptive activities.

Subsequently, several quantitative phytochemical screenings were also reported. The *P. bleo* ethyl acetate extract was found to exhibit higher total contents of phenolics compared to other solvents extracts (Sim et al., 2010b). In other study, the catechin, epicathecin, quercetin and myricetin contents were identified as the major flavonoids in *P. bleo* (Hassanbaglou et al., 2012).

1.2 Rationale of the study

Diabetes mellitus has become one of the major health problems, which is affecting nearly 285 million of the world population (Mustaffa et al, 2011). To date, it has become an increasingly important cause of mortality in population of many countries. Moreover, the need for a new template or lead compound is now important as existing clinical drugs for the treatment of diabetes have been producing

a number of adverse reactions. For example, cardiovascular events in patients with non-insulin dependent diabetes mellitus are prevalent among the widely prescribed sulphonylurea and its derivatives(Smits et al., 1995).

For decades, diabetic patients in less developed nations have relied on oral treatments of traditional medicines sourced by the various types of local plant extract (Singh et al., 2001) even though no scientific data accompanied these medicinal claims. Malaysia which is rich biodiversity forest is endowed with a variety of herbal medicines but yet, the scientific evidence is still lacking. To date, there is a need to investigate *Pereskia bleo* extract as antidiabetic agent based on the traditional medication, as no clear scientific data has linked the plant constituents to its observed antidiabetic effect as local traditional remedy. Hence, this present study will provide valuable data regarding medicinal properties of Malaysia herbal plant extract. Identification of new local medicinal plant bioactives and their mechanism of action will produce new lead compounds or template as effective, highly therapeutic new agents for pharmaceutical industry. This may also contribute to production of potential nutraceutical products in prevention of the chronic disease and to combat against diabetes. All *Pereskia bleo* extract baseline data together with findings on a fully elucidated mechanism of action will also contribute to a complete government Herbal Monograph.

Pereskia bleo, a green leafy cactus with distinctive orange-red flower, has been introduced as an ornamental plant due to its beautiful flowers. This plant is a tropical adapted plant, it grows well in Malaysia climates and can be cultivated extensively in this country.

1.3 Hypothesis of the study

A survey in Kelantan reported that *P. bleo* leaf was widely used to treat diabetes mellitus (Khor et al., 2013). Therefore, it is hypothesized that *P. bleo* leaf extract could lower blood glucose level of diabetic rats with various mechanism of action contributed by active compounds in the leaf.

1.4 Objectives of the study

The objectives of this present study are:

- To determine the antidiabetic effect of *Pereskia bleo* leaf extracts.
- To extract *P. bleo* using solvents of increasing polarity (i.e petroleum ether, chloroform, methanol and aqueous) and investigate the blood glucose lowering activity of the extracts in normal rats, glucose-loaded rats and streptozotocin induced diabetic rats.
- To fractionate the most active extract into different fractions (i.e. from aqueous extract into ethyl acetate, n-butanol and aqueous fractions) and determine the most bioactive fraction associated with the evaluated antidiabetic activity.
- To determine the blood glucose lowering mechanism and antioxidant activity of the most bioactive extract and fraction.
- To identify the potential antidiabetic bioactive compound(s) in the most active fraction.

CHAPTER 2 : THE BIOASSAY GUIDED EVALUATION ON GLUCOSE LOWERING EFFECT OF *PERESKIA BLEO* EXTRACTS AND FRACTIONS

2.1 Introduction

Diabetes mellitus, a serious endocrine syndrome is one of the five world leading cause of death (Alarcon-Aguilara et al., 1998; Chakraborty & Das, 2010). It is a metabolic disease which is characterized by a decrease in insulin production and insulin insensitivity of target organ or both (Edwin et al., 2004). Diabetes condition is highly associated with defect of glucose metabolism which in turn increases the triglyceride and lipoprotein levels (Erciyas et al., 2004). For clinical treatment, a combination of both oral hypoglycaemic and insulin administrations always been used. However, the adverse reaction observed such as coma and liver and kidney failures have always been observed following treatment (Rajesh et al., 2010).

There are many methods to screen for antidiabetic properties in laboratory and one of them involved using chemical agents to induce animal to create a diabetic condition (Nain et al., 2012). Streptozotocin (STZ) is widely used to induce both insulin-dependent and non-insulin-dependent diabetes mellitus (Szkudelski, 2001). STZ acts by selectively destroying the β-cells in pancreas and thus decreasing the insulin production leading to a diabetic condition (Nain et al., 2012).

