In vitro STUDIES ON ALPHA-AMYLASE AND ALPHA-GLUCOSIDASE INHIBITORY ACTIVITIES, PHYTOCHEMICAL SCREENING AND GC-MS ANALYSIS OF EXTRACTS OF *Canavalia ensiformis* (L.) DC. SEEDS

BY

Victor Oluwatosin KOLADE

UNIVERSITY OF BENIN
BENIN CITY

NOVEMBER, 2024

In vitro STUDIES ON ALPHA-AMYLASE AND ALPHA-GLUCOSIDASE INHIBITORY ACTIVITIES, PHYTOCHEMICAL SCREENING AND GC-MS ANALYSIS OF EXTRACTS OF *Canavalia ensiformis* (L.) DC. SEEDS

BY

Victor Oluwatosin KOLADE

(PG/LSC1715472)

B.Sc (ADO-EKITI)

A THESIS WRITTEN IN THE DEPARTMENT OF BIOCHEMISTRY AND SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (M.Sc) IN BIOCHEMISTRY OF THE UNIVERSITY OF BENIN, BENIN CITY

NOVEMBER, 2024

CERTIFICATION

We certify that this work was carried out by Mr. Victor Oluwatosin KOLADE in the Department of Biochemistry, Faculty of Life Sciences, University of Benin, Benin City, Nigeria.

_____ _____

PROF. (MRS.) R. NIMENIBO-UADIA **DATE**
(Project Supervisor)

_____ _____

PROF. E. C. ONYENEKE **DATE**
(Head of Department)

_____ _____

External Examiner **DATE**

CERTIFICATION OF THESIS

We the undersigned attest and declare that the thesis of Mr. Victor Oluwatosin KOLADE titled *in vitro* studies on alpha-amylase and alpha-glucosidase inhibitory activities, phytochemical screening and GC-MS analysis of extracts of *Canavalia ensiformis* (L) DC. seeds has successfully passed the anti-plagiarism test and does not violate any copyright regulations.

_____ _____

PROF. (MRS.) R. NIMENIBO-UADIA **DATE**
(Project Supervisor)

_____ _____

PROF. E. C. ONYENEKE **DATE**
(Head of Department)

_____ _____

External Examiner **DATE**

DEDICATION

This thesis is dedicated to the Almighty God who made it possible and to my beloved family members for their support.

ACKNOWLEDGEMENTS

I want to acknowledge all those who contributed and assisted me throughout the research and the collation of this thesis. A profound gratitude goes to the Almighty God for His grace, provision and wisdom that He emptied on us. And to my supervisor Prof. (Mrs.) R. Nimenibo-Uadia, a woman of great store of knowledge and worth, thank you ma for your guidance, direction and instructions. Also, to all lecturers and technical staff of the Department of Biochemistry, Faculty of Life Sciences, University of Benin, thank you for your help and support. To my lovely parents, Mr. & Mrs. Kolade James Ayotunde and my siblings Paul, Samuel and Mary; who so much believe in me, you've been my backbone, thank you and God bless you all in Jesus' name. To my Senior colleagues; notably Miss Ogedengbe Opeyemi and Mebawondu Charles. To my lovely friends; Mercy, Chiamaka, Irene, Sarah, Mary and my fellow colleagues for their push to make sure I complete this work, you've contributed in one way or the other to the realization of this successful work, thank you and God bless you all.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

ABSTRACT

Diabetes is a common endocrine and metabolic disorder characterized by hyperglycemia or elevated blood sugar proven to be a major health problem. This study was aimed at evaluating *in vitro* inhibitory activities of methanol, n-hexane, dichloromethane, and aqueous extracts of *Canavalia ensiformis* seed on alpha amylase and alpha glucosidase at varying concentrations.

Extraction of methanol, n-hexane, dichloromethane and aqueous extracts of *Canavalia ensiformis* seed was carried out in this study. Phytochemical screening of the extracts of *Canavalia ensiformis* seed was carried out using standard methods. Dichloromethane and n-hexane extracts of *Canavalia ensiformis* seed were used for GC-MS analysis.

Five different concentrations (20, 40, 60, 80, 100µg/ml) of each extract were used in this experiment. The standard drug acarbose was prepared using the same concentration range. The alpha amylase inhibition assay showed that the methanol with IC_{50} value of 13.03 µg/mL was the most potent in inhibiting alpha amylase while for the alpha glucosidase inhibition assay it was the aqueous extract with IC_{50} value of 43.78 µg/mL when compared to the other extracts. The Phytochemical screening results of methanol, n-hexane, dichloromethane and aqueous extracts of *C. ensiformis* seed revealed the presence of starch, glycosides, terpenoids, saponins, alkaloids, and phenols. GC-MS analysis of dichloromethane extract showed the presence of anti-diabetic compounds such as n-Hexadecanoic acid, 3-Eicosene and p-xylene. The results obtained in this study indicate that the extract of the *C. ensiformis* possesses alpha amylase and alpha glucosidase inhibitory activities, thus supporting the traditional use of *C. ensiformis* seed extract by traditional healers for management of type II diabetes. In addition, the alpha amylase and alpha glucosidase activities of *C. ensiformis* seed extracts observed in this study, may be one of the mechanisms of action of the seed in reducing elevated blood glucose levels as observed in diabetes mellitus.

CHAPTER ONE

INTRODUCTION

1.1 Background of Study

Jack bean (*Canavalia ensiformis*) is one of the legume crops that have not been fully exploited. Legumes are rich in many nutrients such as proteins, starch, dietary fibre, oils, vitamins, minerals and phytochemicals that are protected by nature. They provide substantial protein intake support to a significant proportion of the world's population, particularly in most developing countries (Ojo and Ade-Omowaye, 2015). The high crude protein content (20 – 32 %) and amino-acid profile of jack bean seed have been reported to make it suitable for use as a substitute for fish feed, while the fully ripened seeds are occasionally used as a coffee substitute (Osuigwe *et. al.,* 2006). Marimuthu and Gurumoorthi (2013) reported that raw jack bean seeds contain approximately 600 g/kg carbohydrate and 300 g/kg protein which make it a greater potential dietary protein source.

Diabetes mellitus, a chronic metabolic disease, characterized by elevated levels of blood glucose and insufficiency in production and action of insulin is the seventh leading cause of death worldwide. Carbohydrate digestibility has been reported to relate to elevated postprandial blood glucose. One of the strategies to reduce postprandial hyperglycemia is to limit the activity of carbohydrate digestive enzymes in intestinal tract. α-Amylase is the key enzyme that degrades the polymeric substrate into shorter oligomers by catalyzing the hydrolysis of α-1,4-glucan linkages present in starch, maltodextrins, and other related carbohydrates (Truscheit *et. al.,* 2010). Human α-amylases of both salivary (HSA) and pancreatic origins (HPA) were widely researched for clinical chemistry purposes because they are very important in the diagnosis of pancreas and salivary glands (Greenberger and Toskes, 1991; Deenmamode *et. al.,* 1993). Furthermore, they are objectives for drug design to treat some diseases, such as diabetes and hyperlipidemia.

Currently there are some antidiabetic drugs, namely, acarbose, miglitol and voglibose, which act by inhibiting α (alpha) amylase and α-glucosidase activity. While efficient in attenuating the rise in blood glucose levels in many patients, the continuous use of these drugs is often associated with undesirable side effects, such as liver toxicity and adverse gastrointestinal symptoms (Etxeberria *et al.,* 2012). It is for this reason that there is a need for natural α-amylase and α-glucosidase inhibitors which have no adverse or unwanted secondary effects. Phytochemicals derived from natural plants have been used commonly for the prevention and/or treatment of different diseases due to the extended belief of their therapeutic properties and safety. The therapeutic importance of plants has been quoted in the ancient cultures and traditions of many countries and societies and they are believed to be cost effective and safe (Rahmani *et al.,* 2014).

1.2 Justification of the study

Diabetes mellitus has remained a global health and economic challenge due to modern lifestyle and elevated carbohydrate intake.

The basic reason behind the abnormal metabolism of carbohydrate, fat, and protein that occur in people with type II diabetes is deficient action of insulin on target tissues.

Canavalia ensiformis seed extract has been suggested to have antidiabetic potency in treating diabetes mellitus (Nimenibo-Uadia, 2003).

It is therefore important to understand the potential mechanism of hypoglycemic impact of *Cacavalia ensiformis* seed. Using the solvents methanol, n-hexane, dichloromethane, and water for extraction, the *in vitro* alpha amylase and alpha glucosidase inhibitory activities of *Canavalia ensiformis* seed was studied. In order to assess the bio-active compounds present in methanol, n-hexane, dichloromethane, and aqueous extracts, qualitative phytochemical screening and Gas Chromatography-Mass Spectrometry (GC-MS) analysis were also used.

1.3 Aims and Objectives

The aim of the study was to investigate the alpha amylase and alpha glucosidase inhibitory activities of *Canavalia ensiformis* seed extracts *in vitro,* using methanol, n-hexane, dichloromethane and water as solvents.

The objectives of the study were to determine the:

1 phytochemical screening of the methanol, aqueous, dichloromethane and n-hexane extracts of *Canavalia ensiformis* seeds.

2 inhibitory effect of the methanol extract of *Canavalia ensiformis* seed on alpha amylase and alpha glucosidase enzyme activities.

3 inhibitory effect of aqueous extract of the *Canavalia ensiformis* seed on alpha amylase and alpha glucosidase enzymes activities.

4 inhibitory effect of dichloromethane extract of *Canavalia ensiformis* seed on alpha amylase and alpha glucosidase enzymes activities.

5 inhibitory effect of n-hexane extract of *Canavalia ensiformis* seed on alpha amylase and alpha glucosidase enzymes activities.

6 gas chromatography-mass spectrometry analysis of the dichloromethane and n-hexane extracts of *Canavalia ensiformis* seed.

CHAPTER TWO

LITEREATURE REVIEW

2.1 *Canavalia ensiformis* (Jack bean)

Canavalia ensiformis (jack bean) is a legume which is used for animal fodder and human nutrition, especially in Brazil where it is called feijão-de-porco ("pig bean"). It is also the source of concanavalin A. Jack bean is one of African underutilized leguminous crops and scientifically known as *Canavalia ensiformis* which belong to the family of Leguminosae, the sub-family Papilionoideae has about 480 genera and 12,000 species distributed throughout the world (Bamiro *et al.,* 1994). Jack beans can be mildly toxic and copious consumption must be avoided (Yusuf *et al.,* 2010; Carlini *et al.,* 1997). The high demand for starch for various industrial and food applications have recently experienced a hike in its market price in the face of the country's attempt to attain national self-sufficiency in starch production for various industrial applications and national food security. And the use of food crops (such as cassava, maize, potatoes, etc.) highly suitable for human consumption as an industrial raw material for non-food consumption is frowned upon by some stakeholders.

2.1.1 Scientific Classification

Kingdom:	Plantae
Phylum:	Tracheophyta
Class:	Magnoliopsida
Order:	Fabales
Family:	Fabaceae
Genus:	Canavalia
Species:	Ensiformis
Botanica Name:	*Canavalia ensiformis*

(Source: GBIF.org)

Plate 2.1 *Canavalia ensiformis* seeds *(*picture taken by me*)*

2.1.2 Description and Distribution

Jack bean (*Canavalia ensiformis* (L.) DC.) is a climbing perennial legume commonly cultivated as an annual. It grows up to 2 m high with 8-20 cm long trifoliate leaves and a strong root system. Flowers are pink, mauve or white with a red base. Pods are up to 36 cm long and contain 1-2 cm long, ellipsoid seeds. Pods and seeds are edible and used for food, the young pods being cooked as a vegetable. The whole plant, the pods and seeds are also used to feed animals.

The jack bean is native to tropical Africa and to South and Central America but is naturalized and cultivated worldwide (Chee *et al.,* 1992). While it thrives in humid lowland tropics, it can also be found at altitudes up to 1800 m. Optimal annual rainfall is between 800-2000 mm but its deep rooting system allows it to withstand dry periods. It is reported to be tolerant to waterlogging and salinity. Jack bean has been reported as being antagonistic or suppressive of nematodes, particularly when used for intercropping in banana plantations (Vargas-Ayala *et al.,* 2000), but this effect is disputed (Ternisien *et al.,* 1989; Kashaija *et al.,* 2004).

2.1.3 Nutritional Attributes

Jack bean seeds and foliage contain several antinutritional factors: concanavalin A (a lectin protein used in biotechnology), canavanine (a structural analogue of arginine) and canatoxin. Fresh forage and raw seeds are generally detrimental to animals. Cattle consuming too much seed meal develop symptoms such as fever, nasal discharge, lameness and prostration (Chee *et al.,* 1992). It has been shown that 28 g of seed per 0.73 kg body weight are lethal to cattle. Dehulling, heat treatments and soaking have been recommended in order to lower the level of antinutritional factors. The seeds show urease activity likely to release ammonia: it is recommended to avoid using it with urea-treated feed in ruminant diets.

Fresh forage is not palatable to ruminants and is eaten only in small amounts. However, cattle can gradually get used to it and acquire a taste for it (Chee *et al.,* 1992). Drying results in higher intake. In goats, jack bean forage has been found worth considering for a dry season feed strategy in Nigeria (Akingbade *et al.*, 2007).

Seeds. In cattle, diets containing 30 % of jack bean seeds have an adverse effect on average daily gain (8.3 % decrease). Supplementing grazing dairy cows with ground pods had no depressive effect on milk yield (Paredes *et. al.,* 1984; Jimenez *et. al.,* 2021). In sheep, increasing the inclusion rate from 22 % to 32 % resulted in lower rumen fermentation (Mora *et al*., 1986). In pre-weaning calves, supplementation with jack bean seeds resulted in lower daily live-weight gain compared to soybean meal and maize-based diets (Troccoli *et al.*, 1989). In growing pigs, Canavalia seeds were found to have a negative effect on average daily gain however they were processed (raw, alkali-treated, autoclaved or extruded) and the level of inclusion (5 to 15 %) (Risso *et al.,* 1992). However, diets containing up to 20 % toasted seeds (at 194 °C for 18 minutes) were not detrimental to feed intake and weight gain (Michelangeli *et al.*, 2004).

Raw seeds, even at inclusion levels as low as 5 %, have negative effects on broilers (decreased weight gain, increased feed conversion ratio, alterations in the liver, pancreas and kidneys) (Akinmutimi, 2006; Akanji *et al.,* 2007b; Ologhobo *et al.*, 2003). For this reason, it is recommended to process the seeds before feeding them to animals in order to reduce the antinutritional factors. However, autoclaving alone is not sufficient to mitigate deleterious effects of Jack bean. It may thus be useful to combine soaking and autoclaving or boiling, soaking and shaking (Belmar *et al.*, 1999).

2.1.4 Phytochemical Properties of *C. ensiformis* seed

Jack bean has potential medicinal value (Andriati *et al.,* 2018). For example, the methanolic extract of jack bean seeds contain acceptable levels of free phenolics with promising antioxidant and type II diabetes-related enzyme inhibition properties (Vadivel *et al.,* 2012). Moreover, the seed decoction or powdered seeds from jack bean are used as an antibiotic and antiseptic (Andriati *et al.,* 2018). Furthermore, urease-derived peptide from jack bean represents a new example of the membrane-active peptide with insecticidal and fungi toxic activities (Martinelli *et al.,* 2014). Therefore, a further step towards encouraging the idea that jack bean is a suitable ingredient for tempeh production would be to determine its physicochemical characteristic and functional value. Indeed, several investigations have shown that fermentation especially solid-state fermentation during tempeh production can decrease the physicochemical characteristic and functional value of tempeh, including reduction of value e.g., phytic acid and substance that can cause flatulence (Azeke *et al.,* 2007; Duli and Starzy, 2016). The objective of this study was to investigate the physicochemical characteristic and functional property of jack bean, by extracting it with four different types of solvents.

Alkaloids, a heterogeneous group with diverse chemical structures comprises the largest single class of secondary plant substances. Often, they are toxic to man and livestock but many have significant pharmacological activity, hence their wide use in medicine (Paterson, 1993). Some alkaloids from Trigonella foenum-graecum (Fenugreek) seed are reported to lower blood sugar, cholesterol and triacylglycerols (Shani *et al.,* 1974), metabolites which are usually elevated in diabetes mellitus. In a previous study, the presence of alkaloids therefore in *C. ensiformis* might explain its use in diabetic treatment. Plant families such as Leguminosae to which *C. ensiformis* belongs, Amaryllidaceae and Compositae are noted for high levels of alkaloids (Paterson, 1993).

Some of the toxicological manifestations of potato glycoalkaloids involve gastrointestinal upsets and neurological disorders, especially in doses in excess of 20 mg/100 g sample (Osagie, 1998).

The nutritional significance of saponins stems largely from their hypocholesterolaemic action, suggesting that they may prove useful in the control of human cardiovascular disease (Oakenful and Sidhu, 1983; Mohan *et. al.,* 2016). The presence of saponins in C. *ensiformis* seeds thus supports its use as an antidiabetic agent. Nimenibo–Uadia (2003) had reported the antihypercholesterol and antihypertriacylglycerol effects of the aqueous extracts of *C. ensiformis* in diabetic rats. The hypocholesterolaemic activity of dietary saponins may be due to the formation of some complexes with dietary cholesterol or their bile salt precursors which are then made unavailable for absorption (Gee and Johnson, 1988). Besides lowering serum cholesterol, dietary saponins possess immunostimulatory and anticarcinogenic properties (Gemede and Ratta, 2014). The presence of flavonoids suggests *C. ensiformis* seeds to be a source of antioxidants possessing free-radical scavenging abilities (Tiwari and Rao, 2002). Flavonoids have been reported to exhibit numerous pharmacological activities including anti-inflammatory effects, anti-fungal, anti-bacterial, anti-viral and anti-toxic activities (Cook and Samman, 1996). In another studies, Cardiac glycosides also revealed to be present in *C. ensiformis* seeds are a group of triterpenoids. Most are toxic but many have pharmacological activity. They are the active constituents of the major group of cardiovascular drugs (Trease and Evans, 1999).

2.2 α-amylase and α-glucosidase inhibitors

Carbohydrate digestibility has been reported to relate to elevated postprandial blood glucose. One of the strategies to reduce postprandial hyperglycemia is to limit the activity of carbohydrate digestive enzymes in intestinal tract. α-Amylase is the key enzyme that degrades the polymeric substrate into shorter oligomers by catalyzing the hydrolysis of α-1,4-glucan linkages present in

starch, maltodextrins, and other related carbohydrates (Truscheit *et al.,* 2010). α-Glucosidase has been found on the brush border of human intestinal mucosal cells (including maltase, α-dextrinase, and sucrose). This enzyme participates in the body's carbohydrate metabolism and cuts glucose from the nonreducing end of the polysaccharide by hydrolyzing the α-1,4-glycosidic bond. The dietary starch and other related carbohydrates are digested by α-amylase to large number of maltose, which is further digested by α-glucosidase to glucose to be absorbed in human intestine (Vocadlo and Davies, 2008). Therefore, strict control of postprandial blood glucose by inhibiting α-glucosidase and α-amylase is significant for the development of diabetes and the prevention and treatment of diabetic patients (Elbein, 1991; Tundis *et al.,* 2010). α-Amylase inhibitors (Ais) can act as carbohydrate blockers, limiting the digestibility and absorption of carbohydrate in the gastrointestinal diet (Horii *et al.,* 1986). Clinically, Ais can be used to prevent diseases such as diabetes, hyperglycemia, hyperlipemia, and obesity. Moreover, in most cases, the inhibitory mechanism of protein to α-amylase occurs by directly blocking the active centers of several subsites of the enzyme (Françoise, 2004). To determine the inhibition of α-amylase, the most widely used method is the dinitrosalicylic acid (DNSA) assay, which is not selective for the reduction in oligosaccharide ends formed during hydrolysis (Bernfeld, 1955). The α-glucosidase inhibitor (GI) inhibits α-glucosidase activity by reversibly occupying α-glucosidase and sugar-binding sites, thereby reducing polysaccharide degradation, delaying intestinal absorption of carbohydrates, and achieving hypoglycemic effects. The most prominent feature of GIs is the inhibition of α-glucosidase on the rate of intestinal carbohydrate decomposition (Larr, 2008; Seifarth *et al.,* 1998), and it does not stimulate insulin secretion to lower blood sugar, thus not increasing the islet β-cell burden. For α-glucosidase, synthetic chromogenic molecular probes such as p-nitrophenyl-glucoside (pNPG) are widely used assays

because of the ease of measurement. Natural Gis include iminosugars, thiosugars, flavonoids, alkaloids, and terpenes (Ghani, 2015).

2.2.1 α-Amylase

α-Amylase (E.C.3.2.1.1) is a hydrolase enzyme that catalyses the hydrolysis of internal α-1, 4-glycosidic linkages in starch to yield products like glucose and maltose. It is a calcium metalloenzyme i.e., it depends on the presence of a metal co-factor for its activity. There are 2 types of hydrolases: endo-hydrolase and exo-hydrolase. Endo-hydrolases act on the interior of the substrate molecule, whereas exo-hydrolases act on the terminal non reducing ends (Gupta *et al.,* 2003). Hence, terminal glucose residues and α-1, 6-linkages cannot be cleaved by α-amylase. The substrate that α-amylase acts upon is starch. Starch is a polysaccharide composed of two types of polymers – amylose and amylopectin. Amylose constitutes 20-25 % of the starch molecule. It is a linear chain consisting of repetitive glucose units linked by α-1, 4-glycosidic linkage. Amylopectin constitutes 75-80 % of starch and is characterized by branched chains of glucose units. The linear successive glucose units are linked by α1, 4-glycosidic linkage while branching occurs every 15- 45 glucose units where α-1, 6 glycosidic bonds are present. The hydrolysate composition obtained after hydrolysis of starch is highly dependent on the effect of temperature, the conditions of hydrolysis and the origin of enzyme (Gupta *et al.,* 2003). The optimum pH for activity is found to be 7.0. α-Amylase has become an enzyme of crucial importance due to its starch hydrolysis activity and the activities that can be carried out owing to the hydrolysis. One such activity is the production of glucose and fructose syrup from starch. α-Amylase catalyses the first step in this process. Previously, starch was hydrolyzed into glucose by acid hydrolysis. But this method has drawbacks like the operating conditions are of highly acidic nature and high temperatures. These limitations are overcome by enzyme hydrolysis of starch to yield high fructose syrup (Gupta *et al.,* 2010).

2.2.1.1 Production of α-Amylase

Sources

α-Amylase can be isolated from plants, animals or microorganisms. The enzyme has been isolated from barley and rice plants. It has been found that cassava mash waste water is a source of α-Amylase which is active in wide range of pH and temperature (Pandey *et al.,* 2000). In the recent past, there has been extensive research on microbial production of α-Amylase. There are 2 major reasons for the increasing interest in microbial sources:

1) The growth of microorganisms is rapid and this will in turn speed up the production of enzyme. Microorganisms are easy to handle when compared to animals and plants. They require lesser space and serve as more cost-effective sources.

2) Microorganisms can be easily manipulated using genetic engineering or other means. They can be subjected to strain improvement, mutations and other such changes by which the production of α-Amylase can be optimized.

