CHEMICAL GROUP PROFILING AND INHIBITORY POTENTIAL OF ETHANOLIC ROOT EXTRACT OF *Aristolochia ringens* Vahl. (ARISTOLOCHIACEAE) ON THE ACTIVITY OF α-AMYLASE AND α-GLUCOSIDASE

BY

Jamiu Busari AHMAD

B.Sc. (Biochemistry)

(17/27/MBI002)

A DISSERTATION SUBMITTED TO THE

DEPARTMENT OF MEDICAL BIOCHEMISTRY AND PHARMACOLOGY

SCHOOL OF BASIC MEDICAL SCIENCES

COLLEGE OF PURE AND APPLIED SCIENCES

KWARA STATE UNIVERSITY, MALETE, NIGERIA

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE IN BIOCHEMISTRY

MAY 2019

CERTIFICATION

I hereby certify that AHMAD, BUSARI JAMIU with matriculation number 17/27/MBI002 wrote the project 'Chemical profiling and inhibitory potential of ethanolic root extract of *Aristolochia ringens* Vahl. (Aristolochiaceae) on the activity of α-amylase and α-glucosidase' under our supervisions and that it has been read and approved as meeting part of the requirements for the award of Masters of Science in Biochemistry at the Kwara State University.

…… ……

Dr. E.O. Ajani Date
(Head of Department and Major Supervisor)

…… ……

Dr. Sabiu Saheed Date
(Co-Supervisor)

....

Dean, School of Postgraduate Studies Date

....

External Examiner Date

DEDICATION

This research work is dedicated exclusively to the memory of my late parents. May their gentle souls rest in perfect peace.

ACKNOWLEDGEMENT

All adorations are due to Almighty Allah for His Benevolence and Guidance on me. May He continue to shower His unending blessings on the soul of the noblest of mankind: Muhammad (SAW).

I cannot but acknowledge my project supervisor- Dr. E.O. Ajani for his thoroughness and the manner employed in supervising this work. My acknowledgement is incomplete without specially appreciating my co-supervisor: Dr. Sabiu Saheed. I cannot thank him enough. The contributions of Dr. Sulaiman A.O. will not go unnoticed. My acknowledgement is incomplete without appreciating both the academic and non-academic staff of this department. Jazakalahu khair to you all!

I acknowledge the entire AHMAD's family for their support. I want to specially appreciate the contributions of my amiable sister- Hajia Zainab Funmilayo for always been there. I cannot thank you enough. May you reap the fruit of your labour. Amin.

To my colleagues, being one of you is one the best experiences I have had. Your contributions, supports, criticisms and encouragements are unforgettable. Thank you all.

TABLE OF CONTENT

LIST OF TABLE

LIST OF FIGURES AND PLATES

ABSTRACT

Diabetes mellitus (DM) has become a global scourge and the search is ongoing for suitable novel alternatives to the currently used orthodox hypoglycaemic drugs with notable side effects. Phytotherapy is becoming increasingly popular and acceptable due to its ease of accessibility, availability, affordability and relatively lower adverse effect. *Aristolochia ringens* (Vahl) is an ethnopharmacologically valued botanical in the management of degenerative diseases including DM. This study characterized the phytonutrients of *Aristolochia ringens* (Vahl) and evaluated the mechanism(s) of hypoglycaemic potential of its ethanolic root extract through inhibition of the specific activity of α-amylase and α-glucosidase *in vitro and in silico*. The extract was separately incubated with α-amylase and α-glucosidase and subsequently with starch and *p*-nitrophenylglucopyranoside respectively, while the *in silico* molecular docking was performed using PyMOL tool. The mode(s) of inhibition of both enzymes was subsequently determined using Lineweaver-Burk plots. The phytochemical screening revealed the presence of phenol (8.91mg/100g dry weight),flavonoids,(5.22mg/100g dry weight), triterpenes (2.93mg/100g dry weight) and alkaloids(11.62mg/100g dry weight). The extract had respective competitive and uncompetitive inhibitory influence on α-amylase and α-glucosidase with half-maximal inhibitory concentrations of 0.67 and 0.57 mg/mL relative to that of acarbose (0.63 and 0.54 mg/mL). The effect elicited by the extract may be attributed to its phytoconstituents (Asiatic acid, magnoflorine and phenyl-β-D-glucopyranoside) as revealed by LC-MS analysis. Furthermore, the docking results are consistent with the *in vitro* analysis and showed strong binding affinities [(-7.5, -7.3 and -4.5 Kcal/mol for Asiatic acid, magnoflorine and phenyl-β-D-glucopyranoside respectively) and (-8.0, -8.2 and -5.0 Kcal/mol for the respective compounds)] with α-amylase and α-glucosidase. Consequent upon these results, modulation of the specific activity of the enzymes linked to carbohydrate metabolism could be suggested as the probable mechanism of hypoglycaemic potential of ethanolic root extract of *A. ringens* and has further lent credence to its antidiabetic application.

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Diabetes is a group of metabolic diseases characterized by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both. The occurrence of this disorder is on the rise globally and is likely to hit 300 million by 2025 (Gupta and Phatak, 2003). Two major classes of diabetes have been identified. Type 1 diabetes (formerly known as insulin-dependent) occurs when the pancreas fails to produce the insulin which is essential for survival. This form develops most frequently in children and adolescents, but is being increasingly noted later in life. Type 2 diabetes, also known as non-insulin-dependent results from the body's inability to respond properly to the action of insulin produced by the pancreas. Type 2 diabetes is much more common and accounts for around 90% of all diabetes cases worldwide (Inzuchi *et al.,* 2015). It occurs most frequently in adults, but is being noted increasingly in adolescents as well. Gestational diabetes is situational and occurs only in pregnancy but it is a risk factor for the development of type 2 mellitus later in life (Zhu and Zhang, 2016). Insulin-dependent diabetes is treated with exogenous insulin administration (Gbolade*,* 2009). Available management options for type 2 diabetes mellitus include stimulation of endogenous insulin secretion, improvement of the action of insulin at the receptor sites, oral antidiabetic agents, such as biguanides and sulfonylureas and the inhibition of degradation of dietary starch by glycosidases such as α-amylase and α-glucosidase (Grover

et al., 2002; Sulyman *et al.,* 2016). Many useful herbs introduced in pharmacological and clinical trials have confirmed their blood sugar lowering effect, repair of β-cells of islets of Langerhans (Gupta *et al.,* 2007). The final step in the digestion of polysaccharides and disaccharides is the hydrolysis of α-d-glucose residues from the non-reducing end of α-glucoside by α-glucosidase (Gupta *et al.,* 2007). α -Glucosidase (E.C. 3.2.1.20) activity has been linked with increased levels of plasma glucose, and its inhibition is often exploited in down regulating glucose absorption in type 2 diabetes mellitus sufferers (Wang *et al.,* 2013). Most of the α-glucosidase inhibitors earlier reported are sugars or derivatives of sugar moieties such as acarbose which is the first member of α-glucosidase inhibitors approved for the management of type 2 diabetes. Pancreatic α-amylase (E.C. 3.2.1.1) is a key enzyme in the digestive system and catalyses the initial step in hydrolysis of starch to a mixture of smaller oligosaccharides consisting of maltose, maltotriose, and a number of α-(l-6) and α-(1 - 4) oligoglucans. Hence, retardation of starch digestion by inhibition of enzymes such as α-amylase plays a key role in the control of diabetes (Sabiu *et al.,* 2016). Inhibitors of pancreatic α-amylase delay carbohydrate digestion causing a reduction in the rate of glucose absorption and lowering the post-prandial serum glucose levels (Sabiu *et al.,* 2016). Consequently, exploring the binding behaviour and inhibitory effect of inhibitors with α-glucosidase and α-amylase are of great importance for drug–enzyme interactions and therapeutic applications (Grover *et al.,* 2002).

Trigonellafo enumgraecum, Allium sativum, and Aristolochia ringens are well known plants reported to possess antidiabetic compounds (Grover *et al.,* 2002; Eidi *et al.,* 2006 and Sulyman *et al.,* 2016). However, detailed studies on the structures, kinetics and inhibitory mechanisms of these antidiabetic plants are scarce. Aristolochia ringens is a bushy climber native of tropical America, introduced to most West African countries as a garden ornamental, and has become naturalized in roadside bush in Sierra Leone, Ghana, Nigeria, (Burkill, 1985) and Democratic Republic of Congo (De Groot *et al.,* 2006). However, the kinetics, structural elucidation and mechanism of inhibition of the plant with α-glucosidase and α-amylase have not received much attention.

1.2 Problem Statement

In recent years, reports of antidiabetic potential of various plant extracts have been documented. However, the lack of their nature of interaction, mechanism of inhibition, kinetics and lucid structural elucidation of the bioactive principles has retarded the progress ought to have been recorded in the discovery of new lead compounds. These problems were addressed in this work by providing data on the mode of interaction, kinetics and chemical profile of *A. ringens*, thereby contributing to existing knowledge in the management of type 2 diabetes mellitus.

1.3 Justification and significance of the study

Diabetes has assumed the status of a major world killer disease. According to the World Health Organization (WHO), 300 million people are currently living with the disease with annual mortality of 2.2 million (WHO, 1999). Scientifically proven antidiabetic plants envisioned to

serve as alternatives to synthetic drugs are often reported without accurate and rich documentation of their bioactive principles, specific mechanisms of action, and enzyme kinetics. Although many α-glucosidase and α-amylase inhibitors are commercially available, most of them are accompanied with life-threatening side effects including severe hypoglycaemia, weight gain, flatulence and diarrhoea. These side effects have reduced the effective application of these drugs because of their severity on type 2 diabetes patients. It is therefore pertinent to begin the search for alternatives with lesser and milder side effects. One of such alternatives is *Aristolochia ringens* plant being one of the antidiabetic plants for managing diabetes in folklore medicine. Despite its acclaimed antidiabetic efficacy, the probable mechanism through which this happens is still lacking in scientific literature. It is on this background that the present study was designed to provide probable mechanism of action, mode of interaction, inhibition kinetics and complete chemical profiling of *Aristolochia ringens.* This study is therefore envisaged to provide valuable information that could lead to the development of novel antidiabetic compound(s) with clear and well-defined kinetics, structural elucidation and mechanisms of action with a view to improving the management of type 2 diabetes mellitus.

1.4 Starting Hypothesis

H_O: Ethanolic root extract of *Aristolochia ringens* inhibits α-glucosidase and α-amylase as mechanisms for ameliorating diabetes mellitus.

H_1: Ethanolic root extract of *Aristolochia ringens* does not inhibit α-glucosidase and α-amylase as mechanisms for ameliorating diabetes mellitus.

1.5 Aim and objectives

The overall aim of this study is to investigate and establish the enzyme kinetics and probable mechanism of hypoglycaemic action of ethanolic root extract of *Aristolochia ringens*

This aim was achieved through the following specific objectives:

1. To elucidate the structures of the bioactive principles from *Aristolochia ringens* root using chromatographic techniques (Liquid chromatography-mass spectrophotometry).
2. To establish the probable mechanism of action of ethanolic root extract of *Aristolochia ringens* root using molecular docking approach.
3. To investigate the inhibition kinetics of α-glucosidase and α-amylase by ethanolic root extract of *Aristolochia ringens.*

CHAPTER TWO

LITERATURE REVIEW

2.1 Types of Diabetes Mellitus

Two main types of diabetes mellitus have been described. These include the insulin-dependent diabetes (IDD) or Type 1 diabetes and non-insulin dependent diabetes (NIDD) or Type 2 diabetes. Gestational diabetes is conditional and often transient. Type 1 diabetes results from absolute insulin deficiency, usually caused by autoimmune destruction of pancreatic islet cells. The initial clinical presentation may be ketoacidosis with an acute illness, or a more gradual presentation with symptoms of hyperglycaemia. This form of diabetes, which accounts for only 5–10% of those with diabetes, previously encompassed by the terms insulin dependent diabetes, type I diabetes, or juvenile onset diabetes, results from a cellular-mediated autoimmune destruction of the β-cells of the pancreas. On the basis of pathophysiology of the disease, this type of diabetes can be further classified as immune-mediated (Rother, 2007).

Type 2 accounts for 90–95% of those with diabetes. It is previously referred to as non-insulin dependent diabetes, or adult onset diabetes, and encompasses individuals who have insulin resistance and usually have relative (rather than absolute) insulin deficiency. This form of the disease occurs predominantly in adults, especially in persons older than 30 years of age, but it may occur at any age. In recent years there has been a dramatic upsurge of T2DM in children, some younger than 4 years of age (Guthrie and Guthrie, 2009).The global trend to an increase in Type 2 diabetes in African populations is linked to the increase in obesity (Sobngwi, 2001),

longevity and other factors such as: Increase in development, Increase in disposable income, urbanization, mechanization, globalization of food markets, changes in lifestyles and behaviours (Waxman and Norum, 2004). Gestational diabetes mellitus (GDM) is defined as any degree of glucose intolerance with onset or first recognition during pregnancy (Metzger and Coustan, 1998). This form of diabetes is connected with pregnancy. The definition applies whether insulin or only diet modification is used for treatment and whether or not the condition persists after pregnancy. Approximately 7% of all pregnancies are complicated by GDM, resulting in more than 200,000 cases annually. The prevalence may range from 1 to 14% of all pregnancies, depending on the population studied and the diagnostic tests employed. This classification does not refer to the woman with Type 1 or Type 2 diabetes who becomes pregnant, but to the individual whose diabetic condition developed due to pregnancy (Guthrie and Guthrie, 2004).

