PHYTOCHEMICAL AND *IN VITRO* ANTIDIABETIC STUDIES OF CRUDE ETHANOLIC EXTRACTS OF THE STEM OF *Piliostigma thonningii.*

BY

AKANDE, AYOMIDE MARY

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SUPERVISED BY

MR MUSA ABUBAKAR O.

2024/2025 SESSION

CERTIFICATION

This is to certify that this project was carried out by AKANDE, AYOMIDE MARY with matriculation number ND/23/SLT/PT/0578 and it was read and approved as meeting the requirements of Department of Science Laboratory Technology, Institute of Applied Science, Kwara State Polytechnic, Ilorin, for the Award of National Diploma (ND) in Department of Science Laboratory Technology.

_____ _____

MR. MUSA ABUBAKAR O. **DATE**

Project Supervisor

_____ _____

MR. LUKMAN, I. A. **DATE**

PT. Coordinator

_____ _____

DR. USMAN ABDULKAREEM **DATE**

Head of Department

_____ _____

External Examiner **DATE**

DEDICATION

This project is dedicated to the creator of the earth and universe, the Almighty GOD. It is also dedicated to my parents for their moral and financial support.

ACKNOWLEDGEMENT

I give praise to Almighty God for his wisdom, knowledge and understanding given to me for the success of my National Diploma ND. I absolutely appreciate his mercy, blessings and grace over me and my family.

My gratitude goes to the HOD (DR. Usman Abdulkareem) and all the entire staff of Science Laboratory Technology department (SLT) for their care and support throughout this journey.

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Special gratitude to our parents **MR AND MRS AKANDE** who exhibited immeasurable financial, patience, support, prayers and understanding during the periods in which we were busy tirelessly in our studies. Special thanks go to all our lovely siblings. my sincere appreciation also to my friends and classmate.

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ABSTRACT

In the current research, the dried stem weighing 55g and the root weighing 50g of the *Piliostigma thonningii* plant underwent extraction using 95% methanol. A preliminary screening of the crude extracts revealed the presence of various bioactive compounds such as flavonoids, alkaloids, glycosides, tannins, saponins, terpenoids, and steroids. These compounds play a crucial role in the plant's medicinal properties.

To evaluate the anti-diabetic potential of the stem extracts, several tests were conducted. These included non-enzymatic glycosylation of hemoglobin, glucose uptake assays, and enzymatic inhibitory assessments. The results of these tests indicated that the methanolic extract from the stem exhibited notably higher activity, this suggests that the stem extract may hold more promise in terms of managing diabetes mellitus.

In conclusion, the anti-diabetic effects observed in the methanolic crude extracts of the stem of *Piliostigma thonningii* can be attributed to the synergistic interactions among the diverse phytochemicals present in these plant parts. This finding further supports the traditional uses of this plant in diabetes management. The research underscores the importance of exploring natural sources for potential therapeutic agents and highlights the significance of traditional medicine in modern scientific research.

CHAPTER ONE

1.0 INTRODUCTION

Nowadays, plant research has been the Centre of attraction, as they possess the potential to be used in the pharmaceutical industry. A wide variety of natural materials are been used to maintain health of all living things *(Jani et al., 2009*). Plants are rich source of safe and valuable bioactive compounds, and they have used as medicines from ancient time *(Joshi et al., 2009*). Medicinal plants have been used as traditional healing agent for a number of human diseases in many parts of the world (Prabhat *et al.*, 2010). About 80% of world population is still dependent on traditional medicines. Plant materials consist of different phyto-constituents including flavonoids, terpenoids, steroids, glycoside, tannins, saponins and alkaloids. The widespread use of plants as therapeutic agents in the treatment of various ailments can be traced to the occurrence of natural products with medicinal properties.

In more recently, drug discovery from medicinal plants led to the isolation of early drugs such as cocaine, codeine, digitoxin, and quinine, in addition to morphine, of which some are still in use (Newman; *et al.*, 2000. Butler, 2004; and Samuelsson 2004). Isolation and characterization of bioactive compounds from medicinal plants continue till today.

However, there are other organic compounds produced naturally, with some extraordinary complexity, which are not primary metabolites, particularly those compounds can be isolated from plants or are produced by microorganisms. In fact, some compounds may be formed as the result of a "metabolic accident" or are by-products of the synthesis machinery of the cellular enzymes which are value to man as drugs, herbs, flavourings, poisons, dyes, and so on is undisputed (Chin *et al.*,2006).

1.1 Phytochemicals of Medicinal Plants

Medicinal plants are the most important sources of life saving drugs for the majority of world's population. Almost all medicinal plants possess bioactive compounds which are responsible for the various biological activities they possess. Phytochemicals (from the Greek word phyto, meaning plant) are biologically active, naturally occurring compounds present in plants that provide health benefits to humans more than those ascribed to macro-nutrients and micro-nutrients (Blumberg et al., 1999). They protect plants from disease and damage and play a significant role in plant's colour, aroma and flavor. Generally, the plant chemicals that protect plant cells from environmental hazards such as stress, pollution, drought, UV exposure and pathogenic attack must are being referred to as phytochemicals (Mathai, 2000). Recent studies show that they have roles in the protection of human health, when their dietary intake is significant. More than 4,000 phytochemicals have been cataloged (ACS, 2000) and are classified by protective function, physical and chemical characteristics and about 150 phytochemicals have been fully studied (ACS, 2000). Examples of these chemicals found in plants are alkaloids, flavonoids, saponins, tannins, cardiac glycosides, anthraquinones.

1.2 Classification of Phytochemicals

Phytochemicals can be classified as primary or secondary constituents depending on their roles in plant metabolism. Primary constituents include the common sugars, amino acids, proteins, purins and pyrimidines of nucleic acids, chlrophyll's etc, while plant chemicals such as alkaloids, terpenes, flavonoids, steroids, saponins, phenolicsetc constitute the secondary constituents (Mathai, 2000).