Also, the microorganisms can be tailored to cater to the needs of growing industries and to obtain enzymes with desired characteristics like thermostability for example. Thermostable α-Amylases are desired as they minimize contamination risk and reduce reaction time, thus saving considerable amount of energy. Also, when hydrolysis is carried out at higher temperatures, the polymerization of D-glucose to iso-maltose is minimized (Kansoula *et al.,* 2007). α-Amylase is produced by several bacteria, fungi and genetically modified species of microbes. The most widely used source among the bacterial species is the Bacillus spp. B. amyloliquefaciens and B. licheniformis are widely used for commercial production of the enzyme. Other species which have been explored for production of the enzyme include *B. cereus* and *B. subtilis* to name a few. α-Amylases produced from *Bacillus licheniformis*, *Bacillus stearothermophilus*, and *Bacillus*

amyloliquefaciens show promising potential in a number of industrial applications in processes such as food, fermentation, textiles and paper industries (Kansoula *et al.,* 2007). *Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus licheniformis* and *Bacillus amyloliquefaciens* are known to be good producers of thermostable α-Amylase. Thermostability is an important characteristic as enzymatic liquefaction and saccharification of starch are performed at high temperatures (100–110 °C). Thermostable amylolytic enzymes are being investigated to improve industrial processes of starch degradation and are useful for the production of valuable products like glucose, crystalline dextrose, dextrose syrup, maltose and maltodextrins. Use of enzyme produced by thermophiles has the added advantage of reduced risk of contamination by mesophiles. Enzymes produced by some halophilic microorganisms are stable at high salinities and therefore could be used in many harsh industrial processes where the concentrated salt solutions are used. The halophilic nature the enzyme prevents inhibition of its activity under these conditions which would otherwise occur if a normal enzyme is used. In addition, most halobacterial enzymes are considerably tolerant to high temperatures and remain stable at room temperature over long periods. Halophilic amylases from halophilic bacteria such as Chromohalobacter sp., Halobacillus sp., *Haloarcula hispanica, Halomonas* sucrose (Kathiresen and Manivannan, 2006), and *Bacillus dipsosauri* have been characterized.

Genetically modified organisms are also being used for production of α-Amylase. There are various methods by which microorganisms can be manipulated at a genetic level in order to improve and optimize the production of this enzyme. The microbes can be mutated by chemical agents. Nitrous acids or ethyl methane sulphonate (EMS) have been used for genetic manipulation of bacterial strains (Haq *et al.,* 2010). *Bacillus amyloliquefaciens* UNG-16 was subjected to mutation by both the chemical (EMS) and radiation method. The mutant strain

exhibited an activity of 102.78±2.22 U/ml/min which was 1.4 times greater than the parent strain (Yoneda and Maruo, 1975). α-Amylase production in B. subtilis Marburg was improvised by treatment with Nmethyl-N'-nitro-N-nitrosoguanidine. One of the mutants, YN9, showed a threefold increase in production of the enzyme when compared to the parent strain (Pandey *et al.,* 1999).

2.2.1.2 Industrial Applications of α-Amylase

α-Amylase is gaining increased attention due to its starch hydrolyzing properties and the activities that can be carried out owing to this property. There are many potentials and widely used applications of this enzyme on the industrial front. Enzymes have replaced the previously used chemical methods of hydrolysis in various industrial sectors to make the process environment friendly and make processes easier (de Souza and de Oliveir, 2010).

2.2.1.3 Structure–Activity Relationship of Polyphenols Inhibiting α-amylases

Flavonoids Methylation and Methoxylation The methylation and methoxylation of the free hydroxyl groups in the flavonoids dramatically increase their intestinal absorption and metabolic stability by preventing the formation of glucuronic acid and sulfate conjugates (Wen and Walle, 2006). Recently, Walle reported that the oral administration of one methylated flavonoids to rats resulted in high bioavailability and tissue distribution with no detectable levels of its unmethylated analog and it was concluded that the methylation appears to be a simple and effective way to improve metabolic resistance and transport of the flavonoids (Walle, 2007). However, the methylation and methoxylation of flavonoids obviously weakened the inhibitory effects for amylase *in vitro* by amount of reports (Al-Dabbas *et al.,* 2006).

The methylation and methoxylation of flavonoids decreased the hydrogen bond acceptor/donor numbers and the hydrogen bond plays an important role in binding sucrose to amylase. The

methylation and methoxylation of flavonoids decreased the polarity and enhanced the capacity to penetrate into the tryptophan-rich hydrophobic regions of proteins, which are frequently buried in the interior of the folded proteins (Xiao and Kai, 2012).

Hydroxylation of flavonoids

The presence of a C2–C3 double bond on the ring C, a dihydroxyl group (catechol-type) or three adjacent hydroxyl group (pyrogallol-type) on the ring B, and the presence of C-5, and C-7 hydroxyl group on the ring A are usually listed as requirements for antioxidant and antiradical activity of flavonoids (Woodman *et. al.,* 2005).

Glycosylation of flavonoids

The dietary flavonoids in nature exist almost always as βglycosides (Day *et al.,* 1998). The flavonols are found mainly as the 3- and 7-O-glycoside, although the 4 positions mayalso be glycosylated in some plants (Fossen *et al.,* 1998). Other classes of sucrose are found mainly glycosylated in the 7th position (Felgines *et al.,* 2005). However, puerarin is an isoflavone-8-Cglucose. Kim *et al.* compared the inhibitory effects of several flavonoid glycosides on α-amylase (Kim *et al.,* 2000). Baicalin, pectolinarin, and linarin (5 mg/mL) hardly inhibited α-amylase. The inhibitory percentage of luteolin is similar to luteolin7-O-glucoside. The monoglycosides (quercitrin and hyperin) of quercetin are stronger than the polyglycoside form (rutin) as α-amylase inhibitors (Kim *et al.,* 2000). Ye *et al*. (2010) also reported that the inhibitory effect of 200 μg/mL quercetin with an inhibitory percentage of 65.4 % was much stronger than does 200 μg/mL rutin (9.6%) against human pancreatic α-amylase.

Li *et al.* (2009) studied the interaction between α-amylase with quercetin, isoquercetin, and rutin by fluorescence spectroscopy and enzymatic kinetics. It indicated that quercetin, isoquercetin, and rutin could bind to α-amylase to form a new complex and the binding constants (Ka) were

determined as isoquercetin > quercetin > rutin. It was also found that these 3 flavonoids are effective inhibitors against α-amylase and the inhibitory mode was a competitive type (Li *et al.,* 2009).

Catechins

Catechins are the major polyphenols in green tea leaves. The major catechins from green tea are (–)-epicatechin I, (–)- epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECG), (–)-epigallocatechin gallate (EGCG), gallocatechin gallate (GCG), theaflavin (TF1), theaflavin monogallate A (TF2A), theaflavin monogallate B (TF2B), and theaflavin digallate (TF3). Recent catechins as α-amylase inhibitors have attracted great interests among researchers (Hara and Honda, 1990). The inhibition ratio of α-amylase was about 61%, when the concentration of tea polyphenols consisting of 5.1 % C/ EGC, 40.9 % EGCG, 30.4 % ECG, 10.9 % GCG, and 6.3 % EC was 0.05 mg/mL (He *et al.,* 2006). Tadera *et al.* (2006) compared the inhibitory effect of C, EC, EGC, and EGCG against HPA. The inhibitory effects were determined as: EGCG > EC > EGC > C. Hara and Honda (1990) found the inhibitory effects were in the order of TF3 > TF2A > TF2B > TF1 > CG > GCG > ECG > EGCG. These data illustrated that the galloylated catechins have higher inhibition than nongalloylated catechins and the catechol-type catechins (CG and ECG) were twice more than pyrogallol-type catechins (GCG and EGCG). It was found that the inhibition activities of the catechins with 2,3-trans structure (CG and GCG) were 10 times higher than those of the catechins with 2,3-cis structure (ECG and EGCG) (Hara and Honda, 1990). C and sucrose have similar hydroxyl groups including number and position. However, sucrose (IC50 = 4 μmol/L) has much higher inhibition ratio than C (IC50 > 200 μmol/L) (Tadera *et al.,* 2006).

Proanthocyanidins and Anthocyanidins

Proanthocyanidins differ from other natural polyphenols by the polymeric nature. They are made of flavan-3-ol units with the average degree of polymerization from 3 to 11. Lee *et al.* (2007) evaluated the anti-α-amylase effect of polymers and oligomers from proanthocyanidins of perisimmon peel. Polymers showed a stronger inhibitory activity than oligomers. At a concentration of 100 μg/mL, polymers and oligomers showed inhibitory percentages of 53.9 % and 4.6 %, respectively (Lee, 2007). Kawakami *et al.,* (2010) separated water-soluble, proanthocyanidins from perisimmon (Diospyros kaki) and investigated the α-amylase inhibitory activity. The major proanthocyanidins were unique proanthocyanidin sucrose, namely, EGC4β-BTE, EGCG-4β-BTE, EC-4β-BTE, and ECG-4β-BTE, made of four heterogeneous extension units including EGCG, ECG, EC, and EGC (Kawakami *et al.,* 2010).

Anthocyanins are the largest group of water-soluble pigments in the plant kingdom. They have been recently demonstrated to have potential health benefits and disease prevention properties in animals and humans. Anthocyanins are included in the list of natural compounds known as potential antioxidants (Zafra-Stone *et al.,* 2007). Consumption of anthocyanin-enriched foods is associated with a reduced risk of several diseases such as atherosclerosis (Xia *et al.,* 2006), dyslipidemia (Qin *et al.,* 2009), and diabetes (Ghosh and Konishi, 2007). Cyanidin and its glycosides are naturally dietary anthocyanidins, which have been indicated as promising candidates to have potential benefits to humans, especially in the prevention and treatment of diabetes mellitus (Akkarachiyasit *et al.,* 2010).

Tannins

Dietary ellagitannins are the main dietary source of ellagic acid, which has been reported to have antiviral (Corthout *et. al.,* 1991; Yang *et. al.,* 2007) and anticarcinogenic (Rao *et al.,* 1991; MertensTalcott *et. al.,* 2003) properties. Ellagitannins from raspberry have also been found to exert potent vasodilatory properties (Tanaka *et. al.,* 2007). Strawberry and raspberry extracts

showed stronger effect against α-amylase than does blueberry, blackcurrant, or red cabbage (McDougall *et al.,* 2005). The extracts most effective in inhibiting α-amylase (strawberry and raspberry) contain substantial amounts of soluble tannins. Other tannin-rich extracts (red grape, red wine, and green tea) were also effective inhibitors against α-amylase. It was also found that removing tannins from strawberry extracts with gelatin will weaken the inhibition (McDougall *et al.,* 2005). The inhibitory components were identified as ellagitannins, such as sanguiin H_6, sanguiin H_{10}, nobatanin A, lambertianin C, and ellagic acid (McDougall *et al.,* 2005). Ellagitannins inhibit αamylase activity and there is potential for synergistic effects on starch degradation after ingestion of berries containing appreciable amounts of ellagitannins and anthocyanins (McDougall and Stewart, 2005).

2.2.2 α-Glucosidase

α-Glucosidase is an exoenzyme acting in a manner similar to that of glucoamylase on di- and oligo-saccharides and aryl glucosides. It yields glucose. This enzyme can be of animal, plant, bacterial, or fungal origin. All plants contain a-glucosidase as an endocellular enzyme, and it resides in germinated and nongerminated cereals. The neutral α-glucosidase from porcine serum appeared very substrate-specific. It hydrolyzes maltooligosaccharides, phenyl a-maltoside, nigerose, soluble starch, amylose, amylopectin, and b-limit dextrins. Isomaltose and phenyl α-glucoside were hydrolyzed with difficulty. Isomaltooligosaccharides built of 3 and more glucose units were not attacked by that enzyme. α-Glucosidase in plants controls the plant polysaccharide composition already at the stage of plant maturation, as shown, for instance, in the case of rice (Nakai *et al.,* 2006) or potato (Taylor *et. al.,* 2000) Corn α-glucosidase splits glucose from starch as the sole product, with no intermediary compounds. Two glucosidases were isolated from rice. They were strongly activated by KCl and by monovalent and divalent cations. With maltose as the substrate, such activation did not occur and glucose did not inhibit the enzyme activity. Their

behavior resembled that of porcine serum α-glucosidase Along with alpha amylase, two isoform α-glucosidases were isolated from barley kernels. The rate of hydrolysis of native starch granules with them was comparable to the results of its hydrolysis with alpha amylase isoenzymes.

α-Glucosidase secreted by bacilli that contaminate sizing starches decreases the quality of paper and paperboard (Prittijarji *et. al.,* 2001). α-Glucosidase together with alpha amylase and pullulanase, all secreted by *B. subtilis*, after purification produced chiefly maltotetraose. Bacillus sp. APC-9603 produces a novel thermostable α-glucosidase exhibiting isoamylase and pullulanase activity. Optimum conditions for that enzyme are pH 4.5–6.0 and $60–70^0$ C. This enzyme is recommended for a use jointly with glucoamylase for making glucose syrup and with beta amylase for making maltose syrup. The Thermus sucrose bacterium produces thermostable α-glucosidase that works best at pH 6.2 and 85^0 C and is suitable for one-step starch processing, being also remarkably active against maltose and maltotriose (Zdziebbo, 2002). The bacteria Sulfolobus solfataricus and Thermococcus strains also produce a-glucosidase. It has been proposed (Gray *et. al.,* 2004) to use genes of the organisms for making thermostable a-glucosidase for hydrolysis of maltooligosaccharides and liquefied starch. These genes are available from bacteria present in the environment. Mycelia of the Mucor javanicus fungus produce α-glucosidase having glucosyltransferase activity. That enzyme, together with glucoamylase, is also produced by *Lentinus edodes* (Berk.) Sing. Its Molecular weight is 51 kDa, and it hydrolyzes maltose, maltotriose, phenyl α-maltoside, amylase, and soluble starch. Cladosporium resinae produces alpha amylase, α-glucosidase, exopullulanase, and two glucoamylases. *A. niger* and Rhizopus also produce α-glucosidase. Among several enzymes tested, only that enzyme by itself, and more efficiently in combination with pullulanase, could split isomaltose and panose. Optimum conditions for the *A. niger* α-glucosidase are pH 5.5 and

55^0 C. An α-glucosidase from *Paecillomyces lilacinus*, acting as a transferase, synthesizes a-(1!3)- and a-(1!2)-linked oligosaccharides. That enzyme is most active at pH 5.0 and 65^0 C (Zanin *et al.,* 1998).

2.2.2.1 α-Glucosidases and Nonnatural Substrates

Probing α-glucosidases with various substrate analogues, Bock and Pedersen investigated hydrolysis, by the inverting amyloglucosidase (glucoamylase, EC 3.2.1.3; Glucoside Hydrolase Family 15) from *Aspergillus niger*, of all eight possible monodeoxy derivatives of methyl b-maltoside. Hydroxyl groups at positions 40 and 60 (in the nonreducing moiety) as well as 3-OH were found essential for processing by the enzyme. 6-Deoxy and 20 –deoxy derivatives were hydrolyzed faster than the corresponding fully hydroxylated compound, with transition states of similar energy but weaker substrate binding as compared to the parent compound (Bock and Pederson 1987). Such derivatives as the 6-deoxyfluoro analogue were also found to be substrates, but charged groups (6-aminodeoxy as well as 5-carboxy) were not tolerated. Again, 60 –OH was found essential for successful hydrolysis (Bock and Pedersen 1987). In-depth kinetic comparison revealed powerful binding of 40 –OH as well as of 60 –OH to charged partners of the enzyme's active site, providing 17–19 kJ/mol each to the transition-state interactions (Siekrs *et. al.,* 1992). With monodeoxy and mono-O-methyl modified methyl α-isomaltosides, a similarly pronounced significance of 40 –OH as well as 60 –OH was observed (Lemieux *et al.,* 1996). The retaining, high-isoelectric-point α-glucosidase from barley malt (EC 3.2.1.20, Glucoside Hydrolase family 31) was investigated by Frandsen *et al.,* (2000). Again, a strong contribution by 40 –OH (18.9 kJ/mol) as well as by 60 –OH (18.7 kJ/mol) was found, with significantly smaller effects of 20 –OH and 30 –OH, amounting to around 10 kJ/mol each, suggesting charged partners for the former two hydroxyl groups and neutral hydrogen bonds between 20 –OH, 30 –OH, and the enzyme (Frandsen *et. al.,* 2000). For the α-glucosidase of *Aspergillus niger*, the involvement of

2-OH as well as 3-OH in the ionization of the carboxyl groups located in the active site was demonstrated, employing 2-deoxy and 3-deoxy analogues of methyl b-maltoside (Ogawa *et. al.,* 2004). Rice a-glucosidase was probed with all four monodeoxy derivatives (34-37 figure 2.2) of 4-nitrophenyl a-D-glucopyranoside. This enzyme did not hydrolyze the 3-, 4-, or 6-deoxy derivatives but readily processed the 2-deoxy derivative (Hakamata *et. al.,* 1999). In an extended study, the same group probed these substrates, plus the four corresponding mono-O-methyl derivatives of 4-nitrophenyl α-D-glucopyranoside, with a panel of seven α-glucosidases from six different sources, namely the GH 13 enzymes from Saccharomyces cerevisiae, Bacillus stearothermophilus, and two α-glucosidases from the honey bee (honey bee I and III) along with the family 31 α-glucosidases from sugar beet, flint corn, and *Aspergillus niger*. It turned out that the enzymes from sugar beet, flint corn, and *Aspergillus niger* hydrolyzed the 2-deoxy glucosides with a higher rate than the parent a-glucoside. The 3- deoxy glucosides were hydrolyzed by the flint corn and the *Aspergillus niger* enzyme of this GH family, whereas neither the 4-deoxy nor the 6-deoxy sugar served as substrates. The Glucoside Hydrolase family 13 glucosidases invariably required the intact D-gluco configuration, with all hydroxyl groups present. In line with the observations with the α-glucosidase from rice, no hydrolytic activity was observed with any of the mono-Omethyl glucopyranosides, and steric hindrance plus hydrophobic repulsions were postulated as the factors responsible for this nonreactivity. It was therefore concluded that 2-OH is not involved in interactions of the family 31 α-glucosidases with their substrates (Nisho *et. al.,* 2002). The processing α-glucosidases I and II from rat liver were also probed with the monodeoxygenated 4-nitrophenyl α-glucosides (34–37, figure 2.1). Whereas α-glucosidase II readily hydrolyzed the 2deoxy glucoside at a higher rate than the intact substrate, all of the deoxy derivatives inhibited α-glucosidase I (Hakamata, 2004).

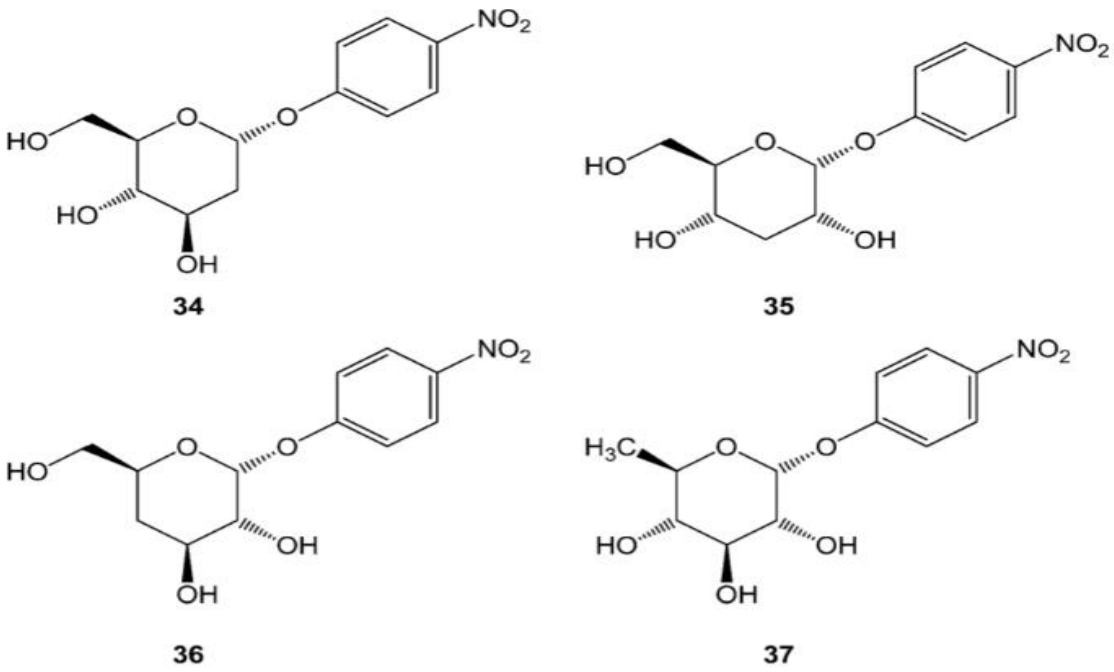


Figure 2.1 Monodeoxy Derivatives of 4-Nitrophenyl A-D-Glucopyranoside (Hakamata, 2004)

2.2.2.2 Pharmacology of Alpha-Glucosidase Inhibitors

Currently available alpha-glucosidase inhibitors include acarbose, voglibose, and miglitol." Acarbose is a nitrogen-containing pseudotetrasaccharide. Voglibose is a valiolamine derivative and miglitol a deoxynojirimycin derivative. Both acarbose and voglibose are of microbial origin, whereas miglitol is synthetically derived (Krause, 1996). Acarbose and voglibose act primarily in the gut because neither is significantly absorbed (acarbose < 2 %; voglibose, approximately 3% to 5%) (Krause, 1996). Miglitol is rapidly absorbed through a transport mechanism in the jejunum which is, in part, identical to that for glucose. Miglitol is excreted quantitatively unchanged by the kidney. Although acarbose is poorly absorbed, cleavage products produced by bacterial enzymes in the colon result in 35 %) absorption of an administered radioactive acarbose.39 Acarbose digestion by bacterial enzymes results in metabolizable intermediates and

4-methypyrogallol which is conjugated and excreted as sulfates or glucuronidates. The mechanisms of action of the alpha-glucosidase inhibitors are similar although not identical. (Puls, 1996) They all bind competitively to the carbohydrate-binding region of alpha-glucosidase enzymes. Therefore, they compete with the binding of oligosaccharides to the enzymes and interfere with their cleavage to monosaccharides. Several aspects of the binding are noteworthy. Acarbose binds to intestinal sucrose with 10^4 to 10^5 greater binding affinity than sucrose. Acarbose inhibits intestinal brush border glucoamylase, maltase, sucrase, and dextrinase, as well (Krause,

1996).

