PHYTOCHEMICAL AND INHIBITION STUDIES OF *GARCINIA KOLA* HECKEL (GUTTIFERAE) SEED EXTRACTS ON SOME KEY ENZYMES INVOLVED WITH DIABETES

BY

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DECLARATION

I declare that the work in this dissertataion entitled Phytochemical and Inhibition Studies of *Garcinia kola,* Heckel (Guttiferae) Seed Extracts on Some Key Enzymes Involved with Diabetes has been carried out by me at the Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences, Ahmadu Bello University Zaria, under the supervision of Dr. U.H. Danmalam, and Dr. A.B. Sallau. The informations drived from literature have been duly acknowledged in the text and a list of references provided. No part of this thesis has been previously presented for another higher degree or diploma at this or any other institution.

______________________	______________________	______________________
Zakariya, Ali Muhammad	Signature	Date

CERTIFICATION

This dissertation entitled PHYTOCHEMICAL AND INHIBITION STUDIES OF *GARCINIA KOLA,* HECKEL (GUTTIFERAE) SEED EXTRACTS, ON SOME KEY ENZYMES INVOLVED WITH DIABETES by Ali Muhammad ZAKARIYA**,** meets the regulations governing the award of the degree of Master`s in Pharmacognosy of Ahmadu Bello University and is approved for its contributions to knowledge and literary presentation.

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DEDICATION

This work is dedicated to my parents, may Allah (SWT) reward them and continue to bless them (Ameen).

ACKNOWLEDGEMENT

All praise is due to Allah (SWT) the Lord of the universe for granting me the oppoturnity to carryout this thesis to completion. My sincere and profound gratitude goes to my supervisors Dr. U.H. Danmalam and Dr. A.B Sallau for their efficient supervision and sound academic contributions to the success of this research work despite their busy and numerous commitments. I also thank Dr. A. U Zezi and the laboratory technologists all of Department of Pharmacology and Therapeutics for their support during the course of this work.

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ABSTRACT

Garcinia kola is an Angiosperm belonging to the family Guttiferae. It is known in commerce as bitter kola. The plant seeds have been used in the treatment of a wide range of diseases including diabetes, and its importance in folkloric medicine as a purgative, mastcatory, aphrodisiac etc. is eminent. Diabetes mellitus is a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia leading to severe complications such as neuropathy, nephropathy, retinopathy and foot ulcer. n-Hexane, ethyl acetate and methanol extracts were prepared successively in a soxhlet apparatus at 50ºC. Qualitative phytochemical screening was carried out. Column chromatographic analysis was carried out on the ethyl acetate extract and the structure of the isolated compound was elucidated via Gas Chromatography-Mass Spectrophotometry and Fourier Transformed-Infra Red spectroscopy. Pancreatic α-amylase and intestinal α-glucosidase were extracted from porcine pancrease and rat small intestine under specified conditions. Steroids/triterpenes, phenolics, flavonoids, cardiac glycosides, alkaloids, coumarins and phlobatannins were detected. Methanol, ethyl acetate and n-hexane extracts inhibited α-amylase with IC_{50} = 0.78 ± 0.32 mg/ml, 3.44 ± 3.46 mg/ml, 4.89 ± 4.62 and α-glucosidase IC_{50} = 2.67 ± 0.74 mg/ml, 1.68 ± 1.27 mg/ml, 10.29 ± 4.08 mg/ml respectively. The compound ZAAK was isolated from ethyl acetate extract. Fourier Transformed-Infrared spectra revealed the presence of carboxylic acid and an ester in ZAAK. Total ion chromatogram of ZAAK revealed three major peaks corresponding to $ZAAK_1$ $ZAAK_2$ and $ZAAK_3$. The mass spectra identified $ZAAK_1$ $ZAAK_2$ and $ZAAK_3$ as 1-pentadecanecarboxylic acid, (Z)-11-Octadecenoic acid and octadecanoic acid, 2-(2-hydroxyethoxy) ethyl ester respectively.

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ACRONYMS AND ABBREVIATIONS

Acronyms	Meanings
Amu	Atomic Mass Unit
Asp	Aspatic acid
DMSO	Dimethylsulfoxide
DNS	3, 5-dinitrosalicylic acid
EC	Enzyme Comission
FT-IR	Fourier Transformed Infra Red Spectroscopy
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GH-Family	Glucanohydrolase Family
Glu	Glutamic acid
HPLC	High Performance Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
NIST	National Institute Standard and Technology
NMR	Nuclear Magnetic Resonance
PNPG	Para-Nitrophhenyl-α-D-glucopyranoside
PNP	Para-Nitrophenyl
TLC	Thin Layer Chromatography
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Medicinal Plants

Since ancient times, people have been exploring nature particularly plants in search of new drugs. This has resulted in the use of large number of medicinal plants with curative properties to treat various diseases (Verpoorte, 1998). Nearly 80% of the world's population relies on traditional medicines for primary health care, most of which involve the use of plant extracts (Sandhya, *et al.,* 2006). Medicinal plants are the richest bio-resources of drugs for traditional medicinal systems, modern medicines, nutraceuticals, food supplement, pharmaceuticals and precursors for synthetic drugs (Hammer *et al.,* 1999).

Secondary metabolites are responsible for the medicinal activity of medicinal plants, hence, due to their large biological activities. These metabolites have been used for centuries in traditional medicine. These secondary metabolites can be classified into three chemically distinct groups viz: alkaloids, terpenoids, and phenolics (Mazid *et al.,* 2011).

1.1.1 Alkaloids

Alkaloids are naturally occurring chemical compounds containing one or more nitrogen atoms (usually in a heterocyclic ring) and are basic in nature (Evans, 2009). Many alkaloids are toxic and often have a pharmacological effect, which makes them to be used as medications and recreational drugs (Guillermo and Victor**,** 1999).

1.1.2 Phenolics

Plants have limitless ability to synthesize aromatic secondary metabolites, most of which are phenols or their oxygen-substituted derivatives (Geissman, 1963). Important subclasses in this group of compounds include phenols, phenolic acids, quinones, flavonoids, tannins and coumarins. These groups of compounds show antimicrobial effect and serves as plant defense mechanisms against pathogenic microorganisms. They are synthesized by plants in response to microbial infection (Dixon *et al.,* 1983) and are often found effective in vitro as antimicrobial substance against a wide array of microorganisms (Bennet and Wallsgrove, 1994). They also show anti-allergic, anti-inflammatory and anticancer activity (Spencer and Jeremy, 2008).

1.1.3 Terpenoids

The terpenoids form a large and structurally diverse family of natural products. They are derived from five-carbon isoprene units, and according to the number of isoprene molecules incorporated, they can be classified into hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}), tetraterpenes (C_{40}), and polyterpenes such as rubber (Dewick, 2002). They occur widely in the leaves and fruits of higher plants, conifers, citrus and eucalyptus (Breitmaier, 2008). Vast majority of the different terpenes structures produced by plants as secondary metabolites are presumed to be involved in defense as toxins and feeding deterrents to a large number of plant feeding insects and mammals (Gershenzon and Croteau, 1991).

1.2 Plants in Traditional Medicine

Plants have formed the basis of sophisticated traditional medicine (TM) practices that have been used for thousands of years by people in China, India, and many other countries (Sneader, 2005). They are important source of medicines, especially in developing countries that still use plant-based TM for their healthcare (Salim *et al.,* 2008). It has been extensively documented that plants still form the bases of traditional medicine system and that plant based system continue to play an essential role in healthcare for over 80% of the world population (WHO, 2002).

1.3 Plants in Modern Medicine

Modern medicine has benefited enormously from plants used in traditional medicine as a source of natural products (Kinghorn, 1992). An estimate showed that over 50% of the best selling pharmaceuticals in use today are derived from or mimics of natural products (Newman and Cragg, 2007).

In more recent history, the use of plants as medicines has involved the isolation of active compounds, beginning with the isolation of morphine **1** from opium poppy (*Papaver somniferum*) in the early 19th century (Samuelsson, 2004). Other early drugs from medicinal plants were also isolated due to drug discovery research of which some are still in use (Butler, 2004). Crude morphine (a pain reliever) was found to be readily converted to codeine **2** (painkiller) when boiled in acetic anhydride (Marderosian and Beutler, 2002). Digitoxin **3**, a cardiotonic glycoside isolated from *Digitalis purpurea* L. (foxglove) enhances cardiac conduction (Marderosian and Beutler, 2002). The anti-malarial drug quinine **4**

isolated from the bark of *Cinchona succirubra* has been used for centuries for the treatment of malaria fever (Marderosian and Beutler, 2002).

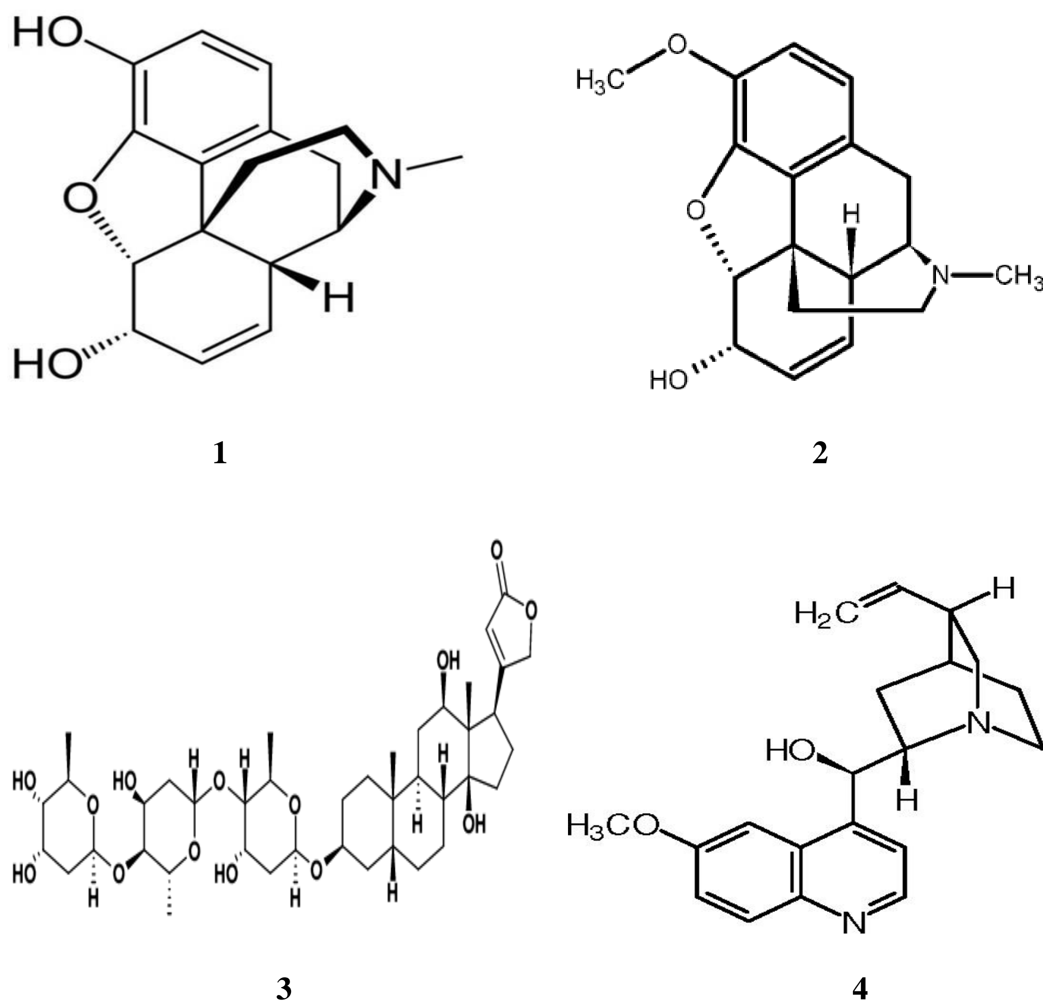


Fig 1.1 Chemical structure of earliest drugs developed from plants

Other drugs that were introduced into western medicine include an L-histidine-derived alkaloid, Pilocarpine **5** isolated from *Pilocarpus jaborandi* (Rutaceae) is used in the treatment of chronic open-angle glaucoma and acute angle-closure glaucoma (Aniszewski, 2007). Also, Vinblastine **6** and vincristine **7** both anti-neoplastic agents isolated from *Catharanthus roseus* (L.) G. Don (Apocynaceae) (formerly Vinca rosea L.) (Van Der Heijden *et al.,* 2004) and artemisinin **8** isolated from *Artemisia annua* L. (Asteraceae)which is now a good remedy against the multidrug resistant strains of *Plasmodium* parasites (Namdeo *et al.,* 2006). Galantamine **9**, a natural product isolated from *Galanthus woronowii* Losinsk., (Amaryllidaceae) is now used in the treatment of Alzheimer's disease (Pirttila *et al.,* 2004). Medicinal plant derived natural products play a dominant role in drug discovery for the treatment of human diseases in modern medicine (Koehn and Carter, 2005).