1.2.1 Secondary Metabolites

Plants contain secondary metabolites derived from primary metabolites and these secondary metabolites are only present in certain plant species. Secondary metabolites do not play a role in primary metabolism but rather function as defense for the plants or play a role in giving color to flowers, which may attract

pollinators (Putnam and Rice, 1983). They are considered as non-essential components and are regarded as by-products of primary metabolism. However, scientists use secondary metabolites as prerequisites for new drugs and now more than ever finding new secondary metabolites is an urgent task due to the rapid spreading of bacterial resistance and emergence of multidrug resistant strain (Dickschat 2010). These metabolites include: flavonoids, terpenoinds, alkaloids, steroids, glycosides, tannins and saponins.

1.2.1.1 Flavonoids

Flavonoids are ubiquitous in nature and play various vital roles. Many of these phenolic compounds have antimicrobial activity and are considered to be antioxidants. An important flavonoid found in red wine is known as resveratrol, in fact, flavonoids are classified into different groups based on the degree of oxidation of the 3-C bridge. This classification results in structures belonging to the following: anthocyanins, flavones, flavonols, and isoflavones. In their natural form, the flavonoids also may exist as their corresponding glycosides. During the extraction process the intact glycoside may be isolated or the glycosidic bond is ruptured due to solvent or hydrolysis conditions (Vermerris and Nicholson, 2006).

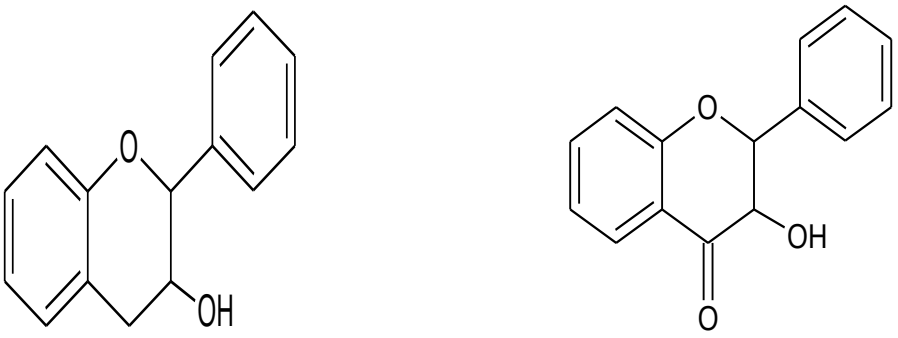


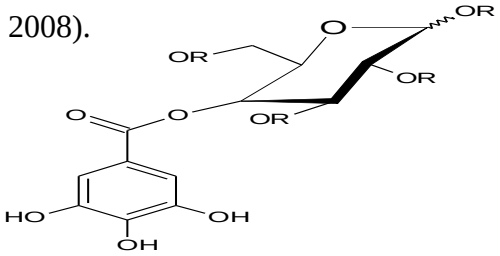
Figure 1: Chemical Structures of some representative Flavonoids

1.2.1.2 Tannins

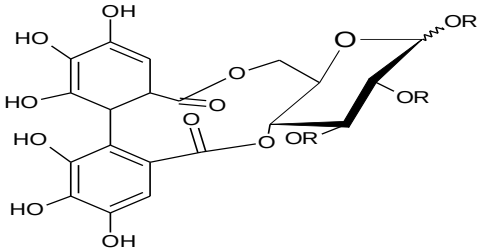
Tannin is the general name given to a large group of complex phenolic substances. The name comes from the leather industry of “tanning” animal hides into leather. Tannins are found in almost every plant part

and are particularly abundant in unripe fruit. It is thought they deter herbivores due to their astringent properties and possess antimicrobial activity (Butler, 2008).

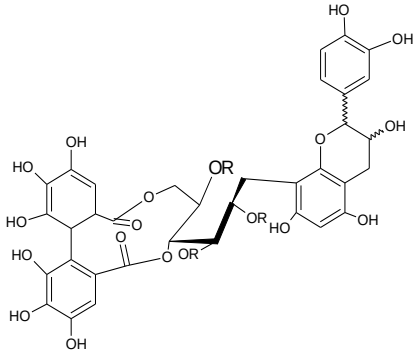
Tannins are naturally occurring polyphenol compounds with molecular weight and ability to precipitate biological molecules like protein, alkaloids, metal ions and other macromolecules like polysaccharide (SalminenJ.P *et al.,* 2001). Based on the basis of their structural characteristics, tannins can be divided into four major groups: Gallotannins, ellagitannins, complex tannins, and condensed tannins (Butler, 2008).



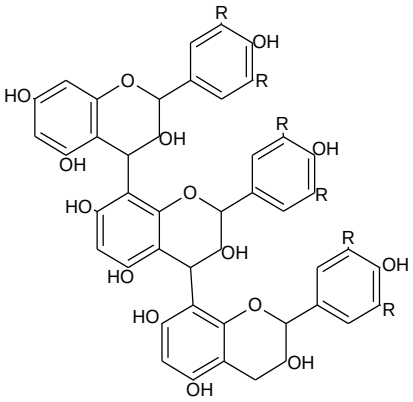
Gallotannin



Egallitannin



Complex tannin
R= Galloyl moiety (G) or other substituents



R= H, OH

Condensed Tannin

Figure 2: Chemical Structures of some representative Tannins.

1.2.1.3 Alkaloids

Alkaloids are a group of organic nitrogenous, which are basically found principally in plant and to a lesser extent in microorganisms and animals (Croteau R *et al*., 2000). Alkaloids are distributed in about 20% of all flowering plants and each plants species accumulate alkaloids in a unique pattern. They are significantly in chemotaxonomy as taxonomic makers (Prachersky, 2000).The most common alkaloids are stimulant caffeine from the coca plants and nicotine from the tobacco plants (Vanwyk and Winkm.2004). Alkaloids are often divided into the following major groups (Fattorusso and Taglialatela, 2008).

1. Alkaloids containing nitrogen in the heterocyclic and originating from amino acids, for example, atropine, nicotine and morphine (Wiley, 2002).

2. "Protoalkaloids" that originate from amino acids, for example, mescaline, adrenaline, and ephedrine (Fattorusso and Taglialatela, 2008).