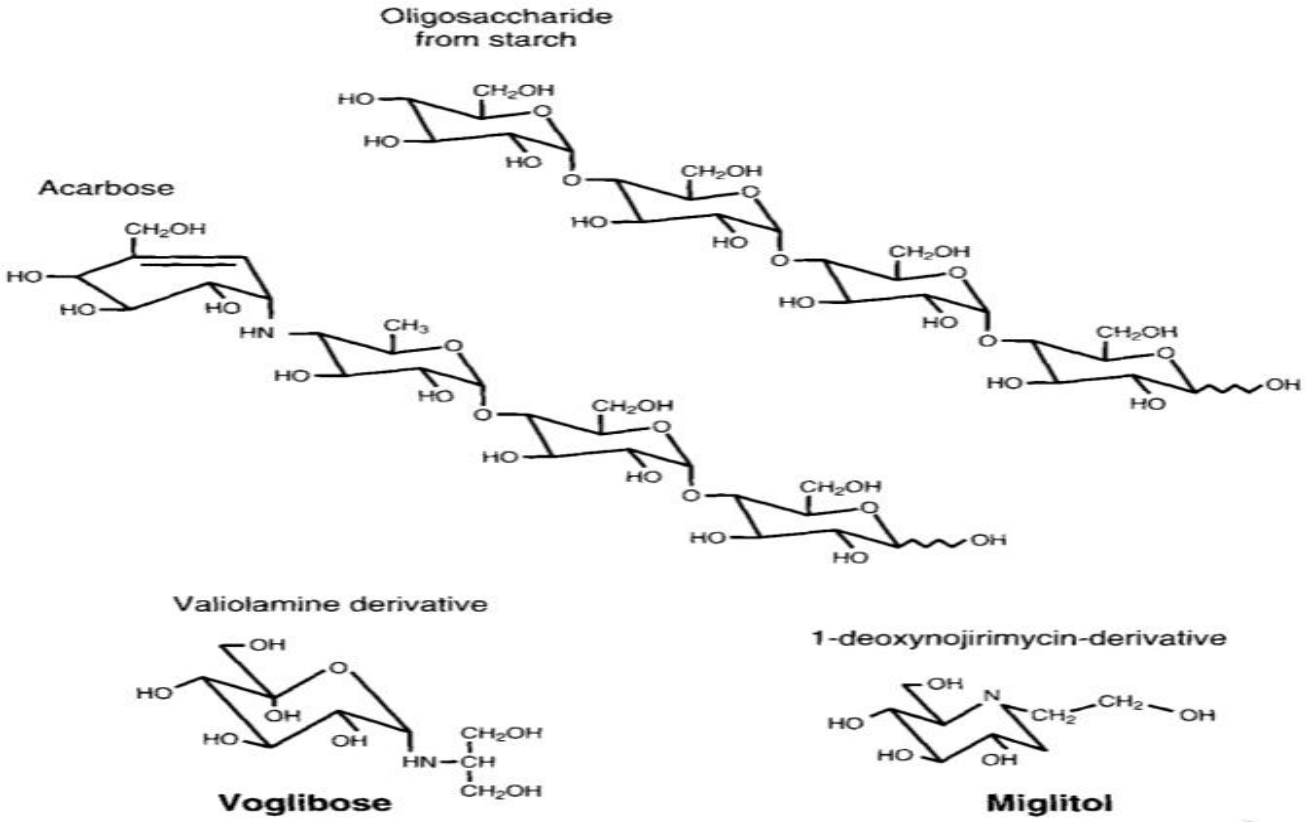


Figure 2.2 Chemical structure of various alpha-glucosidase inhibitors (Puls, 1996)

as pancreatic amylase. It has minimal effects on isomaltase and no effect on lactase, which is a beta-glucosidase enzyme. (Lebovits, 1997) Acarbose does not interfere with the intestinal absorption of glucose because it does not interact with the sodium-dependent glucose transporter of the small intestine. Voglibose is a potent inhibitor of most alpha-glucosidase enzymes but is weaker than acarbose in inhibiting sucrase and has little effect on pancreatic alpha-amylase. Miglitol differs from acarbose in that it does not inhibit pancreatic alpha-amylase but does inhibit intestinal isomaltase and interacts somewhat with the intestinal sodium-dependent glucose transporter. The *in vivo* effects of alpha-glucosidase inhibitors can be predicted from their mechanism of action. Administration of alpha-glucosidase inhibitors with carbohydrate will slow the digestion of carbohydrate. Instead of complete digestion of carbohydrates and absorption of monosaccharides in the proximal jejunum, the digestion of carbohydrate in the jejunum is incomplete. The rise in postprandial glucose is diminished and delayed. The undigested carbohydrate is slowly digested by alpha-glucosidase enzymes in the distal jejunum and ileum. When enzyme activity in the distal small bowel is insufficient, the carbo hydrate spills into the large intestine where bacteria metabolize the carbohydrate to short-chain fatty acids, hydrogen, carbon dioxide, and methane. The magnitude and time course of the postprandial glycemic rise depend on the carbohydrate content of the diet, the extent of inhibition of alpha-glucosidase enzymes in the proximal jejunum, and the alpha-glucosidase enzyme activities in the distal jejunum and ileum. Based on current understanding of the regulation of glucose metabolism, it is anticipated that alpha-glucosidase inhibitor administration would decrease plasma insulin and GIP levels and increase plasma GLP-1 in the late postprandial period. Initial treatment should lead to extensive leakage of carbohydrate into the large intestine unless very small doses are administered and the treatment algorithm provides time for enzyme induction in the distal small

bowel before full therapeutic doses are used. Because leakage of carbohydrate into the large intestine results in metabolism to short-chain fatty acids, no caloric loss occurs, and evidence of carbohydrate malabsorption is obscure. The most reliable method of assessing the leakage of carbohydrate into the colon is the measurement of hydrogen in exhaled air (breath hydrogen measurement). Plasma levels of acetate are elevated during alpha-glucosidase inhibitor treatment, but the magnitude of change is small, and the correlation with breath hydrogen is relatively poor. (Wolver *et. al.,* 1995)

2.2.2.3 Side Effects of Alpha-Glucosidase Inhibitors

The major side effects of acarbose and other alpha-glucosidase inhibitors are gastrointestinal and are a result of the pharmacologic action of the drugs (Standi, 1996). The amount of carbohydrate that escapes into the colon is dependent on dietary carbohydrate content, the dose of alpha-glucosidase inhibitor used, the rapidity with which drug dose is increased, and the duration of treatment. As noted previously, drug therapy should begin with a very low dose that is increased slowly. Gradual exposure to the agent permits the induction of alpha-glucosidase enzymes in those portions of the small intestine which normally have very low concentrations because of their lack of exposure to carbohydrate. The most frequent gastrointestinal symptoms are flatulence, diarrhea, and abdominal discomfort. The initial severity and frequency will depend on the treatment regimen used. All symptoms regress with time, and, after several months of therapy, abdominal discomfort and diarrhea are minimal. A treatment-emergent elevation in liver enzymes may occur but is uncommon with dosages of 300 mg per day or less. Post-marketing surveys have documented only 19 cases of transaminase elevation over 500 IU/L in 500,000 patients (Hollander, 1996). The liver enzyme changes are reversible on withdrawal of the drug.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant material

Dried seeds of *Canavalia ensiformis DC.* was bought from Eggon market, Nasarawa in Nasarawa State. The seed was identified by Dr. H.A. Akinnibosun of the Department of Plant Biology and Biotechnology, University of Benin, Benin City, Nigeria. A sample of the seed was deposited in the herbarium for reference purposes with a voucher number of UBH-C488.

3.1.2 List of Chemicals

Alpha-glucosidase (*Saccharomyces cerevisea*), (Kemlight Lab; PVT Ltd, India), Alpha-amylase kit (Agappe Diagnositics, Switzerland), P-Nitrophenyl –α-D-glucopyranoside (P-NPG) (Elbascience Biotech Inc, China), Acarbose (Tay Pharma, India), Sodium Hydroxide (NaOH) (Loba, Inida), Sodium dihydrogen phosphate (Indiamart, India,), Di-sodium dihydrogen phosphate (JHD, China), N-Hexane (Pharmatrends Nig. Ltd., Nigeria), Dichloromethane (Pharmatrends Nig. Ltd., Nigeria), Methanol (Pharmatrends Nig. Ltd., Nigeria), Chloroform (Pharmatrends Nig. Ltd., Nigeria), Ammonia (Pharmatrends Nig. Ltd., Nigeria), Ferric chloride (E-Merck, Darmstardt), Suphuric acid (Shijiazhung Xilongweil Chemical Co., Ltd., China), Acetic anhydride (BDH Chemical Ltd., Poole England), Glacial acetic acid (Finoric LLC, USA), Hydrochloric acid (May and Baker Ltd., Bagenman, England), Mayer's Reagent (Potassium mercuric iodide) (JHD, China), Benedict solution (BEMAC Scientific & Chemical Co. Ltd., Nigeria), Wagner's Reagent (JHD, China), Fehlings A and B solutions (JHD, China).

3.1.3 List of Apparatus

UV spectrophotometer (Spec. 20D, Techmel & Pechmel, USA), Water bath (HHS_6 – Search Tech., Germany), Rotary evaporator (Searchtech instrument RE52-3, China), Evaporating

crucibles, Refrigerator (HTA319H, Germany), Weighing balance (Kerro BL 2001, Germany), Micropipette (Labline Eco, UK), Mechanical blender (Christy and Norris, England), Gas Chromatography (Agilent Technologies 7890, USA), Mass Spectrometer (Agilent Technologies 5975, USA), (Searchtech instrument RE52-3, China), Muslin cloth.

3.2 Methods

3.2.1 Preparation of Plant Sample

Canavalia ensiformis seeds were washed with distilled water to remove soil and other dirts and then dried under the sun for two days. It was then ground to powdered form with a mechanical blender at the Department of Pharmacognosy, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

3.2.2 Extraction

3.2.3 Extraction of methanol extract of *Canavalia ensiformis* seed

Extraction of methanol extract of *Canavalia ensiformis* seed was done using the method of Francis *et. al*., (2018). Dry powder of *Canavalia ensiformis* seed (1800 g) was macerated using 5 liters of methanol for 72 hours. This mixture was then filtered using muslin cloth and the filtrate was evaporated to dryness using a rotary evaporator at 64.7 ^{0}C, in order to obtain the concentrated form of the sample.

3.2.3 Extraction of n-Hexane extract of *Canavalia ensiformis*

Extraction of n-hexane extract of *Canavalia ensiformis* seed was done using the method of Francis *et al.,* (2018). Dry powder of *Canavalia ensiformis* seed (1000 g) was macerated using 1.8 liter of n-Hexane and was left for 72 hours. The mixture was then filtered using muslin cloth and the filtrate (n-Hexane extract) was evaporated to dryness using rotary evaporator at 68 ^{0}C in order to obtain the concentrated form of the sample.

3.2.4 Extraction of dichloromethane extract of *Canavalia ensiformis* seed

Extraction of dichloromethane extract of *Canavalia ensiformis* seed was done using the method of Francis *et. al*., (2018). Dry powder of *Canavalia ensiformis* (1000 g) seed was macerated using 2.5 liters of dichloromethane for 72 hours and stirred intermittently during this period. This mixture was then filtered using muslin cloth and the filtrate was evaporated to dryness using rotary evaporator at 39.6 ^{0}C in order to achieve the concentrated form of the sample.

3.2.5 Extraction of aqueous extract of *Canavalia ensiformis* seed

Extraction of aqueous extract of *Canavalia ensiformis* seed was done using the method of Francis *et al*. (2018). Dry powder of *Canavalia ensiformis* (1000 g) seed was macerated by using 4.5 liters of distilled water (to recover and isolate most of the bio-active metabolites including primary plant metabolites) for 12 hours. The solution was filtered through the use of muslin cloth. The resulting aqueous extract was evaporated to dryness using a rotary evaporator at 100 ^{0}C, in order to achieve the concentrated form of the sample.

3.3 Enzyme studies

3.3.1 Alpha amylase inhibitory assay

Alpha amylase inhibitory assay was carried out using the method of Rangaswamy and Swathi, (2014) as described in the alpha amylase kit (Agappe Diagnostics, Switzerland). Exactly 1000 µL of reagents (MES (2-(N-morpholino) ethanesulfonic acid) buffer (p^H 6.0) 50 mMol/L, ($CNPG_3$ (2-Chloro-4-nitrophenyl-α-D-maltotrioside)-32.27 mMol/L, Calcium chloride- 60 mMol; Sodium chloride-70 mMol/L and Potassium thiocyanate -900 mMol/L) was added to 25 µL of each sample (methanol extract, n-hexane extract, dichloromethane extract and aqueous extract) and this was done individually. This mixture was pipetted into test tubes of varying

concentrations (20 µg/ml-100 µg/ml) of seed extract (methanol extract, n-hexane extract, dichloromethane extract and aqueous extract) and they were placed in respective test tubes. The mixture was thereafter incubated for 1 minute at 37 ^{0}C. The change in absorbance per minute (ΔOD/min) during 3 minutes was measured at 405 nm. The blank used was distilled water (see Appendix for absorbance readings).

Calculation:

Alpha-amylase activity (µ/L) = (ΔOD/min) x 3178

where 3178 is a factor.

IC_{50} values were obtained

3.3.2 Alpha glucosidase inhibitory assay

Alpha glucosidase inhibitory assay was carried out using the method described by Nair *et al*., (2013). The enzyme solution used for the assay was 0.5mg/ml concentration (0.5 mg of enzyme in 1ml of 100 mM sodium phosphate buffer). It was prepared by dissolving 100 mg (0.1 g) of alpha glucosidase (from *Saccharomyces cerevisiae*; Kem light Lab., PVT Ltd, India) in 200 ml of 100 mM phosphate buffer. 320 µL of 100 mM phosphate buffer at P^H 6.8 was pipetted into each test tube of varying concentration of plant extract from 20 µg/ml – 100 µg/ml and they were placed in respective test tubes. 20 µL of 0.5 mg/ml alpha glucosidase enzyme was added to the test tubes and left to stand for 10 minutes at room temperature. After incubation, 200 µL of the substrate (P-nitrophenyl-alpha-d-glucopyranoside was added. The reaction mixture was thereafter terminated by adding 3 ml of 50 mM NaOH and the absorbance was read at 410 nm (see Appendix II for absorbance readings).

Calculation:

The percentage inhibition was estimated according to the formula shown below (Jung *et al*., 2006).

$$\text{Inhibition (\%)} = \frac{Abs410\ (control) - Abs410\ (extract) x\ 100}{Abs410\ (control)}$$

The IC_{50} values were obtained from the mean inhibitory values using non-linear regression analysis and derived from plots of percentage inhibition versus concentration. The alpha glucosidase inhibitor of choice was acarbose. All of the tests were carried out in triplicate.

3.4 Phytochemicals screening

Phytochemical screening of *Canavalia ensiformis* seed extracts was determined using standard methods (Trease and Evans, 1996). The phytochemicals screened were flavonoids, saponins, reducing sugars, glycosides, steroids, anthraquinones, alkaloids starch, terpenoids, tannins and phenols.

3.4.1 Test for Alkaloids:

Principle: From neutral or slightly acidic solution, alkaloids usually undergo precipitation by Mayer's reagent which is potassium mercuric iodide solution, giving a yellow cream precipitate.

Procedure: Exactly 2.0 ml of the seed extract was evaporated to dryness first. The residue was then filtered after being dissolved in 5 ml of HCl (2 mol/dm^3). Two test tubes were filled with the filtrate. A few drops of Mayer's reagent were added to the first test tube, and the formation of a cream-colored precipitate indicated the presence of alkaloids. A few drops of Wagner's reagent were added to the second test tube, and the formation of a reddish-brown red precipitate confirmed the formation of alkaloids.

3.4.2 Test for Tannins:

Principle: Hydrolysable tannins form blue-black complex with Fe^{3+} while condensed tannins form brownish-green complex with $Fe^{3+.}$

Procedure: A 1 percent gelatin solution containing sodium chloride was added to 1.0ml of the extract. The presence of tannins was indicated by the formation of white precipitate.

3.4.3 Test for Reducing sugars:

Principle: Reducing sugars are converted to powerful reducing species known as enediols when heated in the presence of an alkali. Because the colour of the precipitate gives an indication of the amount of sugar in the solution, the test is referred to as semi-quantitative.

Procedure: About 2 ml of seed extract was mixed with 2 ml of water. The final solution was separated into test tubes. Benedict's reagent was used to treat the first test tube, which was then softly heated. The presence of reducing sugars was suggested by the presence of an orange red precipitate. About 20 drops of boiling Fehling's solution was applied to the second test tube. The presence of reducing sugars was demonstrated by the formation of a brick-red precipitate in the tube's bottom.

3.4.4 Test for Phenols:

Principle: There is a formation of violet complex which is intensely coloured when phenols react with Fe^{3+}. There may be variation in the colour from blue, green or even red which depend on the phenol nature.

Procedure: About 1.0 ml of the seed extract was treated with 4 drops of Ferric chloride solution. Formation of a bluish black colour indicated the presence of phenols.

3.4.5 Test for Saponins:

Principle: Saponins react with water to create foam. The combination of a hydrophobic (fat-soluble) sapogenin and a hydrophilic (water-soluble) sapogenin allows saponins to form foam.

Procedure: The detection of saponins was carried out using the foam and froth test methods. Exactly 0.5 g of plant extract was shaken with 2.0 ml of distilled water in the foam test process. Saponins were detected by the forming of foam that lasted for 10 minutes. In the froth test process, 5.0 ml of the extract was diluted to 20.0 ml with distilled water and shook for 15 minutes in a 50ml graduated cylinder. The formation of 1cm layer of foam indicated the presence of saponins.

3.4.6 Test for Flavonoids:

Principle: Precipitation occurs when flavonoids react with two o-oxygroups in β cycle with lead acetate. Intensive yellow precipitate is produced in the case of flavones.

Procedure: The alkaline reagent test and the lead acetate test were used to do this. The extract was treated with a few drops of a 2 mol/dm^3 sodium hydroxide solution in the alkaline reagent procedure. The presence of flavonoids was shown by the development of an intense yellow colour that turned colourless when dilute hydrochloric acid (2 mol/dm^3) was added. The seed extract was treated with a few drops of lead acetate solution in the lead test. The formation of yellow colour precipitate indicated the presence of flavonoids.

3.4.7 Test for Starch:

Principle: The development of an intermolecular charge-transfer complex converts starch into an intense "blue-black" colour when aqueous solutions of the triiodide anion are applied. The brown colour of the aqueous solution remains in the absence of starch. Iodometry is based on the interaction of starch and triiodide.

Procedure: About 5.0 ml of the seed extract was treated with aqueous solution of iodine. The formation of a blue violet colour indicated the presence of starch.

3.4.8 Test for Steroids:

Principle: This test is used to detect steroids, in which a deep green colour is given. This colour is initially purple, then turns to pink colour and moves through to light green colour and then to a very dark green colour. The formation of the colour is as a result of the reaction of the hydroxyl group (-OH) of steroid with acetic anhydride and increasing the conjugation of the unsaturation in the adjacent fused ring.

Procedure: In 1ml of each sample, 5 drops of concentrated H_2SO_4 were added. A red colouration indicated the presence of steroids.

3.4.9 Test for Terpenoids:

Principle: A mixture of terpenoids, chloroform and concentrated sulphuric acid produces a reddish colouration at the interphase.

Procedure: About 2 ml of the oil was mixed with 2 ml of chloroform and 1ml of concentrated sulphuric acid was carefully added from the sides of the test tube to form a layer. Clear upper and lower layers with a reddish-brown interphase indicated a positive result.

3.4.10 Test for Glycosides:

Principle: When glycosides are hydrolysed with concentrated acid, the hydrolysate forms a brown colour with Fe^{3+}.

Procedure: About 1 ml of the extract solution was dissolved in 1 ml of glacial acetic acid containing a drop of Ferric chloride solution. This was under-layered with 1 ml concentrated H_2SO_4. A brown ring obtained indicated the presence of glycoside.

3.4.11 Test for Anthraquinones:

Principle: The akalization reaction to obtain the phenolate ion can be done by employing weak alkalis, such as ammonium hydroxide. This is used for the identification of anthraquinones in plant extracts and powders.

Procedure: About 0.5 g of the seed extract was shaken with 10ml chloroform, filtered and 5 ml of 10 % ammonia solution added to the filtrate. The mixture was shaken and the presence of a yellow colour in the upper ammonia phase indicated the presence of anthraquinones.

3.5 Analysis using Gas Chromatography and Mass Spectrometry (GC-MS)

Principle: The sample mixture is first separated by the GC before the analyte molecules are eluted into the MS for detection. They are transported by the carrier gas which continuously flows through the GC and into the MS, where it is evacuated by the vacuum system.

Gas chromatography and mass spectrometry was carried out on *Canavalia ensiformis* seed extract (n-hexane extract and dichloromethane extract). This procedure was done to identify the biologically active natural chemical constituents present in the extract.

Procedure: It was analyzed using Gas chromatography (Agilent Technologies 7890, Germany) coupled with Mass spectrometry detector (Agilent Technologies 5975, Germany). Column used was HP5MS Agilent Technologies 30m in length and inner diameter of 0.32 mm and thickness 0f 0.25 mm. The initial column temperature was 80 ^{0}C and final temperature was 240 ^{0}C, with splitless injectors and pressure of 8.267 psi. The flow rate was 2 ml/minutes and the flow within the column was 2 ml/minute. The detector temperature was 250 ^{0}C and helium was used as the gas carrier with mass spectrometric detector and the sample volume injected was 1 ųL.

The identification of compounds was based on comparison of retention indices comparing mass spectra of the compounds with those of authentic samples and stored in NIST (National institute standard and technology) library.

3.6 Data Analysis

Results were reported as mean ± SEM and IC_{50} values were generated from a plot of percentage inhibition against concentration. Statistical analysis was carried out using Statistical Package for

Social Science (SPSS) for windows version 20.0. The analysis of the data was by One Way Analysis of Variance (ANOVA) followed by LSD post HOC test. The level of significance was taken at 5 % confidence interval ($P<0.05$).

CHAPTER FOUR
RESULTS

4.1 Results of *in vitro* anti-diabetic activities of *Canavalia ensiformis* seed Extracts

The percentage inhibition of methanol extract, n-hexane extract, dichloromethane extract and aqueous extract of *Canavalia ensiformis* seed on the activities of alpha amylase at different concentrations (20 µg/mL-100 µg/mL) are shown in Table 4.1.

The result from the table showed that the highest percentage inhibition of alpha amylase was recorded; with methanol extract, having 76.43 % inhibition at 100 µg/mL, followed by the n-hexane extract having 70.35% inhibition at 40 µg/mL, dichloromethane extract having 58.8% inhibition at 40 µg/mL and aqueous extract having 38.16% inhibition at 80 µg/mL. The IC_{50} values of the extracts were methanol: 13.03 µg/ml, n-hexane: 78.87 µg/mL, dichloromethane: 27.10 µg/mL and aqueous: 101.61 µg/mL.

Figure 4.1 shows the percentage inhibition of alpha amylase by *C. ensiformis* seed methanol, n-hexane, dichloromethane and aqueous extracts at varying concentrations.

In the alpha amylase inhibition by acarbose (reference drug), the highest percentage inhibition for the standard drug acarbose showed 40.47% at concentration 60 µg/ml with an IC_{50} value of 21.89 µg/ml (Table 4.2). At the lowest concentration of 20 µg/ml acarbose showed 23.7% inhibition of alpha amylase and 29.09% at the highest concentration 100 µg/ml. Acarbose showed a decrease in percentage alpha amylase inhibition as its concentration was increased from 60-100 µg/ml.