World ethnobotanical information reported that a total of 800 plants have been associated with diabetes control but very few have been scientifically studied (Alarcon-Aguilara et al., 1998). There is a much needed effort for new antidiabetic drugs as the existing clinical oral synthetic agents seems to produce debilitating adverse reaction. Nowadays, researchers worldwide are also approaching natural

product as an alternative to drugs that may produce equal or better efficacy, but less or no side effects and relatively low in cost when compared to the established clinical therapy (Nanu et al., 2008).

Hence, in this chapter the antidiabetic properties of *P. bleo* extracts at their varying polarity will be evaluated in *in vivo* studies on normal rats and diabetic induced rats. The hypoglycaemic properties and glucose tolerance ability of plants extracts will be determined in normoglycaemic rats respectively. Then, the antihyperglycaemic properties will be further evaluated on streptozotocin induced diabetic rats for 12 days. The most active extract will be further fractionated and further tested for the antihyperglycaemic properties in diabetic rats. Dose response relationship of the most active fraction will then be tested on the antihyperglycaemic properties. Lastly, the effect of the most active extract and fraction will be further evaluated on the blood glucose level, insulin level, lipid profile and the body weight of the diabetic rats. This is a bioactivity guided fractionation of the plant extracts where further investigation work of the most potent extract and fraction of the plant extracts will be perform using *in vivo* models.

2.2 Materials and Methods

2.2.1 Chemical

Streptozotocin was purchased from the Sigma-Aldrich (USA) company. Glibenclamide and metformin (standard drugs) used were from Hovid Bhd. Kit for insulin assay namely Ultra Sensitive Rat Insulin ELISA Kit from Crystal Chem Inc. (USA). All other commercial reagents and solvents used were of analytical grade.

2.2.2 Plant sample

The leaves of *P. bleo* were collected from one source at Kubang Semang, Bukit Mertajam, Penang and a voucher has been authenticated by a plant expert, Dr. Rahmad Zakaria and deposited at the Herbarium Unit of the School of Biological Sciences, Universiti Sains Malaysia (USM/Herbarium/11609).

2.2.3 Experimental animal

Healthy male Sprague Dawley (SD) rats weighing between 200 to 250 g obtained from Animal Research and Service Centre (ARASC), Universiti Sains Malaysia (USM), Penang, Malaysia were used in this study. All the animals were kept in Animal Transit Room, School of Pharmaceutical Sciences, USM. They were allowed to access to standard food pellet (Gold Coin Sdn. BHD., Malaysia) and tap water, *ad libitum*. The procedures for this study have been approved by the Animal Ethics Committee of USM, Penang with the number of Animal Ethics Approval is USM/ Animal Ethics Approval/ 2013/ (87) (463) (attached as Appendix 2.1 and 2.2).

2.2.4 The bioassay guided evaluation of antidiabetic effect of *P. bleo* extracts

2.2.4(a) Plant material collection and preparation of extracts

The fresh leaves were washed and dried for several days in 40°C temperature control oven. The dried leaves were milled into powder form using Wiley Laboratory Mill apparatus and then extracted by with petroleum-ether using maceration technique at room temperature for 24 hours. The residues were then re-extracted by chloroform, methanol and water serially using the same technique. Each organic extracts were then filtered using Whatmann no 1 filter paper and concentrated on rotary evaporator (Eyela) in 40°C. Aqueous extracts was evaporated

off at 60°C. The aqueous extract was further dried out completely using freeze drier machine (Labconco) to yield dry powder form. All the four extracts were stored in freezer until it is ready to be used.

2.2.4(b) Hypoglycaemic test in normal rats

A group of 36 overnight fasted Sprague Dawley rats were equally divided into six groups.

Group One (n=6) served as negative control and received 10% tween 20 (10 ml/kg body weight (b.w))

Group Two (n=6) served as positive control and received glibenclamide (10 mg/kg b.w)

Group Three (n=6) received petroleum-ether extract of *P. bleo* (1000 mg/kg1000 mg/kg b.w)

Group Four (n=6) received chloroform extract of *P. bleo* (1000 mg/kg1000 mg/kg b.w)

Group Five (n=6) received methanol extract of *P. bleo* (1000 mg/kg1000 mg/kg b.w)

Group Six (n=6) received aqueous extract of *P. bleo* (1000 mg/kg1000 mg/kg b.w)

The treatments were given orally. Blood samples were withdrawn from tail vein at 0, 1, 2, 3, 5 and 7 hours following treatment and blood glucose levels were determined using glucose meter (ACCU-CHEK® Performa).

2.2.4(c) Intra peritoneal glucose tolerance test (IPGTT)

A group of 36 male Sprague Dawley rats were equally divided into six groups and were fasted overnight.