3. Polyamine alkaloids, for example, spermidine it is a simple linear chain polyamine compound and found in ribosomes and living tissues. Polyamines are polycationic aliphatic amines and serve important roles in cell survival (Wiley, 2002).

4. Peptide and cyclopeptide alkaloids (Fattorusso and Taglialatela, 2008).

Pseudoalkaloids, which do not originate from amino acids, for example, terpene- like and steroid- like alkaloids (Wiley, 2002).

6. Purine- like alkaloids, for example caffeine and theophylline (Wiley, 2002).

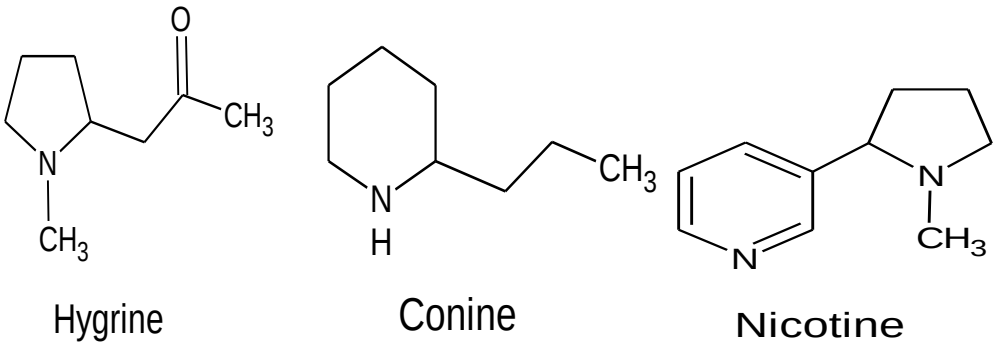


Figure 3: Chemical Structures of some important naturally occurring Alkaloids

1.2.1.4. Saponin

They are also synthesized by lower marine animals and some bacteria *(*Francis *et al.*, 2002*)* they form a stable foam in aqueous solutions such as soap, hence the name "saponin" (Francis *et al.*, 2002). The structure of saponin consist of a sugar moiety (glucose, galactose, glucuronic acid, xylose, rhamnose or methyl pentose) which is linked through a glycosidic linkage and attached to a hydrophobic aglycone (sapogenin) which may be triterpenoid or steroid. Two main types of steroid aglycones are known, spirostan and furostan derivatives

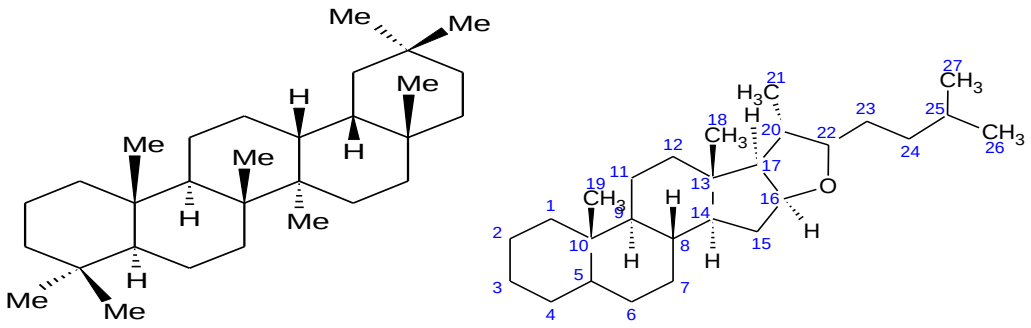


Figure 4: Chemical Structures of some representative Saponins

1.3 Diabetes Mellitus (DM)

The World Health Organization (WHO) defined diabetes mellitus as "a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbances in carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both"(WHO, 1999). Diabetes is a disorder of carbohydrate, fat and protein metabolism attributed to diminished production of insulin or mounting resistance to its action. Its chronic effect occurs either when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin (i.e. hormone that regulates blood sugar) that it produces. Diabetes is becoming the third "killer" of the health of mankind along with cancer, cardiovascular and cerebrovascular diseases (*Chauhan et al., 2010).* Hyperglycemia, or raised blood sugar, is a common effect of uncontrolled diabetes and over the time leads to serious damages to many of the body's systems, especially the nerves and blood vessels. Over the time, diabetes can damage the heart, blood vessels, eyes, kidneys, and nerves. Diabetes increases the risk of heart disease and stroke. 50% of people with diabetes die of cardiovascular disease. Combined with reduced blood flow, neuropathy (nerve damage) in the feet increases the chance of foot ulcers, infection and eventual need for limb amputation. Diabetic retinopathy is an important cause of blindness, and occurs as a result of long-term accumulated damage to the small blood vessels in the retina. One percent of global blindness can be attributed to diabetes. Diabetes is among the leading causes of kidney failure *(WHO, 2016*).

1.4 Classification of Diabetes Mellitus

Insulin is the principal hormone that regulates uptake of glucose into most cells from the blood (primarily muscle and fat cells, but not central nervous system cells), deficiency of insulin or the in sensitivity of its receptors plays a central role in all forms of diabetes mellitus. Therefore, diabetes mellitus as a disease condition is classified into four types depending on their primary causes (*Kenneth, 2006).*

1.4.1 Type 1 Diabetes Mellitus (Insulin dependent Diabetes Mellitus)

This condition may appear at any age, although commonly under 40 years and results due to inadequate production of insulin by the β-cells in the pancreas or abnormality of carbohydrate metabolism, which is linked to low blood insulin level. The hallmark of type 1 diabetes is selective β-cells destruction and severe or absolute insulin deficiency. The impaired insulin action affects fat metabolism, resulting in increased free fatty acid influx and triglyceride levels, and reciprocally low high-density lipoprotein (HDL) levels. Administration of insulin is essential in patients with type 1 diabetes. Insulin dependent diabetes mellitus is further subdivided into those caused by autoimmune and idiopathic factors. The autoimmune form is the most common form of type 1 diabetes. The autoimmune form is mainly triggered by environmental factors such as viruses, diet or chemical exposure in people genetically predisposed. It should be noted that there is no known preventive measure against type 1 diabetes. Diet and exercise cannot reverse type 1diabetes. This type of diabetes can also affect children. The percentage incidence of this type of diabetes in human population ranges between 5 and 15% *(Annette and Jeffrey, 2003; Chauhan et al., 2010).*