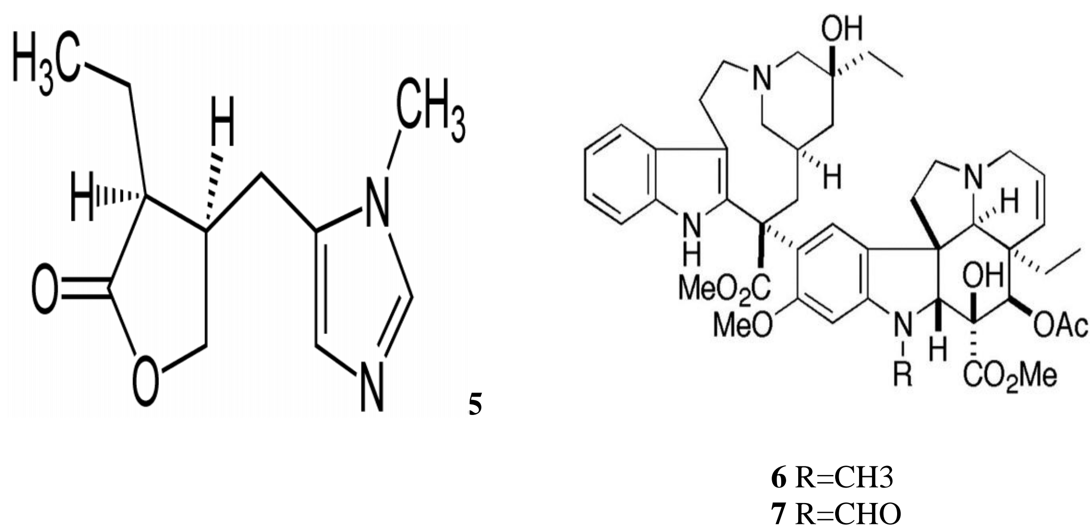


Fig 1.2 Chemical structure of some medicinal natural products of plant origin

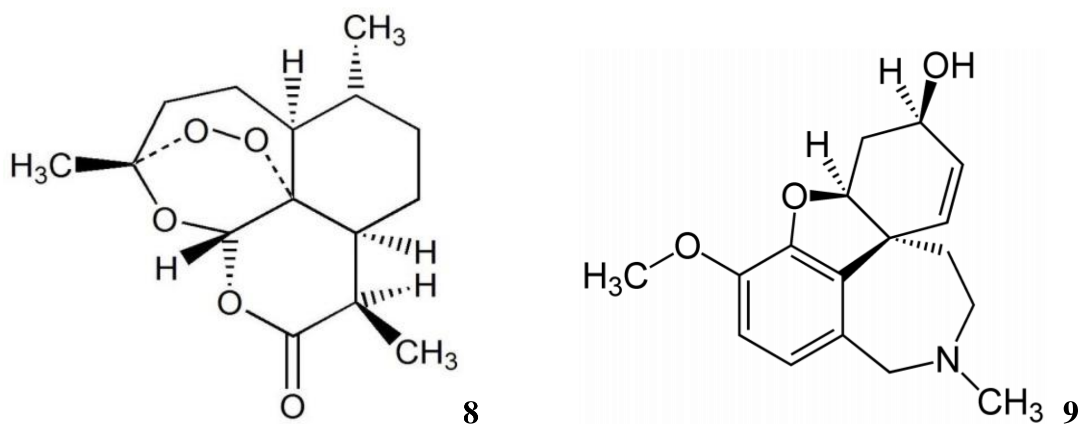
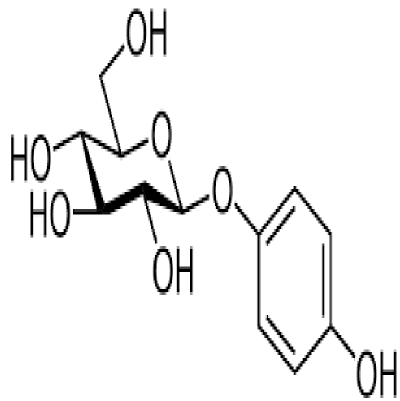


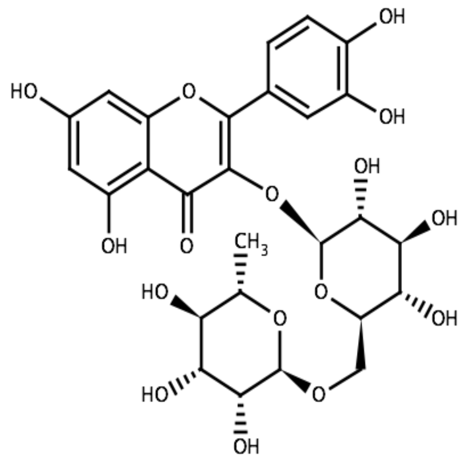
Fig 1.2 Continued

Several plant natural products have made significant impact in cosmetics, perfumes and spices market. Few examples include arbutin **10** from *Arctostaphylos uva-ursi* Spreng for skin whitening and melanin-inhibiting, rutin **11** from *Afrormosia laxiflora* Harms for antioxidant and as emollient.

Plant derived compounds of the terpenoid and phenolic types have also demonstrated their potentials as commercial sweetener (Kinghorn and Soejarto, 2002). Few examples include Hernandulcin [6-(1,5-dimethyl-1-hydroxy-hex-enyl)-3-methylcyclohex-2-enone] **12** from leaves and flowers of *Lipia dulci* Trev (Verbanaceae), periandrin V (3β-O-(β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl)-25-al-olean-18(19)-en-30-oic acid) **13** from the rhizomes of *Periandra dulci* L. (Leguminosae) (Brazilian Licorice) are much sweeter than sucrose (Hashimoto *et al.,* 1983)**.**

Other uses of plant extracts and plant products include pest control for agricultural purposes (Tewary *et al.,* 2005; Asawalam *et al.,* 2007).





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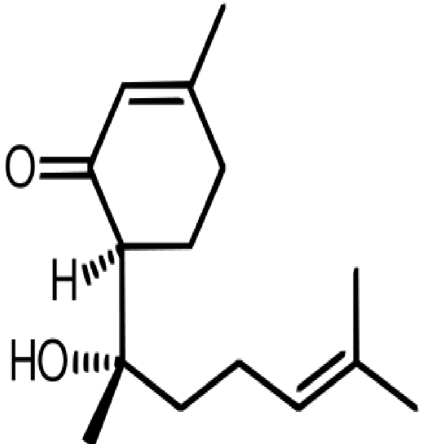

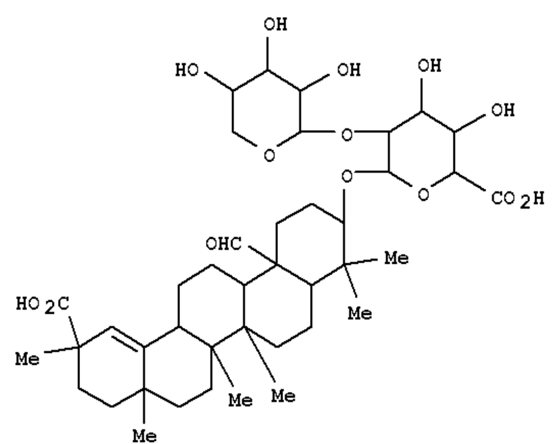

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Fig 1.3 Chemical structure of some natural products of plant origin in use as sweeteners and cosmetics

1.4 Impact of Technological Advancement in Medicinal Plant Research

A number of advances in capability and technology are fostering a renaissance in natural product research and directly or indirectly reducing the historical impediments to development of natural products (Schuster, 2001; McChestney *et al.,* 2007). The advantage in methodologies for separation technologies such as High Performance Liquid Chromatography (HPLC) and countercurrent partition chromatography have further expanded the capacity for separation of plant chemical constituents (Pauli, 2006). Structure elucidation technology has improved especially with the development of high field NMR (Korfmacher, 2005; Phillipson 2007) allowsing rapid and straight forward structure elucidation.

Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC) are now being coupled with detectors in what is known as hyphenated techniques (GC-MS, LC-MS, LC-NMR, LC-MS-NMR etc.) this enable direct (online) identification of plants constituent prior to their isolation (Hostettman and Wolfende, 2004).

Although the active principle isolated from plant may not necessary replace the plant extract (Phillipson, 2001), drugs are now discovered as a result of chemical studies directed at the isolation of the active substances from plants used in traditional medicine. Where the drugs do not possess the optimal properties for their use in human or animal medicine, they would be subjected to structure modification in order to improve their biological properties (Guthikonda *et al.,* 1987).

1.5 Challenges in Medicinal Plant Research

Despite the great contribution of the plant kingdom, it has only been haphazardly investigated. A few plants have been exhaustively studied while many have not been studied at all. It has been estimated that only 5-15% of the approximately 350,000 species of higher plants have been systematically investigated for the presence of bioactive compounds (Cragg, *et al*., 1997). It is well known that some of these important sources of drugs are fast getting extinct due to many factors which include agricultural development, indiscriminate destruction of flora and so on (Rukangira, 2009). This is a result or growing trade demands for cheaper healthcare products and new plant-based therapeutic markets in preference to more expensive target-specific drugs and biopharmaceuticals. This habitat loss is the greatest immediate threat to biodiversity and as such it has stimulated positive legal as well as research interest in order to document the potential of these plants before they disappear completely or results in lose genetic diversity.

Folk healers and their orally transmitted traditions are more vulnerable than medicinal plants themselves because many healers/indigenous knowledge holders aged and are dying or being killed in ethnic, religious or political crises with their knowledge left unrecorded. Traditional medicine is well known to be associated with secrecy (Sofowora, 2008). Families with this knowledge would like to keep their information and knowledge to themselves for fear of being marginalized in the race to exploit the commercial values of their medicine. Nowadays, younger generation show less interest in acquiring knowledge of plants from parents due to expansion of modern education and to some extent modern medicine (Weldegerima, 2009). This is another great challenge to drug discovery and development.

Despite the long history of success in the application of natural products as drugs during the past couple of decades, research into natural products has experienced a steady global decline. This decline could be partly attributed to the introduction of high-throughput synthesis (HTS) and combinatorial chemistry (Eldridge *et al.,* 2002).

1.6 Statement of Research Problem

Diabetes mellitus is a chronic metabolic disease which now afflicts 3% of the world population. Around 95 % of diabetic patients are diagnosed with type 2, diabetes (Attele *et al.,* 2002). The disease is characterized by hyperglycemia. Prevalence of hyperglycemia has been on the increase, not only because of genetic factor/reason but also due to individual lifestyle, thus becoming one of the major causes of death especially in developing countries (Akinloye *et al.,* 2013). It has been reported that long standing hyperglycemia with diabetes mellitus leads to the formation of advanced glycosylated end-products which are involved in the generation of reactive oxygen species, leading to oxidative damage, particularly to heart and kidney (Rolo and Palmeira 2006). The disease has also been reported to be associated with disturbances in learning, memory, and cognitive skills in the diabetic patients (Akram, 2013).

Estimates have shown that at least 150 million people world-wide have diabetes, of which two-thirds live in developing countries (Salisu *et al.,* 2009) such as Nigeria. Based on World Health Organization (WHO) report, the number of diabetic patients is expected to increase from 171 million in year 2000 to 366 million or more by the year 2030 (Wild, *et al.,* 2004). It appears that available treatments, including attempts at lifestyle alterations and drug therapies including insulin, are insufficient to stem the tide.

1.7 Justification

Current oral anti-diabetic agents such as insulin releasers, insulin, sensitizers and α-glucosidase inhibitors, have modest efficacy and limited of modes of action. In addition, current anti-diabetic drugs usually have adverse side effects, decreased efficacy over time, ineffectiveness against some long-term diabetic complications and low cost-effectiveness (Grover *et al.,* 2002).

One therapeutic approach for treating diabetes is to decrease the postprandial hyperglyceamia (Kwon *et al.,* 2007) which can be achieved by retarding the absorption of glucose through the inhibition of the carbohydrate hydrolyzing enzymes such as α-amylase and α-glucosidase in the digestive tract, bringing about delay in carbohydrate digestion and prolongs overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently blunting the postprandial plasma glucose rise (Ranilla *et al.,* 2010). Varieties of therapeutic drugs such as Acarbose, Voglibose and Miglitol, all synthetic inhibitors of microbial origin are available for the management of postprandial hyperglycaemia but are not cost effective and produce adverse side effects such as abdominal distention, flatulence and diarrhea resulting from excessive inhibition of pancreatic α-amylase. Although, food grade α-amylase inhibitors from dietary plant extracts are potentially safer and may be a preferred alternative for modulation of carbohydrate digestion and control of glycaemic index (McCue *et al.,* 2005). Hence, a good strategy to managing diabetes with lesser side effects is to identify the natural inhibitors from diatery sources having mild inhibitory effect on α-amylase and strong inhibitory activity α-glucosidase.

Literature search have shown that *G. kola* seeds have anti-diabetic property. However, there is dearth of information concerning the biochemical pathway and the bioactive constituents that maybe responsible for this activity. Therefore, identifying the possible biochemical pathway and the bioactive constituents that maybe responsible for the reported anti-diabetic property of *G. kola* seeds could be of value in the control of the disease with safe and effective drug of natural product origin.

1.8 Aim and Objectives of the Research

1.8.1 Aim of research

To characterize the bioactive constituents from *Garcinia kola* seeds and evaluate its inhibitory activity on some key enzymes related to diabetes.

1.8.2 Objectives of research

i. To extract and isolate bioactive constituent(s) from *Garcinia kola* seeds.
ii. To identify the constituents via spectroscopic means
iii. To evaluate the inhibitory effects of methanol, ethyl acetate and n-hexane extracts on glycosidase (α-amylase and α-glucosidase) enzymes

1.9 Hypothesis

- *Garcinia kola* seeds contain bioactive constituents with inhibitory activity on glycosidase (α-amylase and α-glucosidase) enzymes related to diabetes.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Taxon Guttiferae/Clusiaceae

Guttiferae (Clusiaceae) is a family of plants that includes 37 genera and 1600 species (Gustafsson *et al.,* 2002). It is also known as Clusiaceae. Most plants belonging to this family are trees and are generally confined to warm humid tropics (Sultanbawa, 1973). Some of the genera and species of this family have been found to be endemic to certain land masses e.g. *Kielmeyera* is confined to Brazil and other South American countries and *Symphonia* is confined to Africa and to the Malagasy Republic (present day, Madagascar) (Hutchinson, 1969). Guttiferae plants also grow as shrubs and herbs with yellow or brightly colored resinous juice which can be used as a source of resins, gums, pigments, dyes, edible oil and fruits (Mabberley, 1987). The leaves are simple, entire, opposite and stipulate with the present of resin or oil gland. The flowers are regular and often bright in color (Dale and Greenway, 1961).

2.1.1 Chemistry of guttiferae

Chemically, plants of the Clusiaceae family constitute a rich source of polyprenylated acylphloroglucinols and xanthones (Dakanali and Theodorakis, 2011); they also constitute a source of coumarins, flavonoids and triterpenes.

The chemical structures of all known polycyclic polyprenylated acylphloroglucinols (PPAPs) can be classified into three types [A, B (I), B (II) and C] (Dakanali and Theodorakis, 2011) on the basis of their biosynthesis and all three types have been reported from Guttiferae. Polycyclic polyprenylated acylphloroglucinols isolated from members of the Guttiferae family

include: Hyperforin **14** (Muller *et al.,* 1998; Mennini and Gobbi, 2004), garsubellin A **15** (Fukuyama *et al.,* 1997), Nemorosone **16** (Cuesta-Rubio *et al.,* 2002) all belonging to the type **A** PPAPs. While clusianone **17** (Piccinelli *et al.,* 2005) and enaimeone A **18** (Winkelmann *et al.,* 2001) belong to the type **B** (I) and **B** (II) respectively. Lastly, the type **C** PPAPs, Garcinielliptone M **19** (Weng *et al.,* 2004) have been reported.