2.2 Complications of Diabetes Mellitus

Microvascular and macrovascular complications are the two categories of diabetic complications. Coronary heart disease and stroke are the greatest cause of morbidity and mortality in diabetes. Preventing these complications in type 2 diabetes which is often associated with other cardiovascular risk factors, are major challenges. In dyslipidaemia, increased levels of low-density lipoprotein (LDL) cholesterol, consisting mostly of small dense particles, promote atherogenesis. Hypertension promotes the development and progression of vascular disease. The characteristic lipid abnormality in patients with type 2 diabetes is dyslipidaemia occasioned by increased levels of both triglycerides and LDL cholesterol, and a low level of high-density lipoprotein (HDL) cholesterol (Bate and Jerums, 2003). Microvascular complications result from

the prolonged toxic and detrimental effects of hyperglycaemia on body tissues such as the cells of the kidney, nerve and eyes. Data from trials over the past 10 years show that controlling hyperglycaemia and hypertension reduces microvascular complications in both type 1 and type 2 diabetes (Bate and Jeroms, 2003). These include nephropathy, peripheral neuropathy and retinopathy. Diabetic nephropathy also known as Kimmelstiel–Wilson syndrome, or nodular diabetic glomerulosclerosis (Berkman and Harold, 1973) and intercapillary glomerulonephritis, is a progressive kidney disease caused by angiopathy of capillaries in the kidney glomeruli. About 20%–30% of patients with diabetes have evidence of overt diabetic nephropathy, defined as persistent clinically detectable proteinuria in association with hypertension and reduced glomerular filtration rate (Marshall, 2003). The earliest sign of diabetic renal disease is the presence of subclinical increases in urinary albumin excretion, termed microalbuminuria (urinary albumin excretion rate, 30–300mg/24 h or 20–200 μg/min; or albumin–creatinine ratio > 2.5 mg/mmol in men and > 3.5 mg/mmol in women). Microalbuminuria is also an independent risk factor for cardiovascular disease (Bate and Jeroms, 2003).

Foot ulcers and amputations are a major cause of morbidity for people with diabetes. Risk factors for these complications are the presence of peripheral neuropathy, altered biomechanics in the foot and peripheral vascular disease. About half of all lower-limb amputations in people with diabetes are preventable. Annual screening for these conditions is recommended. Amitriptyline, carbamazepine and gabapentin are helpful in the symptomatic management of painful peripheral neuropathy (Bate and Jerums, 2003). Diabetic retinopathy is the leading cause of blindness in the adult population (VanNewkrik *et al.,* 2001). In type 1 diabetes, almost all patients develop signs

of retinopathy in the first 20 years. In type 2 diabetes, up to third of patients have retinopathy at diagnosis (Fong *et al.,* 2003), increasing to two-thirds within 20 years. The most important treatable risk factors are hyperglycaemia and hypertension. Diabetic retinopathy progresses silently until visual loss occurs.

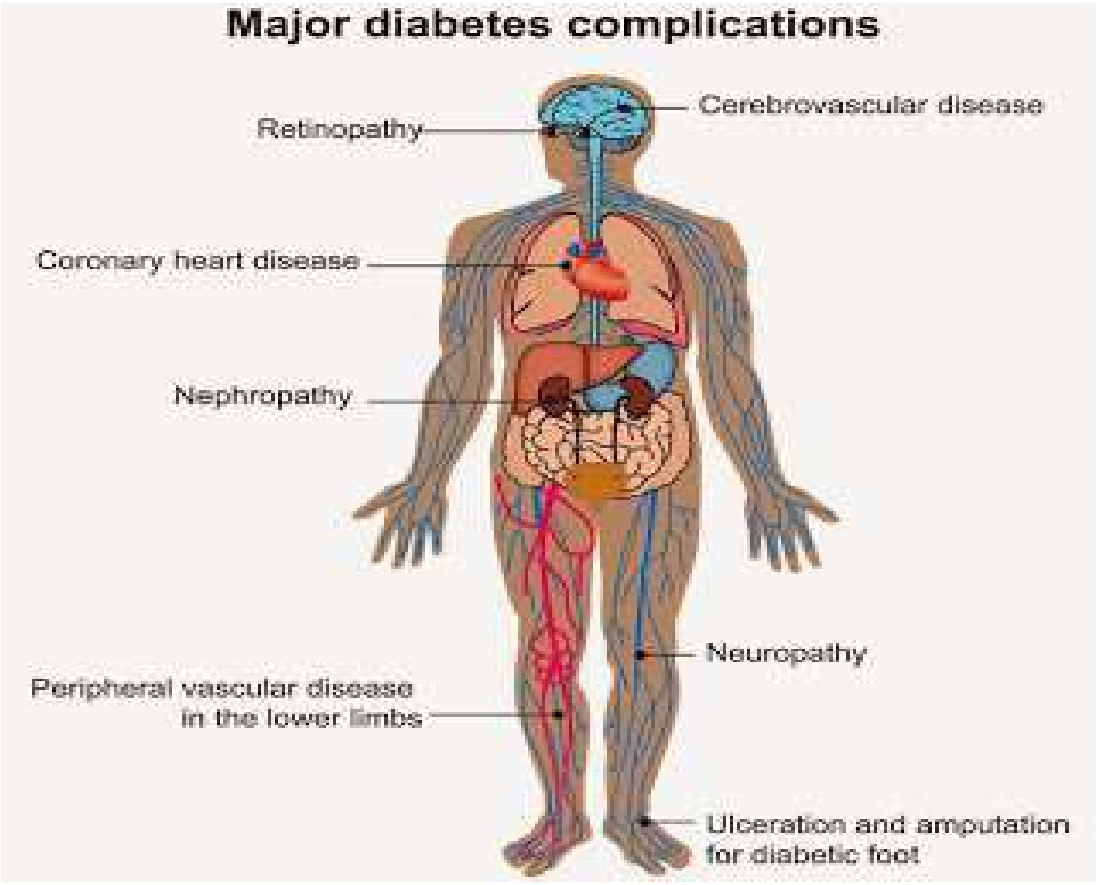


Figure 1: Complications of diabetes mellitus

(Sidney *et al.,* 2012)

2.3 Diagnosis of Diabetes Mellitus

Generally, diabetes mellitus is characterized by recurrent or persistent hyperglycaemia, and is diagnosed by demonstrating any one of the following:

i. Fasting plasma glucose level ≥ 7.0 mmol/L (126 mg/dL).

ii. Plasma glucose ≥ 11.1 mmol/L (200 mg/dL) two hours after a 75 g oral glucose load as in a glucose tolerance test.

iii. Symptoms of hyperglycemia and casual plasma glucose ≥ 11.1 mmol/L (200 mg/dL).

iv. Glycated Hemoglobin (Hb A1C) ≥ 6.5% (WHO, 1999; ADA, 2009).

2.4 Antidiabetic plants

Ethno-botanical data indicates that more than 800 plants are used in folklore medicine as remedies for the treatment of diabetes due to their effectiveness, less side effects and relatively low cost (Ocvirk *et al.,* 2013). Despite the wide usage of pharmaceutical oral hypoglycaemic drugs and insulin therapies as core of diabetes management, they have prominent side effects and fail to significantly alter or amend the course of diabetic complications (Alhassan *et al.,* 2017). Aside high cost, the common side effects linked with oral antihyperglycaemic agents are hypoglycaemia, weight gain, gastrointestinal disorders, peripheral oedema and impaired liver function (Joseph and Jini, 2013). Because natural remedies are comparatively safer and more efficient than orthodox antidiabetic drugs, the practice and study of traditional medicine have become ubiquitous worldwide (Alhassan *et al.,* 2017). Insulin-dependent diabetes mellitus is treated with exogenous insulin (Gbolade*,* 2009) and non insulin-dependent diabetes mellitus with synthetic oral hypoglycemic agents like sulphonylureas and biguanides (Covington, 2001;

Sulyman *et al.,* 2016). While exogenous hormone administration fails as a curative agent for complications of diabetes, synthetic oral drugs produce adverse health effects (Raheja, 1997). Different medicinal systems are using the active plant constituents which were discovered as natural hypoglycemic medicine. The use of crude extracts of medicinal plants in the management of diabetes mellitus is widely practiced in Nigeria (Oguanobi *et al.,* 2012). Plant drugs and herbal formulations are frequently considered to be less toxic and free from side effects than synthetic ones. Anti-hyperglycaemic effects of some of these traditional plants are attributed to their ability to restore the function of pancreatic tissues by causing an increase in insulin production or restore the functions of insulin receptors (Malviya *et al.,* 2010). Some inhibit the intestinal absorption of glucose through the inhibition of digestive enzymes of carbohydrates, mainly α-amylase and α-glucosidase thereby affecting the rate of glucose absorption and subsequently postprandial glucose level (Kazeem *et al.,* 2013; Sabiu *et al.,* 2017). The anti-diabetic properties of these plants could be attributed to their phytochemical constituents which include; glycosides, alkaloids, terpenoids, flavonoids, carotenoids, etc., all of which are frequently implicated as having anti-diabetic effect (Malviya *et al.,* 2010; Irondi *et al.,* 2016). Although many works have been done on the use of phytochemicals in treatment of diabetes through the increase in production of insulin or its receptors, few however have focused on the kinetics of α-amylase and α-glucosidase inhibition.

Different medicinal systems are using the active plant constituents which were discovered as natural hypoglycemic medicine. Herbal drugs are considered free from side effects than synthetic ones. They are less toxic, relatively cheap and popular (Sabiu *et al*, 2017). Plant

derivatives with purported hypoglycemic properties have been used in folk medicine and traditional healing systems around the world e.g., Native American Indian, Jewish (Covington, 2001), East Indian, Mexican and African (Gbolade, 2009). Likewise in this age, the plants and herbs are still being used as decoctions or in other extracted forms for their blood sugar lowering potential. Many useful herbs introduced in pharmacological and clinical trials have been confirmed to elicit their effects by lowering blood sugar level and repairing of β-cells of islets of Langerhans (Sulyman *et al.,* 2016). Many modern pharmaceuticals used in conventional medicine today also have natural plant origins. Among them, metformin was derived from the flowering plant, *Galega officinalis* (Goat's Rue or French Lilac), which was a common traditional remedy for diabetes (Akindele *et al.,* 2015). Traditional antidiabetic plants might provide new oral hypoglycemic compounds, which can counter the high cost and poor availability of the current medicines and present day drugs for many rural populations in developing countries. Yeh *et al.* (2003) reported seven most promising diabetes supplements including five herbs *Coccinia indica,* American ginseng, *Momordica charantia, Gymnema sylvestre,* and *Aloe vera*. Other well known medicinal plants for treatment of diabetes mellitus including *Aristolochia ringens* (Sulyman *et al.,* 2016), *Cochlospermum planchoni* (Abraham *et al.,* 2017), *Cyprus esculentus* (Sabiu *et al.,* 2017), *Mangifera indica* (Irondi *et al.,* 2016) have been reported. However, detailed studies on the efficacy, mechanism of action and safety including inhibition kinetics of the plant extracts are scanty.

2.4.1 *Aristolochia ringens* (Aristolochiaceae)

Aristolochia ringens is a glabrous bushy climber native of tropical America, introduced to most West African countries as a garden ornamental, and has become naturalized in roadside bush in Sierra Leone, Ghana, Nigeria (Burkill, 1985) and DR Congo (De Groot *et al.,* 2006). The plant is commonly called 'Dutchman's pipe' and 'Snakework' but local names in Nigeria include 'Ako-igun' (Yoruba, Southwest Nigeria) and 'Dumandutsee' (Hausa, Northern Nigeria). The plant has been reported for its various medicinal applications. The antidiabetic potential of the ethanolic extract of this plant has been thoroughly investigated and reported (Sulyman et al., 2016). The anti-cancer (Akindele et al., 2015), anti-diarrheal (Dharmalingan *et al.,* 2014), stimulant (Minari and Idris, 2015) and anti-inflammatory (Ruth *et al.,* 2014) potentials of the plant have been reported.

Table 1: List of some reported antidiabetic plants with their proposed mechanisms of action

S/N	Scientific Name/(Family)	Parts Used	Extraction	Active Ingredient	Probable mechanism of action	References
1.	*Aristolochia ringens* (Aristolochiaceae)	Root	Ethanolic	Aristolone	Repair of islet cells; Increased uptake of glucose	Sulyman *et al.,* 2016
2.	*Cyprus esculentus* (Cyperaceae)	Tuber	Aqueous	Allicin, capscicin, tubocurarine, campthecin etc.	Inhibition of α-amylase and α-glucosidase	Sabiu *et al.,* 2017
3.	*Cochlospermum planchoni* (*Cochlospermaceae*)	Leaf	Aqueous	Flavonoids, phenols	Repair of islet cells; Inhibition of α-amylase and activation of G6PDH	Abraham *et al.,* 2017
4.	*Mangifera indica* (Anacardiaceae)	Kernel	--------	Phenols, flavonoids	Inhibition of alpha-amylase and alpha-glucosidase; improvement of β-cell functions	Irondi *et al.,* 2016
5.	*Acanthopanax senticosus* (Araliaceae)	Whole plant	Aqueous	Polysaccharide	Potent antioxidant activity leads to antidiabetic activity.	Fu *et al.,* 2012

6	*Acorus calamus* (Acoraceae)	Rhizome	Methanol	Terpenes, glycosides, flavones, steroids, lignans, and saponins.	Decrease the activity of glucose -6 phosphates and fructose 1, 6 phosphatase enzymes.	Prisilla *et al.,* 2012
7	*Adina cordifolia* (Rubiaceae)	Leaves	Hydro-alcoholic	Tannins, saponins and flavonoids.	Increase the insulin secretion or inhibit the intestinal absorption of glucose.	Chaudary *et al.,* 2012
8	*Cocos nucifera* (Arecaceae)	Spadix	Hydro-methanol	Flavonoid, tannin and saponin.	Potentiating the insulin effect of plasma by increasing either the pancreatic secretion of insulin from the existing β cells or by its release from the bound insulin	Naskar *et al.,* 2011
9	*Afzelia Africana* (Fabaceae)	Stem Bark	Aqueous	Flavonoids, proanthocyanidins, tannins, phenols and flavonols	Potentiating of insulin from β cells or by increasing peripheral glucose uptake.	Moyo *et al.,* 2012
10	*Heliotropium indicum* (Boraginaceae)	Leaves	Aqueous	Flavonoids, tannins, phenols and flavonols	Amelioration of tissue-complications associated with diabetes	Ibrahim *et al.,* 2018

Plate 1: Aristolochia ringens root

2.5 Classes and mechanisms of action of antidiabetic drugs

Different antidiabetic drugs have been described. They include biguanides, sulphonylureas, thiazolidinediones, secratagogues and carbohydrate-linked enzyme inhibitors. The most common biguanide is metformin. Metformin has been available since the 1950s and its historic roots and origin can be traced back to the guanidine-rich *Galega officinalis* (goat's rue or French lilac) which has traditionally been used in Europe to treat diabetes (Rena *et al.,* 2017). Metformin has a variety of clinical actions that extend beyond just the glucose-lowering effects such as weight reduction, improving lipid profiles and vascular effects, which includes improving endothelial function, as well as decreasing PAI-1 levels. With the introduction of thiazolidinediones in 1997, the world watched the peroxisome proliferator activated receptor (PPAR)-γ agonists with anticipation. The net effect of these drugs results from stimulation of a nuclear PPAR-γ that regulates the transcription of genes culminating in an increase in insulin sensitivity. Troglitazone, the forerunner drug, was withdrawn in 2000 following reports of fatal hepatotoxicity, and the future of rosiglitazone currently hangs in the balance, owing to a possible increased risk of myocardial infarction and cardiovascular-related deaths (Rena *et al.,* 2017).