1.4.2 Type 2 Diabetes Mellitus

Type 2 Diabetes mellitus, noninsulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes is a metabolic disorder that is characterized by high blood glucose in the context of insulin resistance and relative insulin deficiency. This is in contrast to diabetes mellitus type 1. in which there is an absolute insulin deficiency due to destruction of islet cells in the pancreas *(Kumar et. al., 2005*) The classic symptoms are excess thirst, frequent urination, and constant hunger. Type 2diabetes makes up about 90% of cases of diabetes with the other 10% due primarily to diabetes mellitus type 1 and gestational diabetes. Obesity is thought to be the primary cause of type 2-diabetes in people who are genetically predisposed to the disease. Type 2-diabetes is initially managed by increasing exercise and dietary modification. If

blood glucose levels are not adequately lowered by these measures, medications such as metformin or insulin may be needed. Rates of type 2 diabetes have increased markedly over the last 50 years in parallel with obesity: As of 2010 there are approximately 285 million people with the disease compared to around 30 million in 1985 (Smyth & Heron , 2006) .

1.4.3 Gestational Diabetes

This type of diabetes involves the combination of inadequate insulin secretion and tissue responsiveness, resembling type 2 diabetes in several respects. It develops during pregnancy and may improve or disappear after delivery. During pregnancy, the placenta and placental hormones create an insulin resistance that is most pronounced in the last trimester. Even though it may be transient, gestational diabetes may damage the health of the foetus or mother and about 20 to 50% of women with gestational diabetes develop type 2- diabetes later in life. Gestational diabetes mellitus (GDM) occurs in about 5.5 to 8.8% of all pregnancies (Kenneth, 2006). It is temporary and fully treatable, but if untreated, may cause problems with the pregnancy such as macrosomia (high birth weight), foetal malformation and congenital heart disease. Foetal and neonatal risks associated with GDM include congenital anomalies such as cardiac, central nervous system and skeletal malformations. Increased foetal insulin may inhibit foetal surfactant production and cause respiratory distress syndrome. Hyperbilirubinaemia may result from red blood cell destruction in this type of diabetes. In severe cases prenatal death may occur, most commonly as a result of poor placental perfusion due to vascular impairment (Kenneth, 2006).

1.4.4 Signs and Symptoms of Diabetes

A lot signs and symptoms can mark the onset of diabetes, although they are not specific to the disease. The known one's symptoms include headache, fatigue, blurry vision, slow healing of cuts and itchy skin. Prolonged high blood glucose can cause glucose absorption in the lens of the eye, resulting to change in its shape, resulting in vision changes. A number of skin rashes that occur in diabetes are collectively

known as diabetic dermadromes. (Cooke and Plotnick, 2008).The classic symptoms of untreated diabetes are weight loss, polyuria (frequent urination), polydipsia (increased thirst), and polyphagia (increased hunger) (Cooke and Plotnick, 2008). Symptoms may develop rapidly (weeks or months) in type 1 diabetes, while they usually develop much more slowly and may be subtle or absent n type 2 diabetes (Cooke and Plotnick 2008).

Figure 5: Overview of the most Significant Symptoms of Diabetes

Source: Cooke and Plotnick (2008**).**

1.5. Medicine for Treatment of Diabetes Mellitus

Basic therapeutic approach to treat diabetes may be to inhibit the absorption of glucose by retarding the action of gastro-intestinal enzymes such as α-glucosidase and α-amylase. Because the complication of disease is mainly due to the higher glucose level in blood which dysfunction the other organs of body. Thus we can say that the effective α-glucosidase inhibitors may serves as chemo- therapeutic agents for clinic use in the treatment of diabetes and obesity (Park *et al.*, 2008).

1.5.1 Insulin

Insulin increases glucose uptake in cells by stimulating the translocation of the glucose transporter GLUT4 from intracellular sites to the cell surface (Saltiel *et al.,* 2001) and Insulin circulates in blood as the free monomer and its half-life in plasma is about 5 - 6 min in normal subjects. Although glucose is the principal stimulus to insulin secretion in human beings, this process is tightly regulated by the coordinated of nutrients, gastrointestinal and pancreatic hormones and autonomic neurotransmitters (Salgueiro *et al.*, 2001). The main drawback of insulin is taken through injection.

1.5.2 Oral hypoglycaemic agents

Oral hypoglycemic drugs are those drugs that lower blood glucose level and taken orally. These drugs are synthetic and complex organic substances and include: sulfonylureas, biguanides, Thiazolidinediones, Alpha glucosidase inhibitors, and DPP-4 inhibitors.

1.5.2.1 Sulfonylureas

The sulfonylurea agents are the oldest class of oral anti-diabetes therapies and are currently used as second-line or add-on treatment options for Type 2 diabetes (SMC, 2013). The agents stimulate pancreatic beta-cells to produce insulin, increase cellular uptake and utilization of glucose, and decrease glucose production in the liver (Powers, 2012, Powers 2012 and Nolte-Kennedy, 2012). Six agents are currently available as oral tablets. The sulfonylureas may be divided into two groups: first-generation agents (chlorpropamide, tolazamide, tolbutamide) and second-generation agents (glimepiride, glipizide, glyburide). The first generation agents have longer half-lives, increased incidence of hypoglycemia, and more drug interactions. The second generation agents have quicker onsets ofaction, shorter half-lives, and lower incidence of hypoglycemia. Glipizide is available in both immediate release and extended release formulations. The therapeutic effects of the sulfonylurea agents can result in hypoglycemia. Slow

initiation and titration of the agents is recommended. The sulfonylurea agents are not used in patients with Type 1 diabetes whose pancreas is unable to produce insulin (Melissa *et al.,* 2013).