Over 80 different xanthones (Carpenter *et al.,* 1971) have been isolated from the different species. Recently, Xanthones from Guttiferae [55 species in 12 genera] (Negi *et al.,* 2013) have been reported. Xanthones with isopentyl and geranyl substituents are common in the family Guttiferae (Sultanbawa, 1973). The occurrence of prenylated xanthones is restricted to species of this family (Negi *et al.,* 2013). Prenylated xanthones such as Xanthonolignoids: Kielcorin **20** (Pinheiro *et al.,* 2003), Cadensin C **21** and Cadensin D **22** (Cardona *et al.,* 1986) have all been reported. Also reported are Bisxanthones such as jacarelhyperols A **23** and B **24** (Ishiguro *et al.,* 2002) and globulixanthone E **25** (Nkengfack *et al.,* 2002).

More than 50 coumarins have been isolated and characterized from various species of Guttiferae. These coumarins have been isolated mostly from the seeds and bark extracts. Examples of such coumarins are Surangin B **26** and mammeisin **27** (Sultanbawa, 1973). Similarly, Flavonoids, such as Kaempferol **28**, Quercetin **29** and Amentoflavone **30** (Shen and Yang, 2006) have been reported from this family. Also reported were biflavonoids such as volkensiflavone **31**, morelloflavone **32** and fugugiside **33** (Jackson *et al.,* 1967; Sultanbawa, 1973).

Many triterpenes and steroids have been reported from several species of the family Guttiferae. These triterpenes include members of the friedelane group [friedelin **34**, canophyllal **35**], oleanane group [β-amyrin **36** and erythrodiol-3-acetate **37**], lupane group [betulinic acid **38** and lupeol **39**], taraxerane group [taraxerol **40** and taraxerone **41**] (Sultanbawa, 1973). Sitosterol **42** and Stigmasterol **43** (Shen and Yang, 2007) have been reported.

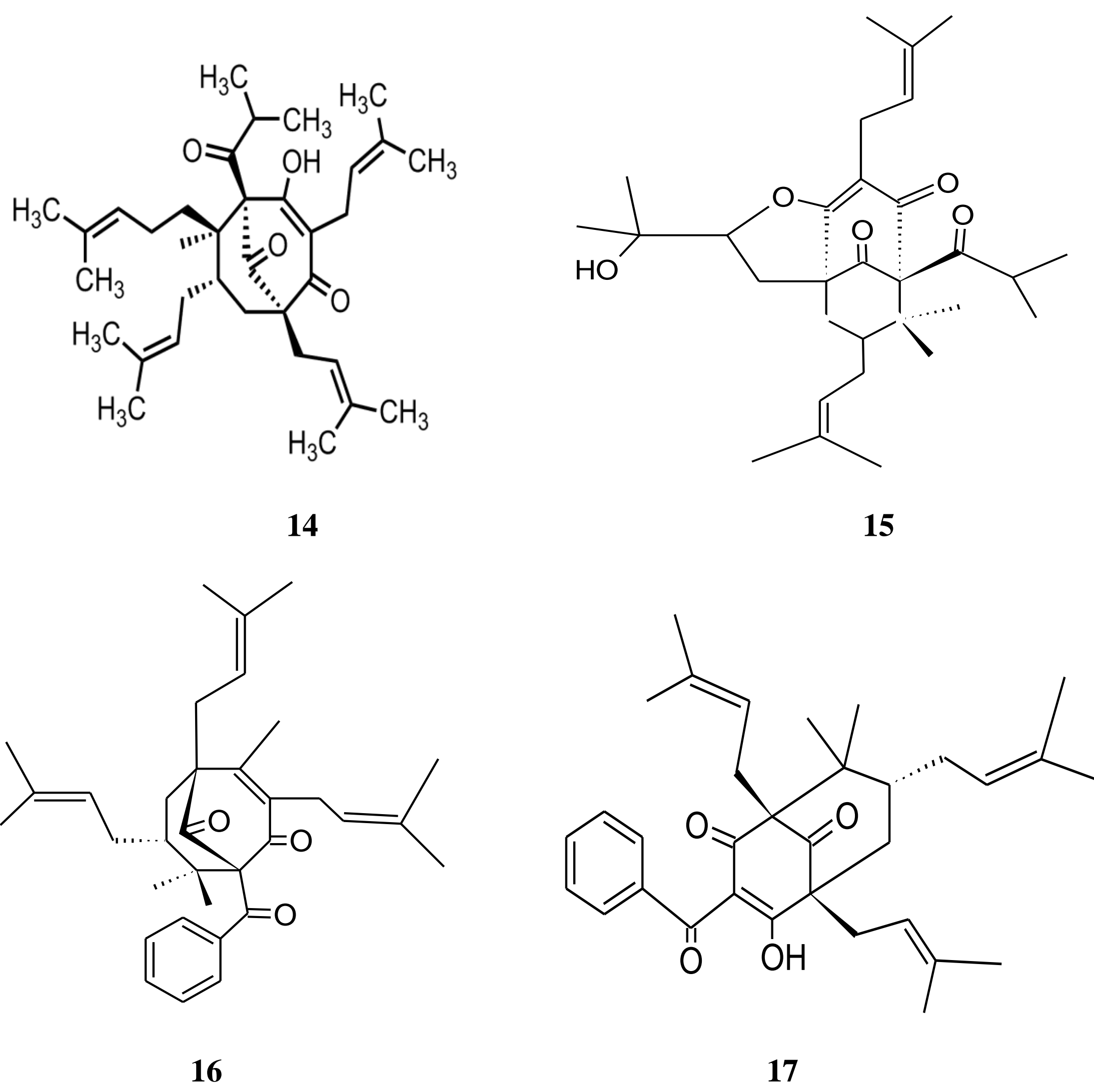
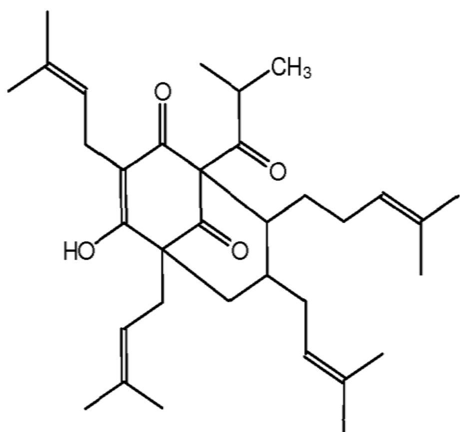
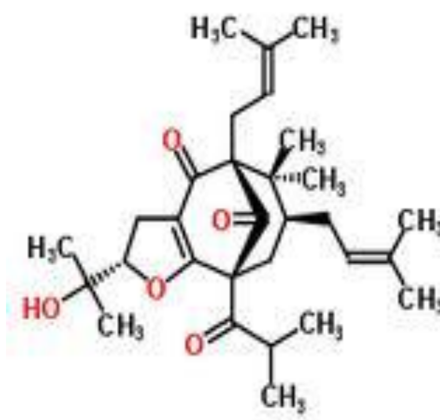


Fig. 2.1Chemical structure of some compounds isolated from Guttiferae (Clusiacea)





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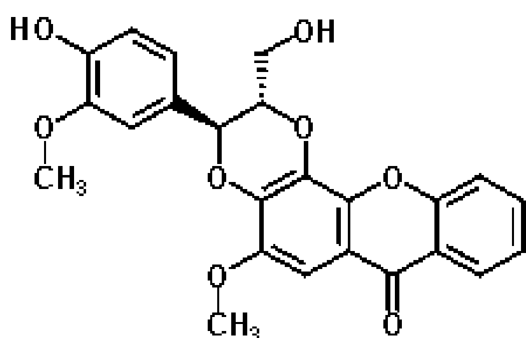

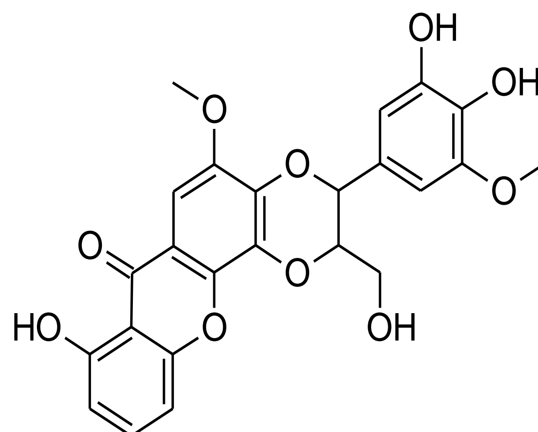

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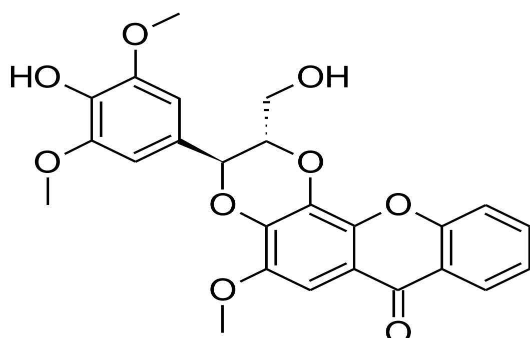

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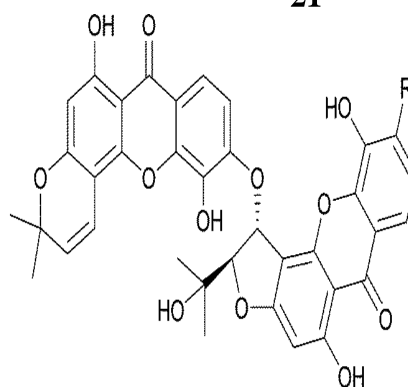
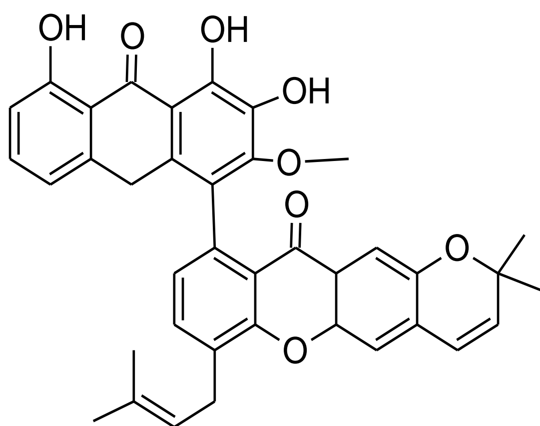


23 R=OH
24 R=H

Fig. 2.1 Continued



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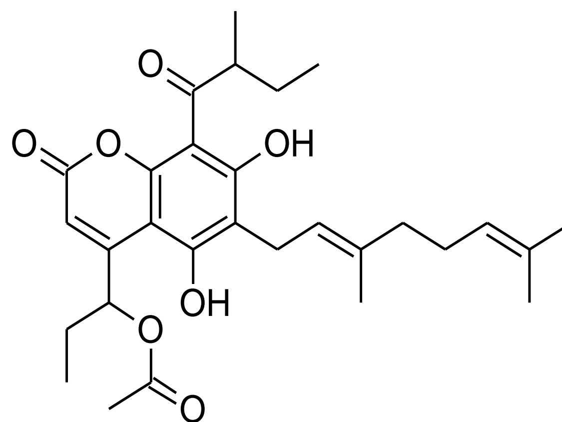

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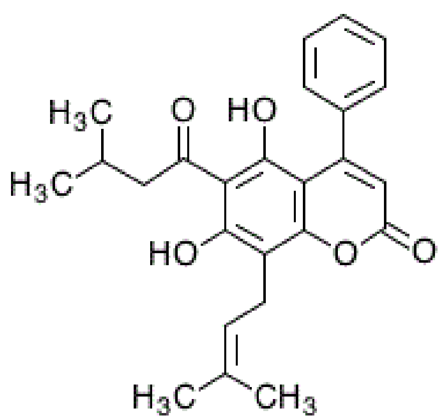

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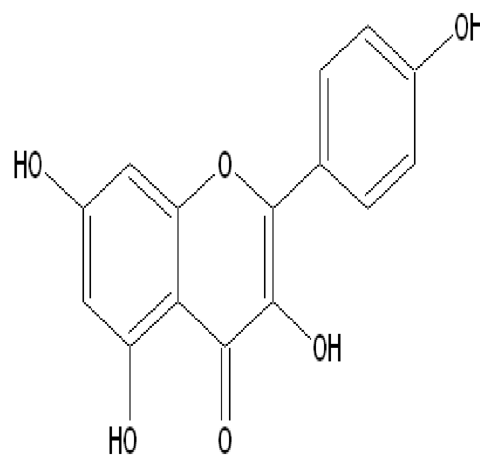

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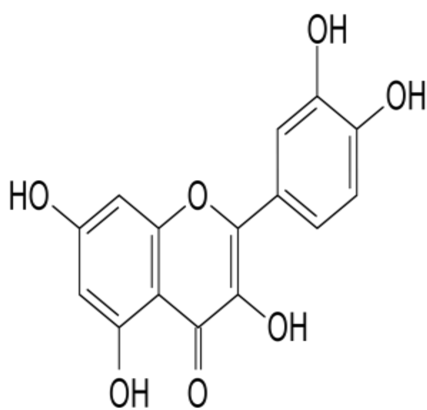

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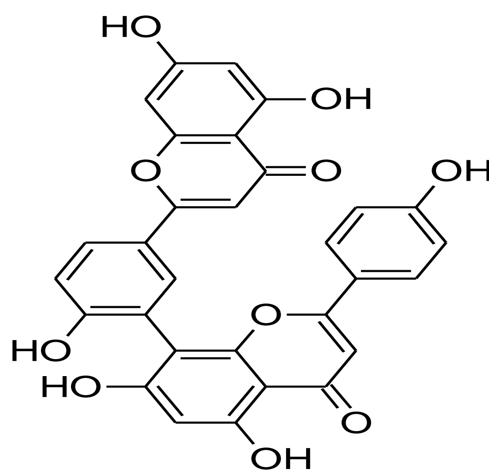

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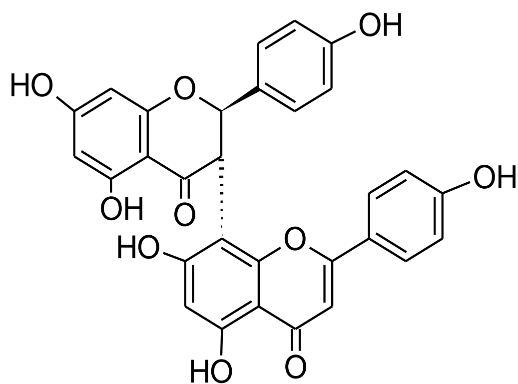