Table 4.1: The percentage inhibition of wheat alpha amylase by methanol, n-hexane, dichloromethane and aqueous extract of *Canavalia ensiformis*

Concentration (µg/mL)	% inhibition by methanol extract	IC_{50} (µg/mL) methanol extract	% inhibition by n-hexane extract	IC_{50} (µg/mL) n-hexane extract	% inhibition by dichloromethane extract	IC_{50} (µg/mL) dichloromethane extract	% inhibition by aqueous extract	IC_{50} (µg/mL) aqueous extract
20	48.56 ± 0.136^{a}		4.85 ± 0.091^{a}		33.35 ± 0.079^{a}		6.76 ± 0.2^{a}	
40	62.95 ± 0.136^{b}	13.03^{d}	70.35 ± 0.091^{b}	78.87	58.8 ± 0.079^{b}	27.10	21.10 ± 0.2^{b}	101.61
60	66.19 ± 0.139^{b}		62.65 ± 0.092^{c}		47.28 ± 0.079^{b}		16.29 ± 0.2^{b}	
80	58.64 ± 0.139^{b}		58.61 ± 0.092^{bc}		10.91 ± 0.079^{c}		38.16 ± 0.2^{c}	
100	76.43 ± 0.139^{c}		59.68 ± 0.092^{c}		34.89 ± 0.079^{c}		18.2 ± 0.2^{b}	

Values are mean ± SEM (n=3). Values with different superscripts are significantly different ($p < 0.05$).

Values with the same superscripts are not significantly different ($p > 0.05$).

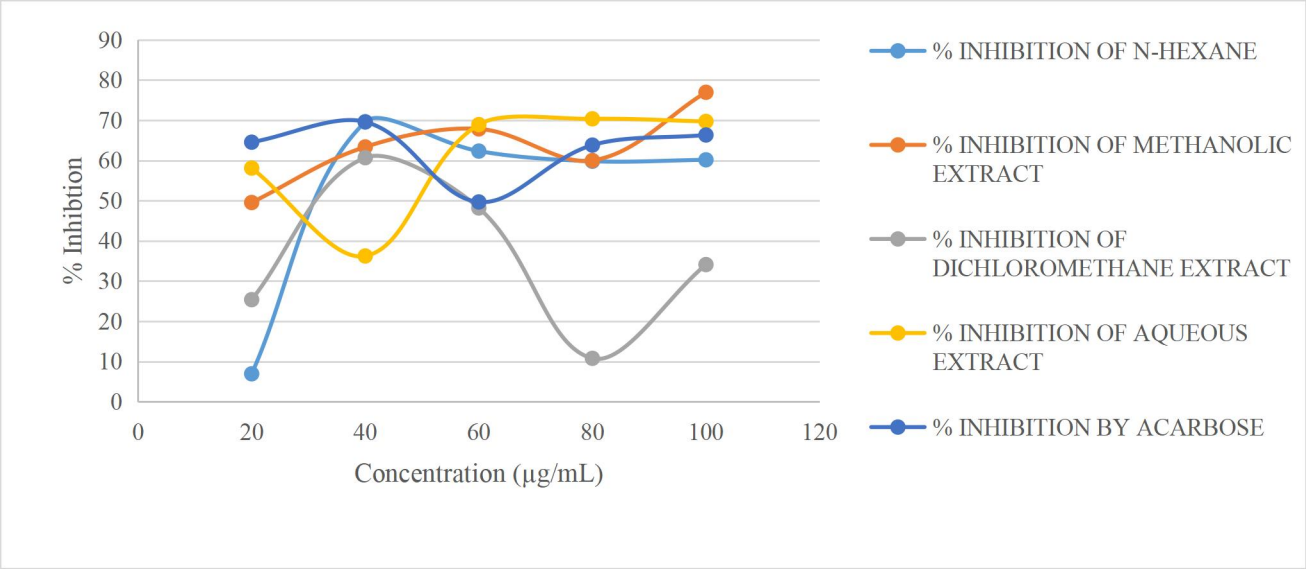


Figure 4.1: Percentage inhibition of alpha amylase by *C. ensiformis* seed methanol, n- hexane, dichloromethane and aqueous extracts at varying concentrations

Table 4.2: The *in vitro* percentage inhibition of alpha amylase by Acarbose (reference drug)

Concentration (μg/mL)	**% inhibition by Acarbose**	**IC_{50} (μg/mL) Acarbose**
20	23.7 ± 0.30^{a}	
40	19.09 ± 0.91^{b}	
60	40.47 ± 0.17^{c}	21.89
80	38.16 ± 0.66^{c}	
100	29.09 ± 0.32^{c}	

Values are mean ± SEM (n=3). Values with different superscripts are significantly different ($p < 0.05$).

Values with the same superscripts are not significantly different ($p > 0.05$).

Table 4.3 shows the mean percentage inhibition of the activity of α-glucosidase of *Canavalia ensiformis* seeds extracts. The results from the table showed that the highest percentage inhibition of α-glucosidase was recorded, aqueous extract having 55.21% inhibition at 20 µg/mL, followed by the n-hexane extract having 44.97% inhibition at 80 µg/mL, followed by methanol extract, having 42.43% at 100 µg/mL and dichloromethane extract having 30.73% inhibition at 100 µg/mL. The IC_{50} values of the extracts were methanol: 127.17 µg/mL, n-hexane: 106.01 µg/mL, dichloromethane: 279.83 µg/mL and aqueous: 43.78 µg/mL.

Figure 4.2 shows the percentage inhibition of alpha-glucosidase by *C. ensiformis* seed methanol, n- hexane, dichloromethane and aqueous extracts at varying concentrations.

In the alpha glucosidase inhibition by acarbose (reference drug), the highest percentage inhibition for the standard drug acarbose was 38.15% with an IC_{50} value of 71.71 µg/ml (Table 4.4). At the lowest concentration of 20 µg/ml acarbose showed 1.1% inhibition of alpha glucosidase and 20.33% at the highest concentration 100 µg/ml. Acarbose showed a decrease in percentage alpha glucosidase inhibition as its concentration was increased from 60-100 µg/ml.

Among the four extracts (methanol, n-hexane, dichloromethane and aqueous), n-hexane extract showed the most potent inhibition of alpha amylase activity while dichloromethane showed the most potent inhibition of alpha glucosidase activity.

Table 4.3: The percentage inhibition of yeast alpha glucosidase by methanol, n-hexane, dichloromethane and aqueous extracts of *Canavalia ensiformis* seed

Concentration (μ g/mL)	% Inhibition by methanol extract	IC_{50} (μg/mL) methanol extract	% Inhibition by n-hexane extract	IC_{50} (μg/mL) n-hexane extract	% inhibition by dichloromethane extract	IC_{50} (μg/mL) dichloromethane extract	% inhibition by aqueous extract	IC_{50} (μg/mL) aqueous extract
20	20.57± 0.048[a]		34.57±0.043 [a]		19.45 ± 0.045[a]		55.21±0.048[a]	
40	41.43 ±0.048[b]	127.17	42.33± 0.043[b]	106.01	17.78 ± 0.045[a]	279.83	49.26±0.048[a]	43.78[d]
60	34.09±0.048[b]		31.96±0.043[a]		12.59 ± 0.045[b]		42.09±0.048[a]	
80	36.45± 0.048[b]		44.97± 0.043[b]		22.64 ± 0.045[c]		27.40±0.048[b]	
100	42.43 ±0.048[b]		34.44± 0.043[a]		30.73 ± 0.045[c]		44.8±0.048[a]	

Values are mean ± SEM (n=3) Values with different superscripts are significantly different ($p < 0.05$).

Values with the same superscripts are not significantly different ($p > 0.05$).

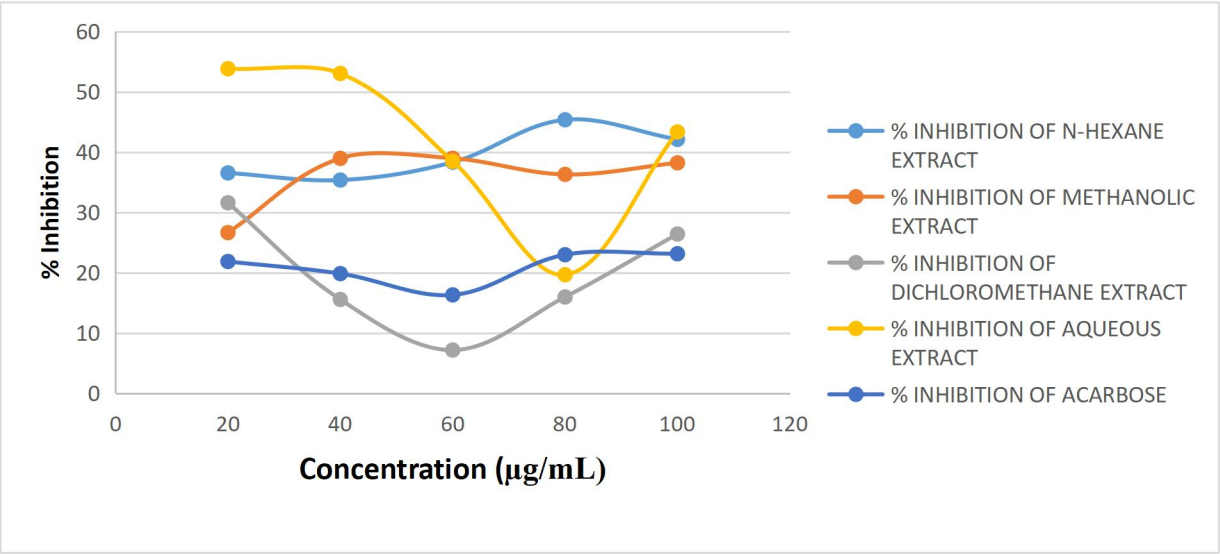


Figure 4.2: Percentage inhibition of alpha-glucosidase by *C. ensiformis* seed methanol, n- hexane, dichloromethane and aqueous extracts at varying concentrations.

Table 4.4: The *in vitro* percentage inhibition of alpha glucosidase by Acarbose (reference drug)

Concentration (ųg/ml)	% inhibition by Acarbose	IC_{50} (ųg/ml) Acarbose
20	1.1 ±1.10[a]	
40	22.62 ± 5.95[b]	
60	38.15 ± 0.82[c]	71.71
80	27.75 ± 3.53[b]	
100	20.33 ± 1.10[b]	

Values are mean ± SEM (n=3). Values with different superscripts are significantly different (p<0.05).

Values with the same superscripts are not significantly different (p > 0.05).

4.2 Phytochemical screening (Qualitative)

Phytochemical screening of *Canavalia ensiformis* seed extracts was determined using standard methods (Trease and Evans, 1996). The phytochemicals screened were flavonoids, saponins, reducing sugars, glycosides, steroids, anthraquinones, alkaloids, starch, terpenoids, tannins and phenols. Saponins, glycosides, alkaloids, starch, terpenoids, and phenols were present in the various extracts, while reducing sugar, steroids, tannins, flavonoids and anthraquinones were not present in any of the extracts.

4.3 Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the n-hexane extract of *C. ensiformis* seed

Thirty-five therapeutically important compounds were identified from the n-hexane extract of *C. ensiformis* seed through GC-MS analysis. The names of the major chemical components are shown in Table 4.6.

4.4 Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the dichloromethane extract of *C. ensiformis* seed

Eight therapeutically important compounds were identified from the dichloromethane extract of *C. ensiformis* seed through GC-MS analysis. The names of the major chemical components are shown in Table 4.7.

4.5 Mass Spectra and molecular structures

The mass spectra and molecular structures of the identified bioactive compounds in n-hexane and dichloromethane extracts of *Canavalia ensiformis* seeds are shown in Figures 4.3-4.6.

Table 4.5: Phytochemical screening of the methanol, n-hexane, dichloromethane and aqueous crude extract of *Canavalia ensifformis* seed

	Methanol extract	**n-Hexane extract**	**Dichloromethane extract**	**Aqueous extract**
Starch	-	-	+	+
Reducing sugars	-	-	-	-
Glycosides	+	-	++	-
Terpenoids	+	++	++	-
Steroids	-	-	-	-
Tannins	-	-	-	-
Saponins	++	-	+	+
Flavonoids	-	-	-	-
Alkaloids	-	+	+	+
Anthraquinones	-	-	-	-
Phenols	++	+	++	++

Key:

Slightly present = +

Moderately present = ++

Not present = -

Table 4.6: Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the n-hexane extract of *C. ensiformis* seed

S/N	Retention time (min)	Name of compound	Peak Area (%)	Molecular Formula	Molecular Weight
1	3.225	Hexane, 3,3,4-trimethyl-	0.54	C_9H_{20}	128.25
2	3.631	Toluene	1.58	C_7H_8	92.141
3	3.825	Cyclohexane, 1,3-dimethyl-, cis-	0.42	C_8H_{16}	112.21
4	4.613	Tetrachloroethylene	1.10	C_2Cl_4	165.8
5	6.114	Ethylbenzene	3.32	C_8H_{10}	106.16
6	6.377	p-Xylene	12.4	C_8H_{10}	106.16
7	7.084	Benzene, 1,3-dimethyl-	5.02	$C_{12}H_{16}$	160.25
8	21.953	5-Tetradecene, (E)-	0.63	$C_{14}H_{28}$	196.37
9	25.350	Phenol, 2,4-bis(1,1-dimethylethyl)	1.24	$C_{17}H_{30}OSi$	278.5
10	25.518	Benzene, (1-butylhexyl)-	2.03	$C_{11}H_{16}$	148.24
11	25.719	Benzene, (1-propylheptyl)-	1.68	$C_{16}H_{26}$	218.38
12	26.138	Benzene, (1-ethyloctyl)-	2.00	$C_{16}H_{26}$	218.3776
13	26.813	9-Eicosene, (E)-	1.52	$C_{20}H_{40}$	280.5
14	26.995	Benzene, (1-methylnonyl)-	3.38	$C_{16}H_{26}$	218.3776
15	27.702	Pyrrolidine, 1-(phenylmethyl)-	2.02	$C_{12}H_{17}N$	175.27
16	27.783	Benzene, (1-butylhexyl)-	4.96	$C_{11}H_{16}$	148.24
17	28.008	Benzene, (1-propyloctyl)-	3.75	$C_{13}H_{20}$	176.30
18	28.452	Benzene, (1-ethylnonyl)-	4.55	$C_{17}H_{28}$	232.4042
19	29.278	Benzene, (1-methyldecyl)-	5.76	$C_{17}H_{28}$	232.4
20	29.853	Benzene, (1-pentylheptyl)-	3.54	$C_{14}H_{22}$	190.32400

21	29.947	Benzene, (1-butyloctyl)-	4.44	$C_{18}H_{30}$	246.4
22	30.204	Benzene, (1-propylnonyl)-	3.15	$C_{15}H_{24}$	204.35
23	30.660	Benzene, (1-ethyldecyl)-	3.19	$C_{18}H_{30}$	246.4
24	31.198	1-Octadecene	2.47	$C_{18}H_{36}$	252.486
25	31.467	Benzene, (1-methylundecyl)	3,71	$C_{17}H_{28}$	232.4
26	31.918	Benzene, (1-hexylheptyl)-	3.29	$C_{16}H_{26}$	218.38
27	32.049	Benzene, (1-butylnonyl)-	2.10	$C_{19}H_{32}$	260.5
28	32.305	Benzene, (1-propylnonyl)-	1.65	$C_{15}H_{24}$	204.35
29	32.762	Benzene, (1-ethylundecyl)-	1.7	$C_{19}H_{32}$	260.5
30	33.563	Benzene, (1-methyldodecyl)-	1.69	$C_{19}H_{32}$	260.4
31	35.170	3-Eicosene, (E)-	2.99	$C_{20}H_{40}$	280.5
32	38.823	Octacosyl acetate	2.55	$C_{17}H_{28}$	232.4
33	42.170	Triacontyl acetate	2.41	$C_{32}H_{64}O_2$	480.8
34	44.610	Phthalic acid, di(2-propylpentyl) ester	1.55	$C_{24}H_{38}O_4$	390.6
35	45.266	Octacosanol	1.63	$C_{28}H_{58}O$	410.8

Table 4.7: Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the Dichloromethane extract of *C. ensiformis* seed

S/N	Retention time (min)	Name of compound	Peak Area (%)	Molecular Formula	Molecular Weight
1	26.820	Carbonic acid	2.7	CH_2O_3	62.025
2	27.007	Benzoic acid	1.51	$C_7H_6O_2$	122.123
3	33.932	Hexadecanoic acid, methyl ester	9.38	$C_{19}H_{38}O_2$	298.5
4	31.204	1-Octadecene	2.91	$C_{18}H_{36}$	252.486
5	29.284	Benzene, (1-methyldecyl)-	2.25	$C_{17}H_{28}$	232.4
6	34.98	n-Hexadecanoic acid	30.72	$C_{21}H_{46}O_2Si_2$	386.8
7	44.622	Phthalic acid	48.78	$C_8H_6O_4$	166.13
8	29.284	Bis[2,4-dimethylbenzyl] sulfone	1.76	$C_{16}H_{18}O_2S$	274.4

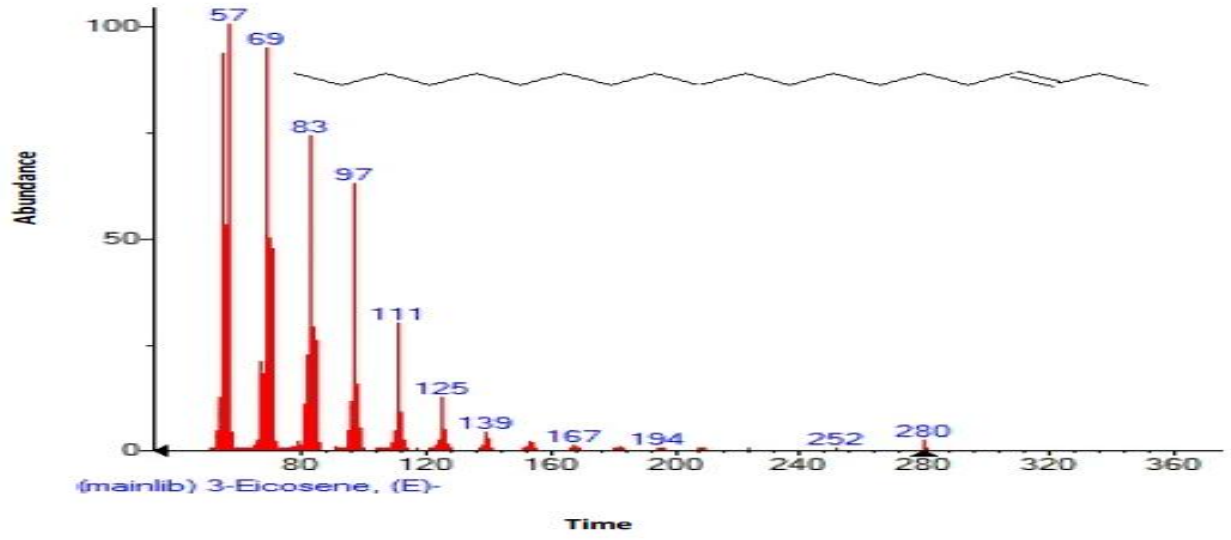


Figure 4.3: GC-MS Spectrum of n-hexane extract of *Canavalia ensiformis* seed and Structure of 3-Eicosene, (E)-

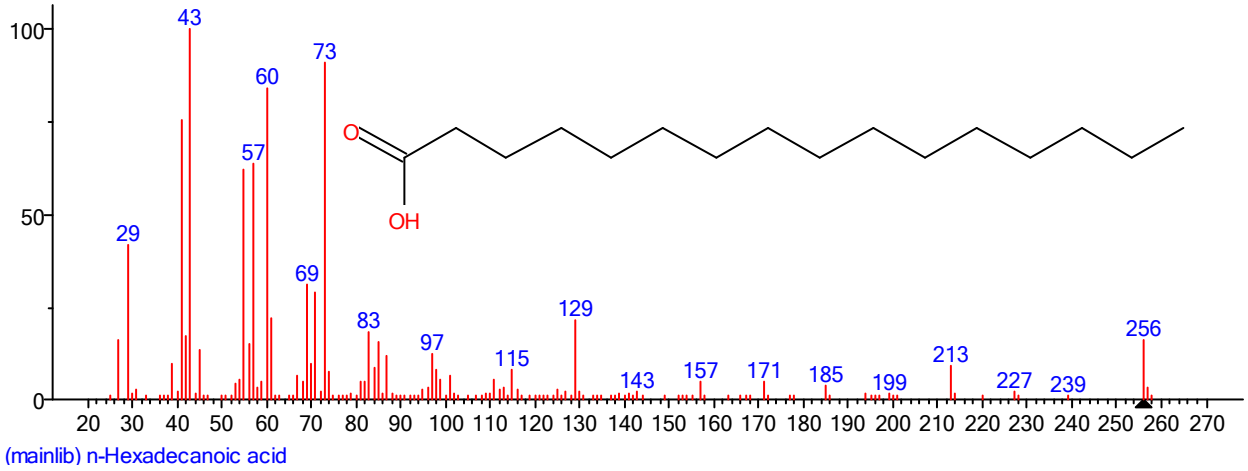


Figure 4.4: GC-MS Spectrum of dichloromethane extract of *Canavalia ensiformis* seed and Structure of n-Hexadecanoic acid

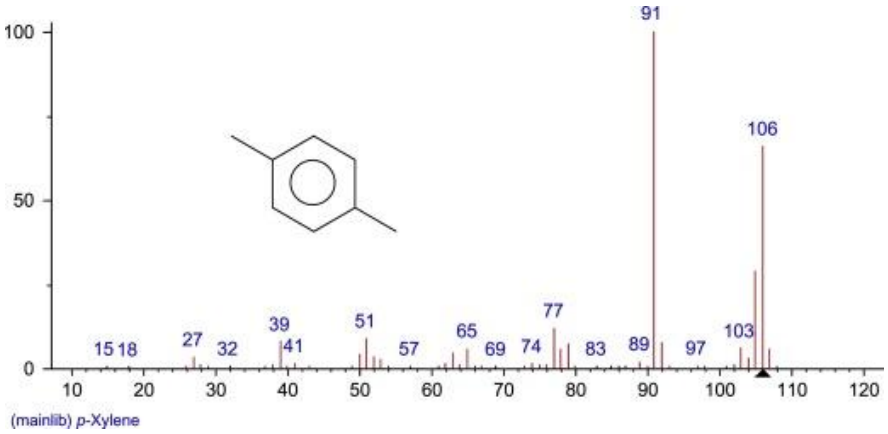


Figure 4.5: GC-MS Spectrum of n-hexane extract of *Canavalia ensiformis* seed and Structure of p-Xylene

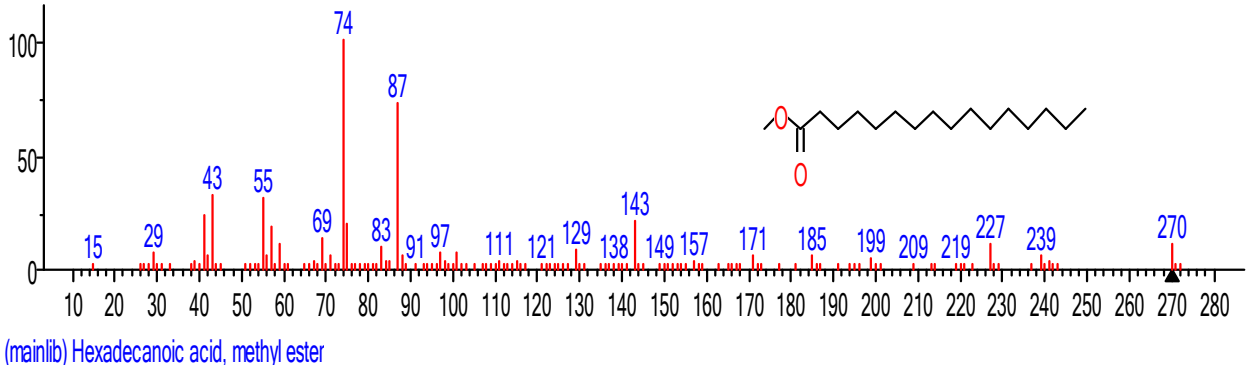


Figure 4.6: GC-MS Spectrum of dichloromethane extract of *Canavalia ensiformis* seed and Structure of Hexadecanoic acid, methyl ester

CHAPTER FIVE
DISCUSSION

Diabetes is a complex metabolic disease characterized by abnormal glycemic control resulting from the body's inability to secrete or utilize insulin that may result from several biochemical processes. An important goal in treatment is to provide the patient with the necessary tools to achieve the best possible control of glycemia, lipidemia, and blood pressure to prevent, delay or arrest the microvascular and macrovascular complications of diabetes (Reka *et al.,* 2017). The approach for controlling postprandial hyperglycemia is to inhibit the carbohydrate hydrolyzing enzymes such as alpha-amylase and alpha-glucosidase in the digestive system (Reka *et al.,* 2017). Alpha-amylase catalyzes the hydrolysis of starch to a mixture of smaller oligosaccharides consisting of maltose, maltotriose, and oligo glucans and it is further degraded to glucose by α-glucosidase after which they are absorbed into the bloodstream (Truscheit *et al.,* 2010). In this study, *C. ensiformis* was subjected to extraction by different solvents; methanol, dichloromethane, n-hexane, and water. The effects of these extracts on alpha-amylase and alpha-glycosidase enzymes were determined.