1.5.2.2 Biguanides

This includes phenformin and metformin (Dichtwald *et al.,* 2012). Metformin reduces blood glucose levels by inhibiting hepatic glucose production and reducing insulin resistance, particularly in liver and skeletal muscle (Giannarelli *et al.,* 2003). Metformin decreases intestinal absorption of glucose, and increases insulin sensitivity by enhanced glucose uptake and utilization in peripheral tissues. Gastrointestinal side effects, i.e. diarrhea, nausea, bloating and metallic taste in the palate common when treatment with metformin is started, affecting 1%-30% of patients *(*Dichtwald *et al.,* 2012).

1.5.2.3 Thiazolidinediones

The thiazolidinediones also known as glitazones, are a class of medications that are used in the treatment of diabetes mellitus type 2. They were introduced in the late 1990s *(Krentz and Friedmann 2006).*

1.5.2.4 Alpha glucosidase inhibitors:

One alternative approach of the treatment of overweight patients with NIDDM is to use drugs which inhibit the enzymes involved in the breakdown of carbohydrates in the intestine. Acarbose is a sham sugar that competitively inhibits α-glucosidase enzymes situated on the brush border of the intestine. As a result, dietary carbohydrates are poorly absorbed, and the postprandial rise in blood glucose is reduced. Undigested starch enters the large intestine where it is broken down by fermentation. Abdominal discomfort, flatulence and diarrhea can result, and dosage needs careful adjustment to avoid these side effects. Very little acarbose enters the circulation, since it is mainly inactivated in the gut, but liver dysfunction may rarely occur with high doses (Chiasson *et al.,* 2003).

1.5.2.5 DPP-4 inhibitors

Drugs targeting the incretin pathway are the latest addition to the available anti-diabetic agents. Incretin-based therapy is either delivered orally (dipeptidyl peptidase-4 [DPP-4]) inhibitors or injected subcutaneously (glucagon-like peptide-1[GLP-1] mimetics and analogues). Dipeptidyl peptidase-4 inhibitors are effective either as a single or combination therapy in loweringglycated hemoglobin, fasting and postprandial glucose levels, with alow incidence of hypoglycemia and no weight gain. There are 3 DPP-4inhibitors currently available (sitagliptin, saxagliptin, and vildagliptin), with more expected to be available in the future. DPP-4 inhibitors are effective in the treatment of patients with type 2 diabetes (Palalau *et al.*, 2009).

1.6 Adverse Effects of Conventional Anti-diabetic Drugs

To control diabetic disease, several conventional-drugs along wth insulin as stated above are available but their prolonged use may lead to other complications like blurred vision, hypoglycaemia and a lingering condition like coma *(*Shukla *et al.,* 2000*).* The anti-diabetic-drugs such as modern oral hypoglycemic agents as depicted in table 1 below are associated with various side effects (Shukla *et al.,* 2000).

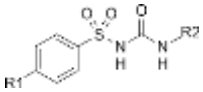
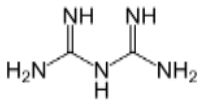
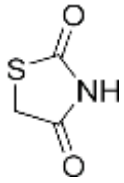
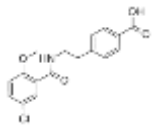
Type	Name	General structure	General side effects	Reference
Sulfonylureas	Glimepiride Glipizide Glyburide		Hypoglycaemia and weight gain, Myocardial infarction, stroke	Sakharove and Inzucchi,(2005) Roumie *et al*
Biquanides	Metformin		Nausea, abdominal pain, weak, dizziness, heavy breathing	www.amc.ed/diabetes
Thiazolidiones	Rosiglitazones		Liver problles, weight gain, swelling of the feet and legs, cough, cold headache.	(Rendell, 2000)
Meglitinides	Repaglinide, Nateglinide		Hypoglycaemia and weight gain	www.amc.ed/diabetes
Alpha-glucosidase inhibitors			Bloating, diarrhea, flatulence	Heffner, (2007).
DPP-4 inhibitors			Upper respiratory infection, stuff or running nose, sore throat, headache, urinary tract infection	Palalau *et al.*,(2009)

Table 1: Conventional drugs for treatment of diabetes mellitus and their side effects

1.7 Alternative Therapy for the Treatment of Diabetes Mellitus

The use of herbal medicines (medicinal plants or phyto-therapy) has recently gained popularity in all over the world for their efficacy in Type II diabetes mellitus. These medicines are used since centuries in Unani system of medicine and they have more efficacy and fewer or no side effects therefore emphasis should be given on herbal medicine because allopathic system of medicine has failed in providing health to all. Herbal medicines are only alternative medicines that can relieve the patients. Various research studies have been carried in all over the world to evaluate the efficacy of herbs in the treatment of Type II diabetes mellitus *(*Patel *et al.,* 2012*).*WHO has recommended the evaluation of traditional plant treatments for diabetes as they are effective, non-toxic, with less or no side effects and are considered to

be excellent aspirants for oral therapy (Shokeen *et al*., 2008).For a long time type II diabetes mellitus has been treated orally with herbal medicines or their extracts, because plant products are frequently prescribed due to their less toxicity than conventional medicines (Shokeen *et al.,* 2008*).*

The use of these plants and phyto-constituents may delay the development of diabetic complications and may regulate the metabolic abnormalities through a variety of mechanisms (Mukherje e *et al.,* 2006). Moreover, during the past few years many phytochemicals responsible for anti-diabetic effects have been isolated from the plants. Several phyto-constituents such as alkaloids, glycosides, flavonoids, saponins, dietary fibers, polysaccharides, glycolipids, peptidoglycans, amino acids and others obtained from various plant sources that have been reported as potent hypoglycemic agents (Switi *et al.,* 2014).

1.8 Justification

Various medications are available for the treatment of diabetes but they have also exhibited a number of undesired side effects associated with their uses, yet the treatment is life-long, due to the chronic nature of disease and thus suggesting other effective alternatives. Because of this, there is therefore necessary to search for medicinal plants with anti-diabetic properties each for therapeutic agents from medicinal plants some patients use affordable and cost effective alternative therapy for management of diabetes in the form of traditional medicines, which are both locally available and cheap.