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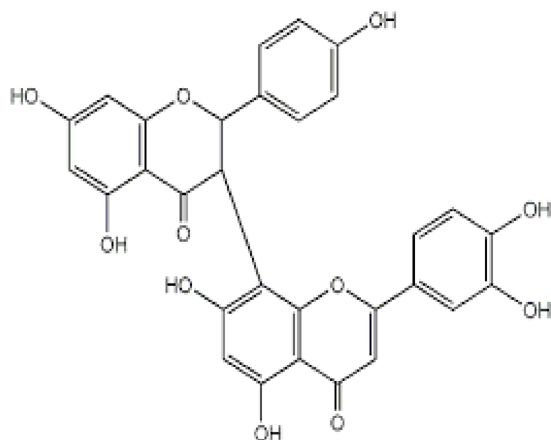

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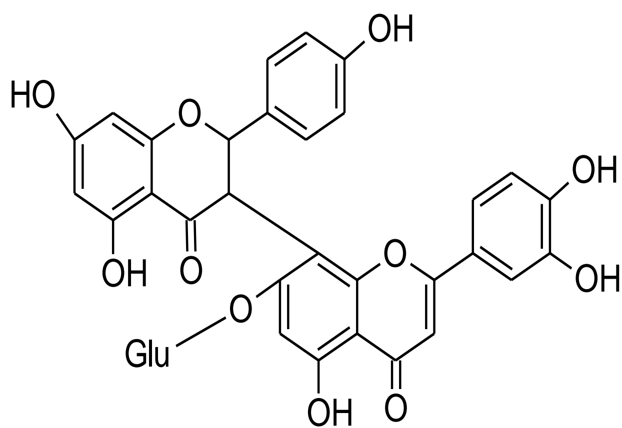

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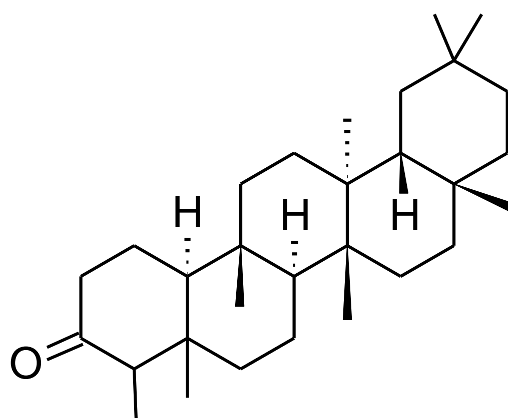

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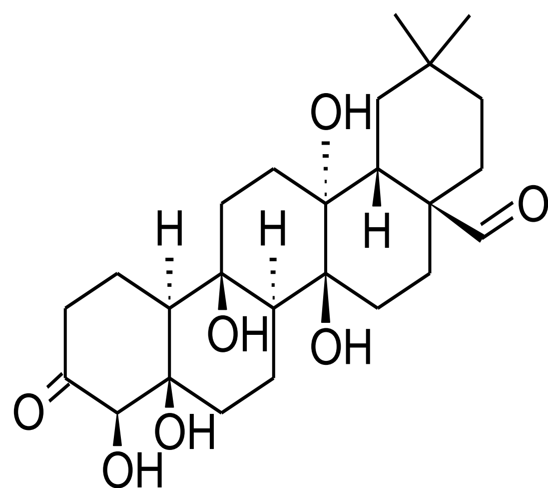

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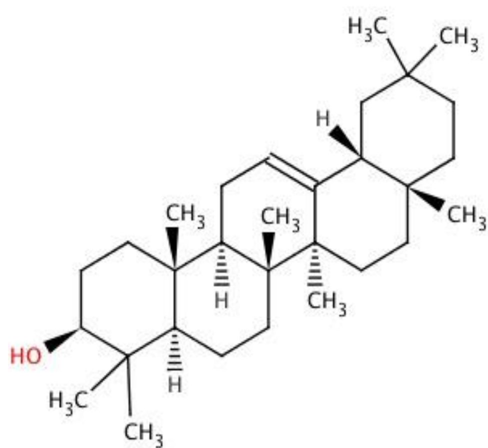

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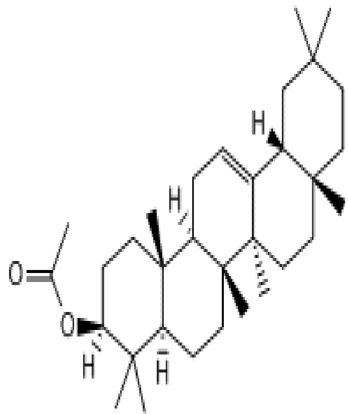

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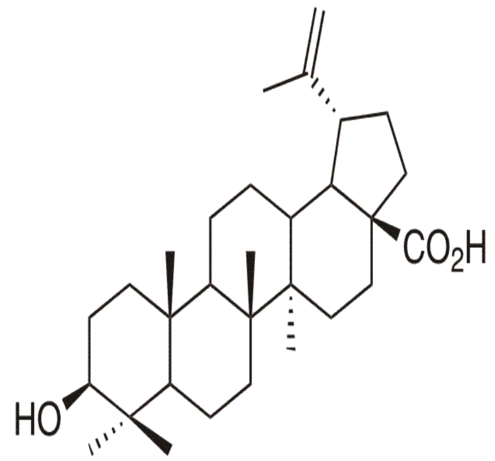

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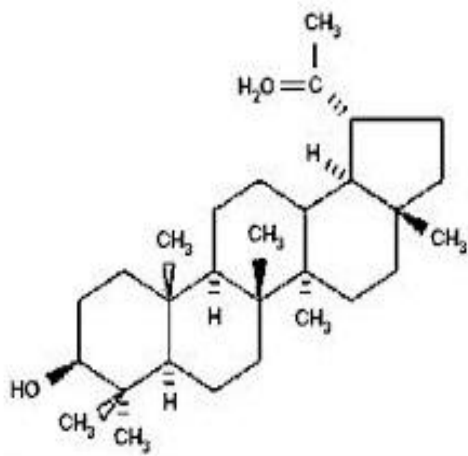

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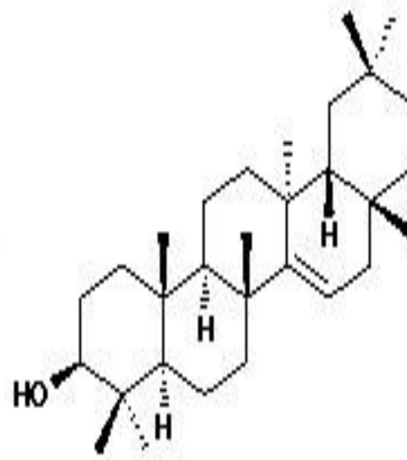

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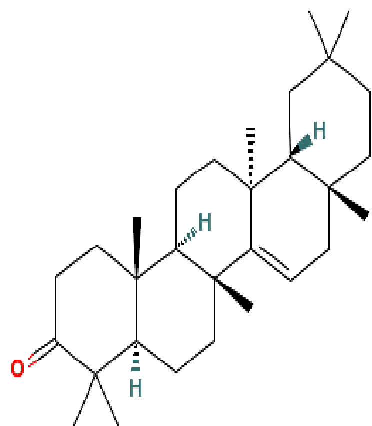

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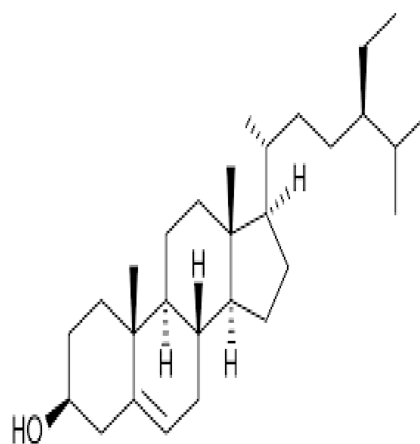

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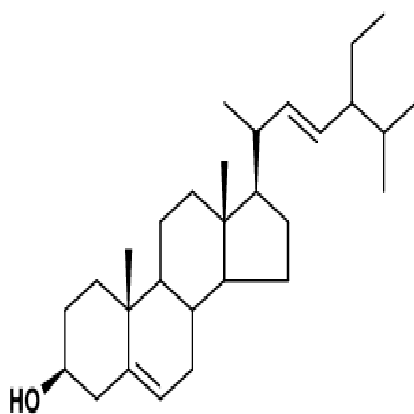

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Fig. 2.1 Continued

2.1.2 The genus *Garcinia*

The Genus *Garcinia*, belonging to the Family Clusiaceae comprises about 300 species (Ritthiwigrom *et al.,* 2013). Native to Asia, Africa, South America and Polynesia, the plants are small to medium sized evergreen trees which may grow up to 30 m in height and are widely distributed in the tropical and temperate regions of the world (Kijjoa and Vieira, 2009). *Garcinia* is a rich source of secondary metabolites, especially triterpenes (Kijjoa and Vieira, 2009), flavonoids (Ansari *et al.,* 1976), xanthones (Rukachaisirikul *et al.,* 2003) and phloroglucinols (Xu *et al.,* 2010). The latter two groups are well recognised as cheomotaxonomic markers for this genus (Han *et al.,* 2008; Nguyen *et al.,* 2011).

2.1.3 Ethnobotany and pharmacological activities of *Garcinia spp*

Garcinia are often used for traditional medicines to treat abdominal pain, dysentery, diarrhoea, infected wound and gonorrhoea (Jayaprakasha *et al*., 2006). The fruits of *Garcinia xanthochymus* have been widely used for bilious condition, diarrhoea and dysentery in Thailand (Perry and Metzger, 1980). Meanwhile the fruit hull of *Garcinia mangostana* L. used for healing skin infections and wound (Mahabusarakum *et al*., 1987).

In Indonesia, the leaves and seeds of *Garcinia dulcis* have been used for the treatment of lymphatitis, parotitis and struma (Kosela *et al*., 2000). In Africa, *Garcinia preussii* Engl. (*syn G. epunctata* Stapf) is traditionally used to treat stomach aches (Bouquet, 1969). The leaves are prepared as a decoction to relieve toothache (Visser, 1975).

Garcinia plants have been reported to show various pharmacological activites including anticancer, anti-inflammatory, antibacterial, antiviral, antifungal, anti-HIV, antidepressant and antioxidant (Hemshekhar *et al.,* 2011).

2.1.4 Isolated compounds from *Garcinia spp*

The family Guttiferae has been extensively investigated from phytochemical point of view. Many genera of this family have been reported to be source of many important compounds with various biological activities such as hyperforin (antibacterial and anticancer properties), nemorosone (antimicrobial, cytotoxic and antioxidant properties), garcinielliptone M, (anti-inflammatory properties), garsubellin A, (activities against neurodegenerative diseases) and clusianone (anti-HIV activities), (Muller *et al.,* 1998; Mennini and Gobbi, 2004; Cuesta-Rubio *et al.,* 2002; Weng *et al.,* 2004; Fukuyama *et al.,* 1997; Piccinelli *et al.,* 2005).

The genus *Garcinia* has been a major source of varieties of oxygenated and prenylated phenol derivatives such as prenylated xanthones, polyisprenylated benzophenones, biflavonoids and triterpenoids (Oliveira *et al.,* 1999). Some of them exhibited novel and intriguing chemical structures (Sordat-Diserens *et al.,* 1989). Some of the compounds isolated from some *Garcinia spp* are as tabulated below:

Table 1.1 Some Phytochemical isolated from the genus *Garcinia*

S/No	*Garcinia* species	Compounds isolated	Reference
1	*G. cowa*	Rubraxanthone, Cowaxanthone, Daucosterol, Oblongifoli D, Garcoowaside A, B and C	Wahyuni *et al.,* 2004; Shen *et al.,* 2007; Panthong *et al.,* 2009; Ritthiwigrom *et al.,*2013
2	*G. cymosa*	α -mangostin, β-mangostin, Epicatehchin	Ernawati and Yusnelti, 2014
3	*G. dulci*	Morelloflavone, 12b-hydroxy-des-D-garcigerrin	Likhitwitayawuid *et al.,* 1998; Zakaria *et al.,* 2012
4	*G. hanburyi*	Gambogenic acid, Gambogic acid, Desoxymorellin, Isomorellinol	Lin *et al.,* 1993; Asano *et al.,* 1996; Jianhong *et al.,* 2013
5	*G. mangostana*	Mangostinone, Garcinone E, Trapezifolixanthone, Toxophyllin	Asai *et al.,* 1995; Susksamrarmetal, 2002
6	*G. morella*	Morellic acid, Isomorellic, Gambogic and Guttiferic acids	Karanjgaokar *et al.,* 1996; Rajagopal *et al.,* 2007

2.2 *Garcinia kola*

Garcinia kola (Heckel) belongs to Order Malpighiales. It is an angiospermae known in commerce as bitter cola. On chewing, *G. kola* seed (Plate I) has a bitter astringent and resinous taste, somewhat resembling that of raw coffee, followed by a slight sweetness (Adesuyi *et al.,* 2012). It is commonly called bitter cola or male kola. Locally, it is called “Orogbo” in Yoruba, “Aku ilu” in Igbo and “Namijin goro” in Hausa (Adegboye *et al.,* 2008).

Nomenclature

Kingdom: Plantae

Phylum: Angiospermae

Order: Malpighiales

Family: Clusiaceae

Genus: Garcinia

Species: *G. kola*

Binomial name: *Garcinia kola* (Heckel)

Plate I: *Garcinia kola* seeds

2.2.1 Description and distribution

Garcinia kola is commonly found in moist rain forests and swamps. It is a medium sized evergreen tree, about 15-17 m tall and with a fairly narrow crown. The leaves are simple, 6-14 cm long and 2-6 cm across, shiny on both surfaces and spotted with resin glands. The small flowers are covered with short, red hairs (Iwu, 1993). It is cultivated through the seedlings or with cuttings. It grows more easily using the cuttings (Adesuyi *et al.,* 2012).

The fruit is a drupe of 5-10 cm in diameter and weighs 30-50g. It is usually smooth and contains a yellow-red pulp. The fruit changes color during maturation from green to orange, and each fruit contains 1-4 seeds (Juliana, *et al.,* 2006). *Garcinia kola* plant is found in countries across west and central Africa and it is distributed by man around the towns and villages of such countries like; Nigeria, Ghana, Cameroon, Sierra Leone, Togo, Congo Democratic Republic, Angola, Liberia, Gambia etc. (Adesuyi *et al.,* 2012). In Nigeria, it is common in the South Western States and Edo State (Otor *et al.,* 2001).