Inhibition of the alpha-amylase and alpha-glucosidase enzymes helps to reduce the rate of carbohydrate digestion and causes a reduction in the rate of absorption thereby lowering the postprandial serum glucose levels (Bhat *et al.,* 2011). The results revealed high inhibition of alpha-amylase by *C. ensiformis* seed extract (Table 4.1). All plant extracts had lesser percentage inhibition when compared with acarbose (standard drug) except for the aqueous extract with an IC_{50} of 209.71 µg/mL. The result indicates that *C. ensiformis* seed possesses moderate alpha-amylase inhibitory activity. The methanol extract with an IC_{50} value of 13.03 µg/mL is the most potent for alpha-amylase and can be considered a potential functional food for diabetic patients.

This is similar to those reported by Zhen *et al.,* (2012), who reported a moderate alpha-amylase inhibitory activity for *Chollrella pyrenoidasa.*

The inhibitory activity of α-glucosidase is one of the useful parameters to show the potential of plant extracts for preventing diabetes (Reka *et al.,* 2017). This enzyme plays a main role in hydrolyzing sugar compounds into monomers that can be easily absorbed in the small intestine, α-glucosidase is a catabolic enzyme responsible for breaking down carbohydrates to the monosaccharide unit glucose, before absorption. As impaired cellular uptake of glucose is a characteristic feature of diabetes, slowing down α-glucosidase activity is beneficial. The resulting slow digestion of carbohydrates delays glucose release and absorption minimizing the glucose concentration surge associated with a carbohydrate meal (Jalili-Safaryan *et al*., 2022). Table 4.3 and Figure 4.3 shows the percentage inhibition of alpha-glucosidase by *C. ensiformis* seed methanol, n-hexane, dichloromethane, and aqueous extracts at varying concentrations, with the aqueous extract with IC_{50} value of 43.78 µg/mL being the most potent. This further shows *Canavalia ensiformis* seeds' ability to slow down postprandial glycemia, which may also be attributed to their high phenol content. Similar inhibitory findings were made by Vadivel *et al.* (2012), where they reported 77.56% of alpha-amylase and 75.45% of alpha-glucosidase enzyme inhibition characteristics under *in vitro* starch digestion bioassay.

Mass spectrometry, incorporated with chromatographic separation such as Gas chromatography (GC-MS) is normally used for direct analysis of components existing in traditional medicines and medicinal plants. In recent years GC-MS studies have been increasingly applied for the analysis of medicinal plants as these techniques have proved to be a valuable method for the analysis of non-polar components and volatile essential oils, fatty acids, lipids, and alkaloids.

Tables 4.6 and 4.7 showed the retention time, peak area percentage of the n-hexane and dichloromethane extracts of *Canavalia ensiformis* seed respectively. Some of the major

compounds identified in the GCMS analysis of the n-hexane extract of *C. ensiformis* seed were p-xylene with 12.4% area peak; benzene, 1,3-dimethyl, with 5.02% area peak; 9-eicosene, (E)-, with 1.52 % area peak; Triacontyl acetate, with 2.41 % area peak.

P-xylene and benzene, 1,3-dimethyl, have been shown to have significant antidiabetic effects (Kumar *et. al.,*2018). P-xylene may improve insulin sensitivity, reducing glucose levels and improving glucose metabolism, while Benzene, 1,3-dimethyl, also known as m-xylene or 1,3-dimethylbenzene may influence glucose metabolism by inhibiting enzymes involved in glucose production or improving insulin signaling (Kumar *et. al.,*2018). 9-Eicosene, (E)- has also been shown to have significant antidiabetic effects (Prananda *et. al.,* 2023). 9-Eicosene, (E)- has anti-diabetic properties (Prananda *et. al.,* 2023). Triacontyl acetate has been shown to inhibit alpha-glucosidase, an enzyme involved in carbohydrate digestion and absorption, which can help regulate blood sugar levels (Zhang *et. al.,* 2019).

Some of the compounds identified in the GCMS analysis of the dichloromethane extract of *C. ensiformis* seed were benzoic acid, with 1.51% area peak; phthalic acid, with 48.78% area peak.

Benzoic acid is a natural metabolite found in various foods and medicinal plants (Jahan *et. al.,* 2017). Some plant-derived compounds containing benzoic acid derivatives like 2,4-Dinitroanilino-benzoic acid demonstrated significant improvement in glucose tolerance and fasting blood glucose levels in diabetic rats, likely by enhancing pancreatic function and insulin secretion (Jahan *et. al.,* 2017). Phthalic acid found to be present in both n-hexane and dichloromethane extracts has been isolated from the plant *Phyllanthus rheedii* and evaluated for its anti-diabetic activity. *In vitro* and *in silico* studies suggested that phthalic acid effectively lowers blood glucose by inhibiting hepatic glucose production and improving insulin resistance (Sivajothi *et. al.,* 2013).

GC-MS analysis on the *C. ensiformis* seed extracts of n-hexane and dichloromethane (Tables 4.6 and 4.7), confirmed the presence of phenols and other bioactive compounds such as phenol, 2,4-bis(1,1-dimethylethyl) in table 4.6 and hexadecanoic acid, methyl ester in table 4.7 with reported antioxidant and anti-inflammatory activities (Majinda and Abubakar, 2016).

Phytochemicals are compounds that alleviate the risk of free radicals that cause oxidative damage to living cells and result in common degenerative disorders like cancer and cardiovascular diseases (Olugboyega and Edem, 2018). Phenolic compounds are considered to be very important as antioxidants. Their antioxidant properties include; anti-carcinogenic, anti-oxidant, anti-tumoral, anti-microbial, anti-ischemic, anti-allergic, anti-mutagenic, and anti-inflammatory effects, as well as being effective in alleviating cardiovascular diseases (Olugboyega and Edem, 2018). The results of phytochemical screening in this study revealed the presence of glycosides, starch, terpenoids, saponins, alkaloids, and phenols in the various extracts, only phenols were detected across all extracts. These findings are similar to that reported by Nimenibo–Uadia (2017), who carried out preliminary studies on *Canavalia ensiformis* (Jack bean) DC. Seeds.

5.1 Contribution to Knowledge

This study has contributed to knowledge in the following ways:

1. The study revealed the mechanism of the anti-diabetic effect of *C. ensifomis* seed through the inhibition of α-amylase and α-glucosidase enzymes activities.
2. This study revealed that dichloromethane and n-hexane extracts of *C. ensiformis* seed had the most inhibitory activities on alpha-amylase and alpha glucosidase enzyme respectively.

3. This study suggests that the rich phytochemical contents; specifically high phenol, contained in *Canavalia ensiformis* seeds may have influenced the alpha-glucosidase inhibitory activities.
4. This knowledge can be used as a guide for further research as *Canavalia ensiformis* seeds could be used as a cheap source of components for production of antidiabetic drug.

5.2 Conclusion and Recommendation

Analysis of the alpha-amylase and alpha-glucosidase inhibitory activities of the extracts demonstrated inhibitory potentials of *C. ensiformis* seed extracts, with dichloromethane and n-hexane extracts having the highest inhibitory potentials for the alpha-amylase and alpha-glucosidase respectively. Phytochemical screening carried out on *C. ensiformis* seed revealed the presence of starch, reducing sugars, glycosides, terpenoids, steroids, saponins, alkaloids, anthraquinones and phenols. GC-MS analysis of dichloromethane extract showed the presence of anti-diabetic compounds such as n-Hexadecanoic acid, 3-Eicosene and p-xylene. Therefore, *C. ensiformis* seed can be inferred to contain powerful inhibitors against alpha-amylase and alpha-glucosidase enzymes that can slow down the breakdown of polysaccharides to liberate glucose; thereby reducing the rate of glucose absorption from the small intestine. This may be one of the mechanisms of action of *C. ensiformis* in ameliorating hyperglycaemia.

This consequently suppresses the postprandial rise of blood glucose. As a result, it is apparent that *C. ensiformis* seed extract with a complement of these phytochemical constituents and bioactive substances exerts inhibition not only to alpha-glucosidase but also alpha-amylase enzymes activities, thereby substantiating its role as a hypoglycaemic agent in herbal medicine. This may be one of the mechanisms of action of *C. ensiformis* seed. This research can be useful for future work in determining the compound(s) responsible for the antidiabetic activity of this seed.

REFERENCES

Akanji, A. M., Ologhobo, A. D. and Emiola, I. A. (2007) Utilisation of some raw tropical legume seeds in diets of exotic adult cockerels. *Journal of Animal and Veterinary Advances*, 6 (4): 485-489.

Akingbade, J. A., Farinu, G. O., Taiwo, A. A., Aderinola, O. A., Adebayo, T. A., Ojebiyi, O. O., Olaniran, O. A., (2007). Agronomic and nutritive evaluation of jack beans (*Canavalia ensiformis*) for fodder in the derived savannah zone of Nigeria. *Int. J. Agric. Res.,* 2 (12): 1059-1063.

Akinlade, J. A., Farinu, G. O., Taiwo, A. A., Aderinola, O. A., Adebayo, T. A., Ojebiyi, O. O. and Olaniran, O. A. (2007). Agronomic and nutritive evaluation of jack beans (Canavalia ensiformis) for fodder in the derived savannah zone of Nigeria. *International Journal of Agricultural Research*, 2 (12): 1059-1063

Akinmutimi, A. H., Abasiekong, S. F. and Shoyinka, V. O. (2006). Raw sword bean meal as a substitute for soyabean meal in broiler finisher diets. *Journal of Animal and Vetinary*. 5 (8): 699-706

Akkarachiyasit, S., Charoenlertkul, P., Yibchok-anun, S., and Adisakwattana, S. (2010). Inhibitory activities of cyanidin and its glycosides and synergistic effect with acarbose against intestinal α-glucosidase and pancreatic α-amylase. *International Journal of Molecular Sciences* 11:3387–3396

Al-Dabbas, M. M., Kitahara, K., Suganuma, T., Hashimoto, F., and Tadera, K. (2006). Antioxidant and α-amylase inhibitory compounds from aerial parts of *Varthemia iphionoides* Boiss *Bioscience, Biotechnology, and Biochemistry*. 70:2178–2184.

Andriati, N., Anggrahini, S., Setyaningsih, W., Sofiana, I., Pusparasi, D. A., & Mossberg, F. (2018). Physicochemical characterization of jack bean (*Canavalia ensiformis*) tempeh. *Food Research*, 2(5), 481–485. https://doi.org/10.26656/fr.2017.2(5).300

Azeke, M.A., Fretzdorff, B., Buening-Pfaue, H. and Betsche, T. (2007). Comparative effect of boiling and solid substrate fermentation using the tempeh fungus (Rhizopus oligosporus) on the flatulence potential of African yambean (Sphenostylis stenocarpa L.) seeds. *Food Chemistry*, 103(4): 1420 –1425.

Bamiro, F.O., Tairu, A.O. and Oderinde, R.A. (1994). The Nutritive values of Nigerian Jack beans (*Canavalia ensiformis*) La Rivista Heliana Delle Sastance citrasse Vol. LXXI, 421 – 424.

Belmar, R., Montero, R., Sandoval-Castro, C. and McNab, J.M. (1999). Jack bean (Canavalia ensiformis L. DC) in poultry diets: antinutritional factors and detoxification studies - a review. *World Poultary Sciences Journals*, 55 (1): 37-59

Bernfeld, P. (1955). Amylases, α and β. Methods in Enzymology, *Advances in Bioscience and Biotechnology***,** 7(31): 149– 158.

Bhat, M., Zinjarde, S.S., Bhargava, S.Y., Kumar, A.R. and Joshi, B.N. (2011).*Evi Basedn Complement Alternate Med.* **2011**:810207

Bock, K. and Pedersen, H. (1987) The substrate specificity of the enzyme amyloglucosidase (AMG). Part I. Deoxy derivatives, *Acta Chemica Scandinavica. Series B*, 41: 617–628.

Carlini C.R., Oliveira, A.E.A., Azambuja, P., Xavier-Filho, J. and Wells, M.A. (1997). Biological effects of canatoxin in different insect models: evidence for a proteolytic activation of the toxin by insect cathepsin- like enzymes. *Journal of Economics and Entomology*, 90: 340-348.

Chee, Y. K., Hacker, J. B., Ramirez, L. and Chen, C. P. (1992). *Canavalia ensiformis* (L.) DC.. Record from Proseabase. Mannetje, L.'t and Jones, R.M. (Editors). PROSEA (Plant Resources of South-East Asia) Foundation, Bogor, Indonesia.

Cook, N.S. and Samman, S. (1996). Flavonoids – chemistry, metabolism, cardioprotective effect and dietary sources. *Journal of Nutritional Biochemistry* 7: 66 – 76.

Corthout, J., Peiters, L. A., Claeys, M., Van den Berghe, D. A., and Vleitinck, A. J. (1991). Antiviral ellagitannins from Spondia mombin. *Phytochemistry* 30:1129–1130

Day, A. J., DuPont, M. S., Ridley, S., Rhodes, M., Rhodes, M. J. C., and Williamson, G. (1998). Deglycosylation of favonoid and isofavonoid glycosides by human small intestine and liver L-glucosidase activity. FEBS Lett. 436:71–75.

de Souza, P, M. and de Oliveira, M. P. (2010). Application of microbial α-amylase in industry - A review. *Brazil Journal Microbiology* (4):850-861

Deenmamode, J. M., Sherwood, R. A., Sherman, D. I., Peters, T. J., (1993). Total pancreatic and salivary serum is α-amylase activities in alcohol misusers in relapse and remission and in alcoholic liver disease. *Clin Chim Acta* **223:** 169–172

Duli, R. and Starzy, A. (2016). Prolonged tempe-type fermentation in order to improve bioactive potential and nutritional parameters of quinoa seeds. *Cereal Science,* 71, 116–121. http://doi.org/10.1016/ j.jcs.2016.08.001

Elbein, A. D. (1991). Glycosidase inhibitors as antiviral and/or antitumor agents. Seminars in Cell Biology, 2, 309– 317.

Etxeberria, U., de la Garza, A. L., Campión, J., Martínez, J. A., and Milagro, F. I. (2012). Antidiabetic effects of natural plant extracts via inhibition of carbohydrate hydrolysis enzymes with emphasis on pancreatic alpha amylase. *Expert Opinion on Therapeutic Targets*, 16: 269–297.

Felgines, C., Talavera, S., Texier, O., Gil-Izquierdo, A., Lamaison, J. L., and Remesy, C. (2005). Blackberry anthocyanins are mainly recovered from urine as methylated and glucuronidated conjugates in humans. *Journal of Agriculture and Food Chemistry*. 53:7721–7727

Fossen, T., Pedersen, A. T., and Anderson, Ø. M. (1998). Flavonoids from red onion (Allium cepa). *Phytochemistry* 47:281–285.

Francis, S., Joseph, S., Koshy, E.P. and Mathew, B., (2018). Microwave assisted green synthesis of silver nanoparticles using leaf extract of elephantopus scaber and its environmental and biological applications. *Artificial cells, nanomedicine, and biotechnology*, 46(4): 795-804.

Françoise, P. (2004). Structural basis for the inhibition of mammalian and insect alpha-amylases by plant protein inhibitors. *Bba—proteins and Proteomics*, 1696, 171– 180.

Frandsen, T. B. Lok, F. Mirgorodskaya, E. Roepstorff, P. and Svensson, B. (2000). Purification, enzymatic characterisation and nucleotide sequence of a high-isoelectric-point a-glucosidase from barley malt. *Plant Physiology*., 123 (2000): 275–286.

GBIF.org (2024) https://www.gbif.org/species/7799711

Gee, J. M., and Johnson, I. T., (1988) Interactions between hemolytic saponins, bile salts and small intestinal mucosa in the rat, *Journal of Nutrition*. 118:1391-1397.

Gemede, H.F. and Ratta, N. (2014). Antinutritional Factors in Plant Foods: Potential Health Benefits and Adverse Effects. *International Journal of Nutrition and Food Science*, 3(4): 284-289.

Ghani, U. (2015). Re-exploring promising α-glucosidase inhibitors for potential development into oral anti-diabetic drugs: Finding needle in the haystack. *European Journal of Medicinal Chemistry*, 103: 133– 162.

Ghosh, D. and Konishi, T. (2007). Anthocyanins and anthocyanin-rich extracts: Role in diabetes and eye function. Asian Pac. *Journal Clinical Nutrition* 16:200–208.

Gray, K., Garrett, J. B., Aboushadi, N. M., Knowles, R., O'Donoghue, E. and Waters, E. (2004) Thermostable a-glucosidase genes from environmental bacteria and their use for hydrolysis of maltooligosaccharides and liquefied starch in food processing and dental care products, EU Patent WO 2004085615 (2004); Chemistry Abstract., 14: 326804.

Greenberger N.J, Toskes P.P (1991). Harrison's principles of internal medicine. McGraw-Hill, New York, NY

Gupta, R., Gigras, P., Mohapatra, H., Goswami, V. K., and Chauhan, B. (2003) "Microbial α-amylases: a biotechnological perspective", *Process Biochemistry*, 38 (11): 1599-1616.

Hakamata, W., Muroi, M., Nishio, T., Oku, T. and Takatsuki, A. (2004). Recognition properties of processing α-glucosidase I and α-glucosidase II, *Journal of Carbohydrate Chemistry*, 23: 27–39.

Hakamata, W., Nishio, T. and Oku, T. (1999) Synthesis of p-nitrophenyl 3- and 6-deoxy-α-D-glucopyranosides and their specificity to rice α-glucosidase, *Journal of Applied Glycoscience*, 46: 459–463.

Haq, I., Ali, S., Javed, M. M., Hameed, U., Saleem, A., Adnan, F., and Qadeer, M. A. (2010). "Introduction of alpha amylase from a randomly induced mutant strain of Bacillus amyloliquefaciens and its application as a desizer in textile industry". *Pak Journal of Botany*, 42 (1): 473- 484,

Hara, Y. and Honda, M. (1990). Inhibition of -amylase by tea polyphenols. *Agriculture Biology and Chemistry* 54:1939–1945.

He, Q., Lv, Y., and Yao, K. (2006). Effects of tea polyphenols on activities of a-amylase, pepsin, trypsin and lipase. *Food Chemistry*, 101, 1178–1182.

Hollander, P., Pi-Sunyer, X. and Coniff, R.F. (1997) Acarbose in the treatment of type 1 diabetes. *Diabetes Care* 20248.

Horii, S., Fukase, H., Matsuo, T., Kameda, Y., Asano, N., and Matsui, K. (1986). Synthesis and alpha-D-glucosidase inhibitory activity of N-substituted valiolamine derivatives as potential oral antidiabetic agents. *Journal of Medicinal Chemistry*, 29: 1038– 1046.

Jahan, H., Choudhary, M. I., Manzoor, M., Khan, K. M., Perveen, S., Atta-Ur-Rahman (2017). Insulinotropic action of 2, 4-dinitroanilino-benzoic acid through the attenuation of pancreatic beta-cell lesions in diabetic rats. *Chemical Biology Interaction.* 273:237-244. doi: 10.1016/j.cbi.2017.06.015. Epub 2017 Jun 15. PMID: 28625491.

Jalili Safaryan, M., Ahmadi, H., Barzegar, M., Tabarsa, M. and Udenigwe, C. (2022). Evaluation of inhibitory effect of alpha-amylase and alpha-glucosidase by interaction phenolic compounds, soluble fiber, and protein extracted from green lentils. *Journal of food science and technology (Iran)*, *19*(122), pp.35-45.

Jimenez, L. E. R., Arni, X. H., Benaouda, M., Avalos, J. O., Corona, L., Castillo-Gallegos, E., Ortega, O. A. C., Gonzalez-Ronquillo, M. (2021). Concentrate supplementation on milk yield, methane and CO2 production in crossbred dairy cows grazing in tropical climate regions. *Journal of Animal Behaviour and Biometeorology*, 9 (1) , art. no. 21018

Jung, M., Park, M., Lee, H.C., Kang, Y.H., Kang, E.S. and Kim, S.K. (2006). Anti-diabetic agents from medicinal plants. *Current Medical Chemistry*, 13: 1203-1208.

Junge, B., Matzke, M., and Stoltefuss, J. (1996) Chemistry and structure-activity relationships of glucosidase inhibitors. In Kuhlman J, Puls W (eds): *Oral Antidiabetics.* Berlin, SpringerVerlag, 1996, p 411

Kashaija, I. N., McIntyre, B. D., Ssali, H. and Kizito, F. (2004). Spatial distribution of roots, nematode populations and root necrosis in highland banana in Uganda. *Nematology* 6 (1):7-12

Kathiresan, K., and Manivannan, S. (2006) "-Amylase production by Penicillium fellutanum isolated from mangrove rhizosphere soil". *African journal of Biotechnology*, 5 (10), (2006).