Acarbose was the first glucosidase inhibitor and was introduced to the market in the early 1990s. This class of drug has the advantage of reducing postprandial hyperglycaemia without associated weight gain. Its usage is at present hampered by unfortunate gastrointestinal side-effects despite a good safety record. The α-glucosidase inhibitors inhibit the activity of the glucosidase enzymes which are present in the brush border of enterocytes in the intestinal villi. Disaccharide and oligosaccharide cleavage is prevented with a net decrease in intestinal carbohydrate absorption. Overall, the α-glucosidase inhibitors reduce postprandial insulin concentrations through the attenuated rise in postprandial glucose levels (Chiasson, 2007).

Inhibition of α-amylase and α-glucosidase has been shown to down regulate the rate glucose absorption (Sabiu *et al.,* 2017). A new class of drug called incretins with novel mechanism of action has also been described (Baggio and Drucker, 2007). The small intestine secretes glucagon-like peptide-1(GLP-1) as well as glucose-dependent insulinotropic polypeptide (GIP, previously called gastric inhibitory peptide) in response to food intake. These hormones stimulate insulin secretion, insulin gene expression and pancreatic beta-cell growth. Furthermore, they mediate the incretin effect which augments insulin secretion following oral administration of glucose. The GLP-1 molecule is subject to rapid degradation by the DPP-IV (dipeptidyl peptidase) enzyme. Patients with type 2 diabetes have greatly impaired or absent incretin-mediated insulin secretion due to a decrease in the level of GLP-1 which leads to a decrease in glucose-dependent secretion of insulin by the pancreatic beta-cells (Drucker and Nauck, 2006; Inzuchi and McGuire, 2008).

2.6 Kinetics of alpha-amylase and alpha-glucosidase

Alpha-amylase is a prominent enzyme found in the pancreatic juice and saliva which breaks down large insoluble starch molecules into absorbable molecules (Afifi *et al.,* 2011). On the other hand, mammalian α-glucosidase in the mucosal brush border of the small intestine catalyzes the end step of digestion of starch and disaccharides that are abundant in human diet (Manohar *et al.,* 2002). Inhibitors of α-amylase and α-glucosidase delay the breaking down of carbohydrates in the small intestine and diminish the postprandial blood glucose excursion (Kwon *et al.,* 2008; Sabiu *et al.,* 2017). An effective means of lowering the levels of postprandial hyperglycaemia have been offered by α-amylase and α-glucosidase inhibitors (Sabiu *et al.,* 2016). Several inhibitors of α-amylase and α-glucosidase have been isolated from medicinal plants to serve as alternative drugs with increased potency and lesser adverse effects than

existing synthetic drugs (Kazeem *et al.*, 2013). α-Glucosidase is a critical enzyme associated with type 2 diabetes mellitus in humans. Inhibition of α-glucosidase is important due to the potential effect of down regulating glucose absorption in patients (Yan *et al.,* 2014; Sabiu *et al.,* 2017). Many α-glucosidase inhibitors have been described. Luteolin inhibited α-glucosidase activity in a concentration dependent-manner (Yan *et al.,* 2014). The significant inhibitory activity of luteolin on α-glucosidase suggested that luteolin may directly bind to the enzyme. Molecular docking studies have shown that the binding site on α-glucosidase for luteolin was not the same as acarbose. Several feed formulations have also been reported to significantly inhibit α-amylase and α-glucosidase (Kwon *et al.,* 2008; Irondi *et al.,* 2016). A combination of inhibition kinetics, interaction mechanism and molecular modelling may facilitate the evaluation of inhibitory mechanism.

Alpha amylase is an endoenzyme that carries out multiple attacks on linear portions of amylose and amylopectin with maltose and maltotriose as the principal short chain products (Rena *et al.,* 2017). Kinetic studies suggested that the active site of porcine pancreatic amylase can accommodate up to five glucose residues, i.e. there are five sub-sites at which glucose residues can become bound (Rena *et al.,* 2017). The existence of multiple sites was confirmed by the production of 3D structures from X-ray crystallography, although a report of the structure of pig pancreatic amylase complexed with the potent carbohydrate inhibitor acarbose provided evidence of a sixth site (Mithieux *et al.,* 1996). The glycosidic bond susceptible to attack is that linking residues 3 and 4 and the catalytically important aspartate and glutamate residues of the active site are suitably located for attack on the susceptible link.

2.7 Characterization of plant extract

Natural products from medicinal plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity (Cos *et al*., 2006). Botanicals and herbal preparations for medicinal usage contain various types of bioactive compounds that can be used in the management of different conditions such as ulcer, diabetes mellitus, infertility and anti-cataract (Saheed *et al.,* 2015; Sulyman *et al.,* 2016; Sabiu *et al.,* 2016 and Ajani *et al.,* 2017). Due to the development of adverse effects and microbial resistance to the chemically synthesized drugs, men turned to ethnopharmacognosy (Sasidharan and Menon, 2010). According to the World Health Organization (WHO), nearly 20,000 medicinal plants exist in 91 countries including 12 mega biodiversity countries. The premier steps to utilize the biologically active compounds from plant resources are extraction, pharmacological screening, isolation and characterization, toxicological and clinical evaluation.

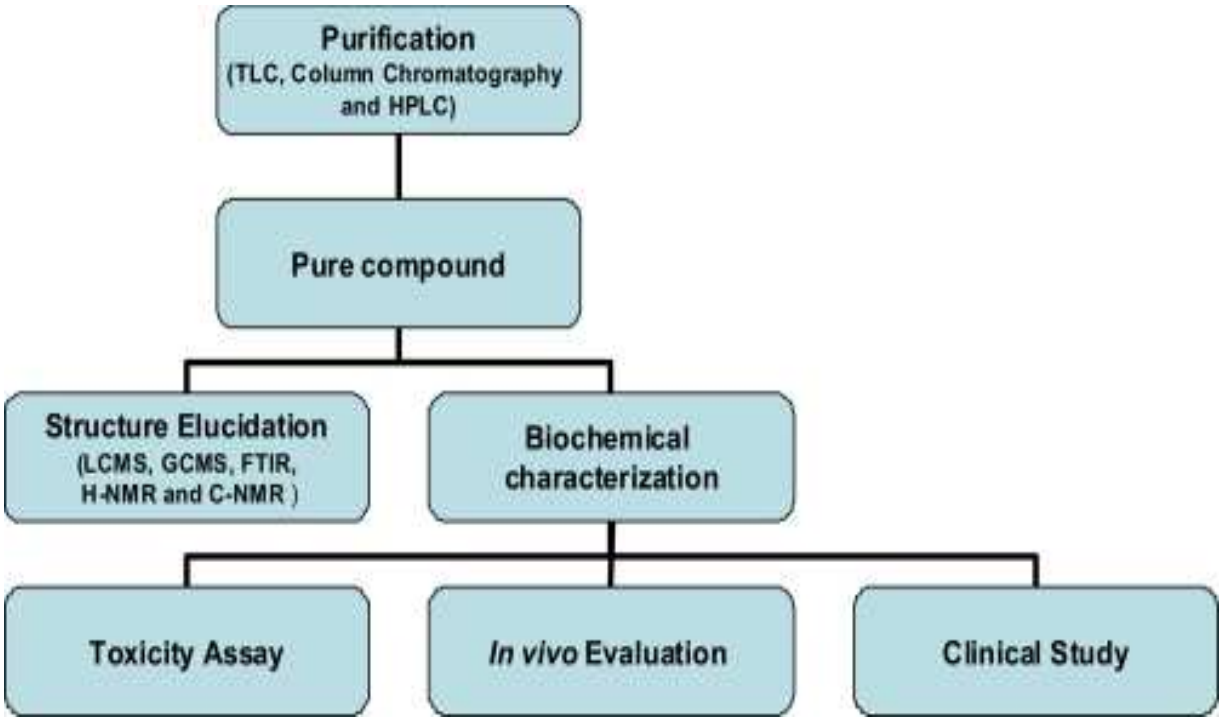


Figure 2: A brief summary of the general approaches in extraction, isolation and characterization of bioactive compound from plants extract

Source: (Sasidharan and Menon, 2010)

Extraction is the first step in the characterization of medicinal plants, because it is necessary to extract the desired phytochemicals from the plant materials for further biochemical assays. Steps involved include pre-washing, drying of plant materials, pulverizing to obtain a fine homogenous sample to improve the kinetics of analytic extraction and also increasing the contact of sample surface with the solvent system. Proper actions must be taken to assure that potential active constituents are not lost, deformed or destroyed during the preparation of the extract from plant samples. If the plant use was on the basis of folkloric application, then it is vital to follow the procedures of preparation of the extract as described by the traditional healer in order to mimic as closely as possible the traditional 'herbal' drug (Fabricant and Farnsworth, 2001). The selection of solvent system largely depends on the specific nature of the bioactive compound being targeted. Different solvent systems are available to extract the bioactive compound from natural products. The extraction of hydrophilic compounds uses polar solvents such as methanol, ethanol or ethyl-acetate. For extraction of more lipophilic compounds, dichloromethane or a mixture of dichloromethane/methanol in ratio of 1:1 are used. In some instances, extraction with hexane is used to remove chlorophyll (Cos *et al.,* 2006).

Due to the fact that plant extracts usually occur as a combination of various types of bioactive compounds or phytochemicals with different polarities, their separation still remains a big challenge for the process of identification and characterization of bioactive compounds. It is a common practice in isolation of these bioactive compounds that a number of different separation techniques such as Liquid chromatography-mass spectrophotometry (LC-MS), thin layer chromatography (TLC), column chromatography, flash chromatography, Sephadex chromatography and high performance liquid chromatography (HPLC) are used to obtain pure compounds. The pure compounds are then used for the determination of structure and biological

activity. Non-chromatographic techniques such as immunoassay, which use monoclonal antibodies (MAbs), phytochemical screening assay, Fourier-transform infrared spectroscopy (FTIR), can also be used to obtain and facilitate the identification of the bioactive compounds (Sasidharan and Menon, 2010).

LC-MS is now a routine technique with the development of electrospray ionisation (ESI) providing a simple and robust interface. It can be applied to a wide range of biological molecules and the use of tandem MS and stable isotope internal standards allows highly sensitive and accurate assays to be developed, although some method optimisation is required to minimise ion suppression effects. Liquid chromatography coupled with mass spectrometry (LC-MS) is a powerful technique for the analysis of complex botanical extracts (He, 2000; Cai *et al.*, 2002). It provides abundant information for structural elucidation of the compounds when tandem mass spectrometry (MS^n) is applied. Therefore, the combination of HPLC and MS facilitates rapid and accurate identification of chemical compounds in medicinal herbs, especially when a pure standard is unavailable (Yeh *et al*., 2003). LC-MS technique has been applied in profiling the chemical identities of *Zanthoxylum zanthoxyloides,* timler extracts and essential oils (Tine *et al.,* 2017). Identification of compounds by HPLC is a crucial part of any HPLC assay. In order to identify any compound by HPLC, a detector must first be selected. Once the detector is selected and is set to optimal detection settings, a separation assay must be developed. The parameters of this assay should be such that a clean peak of the known sample is observed from the chromatograph. The identifying peak should have a reasonable retention time and should be well separated from extraneous peaks at the detection levels which the assay will be performed. UV detectors are popular among all the detectors because they offer high sensitivity (Li *et al*., 2004) and also because majority of naturally occurring compounds encountered have some UV

absorbance at low wavelengths (190–210 nm) (Cannell, 1998). The high sensitivity of UV detection is bonus if a compound of interest is only present in small amounts within the sample. Besides UV, other detection methods are also being employed to detect phytochemicals among which is the diode array detector (DAD) coupled with mass spectrometer (MS) (Tsao and Deng, 2004).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant Material and Authentication

The fresh root of *Aristolochia ringens* used in this study was procured from Oja-oba, Ilorin, Kwara State, Nigeria and authenticated (UIL001/3456) at the Botany Department, University of Ilorin, Ilorin, Nigeria.

3.1.2 Chemicals and Reagents

The following are the materials used to carry out this study:

i. Glucosidase (EC 3.2.1.20) from baker's yeast and porcine pancreatic alpha-amylase (EC 3.2.1.1) obtained from Tokyo Chemical Industry Co. (Shanghai, China).

ii. Sodium phosphate buffer of pH 7.0 prepared at the laboratory of Medical Biochemistry and Pharmacology, Kwara State University, Malete, Nigeria.

iii. Acarbose and p-nitrophenyl-d-glucopyranoside (pNPG) are products purchased from Sigma–Aldrich Co. (St. Louis, MO, USA), starch (Sigma–Aldrich Co. (St. Louis, MO, USA).

iv. Dinitrosalicyclic acid (DNS) colour reagent (Tokyo Chemical Industry Co. (Shanghai, China)

v. Ethanol, distilled water and other reagents used for this study are of high analytical grades.

3.2 Methods

3.2.1 Preparation of Extract

Dried root of *Aristolochia ringens* plant was weighed and pulverized using grinding machine (LP500/200) before extracting with 2000ml of 70% ethanol for 24h with intermittent shaking. The resulting filtrate was concentrated at 60°C using a rotary evaporator (Model R110, Brinkmann Instruments Inc, United States) (Sulyman *et al.,* 2016). Finally, the concentrated extract was dried on a water bath at (TSGP20, Thermoscientific Company, UK) to yield the plant extract.

3.2.2 Phytochemical Analysis

3.2.2.1 Qualitative Phytochemical Screening

Phytochemical analysis extract was carried out using the method described by Odebiyi and Sofowora (1978) for the detection of saponins, tannins, phenolics, alkaloids, steroids, triterpenes, phlobatannins, glycosides and flavonoids.