2.2.2 Ethnobotany and pharmacological activities of *Garcinia kola*

The plant has been used in the treatment of a wide range of diseases and its importance in folkloric medicine is eminent. It is regarded as a wonder plant because every part of the plant (bark, leaves, root and wood) has been found to be of medicinal importance (Adesuyi *et al.,* 2012). *Garcinia kola* is chewed extensively in Southern Nigeria as a masticatory. The root of the plant is used as favorite bitter chew-sticks in West Africa (Otor *et al.,* 2001). The stem bark is used in folklore remedies as a purgative among the natives of Eastern Nigeria and the latex is externally applied to fresh wounds to prevent sepsis, thereby assisting in wound healing (Adesuyi *et al.,* 2012; Ekene and Erhirhie, 2014). A cold water extract of the roots

and bark with salt are administered to cases of "Ukwala" (bronchial asthma or cough) or "agbo" (vomiting) (Iwu *et al.*, 1990). The seeds are chewed as an aphrodisiac or used to cure cough, dysentery, chest colds, liver disorders, diarrhea, laryngitis, bronchitis, and gonorrhea (Adesina, *et al.,* 1995). It is also used to prevent and relieve colic; it can also be used to treat headache, stomach ache and gastritis (Ayensu, 1978). It has also been reported for the treatment of jaundice, high fever, and as purgative (Iwu, 1989). *Garcinia kola* is used as an anti-poison and helps to detoxify the system; it has the ability to repel evil men and spirits (Iwu, 1989). Traditional medicine practitioners in Nigeria, particularly in the Ogoni area use a decoction of *Garcinia kola* stem bark for the treatment of dysmenorrhoea, fever, inflammation and burns (Adesina, *et al.,* 1995).

In Liberia and Congo Democratic Republic, it is used in the treatment of skin infections (Adesuyi *et al.,* 2012). The powdered bark of the plant is applied to malignant tumors, cancers etc. the plants latex is taken internally for gonorrhea (Adesuyi *et al.,* 2012). In Congo, a bark decoction is taken for female sterility and to ease child birth. The bark is added to that of *Sarcocephalus latifolinus*- which has a strong reputation as a strong diuretic, in the treatment of urinary decongestion and chronic urethral discharge (Adesuyi *et al.,* 2012).

In Ivory Coast, a decoction of the bark is taken to induce the expulsion of a dead fetus, while the seed and the bark are taken for stomach pain (Adesuyi *et al.,* 2012). While in Sierra Leone, the roots and bark are taken as a tonic for sexual dysfunction in men. The bark is also added into palm wine to improve its potency (Iwu, *et al*., 1990).

Garcinia kola and its extracts have been shown to elicit a number of biological activities in various experimental models. Powdered seeds of *Garcinia kola* have been

reported to displayed marked inhibition of gastrointestinal motility, protection against castor oil induced diarrhea and prolonged pentobarbital sleeping time (Braide, 1991).

Esimone *et al.,* 2002 reported on the effect of *Garcinia kola* seed extract on the pharmacokinetic and antibacterial activity of ciprofloxacin hydrochloride. In another study, the antimicrobial interaction between *Garcinia kola* seed (GKS) and gatifloxacin (GAT) was evaluated on *Bacillus subtilis* and *Staphylococcus aureus* (Ofokansi, *et al.,* 2008). Trichomonacidal effects of *G. kola* nuts (Gabriel and Emmanuel*,* 2011) were also reported.

The seed powder (GKP) had also been shown to have anti-diabetic, anti-lipidemic and anti-atherogenic properties with a tremendous potential to protect against coronary heart disease (Udenze, *et al.,* 2012). Adaramoye and Adeyemi, (2006) reported on the hypoglycaemic and hypolipidaemic effects of fractions from kolaviron (KV) (a *Garcinia kola* seed extract) in normal and streptozotocin (STZ)-induced diabetic rats. Significant hypoglycaemic and hypolipidemic activity of *Garcinia kola* in alloxan-induced diabetic Wistar rats has also been reported (Nwangwa, 2012).

Hepatoprotective and anti-oxidant activity has been reported on *Garcinia kola* in various studies, including hepatoprotective effect of *Garcinia kola* seed extract against paracetamol induced hepatotoxicity in rats (Alade and Ani, 1990), The protective effects of *Garcinia kola* against a dose of carbon-tetrachloride ($CC1_4$)-induced liver damage in experimental rats (Mathew and Blessing, 2007) and the antioxidant potentials of five fractions (ME1–ME5) of methanolic extract of *Garcinia kola* seeds (Tebekeme, 2009).

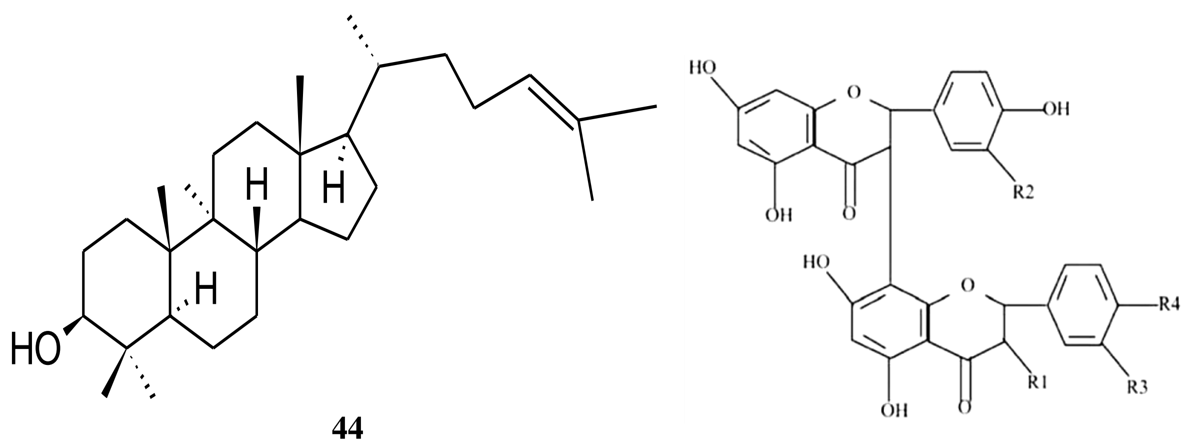
Garcinia kola has also been reported in a number of studies to have effects on fertility: long- term ingestion of *Garcinia kola* seed diet cause a significant reduction in sperm count, sperm motility, and ultimately infertility in male rats (Mesembe, *et al.,* 2013), 200 mg/kg body weight of *G. kola* seed altered oestrous cycle and partly inhibits ovulation in female rats (Akpantah, *et al.,* 2005) while seed meal was reported to have shown improve semen characteristics and sexual drive (libido) in matured rabbit bucks (Iwuji and Herbert, 2012).

Other studies have shown anti-ulcer potential (Onasanwo, *et al.,* 2011), anti-pyretic activity (Kakjing, *et al.,* 2014), intraocular pressure (IOP) activity (Adebukunola, *et al.,* 2010), erythropoiesis in both rabbits and rats (Esomonu, *et al.,* 2005; Unigwe and Nwakpu, 2009) etc. of different exracts and fractions of *G. kola.*

2.2.3 Isolated compounds from Garcinia *kola*

The medicinal importance of *Garcinia kola* is based mainly on the phytochemical components of the plant. From its roots to its leaves, the plant is known to contain several phytochemicals noted for their medicinal importance (Iwu *et al*., 1990).

Some of the phytochemicals compounds that have been isolated from *Garcinia kola* includes, cycloartenol **44** and its 2,4-methylene derivatives (Aplin *et al.,* 1967), C-3/8-link biflavanone GB1 4**5**, GB2 **46** and kolaflavanone **47** (Cotterhill *et al.,* 1978), apigenin-5,7,4'-trimethyl ether **48**, apigenin-4'-methylether **49**, fisetin **50** (Iwu, 1982) have been isolated. Benzophenone **51** and kolanone **52** (Hussain *et al.,* 1982), Garcinia biflavonoid complex containing GB1, GB2 and kolaflavanone collectively called Kolaviron (Iwu, 1985) have also been reported from various parts of the plant. Also reported, are two chromanols: garcioic acid **53** and garcinal **54** together with δ-tocotrienol **55** (Terashima *et al*., 2002).



45 R1= OH, R2= H, R3= OH, R4=H
46 R1=OH, R2=H, R3=OH, R4= OH
47R1=OH, R2=H,R3=OCH_3,R4=OH

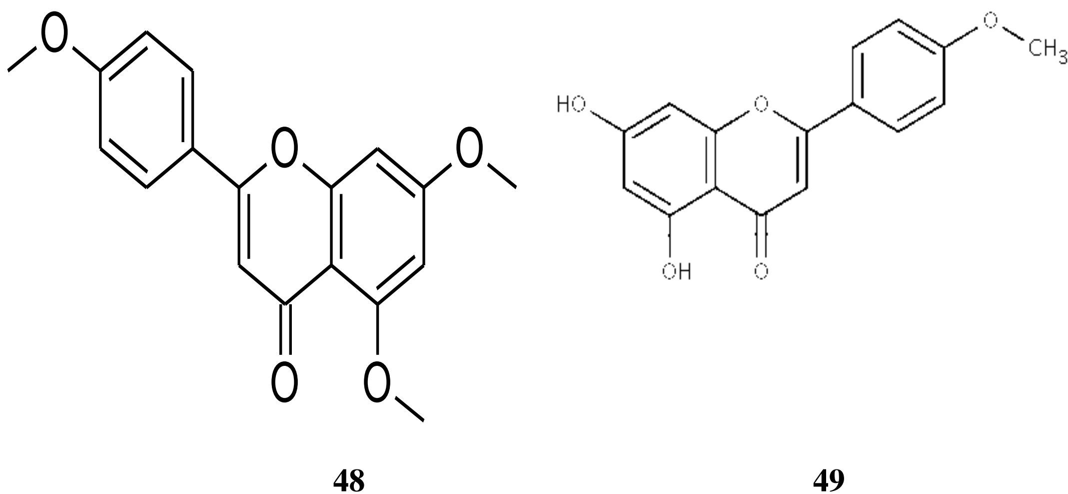
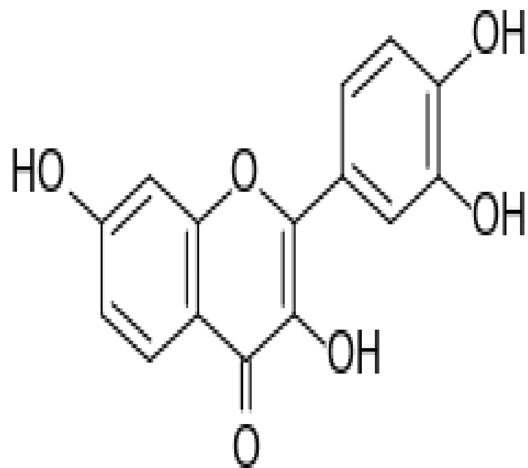
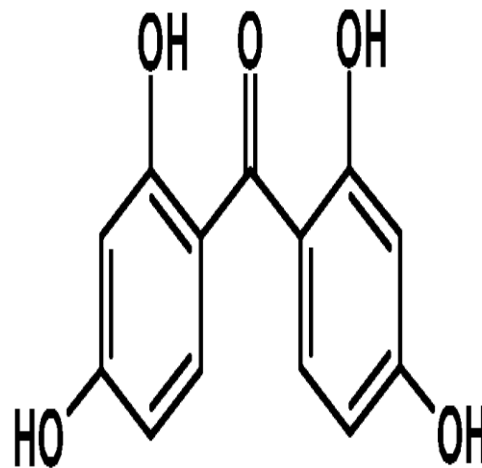


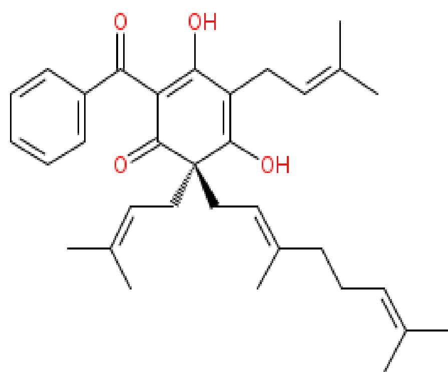
Fig. 2.2 Chemical structures of some compounds isolated from *Garcinia kola*

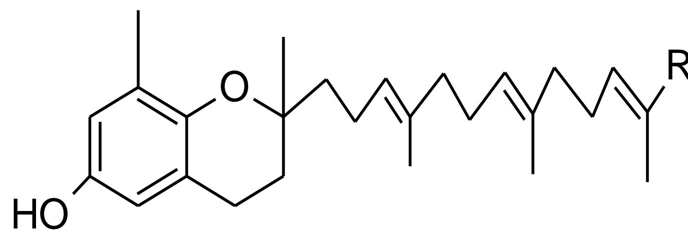




50

51





52

53 **R=CO_2H**
54 **R=CHO**
55 **R=CH_3**

Fig. 2.2 Continued

2.3 Diabetes

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia expressed as abnormal glucose value, and altered lipid and protein metabolism (Pontiroli *et al.,* 1994) leading to severe complications which are classified into acute, sub-acute and chronic (Rang, *et al.,* 1991). Acute complications include hypoglycemia, diabetic ketoacidosis, hyperosmolar and hyperglycaemic non-ketotic syndrome (Knentz and Nattras, 1991) while sub acute complications include thirst, polyuria, lack of energy, visual blurriness and weight loss (Kumar and Clark, 2002). Chronic hyperglycemia causes glycation of body proteins (Sharma, 1993), which in turn leads to complications like arteries diabetic neuropathy, nephropathy leading to end stage renal disease, retinopathy leading to blindness and diabetic foot ulcers necessitating limb amputations (DeFronzo, 2004; Mitra, *et al.,* 2012).

2.4 Glycosidase

Enzymes play a vital role in mediating essential biochemical life processes like metabolism, cell cycling, signal transduction, *etc*. However, hyper or hypo activity of such enzymes leads to malfunctions of the respective biochemical processes which in many cases are the underlying causes of diseases like diabetes, Alzheimer’s disease, myasthenia gravis and Parkinson’s disease, as depicted by their etiopathogenesis at the molecular level. It has been found that enzymes like α-amylase, α-glucosidase contribute significantly to the pathogenesis of type II DM (Dey *et al.,* 2014).

Glycosidases are widespread enzymes that are responsible for the hydrolytic cleavage of glycosidic bonds in contexts ranging from primary metabolism through to glycoprotein glycan assembly (Rempel and Withers, 2008). As some measure of their importance to

biology, around 1–3% of the average genome is dedicated to carbohydrate-active enzymes, many of which are glycosidases (Davies *et al.* 2005). Not surprisingly, therefore, their function or dysfunction has been implicated in a number of different disease states (Butters *et al.,* 2003; De Melo *et al.,* 2006) such as diabetes.