1.9 **Aim and Objectives**

The aim of the present research work is to evaluate the anti-diabetic properties of stem methanolic extracts of *Piliostigma thonningii* plant. This is achieved by the following objectives

- To extract the stem of *Piliostigma thonningii*
- To carry out phytochemical screening on the methanolic crude extracts.
- To evaluate the anti-diabetic properties of the crude extract by alpha-amylase, non-enzymatic glycosylation of hemoglobin and glucose uptake inhibitory assays.

CHAPTER TWO

2.0 LITERATURE REVIEW

Plants have always been a good source of drugs. The ethno botanical information reports about 800 plants that may possess anti-diabetic potential. The beneficial uses of medicinal plants in traditional system of medicine of many cultures have been extensively documented. Several plants have been used as dietary adjuvant and in treating the number of diseases even without any knowledge on their proper functions and constituents. This practice may be attributed to the uncompromised cost and side effects of synthetic hypoglycaemic agent.

Recently, Adeyemi *et al.*, 2015 studied the antihyperglycemic effects of ethanol extracts of *Anchomanes difformis* on normal and alloxan-induced diabetic rats and found that the administration of ethanol extract of this plant significantly lowered the plasma glucose level of the induced rats when compared with the diabetic control rats. Abdullah and Olatunji, (2010) investigated the hypoglycaemic effect of inner bark extract of *Anacardium occidentale* in normal (normoglycemic) and in alloxan-induced diabetic rats and found that the inner bark extract of *A. occidentale* showed significantly anti-hyperglycemic effects. Aloe vera gel extract had been reported to exhibit significant antihyperglycemic activities on streptozotocin-induced hyperglycemia in experimental rats (Rajasekaran *et al*, 2004). Nyunaï *et al.*, 2015 evaluated the sub-acute antidiabetic effect of the aqueous extract of the leaves of *Ageratum conyzoides* on some antidiabetic parameters (blood glucose level, lipid profile, serum insulin, hepatic glycogen, total serum protein, water intake, urine excretion and food intake), and body weight and the results showed that oral administration of *A. conyzoides* produces significant antihyperglycemic effect, lowers both triglyceride levels and, at the same time, increases HDL-cholesterol in STZ-induced diabetic rats. This investigation reveals the potential of *A. conyzoides* for the use as a natural oral agent, with both antihyperglycemic and hypolipidemic effects. These observations taken together suggest that *A. conyzoides* leaves extract has a

potential in the management of diabetes mellitus. Rajasekaran *et al*, 2004 investigated the aqueous fraction of a methanolic extract of *Discorea dumetorum* Linn. And the result showed significant hypoglycemic effect in healthy and alloxan induced- diabetic rabbits when the extract was administered intraperitoneally (20 g/kg). In contrast, the chloroform fraction raised blood glucose level in healthy rabbits.

2.1 Piliostigma thonningii

Piliostigma.thonningii is a leguminous plant belonging to the family *Caesalpiniacea*. It is commonly known as Camel's foot, Monkey bread, Rhodesian bauhinia and locally called "abefe"; "Kaloo" and "Okpoatu" in Yoruba, Hausa and Igbo respectively in Nigeria (Jimoh *et al*., 2005)

Various parts of *P.thoninngii* have been used traditionally for the treatment and management of various diseases in humans and animals (Jimoh *et al*., 2005). The bark is used in the management of cough, stomach infections, malaria, leprosy, sore throat and various forms of inflammation. The roots and twigs are used in treating fever, dysentery, snake bites, hookworms and skin infections, while the leaves decoctions possess antibacterial, antimicrobial and antioxidant activities and used as laxatives for children and for dressing wounds (Alfred, 20013).

Phytochemical screening of the genus *Piliostigma* revealed the presence of flavonoids (Bombardelli, 1973), polyphenols (Bombardelli, 1994) and essential oils (Tira-Pcos *et al*., 2010 and Mustapha *et al*., 2012).

CHAPTER THREE

3.0 MATERIAL AND METHOD

3.1 Materials

3.1.1 Chemicals

Ethyl acetate, Methanol, Tetraoxosulphate (VI) acid (H_2SO_4), Chloroform ($CHCl_3$), Acetic acid, n-hexane, silica gel, ferric chloride, Hydrochloric acid (HCl), silver nitrate ($AgNO_3$), ethanol (C_2H_5OH), glucose, yeast, gentamicin, ascorbic acid, starch, 3,5-dinitrosalycilic acid, α-amylase and distilled water were obtained from Sigma-Aldrich (Germany) and also from Chemistry Unit, Department of Science Laboratory Technology, Institute of Applied Science, Kwara State Polytechnic. Solvent were redistilled before use while reagents were used without further purification.

3.2 Method

3.2.1 Collection of Sample

The stem of *Piliostigma thonningii* plant were collected from Kwara State Polyyechnic, Ilorin environ and identified at the department of Plant and Environmental Biology, Kwara State University Malete, Ilorin, Nigeria.

3.2.2 Preparation of Sample

Fresh stem of *Piliostigma thonningii* plant were collected, washed under running tap water and air dried in the laboratory at a temperature of 25°C for two weeks. They were subsequently pulverized into fine powder weighing 55.5 g.

3.2.3 Extraction

The pulverized stem bark (55.5g) were soaked in distilled methanol for three days. The extracts were decanted; filtered and concentrated using rotary evaporator at 40°C to obtain brown and wine crude extracts (16.8 g and 11.3 g) which were coded as MESP.

3.2.4. Qualitative Phytochemical Screening of (MESP)

The crude plant extracts will be screened for the presence of glycosides, alkaloids, tannins, saponins, terpenoids, carbohydrates, cardiac glycosides, anthraquinones glycosides, flavonoids, and phenols followed the method described (Alamzed *et al.* 2013; Thusa and Mulmi, 2017; Talukdar and Chaudhary, 2010).

3.2.4.1 Test for Reducing Sugar

To 0.5 mL of plant extract, 1 mL of water, and 5-8 drops of Fehling's solution will be added and heated. The presence of reducing sugar shall be indicated by the appearance of brick red precipitation (Thusa & Mulmi, 2017).

3.2.4.2 Test for Glycosides

Molisch's Reagent Test: To the extract, 5 mL Molisch's reagent and concentrated H_2 SO_4 will be added. Violet color will indicate the presence of glycosides (Alamzed *et al*., 2013).