1. Alkaloids: Exactly $1cm^3$ of 1%HCl was added to $3cm^3$ of the extracts in a test tube. The mixture was heated for 20 minutes, cooled and filtered. The filtrate was used in the following tests: 2 drops of Wagner's reagent was added to $1cm^3$ of the extracts. A reddish-brown precipitate indicates the presence of alkaloids

2. Tannins: Exactly $1cm^3$ of freshly prepared 10% KOH was added to $1cm^3$of the extracts. A dirty white precipitate indicates the presence of tannins.

3. Phenolics: Exactly 2 drops of 5% $FeCl_3$ was added to $1cm^3$ of the extracts in a test tube. A greenish precipitate indicates the presence of phenolics.

4. Glycosides: Exactly 10cm^3 of 50% H_2SO_4 was added to 1cm^3 of the extracts, the mixture was heated in boiling water for 15 minutes. 10cm^3 of Fehling's solution was added and the mixture boiled. A brick red precipitate indicates the presence of glycosides.

5. Saponins: Exactly 2cm^3 of the extract in a test tube was vigorously shaken for 2 minutes. Frothing indicates the presence of saponins.

6. Flavonoids: Exactly 1cm^3 of 10% NaOH was added to 3cm^3 of the extracts. A yellow colouration indicates the presence of flavonoids.

7. Steroids: Exactly 5 drops of concentrated H_2SO_4 was added to 1cm^3 of the extracts. Red colouration indicates the presence of steroids

8. Phlobatannins: Exactly 1cm^3 of the extracts was added to 1% HCl. A red precipitate indicates the presence of phlobatannins.

9. Triterpenes: Exactly 5 drops of acetic anhydride was added 1cm^3 of the extracts. A drop of concentrated H_2SO_4 was then added and the mixture was steamed for 1 hour and neutralized with NaOH followed by the addition of chloroform. A blue green colour indicates the presence of triterpenes.

10. Phytosterols: Exactly 50mg is dissolved in 2ml acetic anhydride. To this, one or two drops of concentrated H_2SO_4 is added slowly along the sides of the test tube. An array of colour changes shows the presence of phytosterols (Finar 1986).

11. Terpenoids: Exactly 5ml of aqueous extract of the sample is mixed with 2ml of $CHCl_3$ in a test tube 3ml of concentrated H_2SO_4 is carefully added to the mixture to form a layer. An interface with a reddish-brown coloration is formed if terpenoid is present.

13. Amino acid: Two drops of ninhydrin solution (10mg of ninhydrin in 200ml of acetone) are added to 2 ml of aqueous filtrate. A characteristic purple colour indicates the presence of amino acids (Yasuma and Ichikawa, 1953).

3.2.2.2 Quantitative Phytochemical Screening

Phenolic content: The total phenolic content of sample was estimated according to the method reported by Skotti *et al.* (2014). The aliquot of the extract was taken in a test tube and made up to the volume of 1 ml with distilled water. Then 0.5ml of Folin-Ciocalteu reagent (1:1 with water) and 2.5 ml of sodium carbonate solution (20%) were added sequentially to the test tube. Soon after vortexing the reaction mixture, the tubes were placed in the dark for 40 min and the absorbance was recorded at 725 nm against the reagent blank. Using gallic acid monohydrate, a standard curve was prepared. The linearity obtained was in the range of 1-10 µg/ml. Using the standard curve, the total phenolic content was calculated and expressed as gallic acid equivalent in mg/g of extract.

Total flavonoid assay: Total flavonoid content was measured by aluminium chloride colorimetric assay. 1ml of extracts or standard solution of quercetin (500µg/ml) was added to 10 ml volumetric flask containing 4 ml of distilled water. To the above mixture, 0.3 ml of 5% $NaNO_2$ was added. After 5 minutes, 0.3 ml of 10% $AlCl_3$ was added. At 6th min, 2 ml of 1 M NaOH was added and the total volume was made up to 10 ml with distilled water. The solution was mixed well, and the absorbance was measured against prepared reagent blank at 510 nm. Total flavonoid content of the flower was expressed as percentage of quercetin equivalent per 100 g of the extract.

Saponins: The spectrophotometric method of Bruner (1984) was used for the analysis of saponins. Briefly, 1g of the finely ground dried sample was weighed into a 250ml beaker and 100ml of isobutyl alcohol was added. The mixture was shaken vigorously on to ensure uniform mixing. Thereafter, the mixture was filtered through a Whatman No. 1 filter paper into a 100ml beaker containing 20ml of 40% saturated solution of $MgCO_3$. The resulting mixture was again filtered to obtain a clear colourless solution. Exactly 1 ml of the colourless filtrate was pipetted into a 50ml volumetric flask and 2mlof 5% $FeCl_3$ solution was added and made up to the marked level with distilled water. This was then allowed to stand for 30 min for a blood red colour to develop. Subsequently, 0-10ppm saponins standard was prepared from saponins stock solution. The standard solutions were treated similarly with 2ml of 5% $FeCl_3$ solution as earlier described. The absorbance of the samples as well as standard saponin solutions was read after colour development using a Jenway V6300 spectrophotometer at wavelength of 380nm. The percentage saponin was calculated using the formula:

$$\% \text{ saponin} = \frac{\text{Absorbance of sample} \times \text{Average gradient} \times \text{Dilution factor}}{\text{Weight of sample} \times 100}$$

Alkaloids: The quantitative determination of alkaloids was done by distillation and titrimetric methods. Exactly 2g of finely ground sample was weighed into 100ml beaker and 20ml of 80% alcohol added to give a smooth paste. The mixture was transferred to a 250ml flask and more alcohol added to make it up before 1g of magnesium oxide was then added. The mixture was digested in a boiling water bath for an hour and half under a reflux air condenser with occasional shaking. The mixture was filtered while hot through a Buchner funnel. The residue was poured back into the flask and re-digested for another 30minutes with 50ml alcohol after which the alcohol was evaporated. Distilled water was added to replace the lost alcohol. When all alcohol

has evaporated, 3 drops of 10% HCl was added. The whole solution was later transferred into 250ml volumetric flask; 5ml of zinc acetate solution and 5ml of potassium ferricyanide solution were thoroughly mixed together to give a homogenous mixture. The flask was allowed to stand for a few minutes, filtered through a dry filter paper and 10ml of the filtrate was transferred into a separating funnel and the alkaloids present were extracted vigorously by shaking with five successive portions of chloroform. The residue obtained was dissolved in 10ml of hot distilled water and transferred into a Kjeldahl tube with the addition of 0.2g of selenium for digestion to a clear colourless solution. The clear colourless solution was used to determine nitrogen using Kjeldahl distillation apparatus and the distillate was back titrated with 0.01N HCl and the titre value obtained was used to calculate the % nitrogen using the formulae:

$$\%N = \frac{\text{Titre value} \times \text{Atomic mass of nitrogen} \times \text{Normality of HCl}}{\text{1mg of extract}} \times 100$$

% Alkaloid = % Nitrogen × 3.26

Where 3.26 is a conversion factor (constant)

Tannins: The method of Swain (1979) was used to determine the quantity of tannins. Exactly 0.20g of sample was measured into a 50ml beaker 20ml of 50% methanol was added and covered with paraflim and placed in a water bath at 77-80˚C for 1 hour. It was shaken thoroughly to ensure uniform mixing. The extract was filtered using a double layered Whatman No. 41filter paper into a 100ml volumetric flask.20ml water was added and 2.5ml Folin-Denis reagent and 10ml of 17% Na_2CO_3 were added and mixed properly. The mixture was made up to mark with water mixed well and allowed to stand for 20 minutes. A bluish-green colour will develop at the end of range. 0-10ppm was treated similarly as 1ml sample above.

The absorbance of the Tannic acid standard solutions as well as samples was read after colour development on a spectronic 21D spectrophotometer at a wavelength of 760nm. % Tannin was calculated using the formula:

$$\% \text{ Tannin} = \frac{\text{Absorbance of sample} \times \text{Average gradient} \times \text{Dilution factor}}{\text{Weight of sample}} \times 100$$

Glycosides: Exactly 10ml of extract was pipette into a 250ml conical flask and 50ml of chloroform was added and shaken on a Vortex Mixer for 1 hour. The mixture was filtered into a conical flask. Then, 10ml pyridine and 2ml of 2% sodium nitroprusside were added and shaken thoroughly for 10 minutes. Exactly 3ml of 20% NaOH was later added to develop a brownish yellow colour. Glycoside standard of concentration ranging from 0-5mg/ml were prepared from 100mg/ml stock glycoside standard. The series of standards 0-5mg/ml were treated similarly like the sample above. The absorbance of sample as well as standards was read at a wavelength of 510nm. % Glycoside was calculated using the formula:

$$\% \text{ Glycoside} = \frac{\text{Absorbance of sample} \times \text{Average gradient} \times \text{Dilution factor}}{\text{Weight of sample}} \times 100$$

Steroids: Exactly 0.05g of sample extract was weighed into a 100ml beaker before 20ml of chloroform-methanol (2:1) mixture was added to dissolve the extract upon shaking for 30 minutes on a shaker. Exactly 1ml of the filtrate was pipette into a 30ml test tube and 5ml of alcoholic KOH was added and shaken thoroughly to obtain a homogenous mixture. The mixture was later placed in a water bath set at 37˚C-40˚C for 90 minutes. It was cooled to room temperature and 10ml of petroleum ether added followed by the addition of 5ml distilled water. This was evaporated to dryness on the water bath before 6ml of Liebermann-Buchard reagent

was added to the residue in dry bottle and absorbance taken at a wavelength of 620nm. Standard steroid of concentration of 0-4mg/ml were prepared from 100mg/ml stock steroid solution and treated similarly like the sample as above.

% steroid was calculated using the formula:

$$\% \text{ steroid} = \frac{\text{Absorbance of sample} \times \text{Average gradient} \times \text{Dilution factor}}{\text{Weight of sample}} \times 100$$

3.2.3 α-Amylase Inhibitory Assay

The method reported by Sabiu *et al.* (2016) was adopted for this study. Briefly, 500 µl of *Aristolochia ringens* extract and 500 µl of 0.02 M phosphate buffer pH 6.9, containing porcine α-amylase (Cat. No. 10080, Sigma Aldrich Chemical Co, Steinheim, Germany) at a concentration of 0.5 mg/ml was incubated at 25°C for 10 min. After pre-incubation, 500 µl of 1% starch (R & M Chemicals, Essex, UK) solution in 0.02 M phosphate buffer, pH 6.9, was added. The reaction mixture was then incubated at 25°C for 10 min. The reaction was stopped with 1ml 3,5-dinitrosalicylic acid (DNS) (Cat. No. D 0550, Sigma Aldrich Chemical Co, USA) color reagent. The test tubes were then incubated in a boiling water bath for 5 min and cooled to room temp. Absorbance (A) was measured at 540 nm. Percentage inhibition was calculated as follows:

$$\% \text{ Inhibition} = \frac{A_{540}\ \text{control} - A_{540}\ \text{extract}}{A_{540}\ \text{control}} \times 100$$

* A_{540} control= absorbance of sodium phosphate buffer (0.02 M, pH 6.9)

A_{540} extract= absorbance of *A.ringens*

3.2.4 α-Glucosidase Inhibitory assay

The method described by Elsnoussi *et al.* (2012) and reported by Sabiu *et al.* (2016) was followed. Different concentration (0.1-1.0 mg/mL) of the extract or acarbose were prepared and 50 µL from each stock solution was mixed with 100 µL of 0.1 M phosphate buffer (pH 6.9) containing 1.0 M of α-glucosidase solution and incubated at 25ºC for 10 min. Following this, 50 µL of 5 mM *p*NPG solution in 0.1 M phosphate buffer (pH 6.9) was added and the reaction mixtures were further incubated at 25ºC for 5 min. The absorbance in each case was read at 405 nm and the values compared with a control which contained 50 µL of the buffer instead of the extract. The assay was triplicated and the inhibitory effect of the extract on the activity of α-glucosidase was calculated. Using standard calibration curve, the concentration of the extract causing 50% inhibition (IC50) of α-glucosidase activity was extrapolated.

3.2.5 Inhibitory Kinetic Analysis

3.2.5.1 Alpha-amylase Kinetics

As reported by Sabiu *et al.* (2017), 100 µL of either the extract (at it IC50) or sodium phosphate buffer (pH 6.9, 0.02 M) was pre-incubated (25ºC, 10 min) with α-amylase solution (100 µL) in two sets of test tubes. Following this, varying concentrations (0.3-5.0 mg/mL) of starch (substrate (S) was added to both sets of mixtures to initiate the reaction. Finally, the resulting mixture in each case was further treated with DNS (100 µL), boiled (100 ºC, 5 min) and allowed to proceed as highlighted above. The released reducing sugar was then spectrophotometrically (Beckman, DU 7400, USA) estimated from maltose standard curve. The values obtained were expressed as reaction rates (v). Using Lineweaver-Burk double reciprocal

plot (Lineweaver and Burk, 1934), the kinetic indices (Km and Vmax values) and the tentative mechanism of inhibitory effect of the extract on the activity of α-amylase was predicted.

3.2.5.2 Alpha-glucosidase Kinetics

To determine the inhibitory kinetics of the extract on activity of α-glucosidase, 50 µL of either the extract at its IC_{50} value or phosphate buffer (pH 6.9) was pre-incubated (10 min, 25°C) with solutions of alpha-glucosidase (100 µL) in two sets of tubes. Subsequently, 50 µL each of 0.63 – 2.0 mg/mL (*p*NPG) was introduced into the two set-ups for reaction initiation before allowing an incubation period of 10 min at 25°C. To halt the reaction, Na_2CO_3 (500 µL) was added and the released sugar in each case was colorimetrically estimated from para-nitrophenol calibration curve. The reaction rates (v) were then estimated and Lineweaver-Burk double reciprocal plot was used to predict the nature of inhibition of the enzyme by the extract. This method is as described by Sabiu *et al.* (2017).

3.2.6 Homology modelling and Molecular docking analysis

Homology modelling and molecular docking analysis was carried out following the procedures described by Yan *et al.* (2014). Protein Data Bank (PDB) was searched to find a proper structural template for homology modelling (http://www.ncbi.nlm.nih.gov/protein/), and BLAST algorithms was used with the amino acid sequence of the targets as input. The 3D structure of the bioactive compounds and acarbose was generated in Sybyl × 1.1 (Tripos Inc., St. Louis, USA), and the homology model was used as the receptor model in the virtual screening with docking simulations. Removing all the water molecules, Gasteiger charges and essential hydrogen atoms were added by using the AutoDock tools (version 4.2). The rotatable bonds in

the ligand were assigned with AutoDock Tools, and the ligand docking was performed with the AutoDock Lamar-ckian Genetic Algorithm (LGA, runs 100).