2.5 α-Amylase

α-amylases (1,4- α-D-glucanohydrolases, EC 3.2.1 .1) are members of a class of enzymes called glucosidases, which catalyse the breakdown of carbohydrates (Sogaard *et al.,* 1993; Wong, 1995). The α-amylase constitute a family of endo-amylases that catalyses the initial hydrolysis of starch into shorter oligosaccharides through the cleavage of α-D-(1-4) glycosidic bonds (Iulek *et al.,* 2000; Kandra 2003). The end products of α-amylase action are oligosaccharides with varying length with an α-configuration and α-limit dextrins (Maarel *et al.,* 2002), which constitute a mixture of maltose, maltotriose, and branched oligosaccharides of 6–8 glucose units that contain both *α*-1,4 and *α*-1,6 linkages (Whitcomb and Lowe, 2007). Other amylolytic enzymes participate in the process of starch breakdown, but the contribution of α-amylase is a prerequisite for the initiation of this process (Tangphatsomruang *et al.,* 2005).

2.5.1 Substrate

α-amylases are classified as endo-enzymes. As such, they hydrolyze their substrate at random sites within the molecule rather than from the ends (Truschelt *et al.,* 1981; Wong, 1995). The natural substrates of α-amylase are oligosaccharides and polysaccharides (Sogaard *et al.,* 1993). These carbohydrates are mainly sourced from plants in the form of starch, a major source of dietary carbon for animals.

2.5.2 Structure

The amylase presents a 3D structure capable of binding to a substrate and by the action of highly specific catalytic groups promotes the breakage of the glycoside links (Iulek *et al.,* 2000). The protein contains 3 domains: A, B, and C. Domain A, which has a (β/α) eight (8) barrel fold which constitutes the catalytic core domain. It contains about 280–300 residues. The catalytic triad (Asp, Asp, Glu) is present in domain A (Maarel *et al.,* 2002; Janecek *et al.,* 1997). The B domain is inserted between A and C domains and is attached to the A domain by disulphide bond. The C domain presents a β sheet structure linked to the A domain by a simple polypeptide chain and seems to be an independent domain with unknown function. The active site (substrate binding) of the α-amylase is situated in a long cleft located between the carboxyl end of both A and B domains. The calcium (Ca^{2+}) is situated between A and B domains and may act in stabilizing the three-dimensional structure and as an allosteric activator. The substrate-binding site contains 5 subsites (-3 -2 -1 +1 +2) (Brayer *et al.,* 2000).

2.5.3 Medicinal applications of α-Amylase

The medical interest in α-amylase stems from its involvement in the metabolism of carbohydrates. α-amylase is secreted from the pancreas and salivary glands into the digestive tract where it acts on ingested carbohydrates (Schomburg and Salzmann, 1990; Sogaard *et al.,* 1993). However, in disease states such as acute pancreatitis or salivary lesions, serum amylase levels can increase. Therefore, diagnosis of these conditions routinely includes analysis of a serum sample to

determine the levels of α-amylase activity in the patient (Schomburg and Salzmann, 1990; Sogaard *et al.,* 1993).

The medicinal application of α-amylase is the inhibition of degradation of starch and oligosaccharide (Funke and Melzing, 2006) in the digestive tract by α-amylase, one of the therapeutic strategies in the treatment of type 2 diabetes.

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2.6 α-Glucosidase

α-Glucosidase (α-1,4-glucosidase, α-glucoside glucohydrolase, EC 3.2.1.20) hydrolyses terminal, non-reducing α-linked-D-glucose residues of oligo- and polysaccharides with concomitant release of α-D-glucose (Webb, 1992).

These enzymes play important roles in primary metabolism (e.g human sucrose/isomaltase), in catabolism (e.g human lysosomal α-glucosidase) and glycoprotein processing (e.g Endoplasmic Reticulum glucosidase II). The enzyme also shows the transferring reaction and the condensation as well as the hydration of D-glucal (Yamamoto *et al.,* 2004).

α-Glucosidases from bacteria, Saccharomyces cerevisiae, and insect belong to Gluco-Hydrolase (GH) family 13 (*α*-amylase family) having four conserved sequences of region 1, 2, 3 and 4, in which the amino acids critical for catalytic reaction are observed (Kuriki and Imanaka, 1999; Svensson *et al.,* 2002). The overall amino acid sequence similarity between the GH-family 13 *α*-glucosidase and *α*-amylase is low, whereas that for dextran-glucosidase (EC 3.2.1.10) (Russell & Ferretti, 1990) and trehalose-6-phosphate hydrolase (EC 3.2.1.93) (Rimmele and

Boos, 1994) are high. Plant, animal, mold, and bacteria (two species) α-glucosidases as well as α-glucan lyase (EC 4.2.2.13) (Bojsen *et al.,* 1999) are members of GH-family 31 (Frandsen and Svensson, 1998).

2.6.1 Substrate

α-Glucosidases from various origins show the different substrate recognitions, which divide enzymes into three groups of Type-I, -II, and -III (Chiba, 1997). α-Glucosidase Type I, belonging to GH-family 13, is observed in bacteria, Brewer's yeast (Saccharomyces cerevisiae), and insects, which hydrolyzes the heterogeneous substrate, such as sucrose and p-nitrophenyl (PNP) α-glucoside of synthetic substrate, more rapidly than the homogeneous substrate of malto-oligosaccharide and shows low or no activity towards the polymer substrate (soluble starch, raw starch, and glycogen), this type of α-glucosidase recognizes α-glucosyl-structure of substrate molecule (Nakai *et al.,* 2005).

α-Glucosidases Type II and III are members of GH-family 31. α-glucosidase of Type II, found in mold, prefers homogeneous substrate to heterogeneous and polymer substrates, which means that this group has high recognition of maltosyl-structure (Nakai *et al.,* 2005). Type III α-glucosidase, originated from plant and animal, shows almost no activity to heterogeneous substrate, and high activity to homogeneous and polymer substrates. The member of Type III strongly recognizes maltosyl-structure as well as polymer structure (Nakai *et al.,* 2005).

2.6.2 Structure

The structure of the N-terminal domain of human intestinal maltase-glucoamylase was the first from a eukaryotic member of GH31 (Sim, *et al.,* 2008). These structures reveal a common (β/α) eight (8) barrel catalytic domain. The essential residues D481 and D647 are located in the conservative sequences (designated as "Region A" and "Region B", respectively), which are observed in all the GH-family 31 enzymes (Kimura, 2000; Okuyama *et al.,* 2001). Region A and Region B are present in the central part of amino acid sequence. Between the two catalytic residues, there are about 170 amino acid residues in mold *α*-glucosidase, about 100 residues in plant and animal *α*-glucosidases, and about 70 residues in bacterial *α*-glucosidase.

Most GH-31 members are multi-domain proteins, while the specific function (if any) of these accessory domains is generally unknown (Kimura, 2000; Okuyama *et al.,* 2001).

2.6.3 Medicinal application of α-glucosidase

α-glucosidase has drawn a special interest of the pharmaceutical research community because it was shown in earlier studies that the inhibition of its catalytic activity resulted in the retardation of glucose absorption and the decrease in postprandial blood glucose level (Braun and Brayer, 1995; Dwek and Butters, 2002).

2.7 Glycosidase Mechanisms

Glycosidases can be classified into a number of sequence related families (Henrissat 1991; Henrissat and Bairoch 1993). Enzymes within a sequence related family catalyze the cleavage of the glycosidic bond by the same mechanism and share a similar overall structural fold (Vasella *et al.,* 2002; Davies *et al.,* 2005).

There are two possible stereo-chemical outcomes for the hydrolysis of a glycosidic bond: inversion or retention of anomeric configuration. Both mechanisms involve oxacarbenium-ion-like transition states and a pair of carboxylic acids at the active site (Rye and Withers, 2000).

2.7.1 Inverting mechanism

Inverting glycosidases effect bond cleavage through the action of two carboxylic acid residues (Asp or Glu), typically located on opposite sides of the active site (Zechel and Withers 2000). Of the two carboxylic acids, only one is de-protonated in the enzyme's resting state and acts as a general base, removing a proton from the incoming nucleophile (typically water under the normal glycosidase mechanism) during its attack at the anomeric carbon. The other carboxylic acid acts as a general acid residue, protonating the departing aglycone oxygen atom and assisting in its departure from the anomeric center. The bond-making and bond-breaking steps proceed through a single, concerted oxocarbenium ion-like transition state in which the developing positive charge at the anomeric carbon is partially stabilized by electron donation from the ring oxygen. The truncated sugar product is a hemi-acetal that initially has the opposite configuration at the anomeric center to

that of the starting material; hence, the glycosidase is termed “inverting.” (Rempel and Withers, 2008).

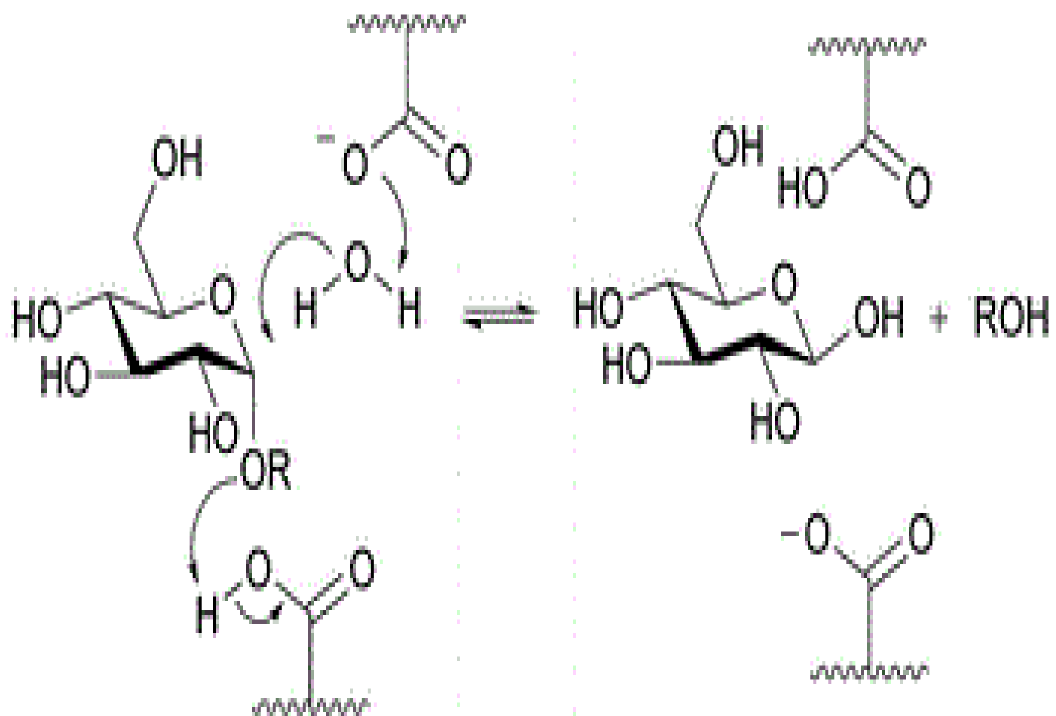


Fig. 2.3 Inverting mechanism of reaction for glycosidase

2.7.2 Retaining mechanism

Most retaining glycosidases as with the inverting glycosidases, also have a pair of essential carboxylic acid residues (Asp or Glu) located on opposite sides of the enzyme active site (Zechel and Withers 2000). One of the residues functions as a general acid in the first mechanistic step by donating a proton during the departure of the aglycone. In the same step, the second, deprotonated carboxylate acts as a nucleophile, attacking the anomeric carbon in a reaction that also proceeds through an oxocarbenium ion-like transition state. This step, referred to as the glycosylation step, leads to the formation of a covalently linked glycosyl-enzyme intermediate that has an anomeric configuration opposite to that of the starting material (Rempel and Withers, 2008).

The second step of this reaction, the deglycosylation step, involves the hydrolytic breakdown of the glycosyl-enzyme intermediate. The carboxylate that first acted as an acid catalyst now acts as a base by abstracting a proton from the incoming nucleophile, normally a water molecule. The water molecule attacks the anomeric center of the sugar, and the carboxylate residue departs via a second oxocarbenium ion-like transition state. The product thus obtained is a hemi-acetal that initially has the same anomeric configuration as the starting material (Rempel and Withers, 2008).