3.2.4.3 Test for Saponins

A 2.0 g of powdered sample will be boiled in 20 mL distilled water. 10 mL of filtrate, 5 mL of distilled water will be quivered vigorously. The appearance of frothing indicated the presence of saponins (Alamzed *et al.*, 2013).

3.2.4.4 Test for Alkaloids

Meyer's Test: To 2 mL of extract, 1 mL of Meyer's reagent will be added. The presence of pale yellow precipitate indicated the presence of alkaloids (Talukdar & Chaudhary, 2010).

Hager Test: To 2 mL of the extract will be treated with few drops of Hager's reagent. A yellow precipitate indicated the presence of alkaloids (Talukdar & Chaudhary, 2010).

Dragendoff's Reagent Test: To 2 mL of extract was warmed with 2% H_2SO_4, Few drops of Dragendroff's reagent were added. Orange-red precipitate indicated the presence of alkaloids (Alamzed et al. 2013).

3.2.5 Quantitative Phytochemicals Screening of (MESP)

The quantitative phytochemical screening will be conducted on the total crude extracts to quantify the phytochemical contents of the extracts. Total phenolic content (TPC), total flavonoid content (TFC), total saponin content and alkaloid contents will be determined followed the standard procedure described by (Senguttuvan *et al.,* 2014).

3.2.5.1 Total Phenolic Content Determination

Folin–Ciocalteu reagent will be adopted for the determination of total phenolic content. 0.5 ml of each extract (5 mg/mL), Folin–Ciocalteu reagent (5 mL, 1:10 v/v diluted with distilled water) and aqueous sodium carbonate (4 ml, 1 M) solution will be mixed together. The mixture will be allowed to stand in the dark for 15 min at room temperature, and the absorbance at 765 nm will be measured with the help of ultraviolet (UV-visible) spectrophotometer. Then, the total phenolic content will be determined in terms of mg GAE/g of dry weight of the extract with the help of a calibration curve prepared with a series of gallic acid standards (10-80 µg/ml) (Senguttuvan *et al.,* 2014).

$$Total\ Phenol = \frac{\text{Absorbance} \times \text{V x D. F}}{\text{Sample weight} \times 1000}$$

Where, D.F=Dilution factor, V = volume

3.2.5.2 Total Flavonoid Content TFC

A 0.5 ml of each extract (50 mg/ml) will be separately mixed with 1.5 mL methanol and 0.1mL aluminum trichloride (10%). Then, 0.1 ml of 1 M potassium acetate and 2.8 ml distilled water will be added into each test tube. Then, absorbance at 415 nm will be measured after it was allowed to stand in the dark for 30 min using a UV-visible spectrophotometer. Finally, a calibration curve will be prepared with a series of quercetin standards.

$$Total\ flavonoid = \frac{\text{Absorbance } \times \text{ V x D. F}}{\text{Sample weight } \times \ 1000}$$

Where, D.F=Dilution factor, V = volume

3.2.5.3 Total Saponin Contents

Test extract will be dissolved in 80% methanol, 2ml of Vanilin in ethanol will be added, mixed well and the 2ml of 72% sulphuric acid solution will be added, mixed well and heated on a water bath at 600 c for 10min, absorbance will be measured at 544nm against reagent blank. Diosgenin is used as a standard material and compared the assay with Diosgenin equivalents (Madhu *et al*., 2016)

3.2.5.4 Total Alkaloids Content

To 1ml of test extract 5 ml pH 4.7 phosphate Buffer will be added and 5 ml BCG solution and shake a mixture with 4 ml of chloroform. The extracts will be collected in a 10-ml volumetric flask and then diluted to adjust volume with chloroform. The absorbance of the complex in chloroform will be measured at 470 nm against blank prepared as above but without extract. Atropine is used as a standard material and compared the assay with Atropine equivalents (Madhu *et al*., 2016)

3.3 *In Vitro* Assessment of Anti-hyperglycemic Activities of Stem Extracts of *Piliostigma thonningii*

3.3.1 Non-enzymatic Glycosylation of Haemoglobin Assay

Anti-diabetic activity of stem and root ethanolic extracts of *Piliostigma thonningii* was assessed by estimating degree of non-enzymatic haemoglobin glycosylation, measured colorimetrically at 520nm. Glucose (2%), haemoglobin (0.06%) and Gentamycin (0.02%) solutions were prepared in phosphate buffer 0.01 M, pH 7.4.

Procedure

I mL of each of the above solution was mixed together, 1 mL of each of concentration of extract (20, 40, 60, 80 and 100µg/mL) was added to the mixture and incubated in dark at room temperature for 72 hrs. The degree of glycosylation of haemoglobin was measured colorimetrically at 520nm. Alpha tocopherol (Trolax) was used as a standard drug for assay. All tests were performed in triplicate.

3.3.2 Alpha amylase inhibition Assay

Different concentration (20µg/ml, 40µg/ml, 60µg/ml, 80µg/ml and 100µg/ml) of ethanolic crude extracts of stem of *P.thonningii* were separately incubated with 500µl of 20mµ phosphate buffer (pH 6.9) containing α-Amylase (0.5µg/ml) at 25ºC for 10mins. Then 500µl of 1% starch solution prepared in phosphate buffer was added to each test-tube. The reaction mixture was further incubated for 10mins at 37ºC. The reaction was terminated with 0.5ml of 3, 5-dinitro salicylic acid (i.e. the colour reagent). The mixture was thereafter incubated in a boiling water for 5mins and cooled to room temperature. The reaction mixture was then diluted with distilled water and absorbance was measured at 540nm. The absorbance was calculated as follow:

$$\text{Percentage inhibition (\%)} = \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{control}}}{\textbf{Abs}_{\textbf{sample}}} \textbf{ x 100}$$

3.3.3 Glucose Uptake in Yeast Cells

Commercial baker's yeast was washed by repeated centrifugation (3000g; 5mins) with distilled water until the supernatant fluids were clean and 10 % (v/v) suspension was prepared in distilled water. Various concentrations of extracts (1-5mg) were added to 1ml of glucose solution (5, 10 and 25 mm) and incubated together for 10 mins at 37 ºC. Reaction started by adding 100ml of yeast suspension, vortex and further incubated at 37 ºC for 60 mins. After 60 mins, the tubes were centrifuged (2500g, 5mins) and glucose was estimated in the supernatant. Metronidazole was taken as standard drug.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Result of Phytochemical Screening

4.1.1 Qualitative Result

The preliminary phytochemical screening conducted on the methanol stem extract of *P.thonningii* demonstrated the presence of flavonoids, phenols, steroids, glycoside, tannins, saponin, terpenoids and other phytochemicals as shown in table 2 below.