3.2.8 LC-MS Data

The phytochemical compositions of *A. ringens* ethanolic root extract were determined using a Hewlett-Packard 1100 chromatograph (Agilent Technologies, Santa Clara, CA) with a quaternary pump and a diode array detector (DAD). The column is coupled with an MSD Ion Trap XCT mass spectrometer (Agilent Technologies, Santa Clara, CA) equipped with an electrospray ionization interface (ESI). Fractions were injected onto a C-18 column (4.6 × 25 cm, 5 μm; Phenomenex UK, Macclesfield, UK). The solvents used were 90% acetic acid–water (A) and 10% MeOH (B). The elution gradient was isocratic 10% B for 5 min, 10–100% B over 20 min, 100% B for 6 min, and re-equilibration of the column, using a flow rate of 200 μL/min. Spectra were recorded in negative and positive ionization mode between *m/z* 50 and 1200.

3.3 Statistical Analysis

The experimental results including alpha glucosidase and alpha amylase activities assay and inhibitory kinetic analysis was expressed as mean value ± standard error of mean (n = 3), and data analyzed using SAS statistical package (version 8.1, SAS Institute, Cary, NC, USA).

CHAPTER FOUR

RESULTS

4.1 Results

4.1.1 Phytochemical Screening

4.1.1.1 Qualitative Phytochemical Screening

Phytochemical screening of ethanolic root extract of *Aristolochia ringens* revealed the presence of alkaloids, tannins, flavonoids, phenolics, terpenoids, saponins, triterpenes and phlobatannins (Table 2). However, glycosides, anthraquinones, steroids, coumarins and amino acids were tested for but not detected.

4.1.1.2 Quantitative Phytochemical Screening

In table 3, the quantitative phytochemical screening of ethanolic root extract of *Aristolochia ringens* was presented. The quantity of the phytochemicals earlier detected was determined in mg/100g of the extract. There is a significant variation in the quantity of the phytochemicals quantified ($p<0.05$).

Table 2: Qualitative Phytochemical Screening of *A. Ringens*

Phytochemicals	**Remark**
Alkaloids	+
Tannins	+
Glycosides	-
Anthraquinones	-
Flavonoids	+
Phenolics	+
Terpenoids	+
Saponin	+
Steroids	-
Triterpenes	+
Coumarins	-
Amino acids	-
Phlobatannins	+

*+=detected; – =not detected.

Table 3: Quantitative phytochemical screening of *A. ringens*

Phytochemicals	**Quantity (mg/100g)**
Flavonoids	5.22±0.05
Phenolics	8.91±0.56
Saponins	14.52±0.30
Alkaloids	11.62±0.05
Tannins	6.52±0.12
Triterpenes	2.93±0.43

*(n=3, SEM), ($p<0.05$).

4.1.2 Inhibitory Assays

4.1.2.1 Alpha-amylase inhibitory assay

The inhibitory potential of ethanolic root extract of *A. ringens* on the activity of alpha-amylase was compared with that of the standard drug (acarbose) as shown in table 4. The inhibition of alpha-amylase is dose-dependent for both acarbose and extract and there exists significant difference between them ($p<0.05$). The highest percentage inhibition was observed at the highest concentration for the extract and acarbose (73.21% and 71.12% respectively). There is however no significant difference between the IC_{50} of acarbose and the extract (0.63 and 0.67mg respectively).

4.1.2.2 Alpha-glucosidase inhibitory assay

The inhibitory potential of ethanolic root extract of *A. ringens* on the activity of alpha-glucosidase is shown in table 5. The inhibition compared favourably with that of the standard drug (acarbose). The inhibition of alpha-glucosidase increases significantly ($p<0.05$) as the concentration increases for both acarbose and extract. The highest percentage inhibition was observed at the highest concentration for the extract and acarbose (81.11% and 84.21% respectively). However, there is no significant difference between the IC_{50} of the acarbose and the extract (0.54 and 0.57mg).

Table 4: Inhibitory potential of *Aristolochia ringens* root extract on the activity of α-amylase

	% Inhibition	
Concentration (mg/ml)	**Acarbose**	**Extract**
0.1	16.25±0.05	14.25±0.01
0.2	19.12±0.03	17.75±0.04
0.4	34.61±0.05	33.10±0.02
0.6	48.1±0.03	45.13±0.02
0.8	62.71±0.15	60.11±0.10
1	73.21±0.25	71.12±0.07
IC_{50}	**0.63±0.03**	**0.67±0.02**

* (n=3, mean ± SEM)

($p>0.05$)

Table 5: Inhibitory potential of *Aristolochia ringens* root extract on the activity of α-glucosidase

	%Inhibition	
Concentration (mg/ml)	**Acarbose**	**Extract**
0.1	19.22±0.04	19.82±0.10
0.2	24.82±0.02	25.52±0.15
0.4	43.55±0.03	41.22±0.02
0.6	55.2±0.05	53.10±0.05
0.8	67.14±0.10	68.24±0.05
1.0	81.11±0.03	84.20±0.01
IC_{50}	**0.54±0.03**	**0.57±0.02**

* (n=3, mean ± SEM)

($p>0.05$)

4.1.3 Inhibitory Kinetics

4.1.3.1 Alpha-amylase Inhibitory Kinetics

In figure 3, the Lineweaver-Burk plot of ethanolic root extract of *Aristolochia ringens* showing competitive inhibition on the activity of α-amylase is presented. The kinetic parameters (Km and Vmax) were extrapolated from the plot and there is a significant difference ($p<0.05$) between the Km values of the extract and control (0.309×10^{-6}mg and 0.238×10^{-6}mg respectively). The Vmax value for the extract and control is the same (0.362×10^{-6} μM/min) as shown in figure 5.

4.1.3.2 Alpha-glucosidase Inhibitory Kinetics

Lineweaver-Burk plot of ethanolic root extract of *Aristolochia ringens* on the activity of α-glucosidase is presented in figure 4. The inhibition pattern is uncompetitive as shown from the kinetic parameters (Km and Vmax) obtained from the plot. There is a significant difference ($p<0.05$) between the Km values of the extract and control (2.683×10^{-7}mg and 2.167×10^{-7}mg respectively). The Vmax values for the extract and control also show significant difference ($p<0.05$) (1.218×10^{-7} μM/min and 1.019×10^{-7} μM/min respectively).

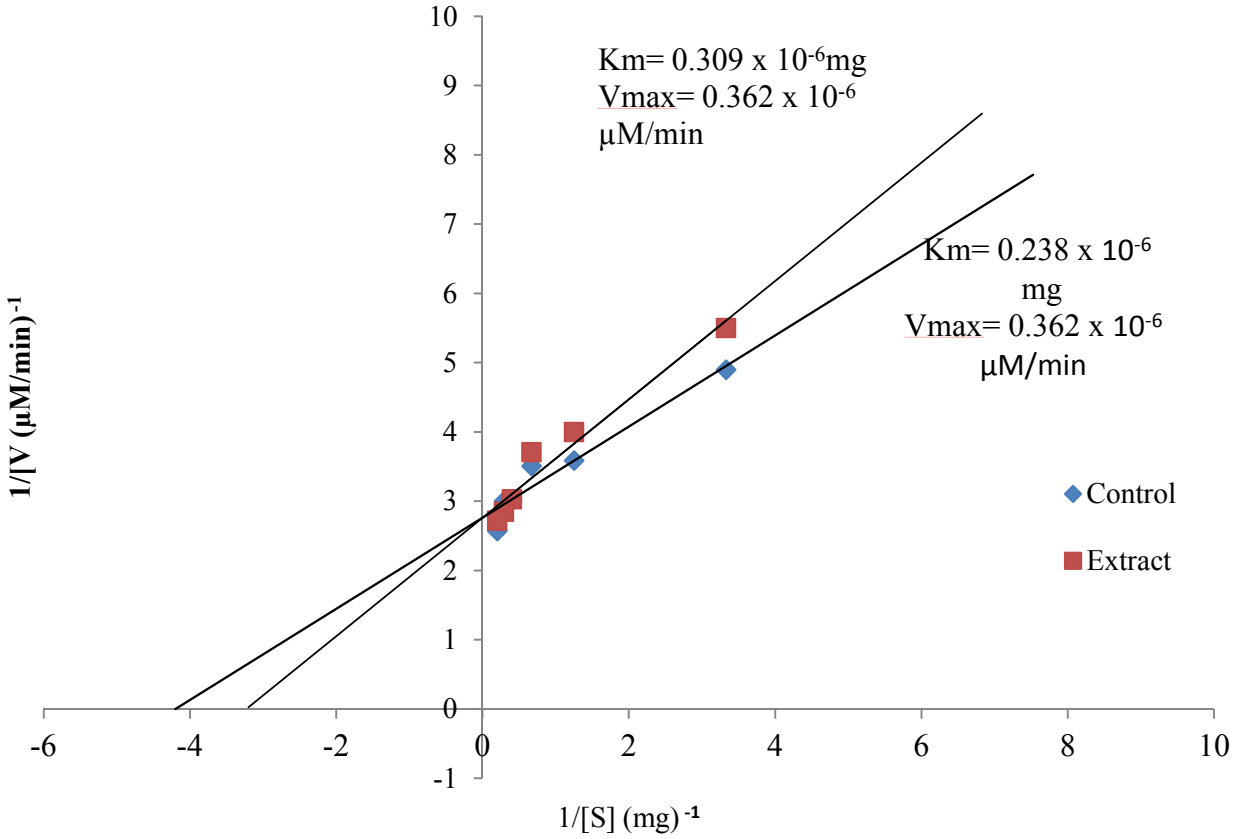


Figure 3: Lineweaver-Burk plot of ethanolic root extract of *Aristolochia ringens* showing competitive inhibition on the activity of α-amylase. Data represent mean ± SEM of three determinations.

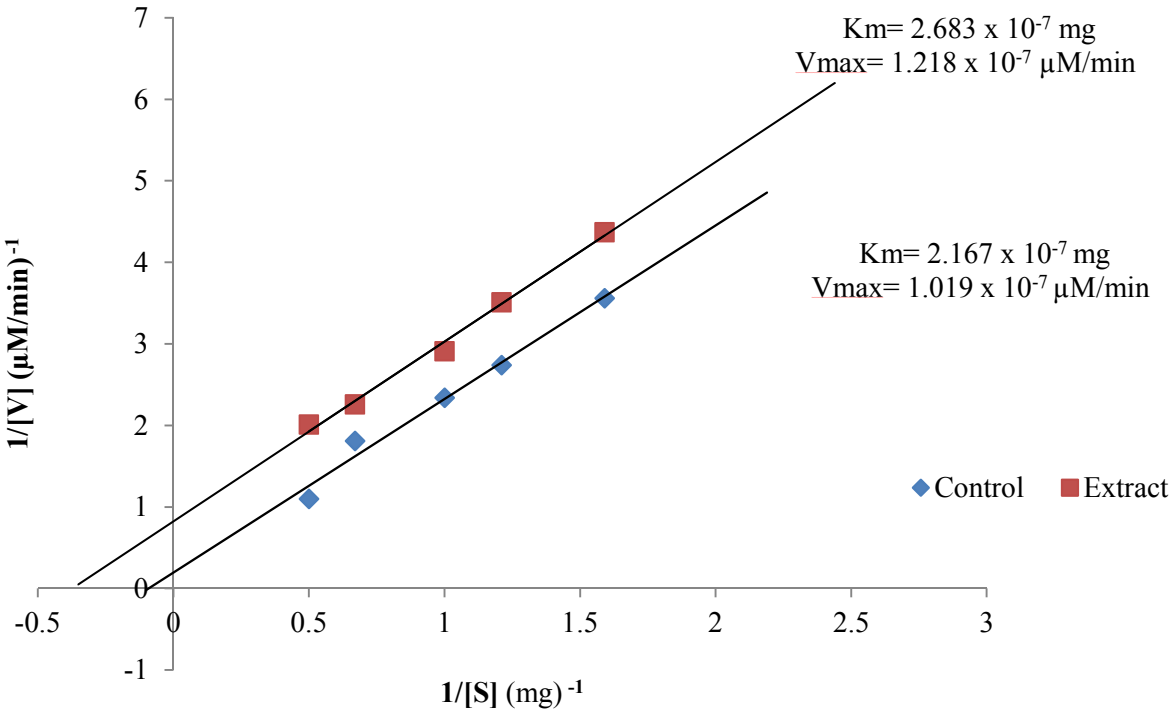


Figure 4: Lineweaver-Burk plot of ethanolic root extract of *Aristolochia ringens* showing uncompetitive inhibition on the activity of α-glucosidase. Data represent mean ± SEM of three determinations.

4.1.4 LC-MS result

The chemical profile of ethanolic root extract of *Aristolochia ringens* as revealed by LC-MS analysis is presented in table 6. The chromatogram revealed the presence of asiatic acid, magnoflorine and phenyl-β-glucopyranoside with retention time of 13.08, 21.60 and 20.15 minutes respectively (figure 5). Other compounds revealed include aristolochic acid, Quercetin-3-*O*-*p*-coumaroyl glucoside, corosolic acid, scrictosidine, fordianoside, trilobine, neoasarinin and two other unidentified compounds (table 6).