Kawakami, K., Aketa, S., Nakanami, M., Iizuka, S., and Hirayama, M. (2010). Major water-soluble polyphenols, proanthocyanidins, in leaves of persimmon (Diospyros kaki) and their alpha-amylase inhibitory activity. *Bioscience Biotechnology and Biochemistry*, 74:1380–1385.

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

Konsoula, Z. and Liakopoulou-Kyriakides, M. (2007) "Co-production of alpha-amylase and beta-galactosidase by Bacillus subtilis in complex organic substrates*." Bioresource Technology*, 98: 150- 157.

Krause, H.P, and Ahr, H.J. (1996): Pharmacokinetics and metabolism of glucosidase inhibitors. In Kuhlmann J, Puls W (eds): *Oral Antidiabetics*. Berlin, Springer-Verlag, p 541

Kumar, V., Kumar, A., Panwar, P., & Kumar, P. (2018). *In vitro* alpha-glucosidase inhibitory activity of p-xylene and its derivatives. *Journal of Enzyme Inhibition and Medicinal Chemistry*, 33(1): 145-160. doi: 10.1080/14756366.2017.1418298.

Laar, F. (2008). α-Glucosidase inhibitors in the early treatment of type 2 diabetes. *Vascular Health and Risk Management*, 4: 1189– 1195

Lebovitz, H. E. (1997). Alpha-Glucosidase Inhibitors. *Endocrinology and Metabolism Clinics of North America*, 26(3): 539–551.

Lee, Y. A., Cho, E. J., Tanaka, T., and Yokozawa, T. (2007). Inhibitory activities of proanthocyanidins from persimmon against oxidative stress and digestive enzymes related to diabetes. *Journal of Nutrition Science and Vitaminology*. 53:287–292

Lemieux, R. U., Spohr, U., Bach, M., Cameron, D. R., Frandsen, T. P., Stoffer, B. B., Svensson, B. and Palcic, M. M. (1996) Chemical mapping of the active site of the glucoamylase of *Aspergillus niger, Canadian Jounal of Chemistry*, 74: 319–335.

Li, Y., Gao, F., Gao, F., Shan, F., Bian, J., and Zhao, C. (2009). Study on the interaction between 3 flavonoid compounds and alpha-amylase by fluorescence spectroscopy and enzymatic kinetics. *Journal Food Science* 74:C199–C203.

Marimuthu, M. and Gurumoorthi, P. (2013). Physicochemical and Functional Properties of Starches from Indian Jack Bean (*Canavalia ensiformis*), an Underutilized Wild Food Legume. *Journal of Chemical and Pharmaceutical Research*, 5(1): 221-225.

Martinelli AH, Kappaun K, Ligabue-Braun R, Defferrari MS, Piovesan AR, Staniscuaski F, Demartini DR, Dal Belo CA, Almeida CGM, Follmer C, Verli H, Carlini CR, Pasquali G. (2014). Structure-function studies on jaburetox, a recombinant insecticidal peptide derived from jack bean (Canavalia ensiformis) urease. BBA-Gen Subjects ;1840(3):935–44.

McDougall, G. J. and Stewart, D. (2005). The inhibitory effects of berry polyphenols on digestive enzymes. *BioFactors* 23:189–195.

McDougall, G. J., Shpiro, F., Dobson, P., Smith, P., Blake, A., and Stewart, D. (2005). Different polyphenolic components of soft fruits inhibit α-amylase and α-glucosidase. *Journal of Agriculture and Food Chemistry*. 53:2760–2766.

Mertens-Talcott, S. U., Talcott, S. T., and Percival, S. S. (2003). Low concentrations of quercetin and ellagic acid synergistically influence proliferation, cytotoxicity and apoptosis in MOLT-4 human leukemia cells. *J. Nutr.* 133:2669–2674.

Michelangeli, C, Perez, G., Mendez, A. and Sivoli, L. (2004). Effect of toasting Canavalia ensiformis seeds on productive performance of growing pigs. *Zootecnia Tropical*, 22 (1): 87-100

Mohan, V. R., Tresina, P. S., and Daffodil, E. D. (2016). Antinutritional Factors in Legume Seeds: Characteristics and Determination.

Mora, M., Parra, R. and Escobar, A. (1986). Canavalia ensiformis: its utilization in feeding of ruminants. Preliminary results. Revista de la Facultad de Agronomia de la Universidad Central de Venezuela, 35: 295-311

Nair, S. S., Kavrekar, V. and Mishra, A. (2013). *In vitro* studies on alpha-amylase and alpha glucosidase inhibitory activities of selected plant extracts. *European Experimental Biology*, 3(1):128-132.

Nakai, H., Ito, T., Tanizawa, S., Matsubara, K., Yamamoto, T., Okuyama, M., Mori, H., Chiba, S., Sano, Y. and Kimura, A. (2006) Plant a-glucosidase: Molecular analysis of rice a-glucosidase and degradation mechanism of starch granules in germination stage, *Journal of Applied Glycoscience*., 53: 137–142.

Nimenibo-Uadia, R. (2003). Effect of aqueous extract of *Canavalia ensiformis* seeds on hyperlipidaemia and hyperketonaemia in alloxan-induced diabetic rats. *BIOCHEMISTRI* 15(1): 7-15.

Nimenibo–Uadia, R. I., (2017). Preliminary studies on *Canavalia ensiformis* (jackbean) dc. seeds: Proximate analysis and phytochemical screening. *Science World Journal*, 12(2): 59-62.

Nishio, T. Hakamata, W. Kimura, A. Chiba, S. Takatsuki, A., Kawachi, R. and Oku, T. (2002) Glycon specificity profiling of a-glucosidases using monodeoxy and mono-O-methyl derivatives of p-nitrophenyl α-D-glucopyranoside, Carbohydrates. *Research*, 337: 629–634.

Majinda, R., and Abubakar, M. (2016). Phytochemical constituents and antimicrobial activity of *Albizia adianthifolia*. *International Journal of Pharmacognosy and Phytochemical Research*. 3(3): 1-7.

Oakenful, D.G. and Sidhu, G.S. (1983). A physicochemical explanation for the effects of dietary saponins on cholesterol and bile salt metabolism. *Nutrition Reports International* 27: 1253 – 1259. http://hdl.handle.net/102.100.100/283664?index=1

Ogawa, M., Nishio, T., Hakamata, W., Matsuishi, Y., Hoshino, S., Kondo, A., Kitagawa, M., Kawachi, R. and Oku, T. (2004). Substrate hydroxyl groups are involved in the ionization of catalytic carboxyl groups of *Aspergillus niger* a-glucosidase, *Journal of Applied Glycoscience*., 51: 9–14.

Ojo, M.A. and Ade-Omowaye, B.I.O. (2015). Some Functional and Physical Properties of Selected Underutilised Hard-To-Cook Legumes in Nigeria. *American Journal of Food Science and Nutrition*, 2(5): 73-81.

Ologhobo, A., Mosenthin, R. and Alaka, O. O. (2003). Histological alterations in the internal organs of growing chicks from feeding raw jack bean or lima bean seeds. *Veterinary and Human Toxicology*, 45 (1): 10-13.

Olugboyega, S.K. and Edem, A.R. (2018). Comparative phytochemicals and in vitro antioxidative effects of jack beans (*Canavalia ensiformis*) and sword beans (*Canavalia gladiata*). *Annals: Food Science & Technology*, *19*(3).

Osagie, A.U. (1998). Antinutritional Factors. In: Nutritional Quality of Plant Foods. Osagie, A.U. and Eka, O. U. (Eds.) Postharvest Research Unit, University of Benin, Benin City. pp. 221 - 244.

Osuigwe, D.I., Obiekezie, A.I. and Onuoha, G.C. (2006). Effects of Jackbean Seed Meal on the Intestinal Mucosa of Juvenile Heterobranchus longifilis. *African Journal of Biotechnology*, 5(13): 1294-1298.

Pandey, A., Nigam, P., Soccol, C. R. V. T., Soccol, V., Singh, D., and Mohan, R. (2000). Advances in microbial amylases. *Biotechnology and Applied Biochemistry*, 31, 135-152.

Pandey, A., Selvakumar, P., Soccol, C. R., and Nigam, P. (1999). Solid state fermentation for the production of industrial enzymes *Current science*, 77 (1): 149-162,

Paredes, L. and Escobar, A. (1984). Canavalia (pods) in rations for cows on pasture. Informe anual '83, Instituto de Produccion Animal, Universidad Central de Venezuela.: 42-43

Paterson, R. T. (1993). Uses of trees by Livestock 4: Antinutritive Factors. National Resources Institute, Chatham, UK. pp 4-8

Pirttijarvi, T. S. M., Wahlstrom. G., Rainey., F. A., Saris, P. E. J. and Salkinoja-Salonen, M. S. (2001). Inhibition of bacilli in industrial starches by nisin, *Journal of Indian Microbiology and Biotechnology*., 26: 107–114.

Prananda, A. T., Dalimunthe, A., Harahap, U., Simanjuntak, Y., Peronika, E., Karosekali, N. E., Hasibuan, P. A. Z., Syahputra, R. A., Situmorang, P. C., and Nurkolis, F., (2023). *Phyllanthus emblica*: a comprehensive review of its phytochemical composition and pharmacological properties. *Frontier Pharmacology*. 14:1288618. doi: 10.3389/fphar.2023.1288618.

Puls, W. (1996). Pharmacology of glucosidase inhibitors. In: Kuhlman, J., Puls, W. (eds): *Oral Antidiabetics*. Berlin, Springer-Verlag, p 497

Qin, Y., Xia, M., Ma, J., Hao, Y., Liu, J., Mou, H., Cao, L., and Ling, W. (2009). Anthocyanin supplementation improves serum LDL- and HDL-cholesterol concentrations associated with the inhibition of cholesteryl ester transfer protein in dyslipidemic subjects. *American Journal of Clinical Nutrition* 90:485–492

Rahmani, A. H., Al Zohairy, M. A., Aly, S. M., and Khan, M. A. (2014). Curcumin: A potential candidate in prevention of cancer via modulation of molecular pathways. *BioMed Research International,* 1–15.

Rangaswamy, R. and Swathi, K., (2014). Serum amylase: an early marker of renal damage in hypertension. *Journal of Evolution of Medical and Dental Sciences*, 3(34): 9090-9094.

Rao, C. V., Tokumo, K., Rigoty, J., Zang, E., Kelloff, G., and Reddy, B. S. (1991). Chemoprevention of colon carcinogenesis by dietary administration of piroxicam, difluromethlornithin, 16-a-fluor-5-androsteron-17-one ˆ and ellagic acid individually and in combination. *Cancer Research*. 51:4528– 4534.

Reka, P., Banu, T. and Seethalakshmi, M. (2017). Alpha amylase and alpha-glucosidase inhibition activity of selected edible seaweeds from south coast area of India. *Int. J. Pharm. Pharm. Sci*, *9*(6), pp.64-68.

Risso, J. F., Montilla, J. J., (1992). Meal of *Canavalia ensiformis* seeds given raw, alkali treated, autoclaved or extruded in diets for growing pigs. *Archivos Latinoamericanos de Nutricion*, 42 (3): 268-274

Seifarth, C., Bergmann, J., Holst, J. J., Ritzel, R., Schmiegel, W., and Nauck, M. A. (1998). Prolonged and enhanced secretion of glucagon-like peptide 1 (7–36 amide) after oral sucrose due to alpha-glucosidase inhibition (acarbose) in Type 2 diabetic patients. *Diabetic Medicine*, 15: 485– 491.

Shani,J., Goldschmied, A., Joseph, B., Abronson, Z. and Sulman, F.G. (1974). Hypoglycaemic effect of Trigonella foenum-graecum and Lupinus terminis seeds and their major alkaloids in alloxan diabetic and normal rats. *Archives Internationales de Pharmacology namie et de Therapie*, 210: 27: 16-24.

Sierks, M. R, Bock, K., Refn, S. and Svensson, A. (1992). Active site similarities of glucose dehydrogenase, glucose oxidase, and glucoamylase probed by deoxygenated substrates, *Biochemistry*, 8972–8977.

Sivajothi, V., & Dr. Shruthi, S. D., (2013). *In vitro* and *in silico* anti-diabetic activity of phthalic acid isolated from *phyllanthus rheedii*. *International Journal of Research in Ayurveda & Pharmacy.* 4. 889-892. 10.7897/2277-4343.04623.

Stand1 E (1996) New drugs for diabetes. In: Marshall, S. M., Home, P. D., Rizza, R. A. (eds): The Diabetes Annual/ 10. Amsterdam, Elsevier, p 228

Tadera, K., Minami, Y., Takamatsu, K., and Matsuoka, T. (2006). Inhibition of α-glucosidase and α-amylase by flavonoids. *Journal of Nutrition Science and Vitaminology* 52:149–153.

Tanaka, T., Zhang, L. H., Yang, Y. J., C. R., and Kouno, I. (2007). Rubusuaviins A-F, monomeric and oligomeric ellagitannins from Chinese sweet tea and their α-amylase inhibitory activity. Chemistry and Pharmacology. *Bull*. 55:1325–1331

Taylor, M. A., Ross, H. A., McRae, D., Stewart, D., Roberts, I., Duncan, G., Wright, F., Millan, S. and Davies, H. V. (2000) A potato a-glucosidase gene encodes a glucoprotein processing a-glucosidase II-like activity. Demonstration of enzyme activity and effects of down regulation in transgenic plants, *Plant Journal,* 24: 305–316.

Ternisien, E. and Melin, P. (1989). Etude des rotations culturales en bananeraie. Première partie: bilan des cultures de rotation. *Fruits,* 44: 373-383

Tiwari, A.K. and Rao, J.M. (2002). Diabetes mellitus and multiple therapeutic approaches of phytochemicals: Present status and future prospects. *Current Science*, 83(1): 30 – 37.

Trease, G.E and Evans, W.C (1996). Pharmacognosy. Macmillan publishers Limited. Pp 213-832.

Trease, G.E. and Evans, W. C. (1999). Pharmacognosy. (14th ed.) W. B. Saunders Company Ltd., London, UK. pp 46-48.

Troccoli, N., Escobar, A. and Gonzalez, A. (1989). Effect of ethanol on the toxicity of Canavalia ensiformis given to calves during the preweaning period. Informe anual, Universidad Central de Venezuela, Facultad de Agronomia, Instituto de Produccion Animal, 45-46

Truscheit, E., Frommer, W., Junge, B., Müller, L., Schmidt, D. D., and Wingender, W. (2010). Chemistry and biochemistry of microbial α-Glucosidase inhibitors. *Angewandte Chemie International Edition in English*, 20, 744– 761.

Tundis, R., Loizzo, M. R., and Menichini, F. (2010). Natural products as α-amylase and α-glucosidase inhibitors and their hypoglycaemic potential in the treatment of diabetes: An update. *Mini Reviews in Medicinal Chemistry*, 10: 315– 331.

Vadivel, V., Cheong, J.N. and Biesalski, H.K. (2012). Antioxidant and type II diabetes related enzyme inhibition properties of methanolic extract of an underutilized food legume, *Canavalia ensiformis* (L.) DC: Effect of traditional processing methods. LWT - *Food Science and Technology*, 47(2): 255– 260.

Vargas-Ayala, R., Rubiano, J. A. and Saavedra, E. D., (2000). Sustainable and integrated management of plantain-parasitic nematodes based on an intercropping system with

tropical legumes. Abstracts of the XXXI annual meeting of ONTA 16-20 April 2000, Auburn, Alabama, USA.

Vocadlo, D. J., and Davies, G. J. (2008). Mechanistic insights into glycosidase chemistry. *Current Opinion in Chemical Biology*, 12: 539– 555.

Walle T. (2007). Methylation of dietary flavones greatly improves their hepatic metabolic stability and intestinal absorption. *Mol Pharm.* Nov-Dec;4(6):826-832.

Wen, X. and Walle, T. (2006). Methylated flavonoids have greatly improved intestinal absorption and metabolic stability. *Drug Metabolism Disposal*. 34:1786–1792.

Wolever, T.M.S., Radmard, R. and Chiasson, J.L. (1995) One-year acarbose treatment raises fasting serum acetate in diabetic patients. *Diabetic Medicine* 12:164.

Woodman, O. L., Meeker, W. F., and Boujaoude, M. (2005). Vasorelaxant and antioxidant activity of flavonols and flavones: Structure activity relationships. *Journal of CardioVascular Pharmacology*. 46:302–309.

Xia, X., Ling, W., Ma, J., Xia, M., Hou, M., Wang, Q., Zhu, H., and Tang, Z. (2006). An anthocyanin-rich extract from black rice enhances atherosclerotic plaque stabilization in apolipoprotein E-deficient mice. *Journal of Nutrition.* **136**: 2220–2225.

Xiao, J. and Kai, G. (2012). A review of dietary polyphenol-plasma protein interactions: characterization, influence on the bioactivity, and structure-affinity relationship. *Critical Reviews in Food Science and Nutrition*; 52(1):85-101. doi: 10.1080/10408398.2010.499017. PMID: 21991992.

Yang, C. M., Cheng, H. Y., Lin, T. C., Chiang, L. C., Lin, C. C. The in vitro activity of geraniin and 1,3,4,6-tetra-O-galloyl-beta-D-glucose isolated from *Phyllanthus urinaria* against herpes simplex virus type 1 and type 2 infection. *Journal of Ethnopharmacology.* 2007;110: 555–558. doi: 10.1016/j.jep.2006.09.039.

Ye, X. P., Song, C. Q., Yuan, P., and Mao, R. G. (2010). α-Glucosidase and αamylase inhibitory activity of common constituents from traditional Chinese medicine used for diabetes mellitus. *Chinese Journal of National Medicine* 8:349–352.

Yoneda Y. and Maruo, B. (1975). "Mutation of Bacillus subtilis Causing Hyperproduction of α-Amylase and Protease, and Its Synergistic Effect", *Journal of Bacteriology*, 124, 48-54,

Yusuf, A. A., Ayedun, H. and Logunleko, G.B. (2010). Functional properties of unmodified and modified Jack bean (*Canavalia ensiformis*) starches. *Nigerian Food Journal*, 25(2): 141-149

Zafra-Stone, S., Yasmin, T., Bagchi, M., Chatterjee, A., Vinson, J. A., and Bagchi, D. (2007). Berry anthocyanins as novel antioxidants in human health and disease prevention. *Molecular Nutrition Food Research*. 51:675–683.

Zanin G. M., De Moraes F. F. (1998). Thermal stability and energy of deactivation of free and immobilized amyloglucosidase in the saccharification of liquefied cassava starch. Appl Biochem Biotechnol. 70–72:383–394. doi: 10.1007/BF02920153.

Zdziebło, A. and Synowiecki, J. (2002) New source of the thermostable a-glucosidase suitable for single step starch processing, *Food Chemistry*, 79 485–49

Zhang, Y., Wang, Y., Liu, X., & Li, Z. (2019). Triacontyl acetate inhibits alpha-glucosidase and improves insulin sensitivity *in vitro*. *Journal of Enzyme Inhibition and Medicinal Chemistry*, 34 (1), 145-152.

Zhen, Sun, Feng. and Chen. (2012). Evaluation of the green algae Chlorella pyrenoidosa for the management of diabetes. *J Food Drug Anal*.**20**:246-249.

APPENDIX

ALPHA-AMYLASE SOLUTION

Alpha amylase inhibitory assay was carried out using the method described by (Rangaswamy and Swathi, 2014) in the alpha amylase kit (Agappe Diagnostics, Switzerland). Exactly 1000 µL of reagents (MES (2-(N-morpholino) ethanesulfonic acid) buffer (pH 6.0) 50 mMol/L, ($CNPG_3$ (2-Chloro-4-nitrophenyl-α-D-maltotrioside)-32.27 mMol/L, Calcium chloride- 60 mMol; Sodium chloride-70 mMol/L and Potassiun thiocyanate -900 mMol/L) was added to 25 µL of the sample (methanol extract, n-hexane extract, dichloromethane extract and aqueous extract) and this was done individually. This mixture was pipetted into test tubes of varying concentrations (20 µg/ml – 100 µg/ml) of plant extract (methanol extract, n-hexane extract, dichloromethane extract and aqueous extract) and they were placed in respective test tubes. The mixture was thereafter incubated for 1 minute at 37^0C.The change in absorbance per minute (ΔOD/min) during 3 minutes was measured.

Calculation:

Alpha-amylase activity (µ/L) = (ΔOD/min) x 3178

Where 3178 is a factor.

The blank used was distilled water. Absorbance was taken at 405 nm

PREPARATION OF ALPHA-GLUCOSIDASE SOLUTION

The concentration of alpha-glucosidase enzyme solution used in this experiment was 0.5 mg/ml-200 ml volume of 0.5 mg/ml enzyme solution was prepared by dissolving 100 mg of alpha-glucosidase enzyme in 100 ml of 1000 mM phosphate buffer.

-Dissolve 100 mg of alpha-glucosidase enzyme in 100 ml of 100 mM phosphate (pH= 6.8) buffer and make it up to 200 ml with 100 mM sodium phosphate buffer.

Concentration→0.5 mg/ml is required

I need a total volume of 200 ml

Thus 0.5 mg→1 ml

X→200 ml

$$X = \frac{0.5\ mg \times 200\ ml}{1\ ml} = 100\ mg$$

Since 1000 mg =1 g

To convert mg to g

Therefore $\frac{100}{1000} = 0.1\ g$

Therefore weigh out 0.1 g of alpha-glucosidase enzyme and dissolve in 190 ml of buffer then make it up to 200 ml with the buffer. Note (measure out 190 ml of sodium phosphate buffer using measuring cylinder).

Preparation of 0.1 M (100 mM) phosphate buffer.

The pH of buffer is 6.8

The salts are;

NaH_2PO_4

Na_2HPO_4

pka= 7.21 at 20 ^{0}C

$$pH = pka + \log \frac{base}{acid}$$

$$pH = pka + \log \frac{NaHPO_4}{NaH_2PO_4}$$

$$6.8 - 7.21 = \log \frac{X}{Y}$$

$$-0.41 = \log \frac{X}{Y}$$

Antilog (-0.41) = $\frac{X}{}$

$$\frac{}{Y}$$

$$0.38905 = \frac{X}{Y}$$

$$\frac{X}{Y} = \frac{0.41}{1}$$

Total ratio= 0.4+1=1.4

To calculate for valve of x that would make 0.1 M

$$X = \frac{0.4}{1.4} \times 0.1$$

$$X = \frac{0.04}{1.4}$$

X=0.02857 M

To calculate the valve of Y that would make 0.1 M

$$Y = \frac{1}{1.4} \times 0.1$$

$$Y = \frac{0.1}{1.4}$$

Y=0.071 M

Total molarity =0.02857+0.071=0.09957 M

=0.1 M

Since 1 M=1 L

1000 ml=1 L

In molarity, CV=N

Where C= Concentration in molarity

V=Volume

N=Number of moles

Therefore, number of moles =

For X =0.02857 × 1=0.02857 moles

For Y= 0.0714×1=0.0714 moles

Number of moles =Reacting mass

Molar mass

Molar mass of Na_2HPO_4

=23(2)+1+30.98+16(4)

=46+1+30.98+64

=141.98 g/mol

Molar mass of NaH_2PO_4

=23+1(2)+30.98+16(4)

=46+2+30.98+64

=119.98 g/mol

Reacting mass for X and Y will be

For X (Base) which is Na_2HPO_4=0.02857moles=$\frac{X}{141.98}$

X=0.02857 × 141.98

X=4.056 g of Na_2HPO_4

For Y (Acid) which is NaH_2PO_4

0.0714=$\frac{Y}{119.98}$

Y=0.0714 × 119.98

Y=8.567 g of NaH_2PO_4

To prepare 0.1 M phosphate buffer weigh out 4.056g of Na_2HPO_4 and 8.567g of NaH_2PO_4 and dissolve in 1000 ml of distilled water, thereafter droplet of 2 M of Na0H was added to get pH 6.8

TO CALCULATE 2 M OF Na0H

Molar mass of Na0H =23+16+1=40 g/mol

To prepare 2 M=40 × 2=80 g

80 g=1000 ml

Xg=200 ml

X=$\frac{80 \times 200}{1000}$

X=$\frac{16000}{1000}$

X=16 g

Therefore, 16 g of Na0H pellet was dissolved in 200 ml of distilled water.