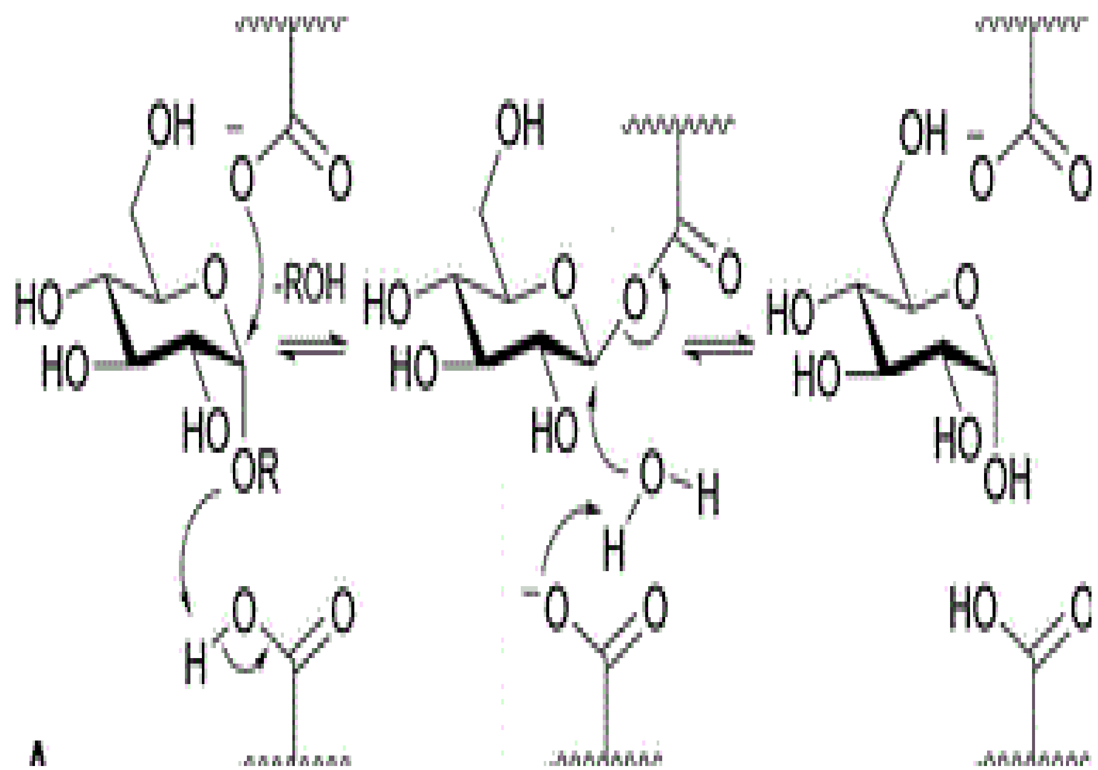


Fig. 2.4 Retaining mechanism of reaction for glycosidase

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Solvents

- Hexane (Loba Chemic, India)
- Ethyl acetate (Sigma Aldrich, USA)
- Methanol (Sigma Aldrich, USA)
- Chloroform (JHD, China)
- Di methyl sulfoxide (DMSO) (SIMAGCHEM Corp., China)
- Distilled water
- Deionised water

3.1.2 Chromatographic adsorbents

- TLC plates (pre-coated silica on aluminium/glass backing) from Merck (Germany)
- Silica gel for column (60-200 mesh) Merck (Germany)

3.1.3 TLC solvents

- Hexane : Ethyl acetate (9:1) and (7:3)
- Chloroform: Methanol (4:1)
- Chloroform: Ethylacetate (9: 1), (4: 1) and (1:1)
- Chloroform: Ethylacetate: Methanol (8:1:1), (5:3:2) and (5:1:3)

3.1.4 TLC spray reagent

- Anisaldehyde spray: alcohol (90ml) + Concentrated H_2SO_4 (5ml) + anisaldehyde (5ml)
- 10% H_2SO_4 in methanol

3.1.5 Extraction/Assay buffer

- 0.15 M sodium phosphate buffer, pH 6.9
- 0.1 M potassium phosphate buffer, pH 6.8

3.1.6 Components of buffer

- Sodium di-hydrogen phosphate monohydrate (NaH_2PO_4)
- Potassium hydrogen phosphate ($KHPO_4$)

3.1.7 Reagents

- 3, 5-dinitrosalicylic acid (DNS)
- Sodium trioxocarbonate IV (Na_2CO_3)

3.1.8 Substrates

- 1% Starch solution
- para-nitropheynyl α-D-glucopyranoside (pNPG)

3.1.9 Machines

- Incubator (Laboratory incubator, series 2000. JP SELECTA, Barcelona, Spain)
- Waterbath (HH-S6 Laboratory double row, six holes digital thermostat, China)

- Spectrophotometer (Microprocessor UV-VIS Spectrophotometer, 2373, Germany)
- Weighing balance (Mettler, AE 260-S, Switzerland)
- Refrigerated centrifuge (LG-25M Ultra high speed cooling centrifuge, China)
- GC-MS machine (GC-MS-QP2010 plus SHIMADZU, England)
- FT-IR machine (FTIR-8400S Fourier transformed infrared spectrophotometer, SHIMADZU, England)
- pH meter (AP71 Acumeter, Singapore)

3.2 Methods

3.2.1 Collection and identification of plant materials

Garcinia kola seeds were purchased from Sabon Gari market at Sabon Gari Local Government Area of Kaduna State in adequate quantity. The seeds were identified at the Herbarium unit, Department of Biological Sciences, Ahmadu Bello University Zaria, Nigeria by Namadi Sunusi. The voucher number 2380 was recorded for further reference.

3.2.2 Preparation of plant material

The purchased seeds of *Garcinia kola* were cut into smaller pieces, after which they were shade-dried for 14 days. They were then pulverized into powder using mechanical grinder.

3.2.3 Preparation of extract from *Garcinia kola* powder

Extraction of the plant material was done using the method described by (Kokate, 2003) with modification. Four hundred and thirty grams of the pulverized plant sample was extracted with n-hexane, ethyl acetate and methanol successively (Fig. 3.1) in a soxhlet apparatus at 50ºC. Extraction with n-hexane was carried out until the solvent became clear to obtain the n-hexane extract. The solvent was then recovered and the exract concentrated and finally dried to a constatnt weight on a rotary evaporator after which it was stored in an air-tight container for subsequent use. The marc was then extracted again with ethyl acetate and methanol solvents, and the extracts treated in the same manner as in n-hexane extract.

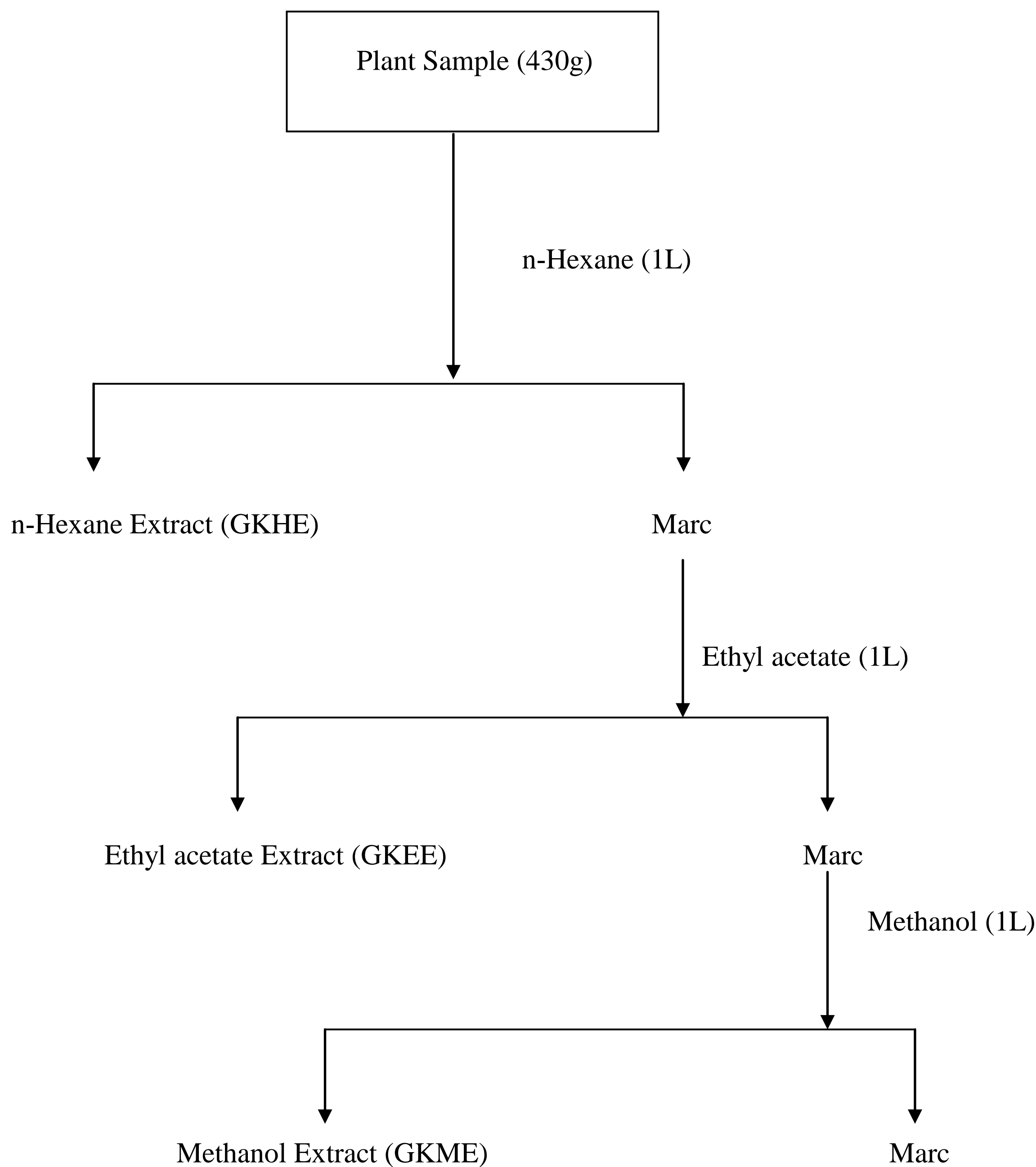


Fig.3.1 Schematic representation of the serial exhaustive extraction of *Garcinia kola* seeds in a Soxhlet apparatus (Kokate, 2003) with modification.

3.2.4 Preliminary phytochemical screening

Crude n-hexane, ethyl acetate and methanol extracts were subjected to preliminary phytochemical screening using standard methods as described below.

3.2.4.1 *Test for steroids/terpenes*

a) Lieberman-Buchard test: a small portion of the sample (extracts) was dissolved in chloroform. Equal volume of acetic anhydride was then added, followed by concentrated sulphuric acid down the side of the test tube. The solution was observed for the presence of a brown ring at the interphase which indicates the presence of steroids/triterpenes (Evans, 2002).

b) Salkowski test: a small quantity of the sample (extracts) was dissolved in 1ml chloroform and to it 1ml of concentrated sulphuric acid was added down the side of the test tube. Formation of red coloration at the interphase was taken as an indication of sterols (Sofowora, 1993).

3.2.4.2 *Test for flavonoids*

a) Shinoda test: A small portion of the sample (extracts) was dissolved in 5ml 95% ethanol, warmed and filtered. Three (3) pieces of magnesium chip were added followed by five drops of concentrated hydrochloric acid. The appearance of a pink, orange or red to purple color was used as an indication for the presence of flavonoids (Evans, 2002).

b) Sodium hydroxide test: A small quantity of the sample (extracts) was dissolved in water and filtered; 2ml of 10% aqueous sodium hydroxide solution was then added. The solution was observed for the presence of

yellow color, a change in color from yellow to colorless on addition of dilute hydrochloric acid was used as an indication for the presence of flavonoids (Evans, 2002).

3.2.4.3 *Test for phenolics compounds*

a) Ferric chloride test: A small portion of the sample (extracts) was stirred with 10 ml distilled water and filtered. Two drops of 1% ferric chloride solution was added to 2 ml of the filtrate. Formation of a blue-black (Hydrolysable/Gallitannins) or green or blue-green (Condensed/Cathehic tannins) precipitate was used as an indication for the presence of phenolic compounds (Evans, 2002).

3.2.4.4 *Test for phlobatannins*

Hydrochloric acid test: A small portion of each plant extract was boiled with 1% aqueous HCl. A red precipitate is expected to be deposited and was taken as evidence for the presence of phlobatannin (Harbourne, 1988).

3.2.4.5 *Test for saponins*

Haemolysis Test: 2 ml of Sodium Chloride (1.8% solution in distilled water) was added to two test tubes A and B. 2 ml of distilled water was added to test tube A and B and 2 ml of extract was added to test tube B. 5 drops of blood was added to each tube and the tubes were inverted gently to mix the contents. Haemolysis in tube B containing the extract but not in tube A (i.e. control), was taken as an indication for the presence of saponins in the extract (Evans, 2002).

3.2.4.6 *Test for alkaloids*

Dragendorff and Wagner test: A small portion of the sample (extracts) was stirred with 5ml of 1% aqueous hydrochloric acid on a water bath and filtered. 3ml of the filtrate was divided into two. To the first portion, few drops of Dragendorff`s reagent was added and observed for formation of orange to brownish coloured precipitate. To the second portion, 1ml of Wagner reagent was added to give a brown or reddish or reddish-brown precipitate (Evans, 2002).

3.2.4.7 *Test for anthraquinones*

a) Free anthraquinones: A small portion of the sample (extracts) was shaken with 10ml of benzene, the content was filtered and 5ml of 10% ammonia solution was added to the filtrate, the mixture was then shaken. Presence of a pink, red or violet colors in the ammoniacal layer (lower phase) was used as an indication for the presence of free anthraquinones (Evans, 2002).
b) Combined anthraquinones: A small portion of the sample (extracts) was boiled with 10ml of aqueous sulphuric acid and filtered hot. The filtrate was then shaken with 5ml benzene, the benzene layer was separated and half of its volume, 10% ammonium hydroxide was added. A pink, red or violet coloration in the ammonia phase (lower phase) was used as an indication for the presence of combined anthraquinone or anthraquinone derivatives (Evans, 2002).

3.2.4.8 *Test for cardiac glycosides*

Keller-Killiani test: A small portion of the sample (Extracts) was dissolved in 1ml of glacial acetic acid containing one drop of ferric chloride solution. This was then under layered with 1ml of concentrated sulphuric acid. A brown ring obtained at the interphase was used as an indication of the presence of a deoxy sugar characteristic of cardenolides (Evans, 1989).

3.2.4.9 *Test for proteins*

Ninhydrin test: To 2ml of plant extract, 1ml of 40% NaOH solution and 2 drops of 1% $CuSO_4$ solution was added. A violet colour was used as an indication of the presence of peptide linkage of the molecule (Harbourne, 1988).

3.2.4.10 *Test for Coumarin*

Flourescence test: A small portion of the sample (5ml) was evaporated to dryness. The residue was dissolved in 2 ml of water by heating. It was then divided in two equal portions. To the non-reference tube, 0.5ml of 10% NH_3 was added and the tubes viewed under UV light. The presence of blue-green fluorescence was used as an indication for the presence of coumarin (Harbourne, 1988).

3.2.5 TLC profile of crude extracts

TLC aluminium sheet of 20×20cm silica gel 60 F_{254} precoated plates using the one way ascending technique was employed for the analysis. The extracts were dissolved in the initial extraction solvent. The plates were cut into size of 5×10cm

and spots were applied manually on the plates using capillary tube after which plates were dried and developed using: Hexane : Ethyl acetate (7:3), Hexane : Chloroform (1:9), Chloroform : Ethyl acetate (9:1), (4:1) and (1:1), Chloroform : Methanol (4:1), Chloroform : Ethyl acetate : Methanol (8:1:1), (6:3:1), (5:3:2) and (5:1:3) as the case maybe in chromatographic tank. Developed plates were sprayed using general detecting reagent (*p*-Anisaldehyde/H_2SO_4, 10% H_2SO_4) and specific detecting reagents: Borntragers, Dragendorf, Ferric chloride, Lieberman-Buchard and aluminium chloride (and viewed under UV, 365 nm) and heated at 110ºC for 2 minutes where applicable. Number of spots, colors and retardation factors (R_f values) for each of the spots were determined and recorded (Gennaro, 2000; Stahl, 2005).

3.3 Biological Activity

3.3.1 Extraction of crude intestinal α-glucosidase

Five Wister rats weighing 180-200 g were procured from the animal house, Department of Pharmacology and Therapeutics, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria. The rats were fasted for a period of twelve hours after which they were sacrificed under chloroform anesthesia. Approval of the Departmental ethical committee for the use of animals for research was obtained before the commencement of the experiment.