Table 6: Chemical profile of ethanolic root extract of *Aristolochia ringens*

S/N	RT (min.)	*m/z*	Fragment ions	Compound	Activity
1	1.42	360 $[M+1]^+$	325 (34), 163 (37), 146 (68), 198 (15).	Aristolochic acid I, III	Carcinogenic (Soon *et al.,* 2015)
2	9.61	611 $[M+1]^+$	303 (97), 465 (70), 197 (63), 372 (30).	Quercetin-3-*O*-*p*-coumaroyl glucoside	Anti-inflammatory (Lesiak *et al.,* 2018)
3	10.79	566 $[M+1]^+$	548 (83), 287 (40), 449 (34).	Unidentified	Antidiabetic (Ramanchandran *et al.,* 2014)
4	13.08	489 $[M+1]^+$	457 (51), 169 (17), 214 (11), 391 (9).	Asiatic acid	Anti-inflammatory (Miura *et al.,* 2012)
5	14.86	473 $[M+1]^+$	457 (50), 169 (17), 214 (11), 391 (9).	Corosolic acid	Antioxidant (Li *et al.,* 2016)
6	13.28	501$[M+Na]^+$	401 (25), 169 (15).	Quercetin-3-*O*-glucoronyl	Anti-inflammatory (Delvin, 2016)
7	14.06	531 $[M+1]^+$	485 (74), 453 (43), 435(5)	Scrictosidine	Anti-inflammatory (Sydney *et al.,* 2012)
8	16.29	485$[M+Na]^+$	473 (43) 419 (38), 183 (38)	Fordianoside	Antimicrobial (Hall *et al.,* 2014)
9	16.68	563 $[M+H]^+$	505 (80), 487 (54), 473 (28).	Trilobine	Anticancer (Liu *et al.,* 2014)

10	17.48	587$[M+Na]^+$	485 (40), 517 (33), 519 (16)	Aristophyll B	Anti-inflammatory (Jang *et al.,* 2017)
11	18.16	387$[M+Na]^+$	267 (15), 183 (10).	Neoasarinin	----------
12	18.48	274 $[M+1]^+$	217 (5), 169 (4).	Unidentified	Antidiabetic (Fujimori *et al.,* 2009)
13	20.15	478$[M+Na]^+$	304 (11), 415 (8), 253 (5).	Phenyl-β-D-glucopyranoside	Antidiabetic (Fujimori *et al.,* 2009)
14	21.60	343 $[M+1]^+$	240 (56), 332 (20), 169 (11).	Magnoflorine	Antidiabetic (Patel & Mishra, 2012)

*RT= Retention time; *m/z* = mass to charge ratio.

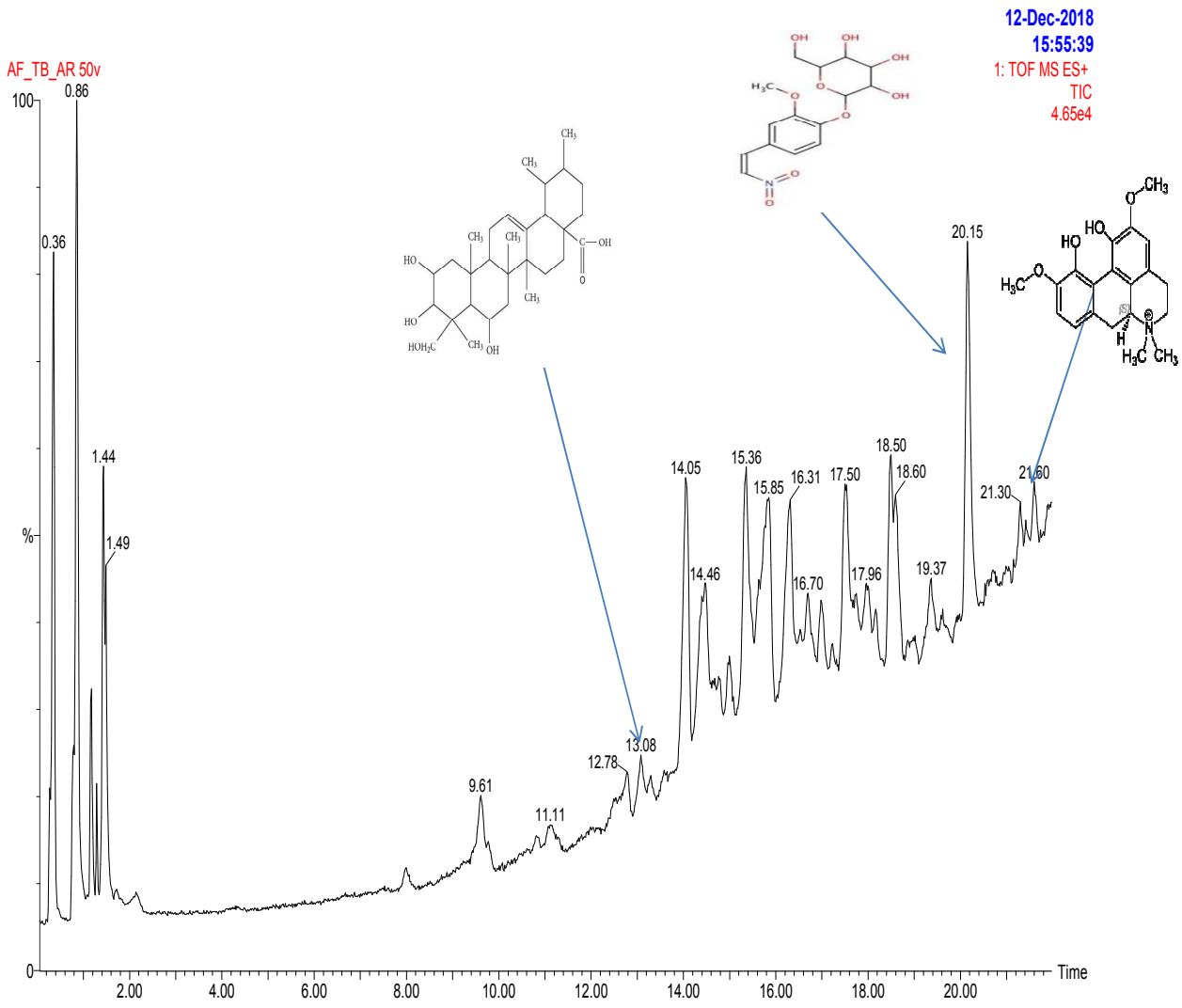


Figure 5: LC-MS chromatogram of root ethanolic extract of *Aristolochia ringens*

4.1.5 Molecular docking

The docking properties of Asiatic acid, phenyl-β-d-glucopyranoside and magnoflorine against α-amylase and α-glucosidase were shown in table 8. The respective binding energy of Asiatic acid, phenyl-β-d-glucopyranoside and magnoflorine against α-amylase are -7.5, -4.6 and -7.3 Kcal/mol). When docked against α-glucosidase, the binding affinity of Asiatic acid, phenyl-β-d-glucopyranoside and magnoflorine are -8.0, -8.2 and -5.0 Kcal/mol (table 8). The respective interactions of Asiatic acid, phenyl-β-d-glucopyranoside and magnoflorine with the amino acids at the active sites of α-amylase and α-glucosidase are shown in table 8.

Table 7: Docking properties of bioactive compounds from ethanolic root extract of *Aristolochia ringens* against α-amylase and α-glucosidase

Enzyme	Ligand molecule	Binding affinity (Kcal/mol)	Amino acids involved in the interactions
α-amylase	Asiatic acid	-7.5	Val400, Trp396, Asn399, Gly9, Arg10
	Magnoflorine	-7.3	Gln187, Arg74, Pro54, Hoh514, Trp357
	Phenyl-β-D-glucopyranoside	-4.6	Asn53, Hoh653, Hoh655, Val51,Thr52, Hoh739, Ala107
	Magnoflorine	-8.2	Phe163, Tyr63
α-glucosidase	Asiatic acid	-8.0	Tyr63, Phe282, Asn258, Phe 44, Phe163, Gln167
	Phenyl-β-d-glucopyranoside	-5.0	Hoh768, Asn277,Trp6, Hoh699, Hoh633, Ile251

CHAPTER FIVE

5.1 Discussion

5.1.1 Chemical profile of *Aristolochia ringens*

The phytochemical screening revealed the presence of diverse groups of secondary metabolites that have been reported to possess hypoglycaemic qualities. Alkaloids, phenolics, and flavonoids have earlier been implicated in anti-hyperglycemic studies (Sulyman *et al.,* 2016, Irondi *et al.,* 2016 and Sabiu *et al.,* 2017). *Aristolochia ringens* is rich in saponin, alkaloid, flavonoids and polyphenols. This is consistent with reports of Alali *et al.* (2006) and Bernaba *et al.,* 2012) on other species of Aristolochiaceae, *Aristolochia maurorum* and *Aristolochia longa* respectively.

LC-MS analysis of the extract further revealed the presence of 14 compounds: 12 known and 2 unknown. Effort is on-going to unravel the identity of the two unknown compounds, with all available information pointing to their novelty. Asiatic acid, magnoflorine, corossolic acid, scrictosidine, fordianoside, trilobine, neoasarinin, and phenyl-β-D-glucopyranoside are among the compounds identified. The antidiabetic potentials of Asiatic acid, magnoflorine and phenyl-β-D-glucopyranoside have earlier been reported in other plants (Ramanchandran *et al.,* 2014; Patel and Mishra, 2012, Fujimori *et al.,* 2008) respectively. These three compounds are the probable compounds responsible for the inhibitory influence of the extract on α-amylase and α-glucosidase. Although many of these compounds are spread across various plant groups, aristolochic acid is peculiar to many plants in Aristolochiaceae family such as *Aristolochia ringens, A. maurorum, A. longa, A. fangchi, A. symbifera* and *A. elegans* (Alali *et al.,* 2006; Bernaba *et al.,* 2012). This however does not agree with the position of Sulyman *et al.* (2016)

who reported that aristolochic acid is absent in *A. ringens* and *A. indica* but adduced it to agro-climatic factor, extraction process and characterization procedure. The anti-inflammatory actions of scrictosidine and corosolic acid have been documented (Sidney *et al.,* 2012).

5.1.2 Enzyme inhibition and kinetics

α-Amylase and α-glucosidase remain the two major enzymes of focus in the regulation of postprandial glucose level in the management of type 2 diabetes mellitus (Krentz and Bailey, 2005). These enzymes are involved in the hydrolysis of starch to glucose resulting into the elevation of systemic concentration of glucose. Microvascular and macrovascular complications of diabetes mellitus are often products of prolonged hyperglycemia mediated through oxidative stress (Kim *et al*., 2000). Inhibition of α-glucosidase and α-amylase has been shown to down regulate glucose absorption (Sabiu and Ashafa, 2016). The inhibitory effect of the extract against α-amylase and α-glucosidase compares well with that of the standard drug (acarbose) as there is no significant difference between their IC_{50} values (0.67, 0.63 and 0.57, 0.54 respectively). This could suggest that the extract and acarbose have similar inhibitory effect on the two enzymes.

The extract competitively inhibits α-amylase with its Km value (0.309×10^{-6}mg) significantly different from that of the control (0.238×10^{-6} mg). According to Sabiu *et al.* (2017), there is a striking resemblance between one of the major compounds in the extract and the original substrate of the enzyme, enabling it to bind with the enzyme at the expense of the substrate. This binding therefore reduces the rate of conversion of starch to free glucose. Furthermore, the mode of inhibition of α-glucosidase by the extract is uncompetitive as revealed by its reduced Km and Vmax values. This may imply a higher affinity of the enzyme for the extract than the substrate leading to its ability to regulate subsequent carbohydrate hydrolysis. This is in agreement with Sabiu *et al.* (2016) and Sabiu *et al.* (2017).

5.1.3 Molecular docking

Docking of inhibitors against known enzymes allows for better appreciation of their nature of interactions (Jamilah *et al.,* 2015). Penna-Coutinho *et al.* (2011) reported the docking scores of itraconazole, atorvastatin and posaconazole inside *Pf*LDH. In this study, the docking properties of asiatic acid, magnoflorine and phenyl-β-D-glucopyranoside were performed. Binding energy values revealed that asiatic acid and magnoflorine show the best interaction with alpha-amylase and alpha-glucosidase respectively. These two compounds have earlier been reported for their hypoglycaemic potentials (Sydney *et al.,* 2012; Patel & Mishra, 2012). Also, phenyl-β-D-glucopyranoside has been implicated in antidiabetic studies (Fujimori *et al.,* 2008). The ability of these compounds to inhibit alpha-amylase and alpha-glucosidase could be as a result of their interactions with various amino acid residues at the active sites of the two enzymes. This agrees with reports of Rasouli *et al.* (2017).

.

CONCLUSIONS

From the results obtained in this research, it is safe to make the following conclusions :

1. Phytochemical results revealed the presence of phenolics, flavonoids, alkaloids and glycosides which are known hypoglycemic phytochemicals.
2. The extract elicited a concentration-dependent inhibition on the specific activity of α-amylase and α-glucosidase.
3. Of the 12 identified compounds, 3 (asiatic acid, magnoflorine and phenyl-β-D-glucopyranoside) were successfully docked with both enzymes and are potent inhibitors of these enzymes.
4. The respective competitive and uncompetitive mode of action of the extract towards α-amylase and α-glucosidase could be attributed to their interaction with asiatic acid, magnoflorine and phenyl-β-D-glucopyranoside.
5. This study has therefore corroborated previous *in vivo* studies on the antidiabetic potential of root extract of *A. ringens* by lending credence to one of its probable mechanisms of action.

RECOMMENDATION .

It is recommended that the complete profiling and structural elucidation of the unidentified compounds be carried out to better understand the mechanisms of action of *Aristolochia ringens.*

REFERENCES

Abraham, B. F., Olarewaju, S. A., Ronke, A. and Oladipo, A. E. (2017). Antidiabetic and Antidyslipidemic Activities of the Aqueous Extract of *Cochlospermum planchonii* Leaves in Streptozotocin-Induced Diabetic Rats. *Iranian Journal of Medical Sciences 42*(6): 553-560.

ADA (2009). Adapted from American Diabetes Association. Diagnosis and classification of diabetes mellitus. *Diabetes care* 3(1): S62- S67.

Afifi, T. D., Granger, D. A., Denes, A., Joseph, A. and Aldeis, D. (2011). Parents' communication skills and adolescents' salivary α-amylase and cortisol response patterns. *Communication Monographs 78*(3): 273-295.

Ajani, E. O., Sabiu, S., Odufuwa, K. T., Ibrahim, T. B. and Salau, B. A. (2017). Evaluation of Lens Aldose Reductase Inhibitory and Free Radical Scavenging Potential of Fractions of *Lonchocarpus cyanescens*: Potential for Cataract Remediation. *Pharmacognosy Journal 9*(1): 62-69.

Akindele, A. J., Otuguor, E., Singh, D., Ota, D. and Benebo, A. S. (2015). Hypoglycemic, antilipidemic and antioxidant effects of valproic acid in alloxan-induced diabetic rats. *European journal of pharmacology 762*: 174-183.

Alali, F. Q., Tawaha, K., Shehadeh, M. B. and Telfah, S. (2006). Phytochemical and biological investigation of *Aristolochia maurorum* L. *Zeitschrift für Naturforschung C 61*(9-10): 685-691.