Preparation of p-nitrophenyl-α-D-glucopyranoside solution (p-NPG) (i.e α glucosidase substrate); molecular weight of p-NPG=301.251 g/mol using 5mM (0.005 M) of p-NPG and 100 ml of the solvent (sodium phosphate buffer).

$$\text{Molarity} = \frac{\text{Mass}}{\text{Molar mass}}$$

$$\text{Therefore } 0.005 = \frac{X}{301.251}$$

$X = 0.005 \times 301.251$

$X = 1.506255$

$X = 1.51$ g

1.51 g is present in 1000 ml

$$100 \text{ ml} = \frac{1.51 \times 100}{1000}$$

0.151 g or 151 mg

Weigh out 151 mg or 0.151 g of p-NPG and dissolve in 90 ml of 100 Mm sodium phosphate buffer and then make up to 100 ml.

Preparation of plant extracts at different concentrations ranging from 20-100 µg/ml i.e. 20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml and 100 µg/ml.

Weigh out 100mg of crude extracts and dissolve in 10ml of distilled water

10mg/ml

1 ml→10 mg

Xml→1 mg

$$X = \frac{(1 \times 1)}{10} \text{ml}$$

=0.1 ml

Thus 0.1ml contains 1mg

To get a concentration of 1mg/ml, pipette 0.1ml of the solution and add 0.9ml of distilled water. This gives a concentration of 1mg/ml (stock) from 1 mg/ml (i.e. 1000µg/1000µl), to prepare:

1) 20 µg/ml

If 1000µg→1000ul

20µg→Xµl

X=$\frac{20\times1000}{1000}$ = 20µl

Then pipette 20µl of the stock into a testube and add 980µl of distilled water to have a concentration of 20µg/ml

2) Pipette 40µl of the stock into a testube and add 960µl of distilled water to have a concentration of 40µg/ml
3) Pipette 60µl of the stock into a testube and add 940µl of distilled water to have a concentration of 60µg/ml.
4) Pipette 80µl of the stock into a testube and add 920µl of distilled water to have a concentration of 80µg/ml
5) Pipette 100µl of the stock into a testube and add 900µl of distilled water to have a concentration of 100µg/ml.

Preparation of Acarbose (positive control)

Preparation of acarbose at concentration of 20, 40, 60, 80 and 100µg/ml, same as the preparation for plant extract concentration.

Preparation of 50mM NaOH

Molar mass of NaOH = 23+16+1=40g/mol

To prepare 50mM= 1m=1000mM

Xm=50mM

Cross multiply

X= $\frac{50}{1000}$ =0.05M

40×0.05=2g

2g=1000ml

g=500ml

cross multiply

$X = \frac{2 \times 500}{1000} = \frac{1000}{1000}$

X=1g

Therefore, 1g of NaOH pellet was dissolved in 500ml of distilled water.

Absorbance values at 405nm for alpha-amylase inhibition assay

Acarbose

Concentration(µg/ml)	1	2	3
20	0.090	0.092	0.094
40	0.090	0.092	0.094
60	0.090	0.092	0.094
80	0.090	0.092	0.094
100	0.090	0.092	0.094

Methanol extract

Concentration(µg/ml)	1	2	3
20	0.134	0.138	0.140
40	0.134	0.138	0.140
60	0.134	0.138	0.140
80	0.134	0.138	0.140
100	0.134	0.138	0.140

n-Hexane extract

Concentration(µg/ml)	1	2	3
20	0.090	0.092	0.094
40	0.090	0.092	0.094
60	0.090	0.092	0.094
80	0.090	0.092	0.094
100	0.090	0.092	0.094

Dichloromethane extract

Concentration(µg/ml)	1	2	3
20	0.076	0.082	0.083
40	0.076	0.085	0.083
60	0.076	0.085	0.083
80	0.076	0.085	0.083
100	0.076	0.085	0.083

Aqueous extract

Concentration(µg/ml)	1	2	3
20	0.197	0.203	0.204
40	0.197	0.203	0.204
60	0.197	0.203	0.204
80	0.197	0.203	0.204
100	0.197	0.203	0.204

Absorbance values at 410nm for alpha-glucosidase

Acarbose

Concentration(µg/ml)	1	2	3
20	0.045	0.045	0.043
40	0.045	0.045	0.043
60	0.045	0.045	0.043
80	0.045	0.045	0.043
100	0.045	0.045	0.043

Methanol extract

Concentration(µg/ml)	1	2	3
20	0.047	0.049	0.050
40	0.047	0.049	0.050
60	0.047	0.049	0.050
80	0.047	0.049	0.050
100	0.047	0.049	0.050

n-Hexane extract

Concentration(µg/ml)	1	2	3
20	0.039	0.047	0.042
40	0.039	0.047	0.042
60	0.039	0.047	0.042
80	0.039	0.047	0.042
100	0.039	0.047	0.042

Dichloromethane extract

Concentration(µg/ml)	1	2	3
20	0.045	0.045	0.043
40	0.045	0.045	0.043
60	0.045	0.045	0.043
80	0.045	0.045	0.043
100	0.045	0.045	0.043

Aqueous extract

Concentration(µg/ml)	1	2	3
20	0.047	0.048	0.048
40	0.047	0.048	0.048
60	0.047	0.048	0.048
80	0.047	0.048	0.048
100	0.047	0.048	0.048

Analysis of variance for percentage inhibition values for alpha amylase between methanol extract, n-hexane extract, dichloromethane extract, aqueous extract and Acarbose

Multiple Comparisons

Dependent Variable		(I) AMYLASE	(J) AMYLASE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
ACA	LSD	20.00	40.00	-5.05333	2.48538	.069	-10.5911	.4844
			60.00	14.86333*	2.48538	.000	9.3256	20.4011
			80.00	.72667	2.48538	.776	-4.8111	6.2644
			100.00	-1.79333	2.48538	.487	-7.3311	3.7444
		40.00	20.00	5.05333	2.48538	.069	-.4844	10.5911
			60.00	19.91667*	2.48538	.000	14.3789	25.4544
			80.00	5.78000*	2.48538	.042	.2422	11.3178
			100.00	3.26000	2.48538	.219	-2.2778	8.7978
		60.00	20.00	-14.86333*	2.48538	.000	-20.4011	-9.3256
			40.00	-19.91667*	2.48538	.000	-25.4544	-14.3789
			80.00	-14.13667*	2.48538	.000	-19.6744	-8.5989
			100.00	-16.65667*	2.48538	.000	-22.1944	-11.1189
		80.00	20.00	-.72667	2.48538	.776	-6.2644	4.8111
			40.00	-5.78000*	2.48538	.042	-11.3178	-.2422
			60.00	14.13667*	2.48538	.000	8.5989	19.6744
			100.00	-2.52000	2.48538	.335	-8.0578	3.0178
		100.00	20.00	1.79333	2.48538	.487	-3.7444	7.3311
			40.00	-3.26000	2.48538	.219	-8.7978	2.2778
			60.00	16.65667*	2.48538	.000	11.1189	22.1944
			80.00	2.52000	2.48538	.335	-3.0178	8.0578
HEX	LSD	20.00	40.00	-62.66000*	1.80606	.000	-66.6841	-58.6359
			60.00	-55.39667*	1.80606	.000	-59.4208	-51.3725
			80.00	-52.87667*	1.80606	.000	-56.9008	-48.8525
			100.00	-53.22333*	1.80606	.000	-57.2475	-49.1992
		40.00	20.00	62.66000*	1.80606	.000	58.6359	66.6841
			60.00	7.26333*	1.80606	.002	3.2392	11.2875
			80.00	9.78333*	1.80606	.000	5.7592	13.8075
			100.00	9.43667*	1.80606	.000	5.4125	13.4608
		60.00	20.00	55.39667*	1.80606	.000	51.3725	59.4208
			40.00	-7.26333*	1.80606	.002	-11.2875	-3.2392

			80.00	2.52000	1.80606	.193	-1.5041	6.5441
			100.00	2.17333	1.80606	.257	-1.8508	6.1975
		80.00	20.00	52.87667*	1.80606	.000	48.8525	56.9008
			40.00	-9.78333*	1.80606	.000	-13.8075	-5.7592
			60.00	-2.52000	1.80606	.193	-6.5441	1.5041
			100.00	-.34667	1.80606	.852	-4.3708	3.6775
		100.00	20.00	53.22333*	1.80606	.000	49.1992	57.2475
			40.00	-9.43667*	1.80606	.000	-13.4608	-5.4125
			60.00	-2.17333	1.80606	.257	-6.1975	1.8508
			80.00	.34667	1.80606	.852	-3.6775	4.3708
ACA2	LSD	20.00	40.00	-46.10000*	.94717	.000	-48.2104	-43.9896
			60.00	-21.08667*	.94717	.000	-23.1971	-18.9762
			80.00	-50.93333*	.94717	.000	-53.0438	-48.8229
			100.00	-50.70000*	.94717	.000	-52.8104	-48.5896
		40.00	20.00	46.10000*	.94717	.000	43.9896	48.2104
			60.00	25.01333*	.94717	.000	22.9029	27.1238
			80.00	-4.83333*	.94717	.000	-6.9438	-2.7229
			100.00	-4.60000*	.94717	.001	-6.7104	-2.4896
		60.00	20.00	21.08667*	.94717	.000	18.9762	23.1971
			40.00	-25.01333*	.94717	.000	-27.1238	-22.9029
			80.00	-29.84667*	.94717	.000	-31.9571	-27.7362
			100.00	-29.61333*	.94717	.000	-31.7238	-27.5029
		80.00	20.00	50.93333*	.94717	.000	48.8229	53.0438
			40.00	4.83333*	.94717	.000	2.7229	6.9438
			60.00	29.84667*	.94717	.000	27.7362	31.9571
			100.00	.23333	.94717	.810	-1.8771	2.3438
		100.00	20.00	50.70000*	.94717	.000	48.5896	52.8104
			40.00	4.60000*	.94717	.001	2.4896	6.7104
			60.00	29.61333*	.94717	.000	27.5029	31.7238
			80.00	-.23333	.94717	.810	-2.3438	1.8771
METH	LSD	20.00	40.00	-13.82333*	1.53419	.000	-17.2417	-10.4049
			60.00	-18.30667*	1.53419	.000	-21.7251	-14.8883
			80.00	-10.45000*	1.53419	.000	-13.8684	-7.0316
			100.00	-27.41333*	1.53419	.000	-30.8317	-23.9949
		40.00	20.00	13.82333*	1.53419	.000	10.4049	17.2417
			60.00	-4.48333*	1.53419	.015	-7.9017	-1.0649
			80.00	3.37333	1.53419	.053	-.0451	6.7917
			100.00	-13.59000*	1.53419	.000	-17.0084	-10.1716

		60.00	20.00	18.30667*	1.53419	.000	14.8883	21.7251
			40.00	4.48333*	1.53419	.015	1.0649	7.9017
			80.00	7.85667*	1.53419	.000	4.4383	11.2751
			100.00	-9.10667*	1.53419	.000	-12.5251	-5.6883
		80.00	20.00	10.45000*	1.53419	.000	7.0316	13.8684
			40.00	-3.37333	1.53419	.053	-6.7917	.0451
			60.00	-7.85667*	1.53419	.000	-11.2751	-4.4383
			100.00	-16.96333*	1.53419	.000	-20.3817	-13.5449
		100.00	20.00	27.41333*	1.53419	.000	23.9949	30.8317
			40.00	13.59000*	1.53419	.000	10.1716	17.0084
			60.00	9.10667*	1.53419	.000	5.6883	12.5251
			80.00	16.96333*	1.53419	.000	13.5449	20.3817
ACA3	LSD	20.00	40.00	7.47667*	2.54591	.015	1.8040	13.1493
			60.00	15.79667*	2.54591	.000	10.1240	21.4693
			80.00	28.40000*	2.54591	.000	22.7274	34.0726
			100.00	43.22667*	2.54591	.000	37.5540	48.8993
		40.00	20.00	-7.47667*	2.54591	.015	-13.1493	-1.8040
			60.00	8.32000*	2.54591	.008	2.6474	13.9926
			80.00	20.92333*	2.54591	.000	15.2507	26.5960
			100.00	35.75000*	2.54591	.000	30.0774	41.4226
		60.00	20.00	-15.79667*	2.54591	.000	-21.4693	-10.1240
			40.00	-8.32000*	2.54591	.008	-13.9926	-2.6474
			80.00	12.60333*	2.54591	.001	6.9307	18.2760
			100.00	27.43000*	2.54591	.000	21.7574	33.1026
		80.00	20.00	-28.40000*	2.54591	.000	-34.0726	-22.7274
			40.00	-20.92333*	2.54591	.000	-26.5960	-15.2507
			60.00	-12.60333*	2.54591	.001	-18.2760	-6.9307
			100.00	14.82667*	2.54591	.000	9.1540	20.4993
		100.00	20.00	-43.22667*	2.54591	.000	-48.8993	-37.5540
			40.00	-35.75000*	2.54591	.000	-41.4226	-30.0774
			60.00	-27.43000*	2.54591	.000	-33.1026	-21.7574
			80.00	-14.82667*	2.54591	.000	-20.4993	-9.1540
DCM	LSD	20.00	40.00	-35.31333*	1.78090	.000	-39.2814	-31.3452
			60.00	-22.81000*	1.78090	.000	-26.7781	-18.8419
			80.00	14.59000*	1.78090	.000	10.6219	18.5581
			100.00	-8.72667*	1.78090	.001	-12.6948	-4.7586
		40.00	20.00	35.31333*	1.78090	.000	31.3452	39.2814

			60.00	12.50333*	1.78090	.000	8.5352	16.4714
			80.00	49.90333*	1.78090	.000	45.9352	53.8714
			100.00	26.58667*	1.78090	.000	22.6186	30.5548
		60.00	20.00	22.81000*	1.78090	.000	18.8419	26.7781
			40.00	-12.50333*	1.78090	.000	-16.4714	-8.5352
			80.00	37.40000*	1.78090	.000	33.4319	41.3681
			100.00	14.08333*	1.78090	.000	10.1152	18.0514
		80.00	20.00	-14.59000*	1.78090	.000	-18.5581	-10.6219
			40.00	-49.90333*	1.78090	.000	-53.8714	-45.9352
			60.00	-37.40000*	1.78090	.000	-41.3681	-33.4319
			100.00	-23.31667*	1.78090	.000	-27.2848	-19.3486
		100.00	20.00	8.72667*	1.78090	.001	4.7586	12.6948
			40.00	-26.58667*	1.78090	.000	-30.5548	-22.6186
			60.00	-14.08333*	1.78090	.000	-18.0514	-10.1152
			80.00	23.31667*	1.78090	.000	19.3486	27.2848
ACA4	LSD	20.00	40.00	-39.09000*	.58070	.000	-40.3839	-37.7961
			60.00	-46.53333*	.58070	.000	-47.8272	-45.2395
			80.00	-50.66333*	.58070	.000	-51.9572	-49.3695
			100.00	-52.82000*	.58070	.000	-54.1139	-51.5261
		40.00	20.00	39.09000*	.58070	.000	37.7961	40.3839
			60.00	-7.44333*	.58070	.000	-8.7372	-6.1495
			80.00	-11.57333*	.58070	.000	-12.8672	-10.2795
			100.00	-13.73000*	.58070	.000	-15.0239	-12.4361
		60.00	20.00	46.53333*	.58070	.000	45.2395	47.8272
			40.00	7.44333*	.58070	.000	6.1495	8.7372
			80.00	-4.13000*	.58070	.000	-5.4239	-2.8361
			100.00	-6.28667*	.58070	.000	-7.5805	-4.9928
		80.00	20.00	50.66333*	.58070	.000	49.3695	51.9572
			40.00	11.57333*	.58070	.000	10.2795	12.8672
			60.00	4.13000*	.58070	.000	2.8361	5.4239
			100.00	-2.15667*	.58070	.004	-3.4505	-.8628
		100.00	20.00	52.82000*	.58070	.000	51.5261	54.1139
			40.00	13.73000*	.58070	.000	12.4361	15.0239
			60.00	6.28667*	.58070	.000	4.9928	7.5805
			80.00	2.15667*	.58070	.004	.8628	3.4505
AQ	LSD	20.00	40.00	21.86333	15.82811	.197	-13.4039	57.1306
			60.00	-10.80667	15.82811	.510	-46.0739	24.4606
			80.00	-12.25333	15.82811	.457	-47.5206	23.0139

	100.00	-11.60333	15.82811	.480	-46.8706	23.6639
40.00	20.00	-21.86333	15.82811	.197	-57.1306	13.4039
	60.00	-32.67000	15.82811	.066	-67.9372	2.5972
	80.00	-34.11667	15.82811	.057	-69.3839	1.1506
	100.00	-33.46667	15.82811	.061	-68.7339	1.8006
60.00	20.00	10.80667	15.82811	.510	-24.4606	46.0739
	40.00	32.67000	15.82811	.066	-2.5972	67.9372
	80.00	-1.44667	15.82811	.929	-36.7139	33.8206
	100.00	-.79667	15.82811	.961	-36.0639	34.4706
80.00	20.00	12.25333	15.82811	.457	-23.0139	47.5206
	40.00	34.11667	15.82811	.057	-1.1506	69.3839
	60.00	1.44667	15.82811	.929	-33.8206	36.7139
	100.00	.65000	15.82811	.968	-34.6172	35.9172
100.00	20.00	11.60333	15.82811	.480	-23.6639	46.8706
	40.00	33.46667	15.82811	.061	-1.8006	68.7339
	60.00	.79667	15.82811	.961	-34.4706	36.0639
	80.00	-.65000	15.82811	.968	-35.9172	34.6172

*. The mean difference is significant at the 0.05 level.

Analysis of variance for percentage inhibition values for alpha glucosidase between methanol extract, n-hexane extract, dichloromethane extract, aqueous extract and Acarbose.

Multiple Comparisons

Dependent Variable		(I) GLUCOSIDASE	(J) GLUCOSIDASE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
DCM	LSD	20.0	40.0	16.03667*	6.38945	.031	1.8001	30.2732
			60.0	24.43333*	6.38945	.003	10.1968	38.6699
			80.0	15.62667*	6.38945	.035	1.3901	29.8632
			100.0	5.18333	6.38945	.436	-9.0532	19.4199
		40.0	20.0	-16.03667*	6.38945	.031	-30.2732	-1.8001
			60.0	8.39667	6.38945	.218	-5.8399	22.6332
			80.0	-.41000	6.38945	.950	-14.6466	13.8266
			100.0	-10.85333	6.38945	.120	-25.0899	3.3832
		60.0	20.0	-24.43333*	6.38945	.003	-38.6699	-10.1968
			40.0	-8.39667	6.38945	.218	-22.6332	5.8399
			80.0	-8.80667	6.38945	.198	-23.0432	5.4299
			100.0	-19.25000*	6.38945	.013	-33.4866	-5.0134
		80.0	20.0	-15.62667*	6.38945	.035	-29.8632	-1.3901
			40.0	.41000	6.38945	.950	-13.8266	14.6466
			60.0	8.80667	6.38945	.198	-5.4299	23.0432
			100.0	-10.44333	6.38945	.133	-24.6799	3.7932
		100.0	20.0	-5.18333	6.38945	.436	-19.4199	9.0532
			40.0	10.85333	6.38945	.120	-3.3832	25.0899
			60.0	19.25000*	6.38945	.013	5.0134	33.4866
			80.0	10.44333	6.38945	.133	-3.7932	24.6799
ACA	LSD	20.0	40.0	-8.75000	8.37146	.321	-27.4028	9.9028
			60.0	-8.01333	8.37146	.361	-26.6661	10.6394
			80.0	-10.23333	8.37146	.250	-28.8861	8.4194
			100.0	-7.42667	8.37146	.396	-26.0794	11.2261
		40.0	20.0	8.75000	8.37146	.321	-9.9028	27.4028
			60.0	.73667	8.37146	.932	-17.9161	19.3894
			80.0	-1.48333	8.37146	.863	-20.1361	17.1694
			100.0	1.32333	8.37146	.878	-17.3294	19.9761
		60.0	20.0	8.01333	8.37146	.361	-10.6394	26.6661
			40.0	-.73667	8.37146	.932	-19.3894	17.9161
			80.0	-2.22000	8.37146	.796	-20.8728	16.4328

			100.0	.58667	8.37146	.946	-18.0661	19.2394
		80.0	20.0	10.23333	8.37146	.250	-8.4194	28.8861
			40.0	1.48333	8.37146	.863	-17.1694	20.1361
			60.0	2.22000	8.37146	.796	-16.4328	20.8728
			100.0	2.80667	8.37146	.744	-15.8461	21.4594
		100.0	20.0	7.42667	8.37146	.396	-11.2261	26.0794
			40.0	-1.32333	8.37146	.878	-19.9761	17.3294
			60.0	-.58667	8.37146	.946	-19.2394	18.0661
			80.0	-2.80667	8.37146	.744	-21.4594	15.8461
AQUEOUS	LSD	20.0	40.0	.78000	7.34687	.918	-15.5899	17.1499
			60.0	15.35000	7.34687	.063	-1.0199	31.7199
			80.0	34.17333*	7.34687	.001	17.8035	50.5432
			100.0	10.48667	7.34687	.184	-5.8832	26.8565
		40.0	20.0	-.78000	7.34687	.918	-17.1499	15.5899
			60.0	14.57000	7.34687	.075	-1.7999	30.9399
			80.0	33.39333*	7.34687	.001	17.0235	49.7632
			100.0	9.70667	7.34687	.216	-6.6632	26.0765
		60.0	20.0	-15.35000	7.34687	.063	-31.7199	1.0199
			40.0	-14.57000	7.34687	.075	-30.9399	1.7999
			80.0	18.82333*	7.34687	.028	2.4535	35.1932
			100.0	-4.86333	7.34687	.523	-21.2332	11.5065
		80.0	20.0	-34.17333*	7.34687	.001	-50.5432	-17.8035
			40.0	-33.39333*	7.34687	.001	-49.7632	-17.0235
			60.0	-18.82333*	7.34687	.028	-35.1932	-2.4535
			100.0	-23.68667*	7.34687	.009	-40.0565	-7.3168
		100.0	20.0	-10.48667	7.34687	.184	-26.8565	5.8832
			40.0	-9.70667	7.34687	.216	-26.0765	6.6632
			60.0	4.86333	7.34687	.523	-11.5065	21.2332
			80.0	23.68667*	7.34687	.009	7.3168	40.0565
ACA2	LSD	20.0	40.0	.69333	5.74413	.906	-12.1054	13.4920
			60.0	6.26667	5.74413	.301	-6.5320	19.0654
			80.0	28.58000*	5.74413	.001	15.7813	41.3787
			100.0	30.82000*	5.74413	.000	18.0213	43.6187
		40.0	20.0	-.69333	5.74413	.906	-13.4920	12.1054
			60.0	5.57333	5.74413	.355	-7.2254	18.3720
			80.0	27.88667*	5.74413	.001	15.0880	40.6854
			100.0	30.12667*	5.74413	.000	17.3280	42.9254
		60.0	20.0	-6.26667	5.74413	.301	-19.0654	6.5320