Phtyochemicals	**Methanolic stem extract** of *P.thonningii*
Tannins	+
Flavonoids	++
Steroids	+
Terpenoids	+
Glycoside	++
Carbohydrate	+
Alkaloids	++
Saponins	++
Test for reducing sugars	++
Phenolic	++

Key: +=trace amount, ++= strongly present, -= absent

Table 2: Result of phytochemical screening of methanolic stem extract of *Piliostigma thonningii*

4.1.2 Quantitative Result

Table 3: The quantitative phytochemical result of the stem methanolic extract of *P.thonningii*

Phytochemicals	**Result (mg/g)**
Total phenols	137.09 ± 0.50
Total flavonoids	97.14 ± 0.98
Tannins	24.41 ± 0.89

Results are expressed as mean ± standard deviations (SD) of replicate analysis

The result shows that the sample contains high phenol compound then flavonoids and tannins

4.2 Results of *In vitro* Assessment of Anti-hyperglycemic Activities

4.2.1 Result of Non-enzymatic Glycosylation of Haemoglobin Assay of Methanolic Stem Extract of *P.thonningii*

CONCENTRATION	STANDARD		METHANOLIC STEM EXTRACT	
mg/ml	Abs Absorbance	% Inhibition	Abs Absorbance	% Inhibition
20	0.26 ± 0.02	5.38	0.31 ± 0.01	20.60
40	0.27 ± 0.01	8.80	0.34 ± 0.01	24.60
60	0. 29 ± 0.01	17.80	0.34 ± 0.02	24.60
80	0.32 ± 0.00	23.10	0.36 ± 0.01	31.70
100	0.35 ± 0.01	29.70	0.39 ± 0.02	36.90

Values are expressed as mean ± SEM. Absorbance of blank=0.246

Table 4: Result of Non-enzymatic glycosylation of haemoglobin assay of methanolic stem extract

4.2.2 Result of Glucose Uptake Assay of Methanolic Stem Extract of *P.thonningii*

CONCENTRATION	STANDARD		METHANOLIC STEM EXTRACT	
mg/ml	Abs Absorbance	% Inhibition	Abs Absorbance	% Inhibition
20	0.26 ± 0.02	5.38	0.31 ± 0.01	20.60
40	0.27 ± 0.01	8.80	0.34 ± 0.01	24.60
60	0. 29 ± 0.01	17.80	0.34 ± 0.02	24.60
80	0.32 ± 0.00	23.10	0.36 ± 0.01	31.70
100	0.35 ± 0.01	29.70	0.39 ± 0.02	36.90

Values are expressed as mean ± SEM. Absorbance of blank=0.207

The table 5: The result of glucose uptake assay of methanolic stem extract of *P.thonningii*

4.2.3 Result of Alpha Amylase Inhibition Assay of Methanolic Stem Extract

CONCENTRATION mg/ml	METHANOLIC STEM EXTRACT Abs	
	Absorbance	% Inhibition
20	0.23 ± 0.00	5.22
40	0.24 ± 0.01	9.17
60	0. 26 ± 0.00	16.22
80	0.28 ± 0.01	22.10
100	0.30 ± 0.00	27.30

Values are expressed as mean ± SEM. Absorbance of blank=0.217±0.00

The table 6: The result of alpha amylase inhibition assay of methanolic stem extract of *P.thonningii*

4.3 DISCUSSION

In the present study, the results of phytochemical screening had demonstrated the presence of flavonoids, terpenoids, steroids, cardiac glycoside, saponin, tanins and phenols (table 2). Thus the presence of these phytochemicals in stem of *P.thonningii* plant confirms its folklore uses for the treatment of various ailments.

Table 3 also shows the quantitative screening results of methanolic extracts of stem of *P.thonningii* sample show high present of total phenols in agreed to the qualitative revealing much present of phenol, followed by total flavonoids present in similarity to the qualitative result which shows flavonoids is much present in the methanolic extract stem of *P.thonningii* and total tanins result of qualitative show same as qualitative results.

The methanolic extracts of stem back *of P.thonningii* plant were investigated for their potential to inhibit non-enzymatic glycosilation of haemoglobin, glucose uptake and α-amylase activities. The methanolic stem bark extract demonstrated the highest percentage inhibition (36.9%) in non-enzymatic glycosilation

of haemoglobin and α-amylase assays when compared with the standard drugs. It also showed good percentage inhibition (25.2 %) with in glucose uptake inhibition assay than the standard. The activities of the methanolic stem extracts of this plant to inhibit the non-enzymatic glycosilation of haemoglobin, glucose uptake and α-amylase may be due to the presence of the phytochemicals identified in the crude extract of this plant.

4.4 Conclusion and Recommendation

Natural products could provide a sustainable solution to high alarming of diabetes compared to synthetic drug that have enamours side effect. The literature review shows that the uses of many herbal extract can represent promising valuable methods for the curing of diseases. Therefore, the use of plants as natural sources of compounds for the management of diseases can contribute to minimize the risk of health problems most especially the threaten of diabetes.

Also, results of this work showed that the phytochemical constituents of the stem of *P.thonningii* are responsible for the anti-diabetic's activities of this plant. It is therefore revealed that these studies have established the scientific proof that this plant as anti-diabetics agent due to the present of bio active compound present.

Further studies should be done on the isolation, characterization and *in vivo* studies of the bioactive compounds responsible for the anti-diabetics properties.

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