Alhassan, A. J., Lawal, T. A. and Dangambo, M. A. (2017). Antidiabetic properties of thirteen local medicinal plants in Nigeria, a review. *World Journal of Pharmaceutical Research* 2(8): 2170-2189.

American Diabetes Association. (2015). Standards of medical care in diabetes—2015 abridged for primary care providers. *Clinical diabetes: a publication of the American Diabetes Association 33*(2): 97-111.

Baggio, L. L. and Drucker, D. J. (2007). Biology of incretins: GLP-1 and GIP. *Gastroenterology 132*(6): 2131-2157.

Bate, K. L. and Jerums, G. (2003). Preventing complications of diabetes. Aggressive management of hyperglycaemia and other risk factors can prevent many complications. *MJA Practice Essentials Endocrinology* 179: 498–503.

Benarba, B., Ambroise, G., Aoues, A., Meddah, B. and Vazquez, A. (2012). *Aristolochia longa* aqueous extract triggers the mitochondrial pathway of apoptosis in BL41 Burkitt's lymphoma cells. *International Journal of Green Pharmacy (IJGP) 6*(1): 45-49.

Berkman, J. and Harold, R. (1973). Unilateral nodular diabetic glomerulosclerosis (Kimmelstiel–Wilson): Report of a case. *Metabolism, 22* (5): 715–722.

Bruner, J. H. (1984). Direct determination of saponins. *Analytical Chemistry* 34: 1314-1326

Burkill, H. M. (1985). The Useful Plants of West Tropical Africa. Vol 1. Kew: Royal Botanic Gardens. Kew, UK.

Cai, Z., Lee, F. S. C., Wang, X. R. and Yu, W. J. (2002). A capsule review of recent studies on the application of mass spectrometry in the analysis of Chinese medicinal herbs. *Journal of Mass Spectrometry 37*(10): 1013-1024.

Cannell, R. J. (Ed.). (1998). *Natural products isolation* (Vol. 4). Springer Science & Business Media.

Chaudhary, P., Goel, B. and Ghosh, A. K. (2012). Antidiabetic activity of *Adina cordifolia* (Roxb) leaves in alloxan induced diabetic rats. *Asian Pacific Journal of Tropical Biomedicine 2*(3): S1630-S1632.

Chiasson, J. L. (2007). Prevention of Type 2 diabetes: fact or fiction? *Expert Opinion on Pharmacotherapy 8*(18): 3147-3158.

Cos, P., Vlietinck, A. J., Berghe, D. V. and Maes, L. (2006). Anti-infective potential of natural products: how to develop a stronger in vitro 'proof-of-concept'. *Journal of ethnopharmacology 106*(3): 290-302.

Covington, M. B. (2001). Traditional Chinese medicine in the treatment of diabetes. *Diabetes Spectrum* 14: 154–159.

De Groot, H., Wanke, S. and Neinhuis, C. (2006). Revision of the genus *Aristolochia (Aristolochiaceae)* in Africa, Madagascar and adjacent islands. *Botanical Journal Linnean Society* 151: 219-238.

Dharmalingam, S. R., Madhappan, R., Ramamurthy, S., Chidambaram, K., Srikanth, M. V., Shanmugham, S. and Kumar, S. K. (2014). Investigation on antidiarrhoeal activity of *Aristolochia indica* Linn. root extracts in mice. *African Journal of Traditional, Complementary and Alternative Medicines 11*(2): 292-294.

Drucker, D. J. and Nauck, M. A. (2006). The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *The Lancet 368*(9548): 1696-1705.

Eidi, A., Eidi, M. and Esmaeili, E. (2006). Antidiabetic effect of garlic (*Allium sativum* L.) in normal and streptozotocin-induced diabetic rats. *Phytomedicine 13*(9-10): 624-629.

Elsnoussi, A.H.M., Mohammad, J.A.S., Lee, F.A., Amirin, S., Sue, H.C. and Soo, C.T. (2012). Potent α glucosidase and α- amylase inhibitory activities of standardized 50% ethanolic extracts and sinensetin from *Orthosiphon stamineus* Benth as anti-diabetic mechanism. *BMC Complementary Alternative Medicine* 12: 176-182.

Fabricant, D. S. and Farnsworth, N. R. (2001). The value of plants used in traditional medicine for drug discovery. *Environmental health perspectives 109*(1): 69-75.

Finar, G. (1986). Plants of economic importance. Medicinal Plants and Medicine in Africa. *Spectrum Books Ltd. Ibadan 78*: 150-153.

Fong, D. S., Aiello, L. and Gardner, T. W. (2003). Diabetic retinopathy. *Diabetes Care* 26(1): S99-S102

Fu, J., Fu, J., Yuan, J., Zhang, N., Gao, B., Fu, G. and Zhang, Y. (2012). Anti-diabetic activities of *Acanthopanax senticosus* polysaccharide (ASP) in combination with metformin. *International Journal of Biological Macromolecules 50*(3): 619-623.

Fujimori, Y., Katsuno, K., Nakashima, I., Ishikawa-Takemura, Y., Fujikura, H. and Isaji, M. (2008). Remogliflozin etabonate, in a novel category of selective low-affinity sodium

glucose cotransporter (SGLT2) inhibitors, exhibits antidiabetic efficacy in rodent models. *Journal of Pharmacology and Experimental Therapeutics 327*(1): 268-276.

Gbolade, A. A. (2009). Inventory of antidiabetic plants in selected districts of Lagos State, Nigeria. *Journal of Ethnopharmacology* 12: 135–139

Grover, J. K., Yadav, S. and Vats, V. (2002). Medicinal plants of India with anti-diabetic potential. *Journal of ethnopharmacology 81*(1): 81-100.

Gupta, O. P. and Phatak, S. (2003). Pandemic trends in prevalence of diabetes mellitus and associated coronary heart disease in India-their causes and prevention. *International Journal of Diabetes in Developing Countries 23*(2): 37-45.

Gupta, R., Bajpai, K.G., Johri, S. and Saxena, A.M. (2007). An overview of Indian novel traditional medicinal plants with anti-diabetic properties. *African Journal of Complementary and Alternative Medicine* 5(1): 1–17.

Guthrie, D. W. and Guthrie, R.A. (2009). Management of diabetes mellitus: A guide to the pattern approach. 5th edition. Springer, New York.

He, W., Cik, M., Lesage, A., Van der Linden, I., De Kimpe, N., Appendino, G. and Van Puyvelde, L. (2000). Kirkinine, a new daphnane orthoester with potent neurotrophic activity from *Synaptolepis kirkii*. *Journal of Natural Products 63*(9): 1185-1187.

Ibrahim, R. B., Akolade, J. O., Sulyman, A. O., Sabiu, S., Yusuf, B. O. and Yusuf, L. B. (2018). Aqueous Leaf Extract of *Heliotropium indicum* Ameliorates Hyperglycaemia-Induced Tissue Complications in Albino Rats. *Wounds 6*(11): 19-26.

Inzucchi, S. E. and McGuire, D. K. (2008). New drugs for the treatment of diabetes: part II: Incretin-based therapy and beyond. *Circulation 117*(4): 574-584.

Inzucchi, S. E., Bergenstal, R. M., Buse, J. B., Diamant, M., Ferrannini, E., Nauck, M. and Matthews, D. R. (2015). Management of hyperglycemia in type 2 diabetes, 2015: a patient-centered approach: update to a position statement of the American Diabetes Association and the European Association for the Study of Diabetes. *Diabetes care 38*(1): 140-149.

Irondi, E. A., Oboh, G. and Akindahunsi, A. A. (2016). Antidiabetic effects of *Mangifera indica* Kernel Flour-supplemented diet in streptozotocin-induced type 2 diabetes in rats. *Food science & nutrition 4*(6): 828-839.

Jamila, N., Yeong, K. K., Murugaiyah, V., Atlas, A., Khan, I., Khan, N. and Osman, H. (2015). Molecular docking studies and *in vitro* cholinesterase enzyme inhibitory activities of chemical constituents of *Garcinia hombroniana*. *Natural product research 29*(1): 86-90.

Joseph, B. and Jini, D. (2013). Antidiabetic effects of *Momordica charantia* (bitter melon) and its medicinal potency. *Asian Pacific Journal of Tropical Disease 3*(2): 93-102.

Kazeem, M. I., Abimbola, S. G. and Ashafa, A. O. T. (2013). Inhibitory potential of *Gossypium arboreum* leaf extracts on diabetes key enzymes, α-amylase and α-glucosidase. *Bangladesh Journal of Pharmacology 8*(2): 149-155.

Kim, J.S., Kwon, C.S. and Son, K.H. (2000). Inhibition of alpha glucosidase and amylase by luteolin, a flavonoid. *Bioscience, Biotechnology and Biochemistry* 64: 2458–2461.

Krentz, A.J. and Bailey, C.J. (2005). Oral antidiabetic agents: Current role in type 2 diabetes mellitus. *Drug* 65: 385-411

Kwon, Y. I., Apostolidis, E. and Shetty, K. (2008). *In vitro* studies of eggplant (*Solanum melongena*) phenolics as inhibitors of key enzymes relevant for type 2 diabetes and hypertension. *Bioresource Technology 99*(8): 2981-2988.

Li, H.B., Jiang, Y. and Chen, F. (2004). Separation methods used for *Scutellaria baicalensis* active components. *Journal of Chromatographic Science* 812: 277–290.

Lineweaver, H. and Burk, D. (1934). The determination of enzyme dissociation constants. *Journal of American Chemical Society* 56: 658-666.

Lo Piparo, E., Scheib, H., Frei, N., Williamson, G., Grigorov, M. and Chou, C. J. (2008). Flavonoids for controlling starch digestion: structural requirements for inhibiting human α-amylase. *Journal of Medicinal Chemistry 51*(12): 3555-3561.

Malviya, N., Jain, S. and Malviya, S. A. P. N. A. (2010). Antidiabetic potential of medicinal plants. *Acta Poloniae Pharmaceutica 67*(2): 113-118.

Manohar, V., Talpur, N. A., Echard, B. W., Lieberman, S. and Preuss, H. G. (2002). Effects of a water-soluble extract of maitake mushroom on circulating glucose/insulin concentrations in KK mice. *Diabetes, Obesity and Metabolism 4*(1): 43-48.

Marshall, S.M. (2003). Clinical features and management of diabetic nephropathy. In: Pickup JC, Williams G, editors. *Textbook of diabetes*. Boston: Blackwell Publishing.

Metzger, B.E. and Coustan, D.R. (1998). Conference on Gestational Diabetes Mellitus. (Eds.): Proceedings of the Fourth International Workshop. *Diabetes Care,* 21(2): B1–B167.

Minari, J. B. and Idris, M. A. (2015). Forensic and Pharmacognostic Study of *Aristolochia ringens* Stem. *Journal of Forensic Research 6*(1): 252-261.

Mithieux, G., Vidal, H., Zitoun, C., Bruni, N., Daniele, N. and Minassian, C. (1996). Glucose-6-phosphatase mRNA and activity are increased to the same extent in kidney and liver of diabetic rats. *Diabetes 45*(7): 891-896.

Moyo, B., Oyedemi, S., Masika, P. J. and Muchenje, V. (2012). Polyphenolic content and antioxidant properties of *Moringa oleifera* leaf extracts and enzymatic activity of liver from goats supplemented with *Moringa oleifera* leaves/sunflower seed cake. *Meat science 91*(4): 441-447.

Naskar, S., Mazumder, U. K., Pramanik, G., Gupta, M., Kumar, R. S., Bala, A. and Islam, A. (2011). Evaluation of antihyperglycemic activity of *Cocos nucifera* Linn. on streptozotocin induced type 2 diabetic rats. *Journal of ethnopharmacology 138*(3): 769-773.

Ocvirk, S., Kistler, M., Khan, S., Talukder, S. H. and Hauner, H. (2013). Traditional medicinal plants used for the treatment of diabetes in rural and urban areas of Dhaka, Bangladesh–an ethnobotanical survey. *Journal of Ethnobiology and Ethnomedicine 9*(1): 43-50.

Odebiyi, O. O. and Sofowora, E. A. (1978). Phytochemical screening of Nigerian medicinal plants II. *Lloydia 41*(3): 234-246.

Oguanobi, N. I., Chijioke, C. P. and Ghasi, S. (2012). Anti-diabetic effect of crude leaf extracts of *Ocimum gratissimum* in neonatal streptozotocin-induced type-2 model diabetic rats. *International Journal of Pharmacology and Pharmaceutical Science 4*(5): 77-83.

Patel, M. B. and Mishra, S. M. (2012). Magnoflorine from *Tinospora cordifolia* stem inhibits α-glucosidase and is antiglycemic in rats. *Journal of Functional Foods 4*(1): 79-86.

Penna-Coutinho, J., Cortopassi, W. A., Oliveira, A. A., França, T. C. C., and Krettli, A. U. (2011). Antimalarial activity of potential inhibitors of *Plasmodium falciparum* lactate dehydrogenase enzyme selected by docking studies. *PloS one 6*(7): 212-220.

Prisilla, D. H., Balamurugan, R. and Shah, H. R. (2012). Antidiabetic activity of methanol extract of *Acorus calamus* in STZ induced diabetic rats. *Asian Pacific Journal of Tropical Biomedicine 2*(2): S941-S946.

Raheja, G. G. (1997). Introduction: the paradoxes of power and community: women's oral traditions and the uses of ethnography. *Oral Tradition* 12(1): 1-22.

Ramachandran, V., Saravanan, R. and Senthilraja, P. (2014). Antidiabetic and antihyperlipidemic activity of asiatic acid in diabetic rats, role of HMG CoA: *in vivo* and *in silico* approaches. *Phytomedicine 21*(3): 225-232.

Rasouli, H., Hosseini-Ghazvini, S. M. B., Adibi, H. and Khodarahmi, R. (2017). Differential α-amylase/α-glucosidase inhibitory activities of plant-derived phenolic compounds: a virtual screening perspective for the treatment of obesity and diabetes. *Food & function 8*(5): 1942-1954.

Rena, G., Hardie, D. G. and Pearson, E. R. (2017). The mechanisms of action of metformin. *Diabetologia 60*(9): 1577-1585.

Rother, K.I. (2007). Diabetes treatment—bridging the divide. *The New England Journal of Medicine* 356 (15): 1499–1501.