			40.0	-5.57333	5.74413	.355	-18.3720	7.2254
			80.0	22.31333*	5.74413	.003	9.5146	35.1120
			100.0	24.55333*	5.74413	.002	11.7546	37.3520
		80.0	20.0	-28.58000*	5.74413	.001	-41.3787	-15.7813
			40.0	-27.88667*	5.74413	.001	-40.6854	-15.0880
			60.0	-22.31333*	5.74413	.003	-35.1120	-9.5146
			100.0	2.24000	5.74413	.705	-10.5587	15.0387
		100.0	20.0	-30.82000*	5.74413	.000	-43.6187	-18.0213
			40.0	-30.12667*	5.74413	.000	-42.9254	-17.3280
			60.0	-24.55333*	5.74413	.002	-37.3520	-11.7546
			80.0	-2.24000	5.74413	.705	-15.0387	10.5587
HEX	LSD	20.0	40.0	1.18000	8.38587	.891	-17.5049	19.8649
			60.0	-1.78667	8.38587	.836	-20.4715	16.8982
			80.0	-8.82000	8.38587	.318	-27.5049	9.8649
			100.0	-5.56667	8.38587	.522	-24.2515	13.1182
		40.0	20.0	-1.18000	8.38587	.891	-19.8649	17.5049
			60.0	-2.96667	8.38587	.731	-21.6515	15.7182
			80.0	-10.00000	8.38587	.261	-28.6849	8.6849
			100.0	-6.74667	8.38587	.440	-25.4315	11.9382
		60.0	20.0	1.78667	8.38587	.836	-16.8982	20.4715
			40.0	2.96667	8.38587	.731	-15.7182	21.6515
			80.0	-7.03333	8.38587	.421	-25.7182	11.6515
			100.0	-3.78000	8.38587	.662	-22.4649	14.9049
		80.0	20.0	8.82000	8.38587	.318	-9.8649	27.5049
			40.0	10.00000	8.38587	.261	-8.6849	28.6849
			60.0	7.03333	8.38587	.421	-11.6515	25.7182
			100.0	3.25333	8.38587	.706	-15.4315	21.9382
		100.0	20.0	5.56667	8.38587	.522	-13.1182	24.2515
			40.0	6.74667	8.38587	.440	-11.9382	25.4315
			60.0	3.78000	8.38587	.662	-14.9049	22.4649
			80.0	-3.25333	8.38587	.706	-21.9382	15.4315
ACA3	LSD	20.0	40.0	2.00667	11.68359	.867	-24.0260	28.0393
			60.0	5.53333	11.68359	.646	-20.4993	31.5660
			80.0	-1.12667	11.68359	.925	-27.1593	24.9060
			100.0	-1.29333	11.68359	.914	-27.3260	24.7393
		40.0	20.0	-2.00667	11.68359	.867	-28.0393	24.0260
			60.0	3.52667	11.68359	.769	-22.5060	29.5593
			80.0	-3.13333	11.68359	.794	-29.1660	22.8993

			100.0	-3.30000	11.68359	.783	-29.3327	22.7327
		60.0	20.0	-5.53333	11.68359	.646	-31.5660	20.4993
			40.0	-3.52667	11.68359	.769	-29.5593	22.5060
			80.0	-6.66000	11.68359	.581	-32.6927	19.3727
			100.0	-6.82667	11.68359	.572	-32.8593	19.2060
		80.0	20.0	1.12667	11.68359	.925	-24.9060	27.1593
			40.0	3.13333	11.68359	.794	-22.8993	29.1660
			60.0	6.66000	11.68359	.581	-19.3727	32.6927
			100.0	-.16667	11.68359	.989	-26.1993	25.8660
		100.0	20.0	1.29333	11.68359	.914	-24.7393	27.3260
			40.0	3.30000	11.68359	.783	-22.7327	29.3327
			60.0	6.82667	11.68359	.572	-19.2060	32.8593
			80.0	.16667	11.68359	.989	-25.8660	26.1993
METH	LSD	20.0	40.0	-12.32667	5.91883	.064	-25.5146	.8613
			60.0	-12.34333	5.91883	.064	-25.5313	.8446
			80.0	-9.66000	5.91883	.134	-22.8480	3.5280
			100.0	-11.57667	5.91883	.079	-24.7646	1.6113
		40.0	20.0	12.32667	5.91883	.064	-.8613	25.5146
			60.0	-.01667	5.91883	.998	-13.2046	13.1713
			80.0	2.66667	5.91883	.662	-10.5213	15.8546
			100.0	.75000	5.91883	.902	-12.4380	13.9380
		60.0	20.0	12.34333	5.91883	.064	-.8446	25.5313
			40.0	.01667	5.91883	.998	-13.1713	13.2046
			80.0	2.68333	5.91883	.660	-10.5046	15.8713
			100.0	.76667	5.91883	.900	-12.4213	13.9546
		80.0	20.0	9.66000	5.91883	.134	-3.5280	22.8480
			40.0	-2.66667	5.91883	.662	-15.8546	10.5213
			60.0	-2.68333	5.91883	.660	-15.8713	10.5046
			100.0	-1.91667	5.91883	.753	-15.1046	11.2713
		100.0	20.0	11.57667	5.91883	.079	-1.6113	24.7646
			40.0	-.75000	5.91883	.902	-13.9380	12.4380
			60.0	-.76667	5.91883	.900	-13.9546	12.4213
			80.0	1.91667	5.91883	.753	-11.2713	15.1046
ACA4	LSD	20.0	40.0	1.28667	5.37912	.816	-10.6988	13.2721
			60.0	-.70000	5.37912	.899	-12.6854	11.2854
			80.0	6.11000	5.37912	.282	-5.8754	18.0954
			100.0	7.96667	5.37912	.169	-4.0188	19.9521

	40.0	20.0	-1.28667	5.37912	.816	-13.2721	10.6988
		60.0	-1.98667	5.37912	.720	-13.9721	9.9988
		80.0	4.82333	5.37912	.391	-7.1621	16.8088
		100.0	6.68000	5.37912	.243	-5.3054	18.6654
	60.0	20.0	.70000	5.37912	.899	-11.2854	12.6854
		40.0	1.98667	5.37912	.720	-9.9988	13.9721
		80.0	6.81000	5.37912	.234	-5.1754	18.7954
		100.0	8.66667	5.37912	.138	-3.3188	20.6521
	80.0	20.0	-6.11000	5.37912	.282	-18.0954	5.8754
		40.0	-4.82333	5.37912	.391	-16.8088	7.1621
		60.0	-6.81000	5.37912	.234	-18.7954	5.1754
		100.0	1.85667	5.37912	.737	-10.1288	13.8421
	100.0	20.0	-7.96667	5.37912	.169	-19.9521	4.0188
		40.0	-6.68000	5.37912	.243	-18.6654	5.3054
		60.0	-8.66667	5.37912	.138	-20.6521	3.3188
		80.0	-1.85667	5.37912	.737	-13.8421	10.1288

*. The mean difference is significant at the 0.05 level.

0·083
0·076
6.

AGAPPE

AMYLASE

4 x 5 mL, 4 x 10 mL, 1 x 50 mL, 1 x 100 mL
51402005, 51402002, 51402006, 51402007

Intended Use

This reagent is intended for *in vitro* quantitative determination of amylase in serum, plasma & urine.

- CNPG3 methodology

- Linear up to 2000 U/L

Clinical Significance

Amylase occurs in the salivary glands, fallopian tubes & in pancreas. Alpha-amylase is secreted by the pancreas from where it enters the duodenum, through the pancreatic duct. Any obstruction to these ducts causes alpha-amylase enzyme to enter the blood stream.

Elevated levels seen in acute pancreatitis, peptic ulcers, biliary disease, parotitis & other intestinal obstructions.

Decreased levels are seen in chronic pancreatic disorders having pancreatic cell destruction.

Principle

$$5CNPG3 \xrightarrow{\text{Amylase}} 3\ CNP + 2CNPG2 + 3\ Maltotriose + 2\ Glucose.$$

CNP = 2-Chloro-4-nitrophenol

CNP-G2= 2-chloro -4-nitrophenyl-a-maltoside

Kit Components

Reagent/ Component	Product Code				Description
	51402005	51402002	51402006	51402007	
Alpha Amylase (R.L) R1	4 x 5 mL	4 x 10 mL	1 x 50 mL	1 x 100 mL	MES Buffer (pH6.0) 50 mmol/L CNPG3 2.27 mmol/L Calcium chloride 60 mmoL Sodium chloride 70 mmol/L Activator 900 mmol/L

Risk & Safety

Material Safety data sheet (MSDS) will be provided on request.

Reagent Preparation

Amylase reagent is ready to use.

Reagent Storage and Stability

The sealed reagents are stable up to the expiry date stated on the label, when stored at 2- 8°C and protected from light.

Open Vial Stability

Once opened, the reagent is stable up to 4 weeks if contamination is avoided.

On-board Calibration Stability

On-board Calibration stability is 20 days.

Reagent Deterioration

Turbidity or precipitation in any kit component indicates deterioration and the component must be discarded. Values outside the recommended acceptable range for the Agappe Qualichek Norm & Path control may also be an indication of reagent instability and associated results are invalid. Sample should be retested using fresh vial of reagent.

Precaution

To avoid contamination, use clean laboratory wares. Use clean, dry disposable pipette tips for dispensing. Close reagent bottles immediately after use.

Avoid direct exposure of reagent to light. Do not blow into the reagent bottles.

This reagent is only for IVD use and follow the normal precautions required for handling all laboratory reagents.

Waste Management

Reagents must be disposed off in accordance with local regulations.

Sample

Fresh serum / plasma (free of hemolysis) / Urine (1/3 diluted).

Interferences

No interference for

Bilirubin up to	10 mg/dL
Ascorbic acid up to	50 mg/dL
Hemoglobin up to	1000 mg/dL

Materials Provided

Amylase Reagent .

Materials required but not provided

- Pipettes & Tips
- Test Tubes & racks
- Timer → Timer
- Incubator
- Analyzer

Test Parameters

Mode of Reaction	Kinetic
Slope of reaction	Increasing
Wavelength	405 nm
Temperature	37°C
Factor	3178
Linearity	2000 U/L
Blank	DI water
Delay time	60 sec
No.of readings	3
Interval	60 sec
Sample volume	25 µL
Reagent volume	1000 µL
Cuvette	1 cm light path

* Full meaning of MES.
* Full meaning of CNPG3
* 3178 is a factor.
* What type of Activator.

Application parameters for various instrument are available. Please contact customer support department for specific information.

Unit Conversion

Traditional Unit	SI Unit	Conversion from Traditional to SI
U/L	mKat/L	x 0.017

Calibration

Agappe multicalibrator is recommended for calibration of this assay on fully auto analyzer.

Use provided factor (3178) for estimation of Amylase on semi auto analyzer.

Procedure notes

Laboratory Procedure for Semi Auto Analyzer

Reagent (R1)	1000 µL
Sample	25 µL
Mix and incubate for 1 min. at 37°C. Measure the change in absorbance per minute (△ OD/min) during 3 minutes.	

Calculation

Alpha-Amylase activity (U/L) = (△OD/ min.) x 3178.

Quality Control

It is recommended to use Agappe Qualicheck Norm & Path (51601001) to verify the performance of the assay. Each laboratory has to establish its own internal quality control scheme and procedure for corrective action, if control do not recover within the acceptable range.

Reference Range

It is recommended that each laboratory establish its own reference values. The following value may be used as guide line.

Serum / plasma : 25 - 86 U/L

Urine : < 470 U/L

Results obtained for patient samples are to be correlated with clinical findings of patient for interpretation and diagnosis.

SYMBOLS USED ON THE LABELS

IVD IN VITRO DIAGNOSTIC USE | SEE PACKAGE INSERT FOR PROCEDURE | LOT LOT NUMBER | MANUFACTURER'S ADDRESS | MANUFACTURING DATE | EXPIRY DATE | TEMPERATURE LIMIT

AGAPPE DIAGNOSTICS SWITZERLAND GmbH
Knonauerstrasse 54 - 6330 Cham Switzerland
Tel. +41 41 780 60 10 Fax: +41 41 780 60 11
info@agappeswiss.com | www.agappeswiss.com

REV. NO.: ADS/IFU/AMY/LIQ/R01 | CE ISO 9001 : 2015 EN ISO 13485: 2016

AGAPPE

4 x 5 mL, 4 x 10 mL, 1 x 50 mL, 1 x 100 mL
51402005, 51402002, 51402006, 51402007

AMYLASE

Performance

1. Linearity

The reagent is linear, upto 2000 U/L.

If the concentration is greater than linearity (2000 U/L), dilute the sample with normal saline & repeat the assay. Multiply the result with dilution factor.

2. Comparison

A comparison study has been performed between Agappe reagent and another internationally available reagent yielded a correlation coefficient of $r^2 = 0.9909$ and a regression equation of $y = 1.0464x$.

3. Precision

Intra Run

	Control Level 1	Contro Level 2
n	20	20
Mean (U/L)	73.9	860.8
SD	2.0	10.1
CV(%)	2.7	1.2

Inter Run

	Control Level 1	Control Level 2
n	20	20
Mean (U/L)	74.3	854.9
SD	2.0	22.6
CV(%)	2.7	2.6

Accuracy (U/L)

Control	Expected Value	Measured Value
Control Level 1	64.7 ± 23	65
Control Level 2	425 ± 75	430.3
Qualichek Norm	70 ± 14.80	73
Qualichek Path	175 ± 23	183

4. Sensitivity

Lower detection Limit is 2.0 U/L.

Bibliography

1. Junge, W., *et al.;* Clin. Biochem. 22, 109(1989)
2. Hohenwallnern, W.; J.Clin. chem. Clin. Biochem. 27,97(1989)

0.2 - 20 ml
0.4 - 10 ml.
0.1 - 0.18 ml

SYMBOLS USED ON THE LABELS

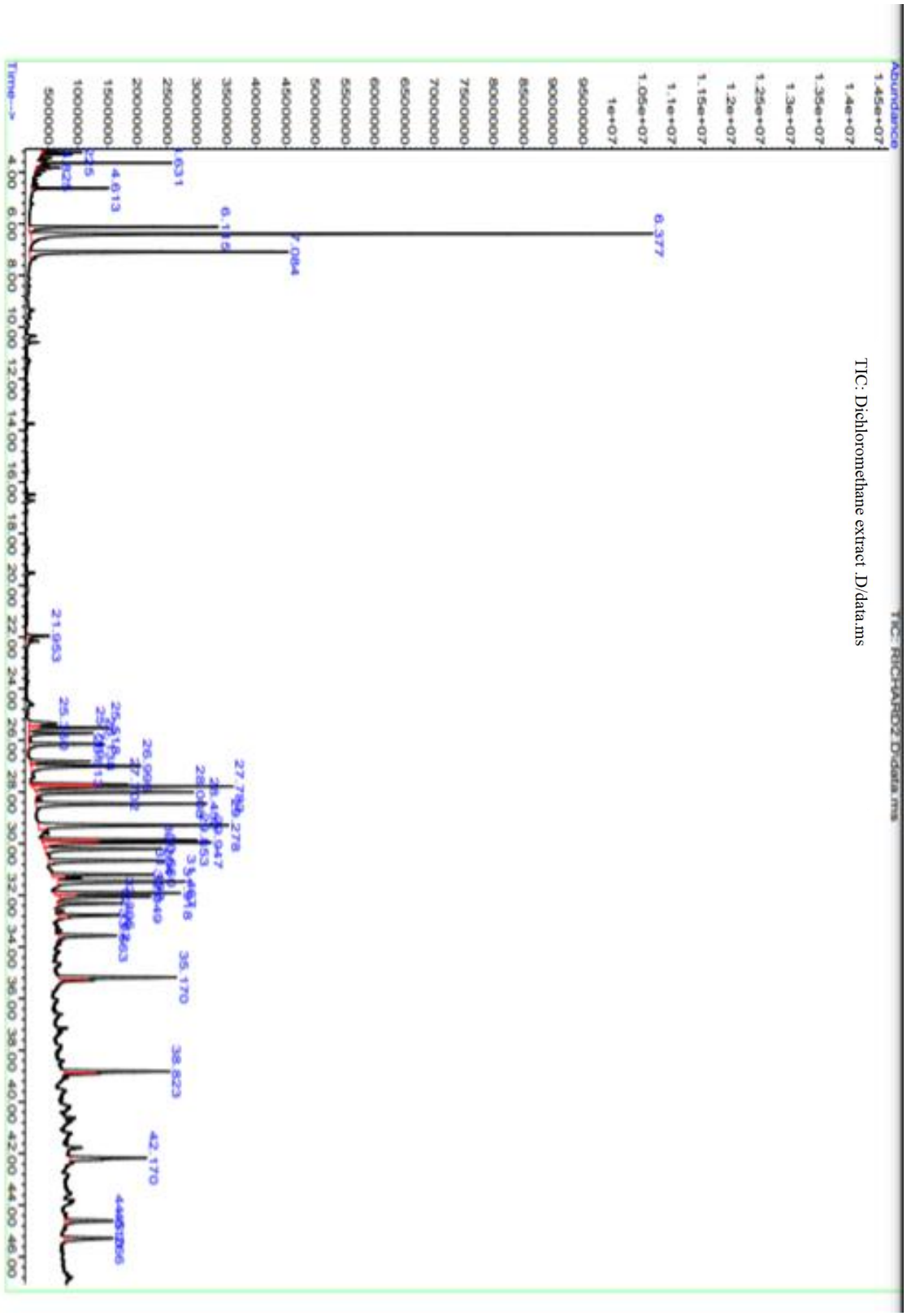
IVD IN VITRO DIAGNOSTIC USE | SEE PACKAGE INSERT FOR PROCEDURE | LOT LOT NUMBER | MANUFACTURER'S ADDRESS | MANUFACTURING DATE | EXPIRY DATE | TEMPERATURE LIMIT

AGAPPE DIAGNOSTICS SWITZERLAND GmbH
Knonauerstrasse 54 - 6330 Cham Switzerland
Tel. +41 41 780 60 10 Fax: +41 41 780 60 11
info@agappeswiss.com | www.agappeswiss.com

REV. NO.: ADS/IFU/AMY/LIQ/R01

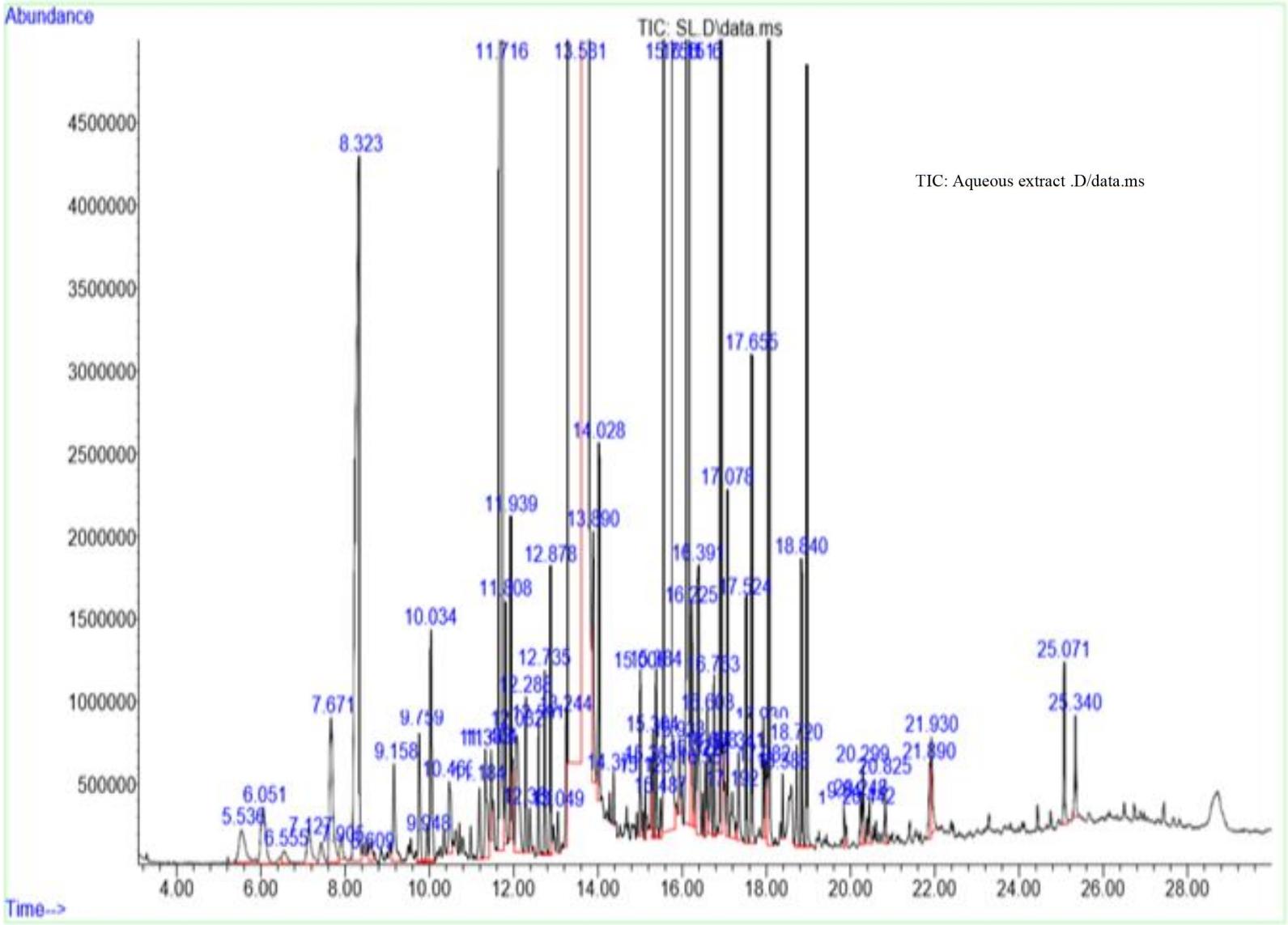
CE ISO 9001 : 2015
EN ISO 13485: 2016

Chromatogram of Dichloromethane extract of *Canavalia ensiformis* seed



TIC: Dichloromethane extract .D/data.ms

Chromatogram of Aqueous extract of *Canavalia ensiformis* seed



TIC: Aqueous extract .D/data.ms