Ruth, A. F., Olaide, A. O. and Oluwatoyin, S. M. (2014). The aqueous root extract of *Aristolochia ringens* (Vahl.) *Aristolochiaceae* inhibits chemically-induced inflammation in rodents. *Pakistani Journal of Pharmaceutical Science 27*(6): 1885-1889.

Sabiu, S. and Ashafa, A.O.T. (2016). Membrane stabilization and kinetics of carbohydrate metabolizing enzymes (*α*-amylase and *α*-glucosidase) inhibitory potentials of *Eucalyptus obliqua* L. Her. (Myrtaceae) Blakely ethanolic leaf extract: An *in vitro* assessment. *South African Journal of Botany* 105: 264-269.

Sabiu, S., Ajani, E. O., Sunmonu, T. O. and Ashafa, A. O. T. (2017). Kinetics of modulatory role of *Cyperus esculentus* L. on the specific activity of key carbohydrate metabolizing enzymes. *African Journal of Traditional, Complementary and Alternative Medicines 14*(4): 46-53.

Sabiu, S., O'neill, F. H., & Ashafa, A. O. T. (2016). Kinetics of α-amylase and α-glucosidase inhibitory potential of *Zea mays* Linnaeus (Poaceae), *Stigma maydis* aqueous extract: An *in vitro* assessment. *Journal of Ethnopharmacology 183*: 1-8.

Saheed, S., Oladipipo, A. E., Abdulazeez, A. A., Olarewaju, S. A., Ismaila, N. O., Emmanuel, I. A. and Aisha, A. Y. (2015). Toxicological evaluations of *Stigma maydis* (corn silk) aqueous extract on hematological and lipid parameters in Wistar rats. *Toxicology Reports 2*: 638-644.

Sasidharan, I. and Menon, A. N. (2010). Comparative chemical composition and antimicrobial activity fresh & dry ginger oils (*Zingiber officinale* Roscoe). *International Journal of Current Pharmaceutical Research 2*(4): 40-43.

Sidney, J., Stohs, H. M. and Gilbert, R. (2012). A Review of the Efficacy and Safety of Banaba (*Lagerstroemia speciosa* L.) and Corosolic Acid. *Phytotherapy Research* 26(3): 317-324.

Skotti, E., Anastasaki, E., Kanellou, G., Polissiou, M., & Tarantilis, P. A. (2014). Total phenolic content, antioxidant activity and toxicity of aqueous extracts from selected Greek medicinal and aromatic plants. *Industrial Crops and Products 53*: 46-54.

Sobngwi, E. (2001). Diabetes in Africans. Part 1: epidemiology and clinical specificities. *Diabetes Metabolism* 27(6): 628-634.

Sulyman, A. O., Akolade, J. O., Sabiu, S. A., Aladodo, R. A. and Muritala, H. F. (2016). Antidiabetic potentials of ethanolic extract of *Aristolochia ringens* (Vahl.) roots. *Journal of Ethnopharmacology 182*: 122-128.

Swain, T. (1979). Tannins and Lignins Herbivores: Their interaction with Secondary Plant Metabolites. New York: Academic Press

Tine, Y., Renucci, F., Costa, J., Wélé, A. and Paolini, J. (2017). A Method for LC-MS/MS profiling of coumarins in *Zanthoxylum zanthoxyloides* (Lam.) B. Zepernich and Timler extracts and essential oils. *Molecules 22*(1): 174-186.

Tsao, R. and Deng, Z. (2004). Separation procedures for naturally occurring antioxidant phytochemicals. *Journal of Chromatography B 812*(1-2): 85-99.

VanNewkirk, M. R., Weih, L., McCarty, C. A. and Taylor, H. R. (2001). Cause-specific prevalence of bilateral visual impairment in Victoria, Australia: the Visual Impairment Project. *Ophthalmology 108*(5): 960-967.

Wang, X., Bao, W., Liu, J., OuYang, Y. Y., Wang, D., Rong, S. and Liu, L. G. (2013). Inflammatory markers and risk of type 2 diabetes: a systematic review and meta-analysis. *Diabetes Care 36*(1): 166-175.

Waxman, A. and Norum, K. (2004). Why a global strategy on diet, physical activity and health? The growing burden of non-communicable diseases. *Public Health Nutrition* 7(3): 381-383.

WHO (1999). A Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications (PDF). Adapted from World Health Organisation Department of Noncommunicable Disease Surveillance http://whqlibdoc.who.int/hq/1999/WHO_NCD_NCS_99.2.pdf.

Yan, J., Zhang, G., Pan, J. and Wang, Y. (2014). α-Glucosidase inhibition by luteolin: Kinetics, interaction and molecular docking. *International Journal of Biological Macromolecules 64*: 213-223.

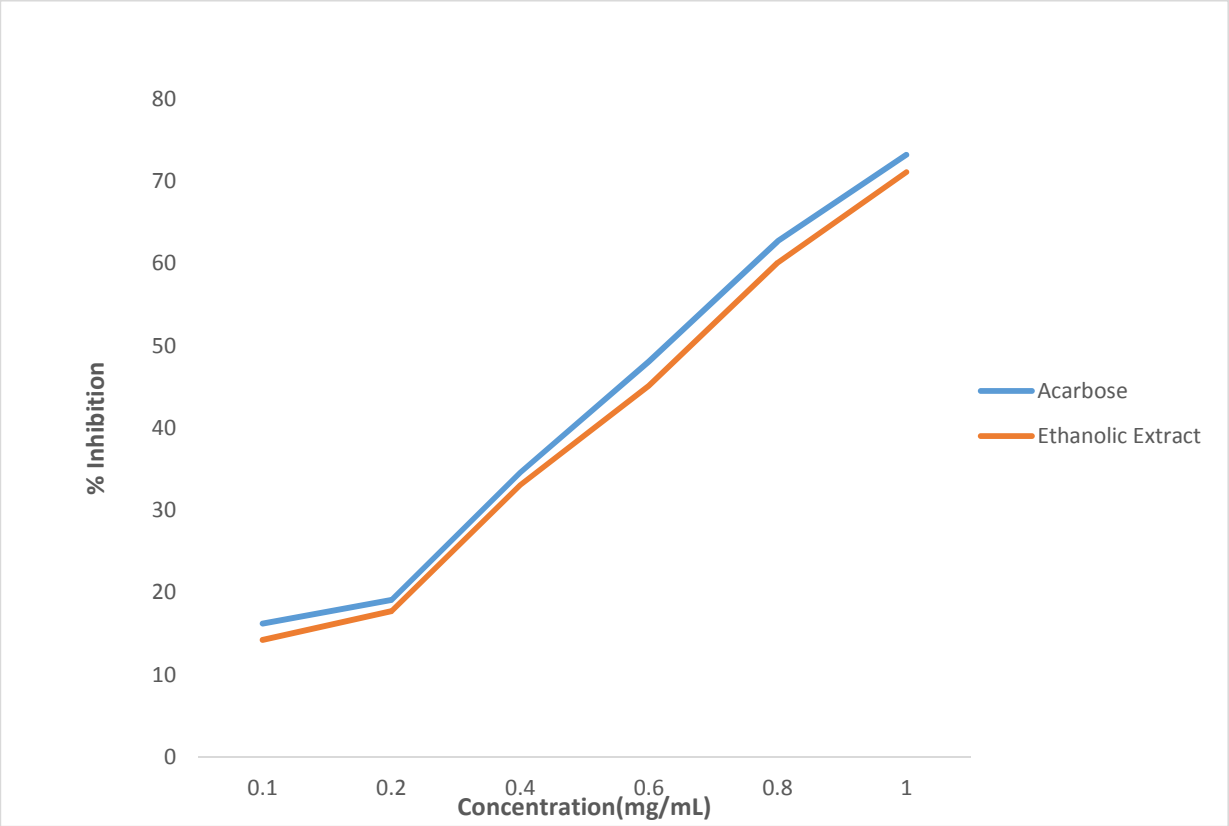
Yasuma, A. an Ichikawa, T. (1953). Ninhydrin-Schiff and alloxan-Schiff staining: a new histochemical staining method for protein. *The Journal of Laboratory and Clinical Medicine 41*(2): 296-299.

Yeh, G. Y., Eisenberg, D. M., Kaptchuk, T. J. and Phillips, R. S. (2003). Systematic review of herbs and dietary supplements for glycemic control in diabetes. *Diabetes Care 26*(4): 1277-1294.

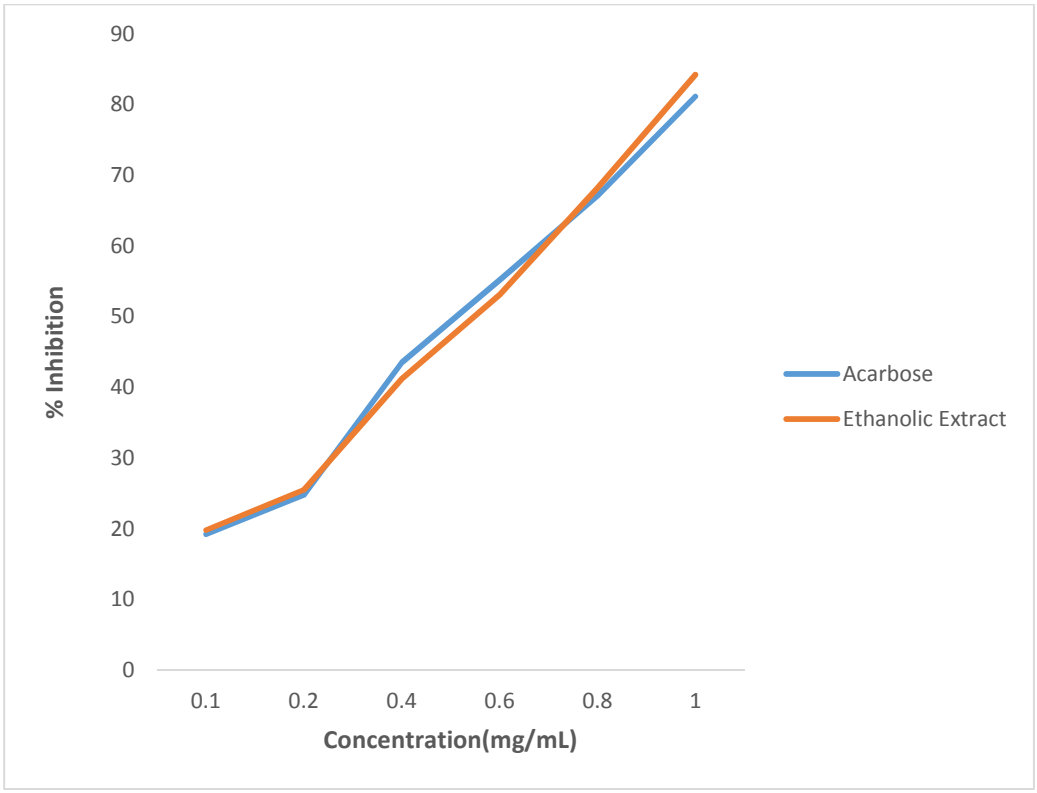
Zhu, Y., & Zhang, C. (2016). Prevalence of gestational diabetes and risk of progression to type 2 diabetes: a global perspective. *Current Diabetes Reports 16*(1): 7.

APPENDICES

Appendix A: Inhibitory curve of *Aristolochia ringens* root extract on the activity of α-amylase for IC_{50} determination



APPENDIX B: Inhibitory curve of *Aristolochia ringens* root extract on the activity of α-glucosidase for IC_{50} determination



Appendix C: Maltose standard curve for alpha-amylase kinetics

[S] (mg/ml)	Abs at 540nm	Amount of Maltose (µg)	Velocity,V (µmoles/min)	1/[V]	1/[S]
0.3	0.080	1.092	3364094.916	$2.97x10^{-7}$	3.33
0.8	0.083	1.079	3324046.167	$3.00x10^{-7}$	1.25
1.5	0.092	1.072	3302481.456	$3.03x10^{-7}$	0.67
2.5	0.096	1.062	3272290.861	$3.05x10^{-7}$	0.40
3.5	0.103	1.049	3231625.977	$3.09x10^{-7}$	0.29
5.0	0.111	1.036	3192501.43	$3.13x10^{-7}$	0.20

*Abs=Absorbance

Appendix D: p-Nitrophenol standard curve for alpha-amylase kinetics

[S] (mg/ml)	Abs at 540nm	Amount of p-NP (µg)	Velocity,V (µmoles/min)	1/[V]	1/[S]
0.3	0.080	0.293	367083.468	2.72 $x10^{-6}$	3.33
0.8	0.083	0.280	350807.598	2.85 $x10^{-6}$	1.25
1.5	0.092	0.273	342294.066	2.92 $x10^{-6}$	0.67
2.5	0.096	0.263	329774.166	3.03 $x10^{-6}$	0.40
3.5	0.103	0.251	313999.092	3.18 $x10^{-6}$	0.29
5.0	0.111	0.238	297347.625	3.36 $x10^{-6}$	0.20

*Abs=Absorbance

Appendix E: Maltose standard curve for alpha-glucosidase kinetics determination

[PNPG] (mg/ml)	**Abs at 540nm**	**Amount of Maltose (µg)**	**Velocity,V (µmoles/min)**	**1/[V]**	**1/[S]**
0.63	0.085	1.157	3565570.93	2.80×10^{-7}	1.59
0.83	0.087	1.152	3549551.43	2.81×10^{-7}	1.21
1.00	0.092	1.144	3525522.181	2.84×10^{-7}	1.00
1.50	0.094	1.144	3525522.181	2.84×10^{-7}	0.67
2.00	0.104	1.1364	3500876.797	2.86×10^{-7}	0.50

*Abs=Absorbance

Appendix F: p-Nitrophenol standard curve for alpha-glucosidase kinetics determination

[PNPG] (mg/ml)	**Abs at 540nm**	**Amount of p-NP(µg)**	**Velocity,V (µmoles/min)**	**1/[V]**	**1/[S]**
0.63	0.085	0.359	448963.614	2.22×10^{-6}	1.59
0.83	0.087	0.354	442453.266	2.26×10^{-6}	1.21
1.00	0.092	0.346	432687.744	2.31×10^{-6}	1.00
1.50	0.094	0.346	432687.744	2.31×10^{-6}	0.67
2.00	0.104	0.338	422671.824	2.37×10^{-6}	0.50

*Abs=Absorbance