Small intestines (9 g) was removed from the rats' gastrointestinal tract and washed with 30ml of 0.9 % sodium chloride solution and placed in ice cold 0.9 % sodium chloride solution. The intestines were later minced with a surgical knife and

homogenized (1:4 w/v) in a mechanical tissue grinder under ice cold condition with 0.1 M potassium phosphate buffer, pH 6.8. After 30 min, the homogenate was centrifuged for 30 min at 10,000 rpm at 4°C. The supernatant was designated as crude enzyme source (Lossow *et al.,* 1964; Kathirvel *et al.,* 2012) with modifications.

3.3.2 Extraction of crude pancreatic α-amylase

Porcine pancreas was obtained from five different adult pigs at a slaughter house in Samaru, Zaria.

The pancreas (8 g) was removed and trimmed free of fat. The tissue was sliced into smaller pieces and homogenized (1:5 w/v) in a mechanical tissue grinder under ice cold condition in 0.02 M sodium phosphate buffer, pH 6.9 containing 0.15 M sodium chloride. The tissue homogenate was then centrifuged at 13,500 rpm for 60min at 4ºC. The supernatant was taken as crude enzyme source (Reddy *et al.,* 1987) with modification.

3.3.3 Inhibitors

Each extract tested was prepared by dissolving 1.09 g of the extract in 4 drops of DMSO. Thereafter 3 ml of the different concentrations of the extracts for the assay was prepared by serial dilution using distilled water as the diluents.

3.3.4 Enzymes

Aliquots (2ml) each of the enzymes extracted were made and stored under ice cold condition until required.

3.3.5 Enzyme Assay

3.3.5.1 *Assay principle for α-glucosidase*

Under specified conditions (pH = 6.8; T = 37 °C), α-glucosidase will catalyze the conversion of the substrate 4-nitrophenyl- α -D-glucopyranoside (PNPG) to α-D-glucopyranoside and p-nitrophenol (PNP), as shown below. The yellow colour of the product (PNP) is then measured spectrophotometrically at 405 nm (Paquin, *et al.,* 1984).

PNPG + α-glucosidase → α-D-glucopyranoside + PNP (yellow)

3.3.5.2 *Assay procedure*

A pipette was used to measure 100 µL of α-glucosidase into triplicate test tubes including a blank with 100 µL distilled water. The test tubes were pre-incubated in a laboratory incubator for 10 mins. Then 50 µL of 3.0 mM pNPG as a substrate was added to start the reaction. The reaction mixture was incubated at 37ºC for 20 mins, stopped by adding 2 ml of 0.1M Na_2CO_3 and mixed by inversion. The α-glucosidase activity was determined by measuring the yellow coloured para-nitrophenol released from pNPG at 405 nm on a spectrophotometer. The enzyme activity, defined as the amount of enzyme that will convert a substrate (S) to product (P) in a specified period of time under conditions of constant temperature and pH was calculated by the equation given below One unit of α-glucosidase was defined as the amount of enzyme that will liberate 1.0 µmole of D-glucose from p-nitrophenyl α-D-glucoside per minute at pH 6.8 at 37°C (Paquin, *et al.,* 1984) with modofication..

$$U/ml\ Enzyme = \Delta A V_1 V_2 / M \square T V_3 V_4 \times df$$

Where:

ΔA = Change in absorbance

V_1 = Volume of reaction mixture

M□ = Millimolar extinction coefficient of p-Nitrophenol at 405 nm

T = Time (in minutes) of the assay

V_2= Volume of Colorimetric Determination

V_3= Volume of reaction mix used in the colorimetric determination

V_4 = Volume of enzyme used

df= dilution factor

3.3.5.3 *Preparation of maltose standard curve*

In numbered test tubes, 10 maltose dilutions ranging from 0.07 mg/ml to 5 mg/ml was prepared including a blank test tube with disttilled water only. Into the corresponding numbered test tubes 1 ml of each dilution of maltose was added, after which 1 ml of 3, 5-dinitrosalicylic acid color reagent was then added. The mixture was then Incubated in boiling water bath for 5 minutes and cooled to room temperature. Then 10 ml of distilled water was added to each tube and mixed well. Absorbance was then taken at 540 nm. The change in absorbance was calculated using the equation below and a standard curve graph was ploted (Worthington, 1993).

$$\Delta A_{standard} = A_{540} \text{ of standard (maltose)} - A_{540} \text{ of standard blank}$$

3.3.5.4 *Assay principle for α-amylase*

Under specified conditions (pH = 6.9; T = 25°C), α-amylase will catalyze the conversion of the substrate soluble starch to a reducing group (Maltose) wherein the

reducing groups released from the starch are measured by the reduction of 3,5-dinitrosalicylic acid (Bernfield, 1955).

Starch + H_2O + α-amylase ⟶ Maltose + 3, 5-dinitrosalicylic acid

3.3.5.5 ***Assay procedure***

A pipette was used to measure 0.5 ml of crude enzyme (α-amylase) into triplicate test tubes, including a blank with 0.5 ml distilled water. The tubes were incubated at 25ºC in a laboratory incubator for 3 min to achieve temperature equilibration. At timed intervals of 3 min, 0.5 ml starch solution was added and incubated for another 3 min. after which 1 ml of 3, 5-dinitrosalicylic acid was added to each tube. The tubes were then further incubated in a boiling water bath for 5 min. They were cooled to room temperature and 10 ml of distilled water was added and mixed by inversion. The absorbance of the content of each test tube was taken using a spectrophotometer. The enzyme activity, defined as the amount of enzyme that will convert a substrate (S) to product (P) in a specified period of time under conditions of constant temperature and pH was calculated by the equation given below. One unit of α-amylase was defined as the amount of enzyme that releases from soluble starch one micromole of reducing groups per minute at 25°C and pH 6.9 under the specified conditions (Bernfiel, 1955).

U/ml Enzyme = mg of maltose released/volume (ml) of enzyme used

3.3.5.6 *α-glucosidase inhibition assay*

α-glucosidase activity was determined according to the method described by Kim *et al*. (2005). Briefly, 100 µL of α-glucosidase was pre-incubated with 50 µL of the different concentration of the extracts and a control (distilled water) for 10 mins. Then 50 µL of 3.0 mM pNPG as a substrate dissolved in 0.1 M phosphate buffer (pH 6.8) was then added to start the reaction. The reaction mixture was incubated at 37ºC for 20 mins and stopped by adding 2 ml of 0.1 M Na_2CO_3. The α-glucosidase activity was determined by measuring the yellow coloured para-nitrophenol released from pNPG at 405 nm. The results were expressed as percentage inhibition. Concentration of extracts resulting in 50 % inhibition of enzyme activity (IC_{50}) was determined graphically using the least squares curve fits.

$$\% \text{ Inhibition} = [(A_{Control} - A_{Samples})/A_{Control}] \times 100$$

3.3.5.7 *α-amylase inhibition assay*

The assay was carried out using the modified procedure of McCue and Shetty (2004). A total of 250 µL of different concentrations of the extracts and a control (distilled water) were placed in a tubes and 250 µL of 0.02 M sodium phosphate buffer (pH 6.9) containing α-amylase solution was added. This solutions were pre-incubated at 25ºC for 10 mins, after which 250 µL of 1 % starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added at timed intervals of 2 min and then further incubated at 25°C for 10 mins. The reaction was terminated after incubation by adding 500 µL of 3, 5 dinitrosalicylic acid reagent. The tubes were then incubated in boiling water for 5 mins and cooled to room temperature. The reaction mixture

was diluted with 5ml distilled water and the absorbance measured at 540 nm using spectrophotometer. A control was prepared using the same procedure replacing the extract with distilled water. The α-amylase inhibitory activity was calculated as percentage inhibition. Concentration of extracts resulting in 50% inhibition of enzyme activity (IC_{50}) was determined graphically using the least squares curve fits.

$$\% \text{ Inhibition} = [(A_{Control} - A_{Samples})/A_{Control}] \times 100$$

3.4 Column Chromatographic

3.4.1 Development of solvent system

The extract with the lowest IC_{50} (ethyl acetate extract) was subjected to TLC to determine suitable solvent system for column chromatography. The extract was developed in hexane (100%) hexane: ethyl acetate (7:3), chloroform (100%) and Chloroform: Ethyl acetate (9:1). The plates were sprayed with *p*-anisaldehyde/sulphuric acid as detecting reagent followed by heating at 110ºC. Chromatograms were scanned accordingly.

3.4.2 Silica-gel column chromatography of ethyl acetate extract

A glass tube, 50 cm high with a diameter of 50 mm was used for column chromatography. The adsorbent, silica gel (100 g, 60-120 µm) was carefully packed using wet slurry method to about 25 cm in the glass tube. The extract, 3 g was loaded on to packed adsorbent and allowed to stabilize for 3 hours before elution started. Chloroform (100%) was used as initial eluent followed by addition of ethyl acetate

gradient wise. Volumes of 20 ml each were collected per fraction and allowed to concentrate under room temperature. The fractions collected were monitored on TLC plates and visualized with 10% H_2SO_4/methanol as spraying reagent. Similar fractions were pooled and coded (Tomer *et al.,* 2009; Patra *et al.,* 2012).

3.4.3 Isolation of compound ZAAK

Fractions 30-36 obtained from column chromatography, coded B weighing 213mg was purified by preparative TLC (PTLC) developed in Chloroform: Ethyl acetate (9:1) and Hexane: Ethyl acetate (7:3). Developed plates were scraped, washed and observed on TLC (Merck F_{254}) for the appearance of a single spot which was then coded ZAAK.

3.5 Physico-Chemical Studies

3.5.1 Melting point determination for ZAAK

The melting point of isolated compound was determined using Gallenkamp melting point apparatus and the melting point was recorded from the thermometer when the sample melts.

3.5.2 Chemical tests

The isolate, ZAAK was spotted on TLC plates and developed in Chloroform: Ethyl acetate (9:1). Developed plates were visualized with Lieberman-Buchard and Ferric chloride spray reagents at 110ºC were applicable.

3.6 Structural Elucidation of ZAAK

3.6.1 Fourier-Transformed Infrared Spectroscopy of ZAAK

The isolate ZAAK was mixed with 5 mg of KBr and ground to a very fine powder. The powder was compressed under high pressure using a press to produce pellets of the compound to be analyzed. The pellets were then analyzed on FTIR-8400S Fourier transform infrared spectrophotometer. The bands were compared with those reported in literatures.

3.6.2 Gas Chromatography-Mass Spectrophotometry of ZAAK

Gas Chromatography-Mass Spectrometry (GC-MS) was carried out on the isolate ZAAK on a GC-MS-QP2010 plus SHIMADZU with SGE BPX5 column (30 m × 0.25 mm, I.D × 0.25 μm) at the Basic Reasearch Department, National Research Institute for Chemical Technology (NARICT), Zaria, Kaduna State, Nigeria. Oven temperature was at 80°C to 280°C at 30°C/min. Injection temperature was set at 250°C using a split mode. Helium gas (99.9%) was used as the carrier. The flow rate of helium gas was 1.58 ml/min with a total GC-MS running time of 27 minutes. The ionization mass spectroscopic analysis was done with 70 eV. Mass spectra were recorded across the range of 40 to 600 m/z for the duration of 28 min. Peak areas were compared with the database in the GC-MS library version NIST 05-S and reported literatures.

3.7 Statistical analysis

Results were expressed as mean ± standard errors of the mean for all values. SPSS was used for the Analysis of Variance (ANOVA) to determine variation or difference between extracts and among treatments. Duncan Multiple Range Test was used to compare the means.

CHAPTER FOUR

4.0 RESULTS

4.1 Percentage Yield

The yield of the powdered seeds of *G. kola* extracted with n-hexane, ethyl acetate and methanol solvents is presented in the table 4.1 below.

Table 4.1 Percentage Yield for the Crude Extracts of *G. kola*

S/No.	Crude Extracts	Yield (g)	% Yield (w/w)	Colour of Extract
1.	GKHE	8.10	1.88	Brown
2.	GKEE	37.77	8.78	Black
3.	GKME	44.42	10.33	Brownish-Black

Key:

GKHE= *G. kola* Hexane Extract

GKEE= *G. kola* Ethyl acetate Extract

GKME= *G. kola* Methanol Extract

4.2 Preliminary Phytochemical Screening

The result of the preliminary phytochemical screening on the crude n-hexane, ethyl acetate and methanol extracts using standard methods are as shown in table 4.2

Table 4.2 Preliminary Phytochemical Screening for the Crude Extracts from *G. kola* Seeds

Phytochemicals	GKHE	GKEE	GKME
Steroids/Triterpenes	+	+	+
Phenolic Compounds	+	+	+
Phlobatannin	−	+	−
Flavonoids	−	+	+
Saponin	−	−	+
Alkaloids	+	+	+
Anthraquinones	−	−	−
Glycoside	−	+	+
Protein	−	−	−
Coumarin	−	+	+

Key:

GKHE= *G. kola* Hexane Extract,

GKEE= *G. kola* Ethyl acetate Extract,

GKME= *G. kola* Methanol Extract,

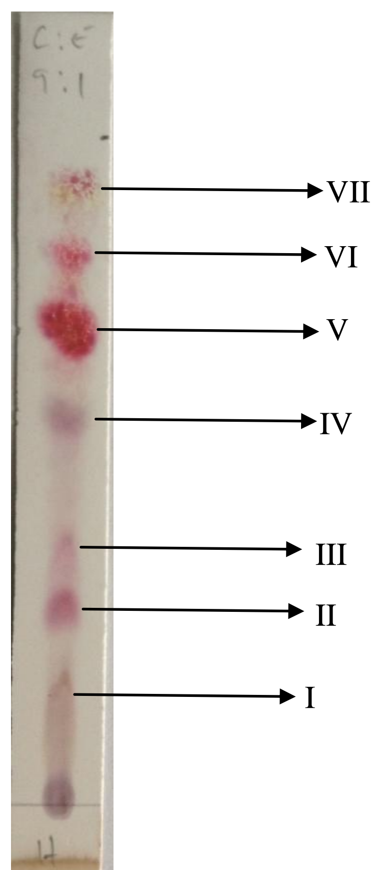
+ = Present,

− =Absent

4.3 TLC profile of crude extracts

4.3.1 TLC profile of *Garcinia kola* hexane extract

Garcinia kola Hexane Extract (GKHE) was noted to have eleven (11) spots in Hexane: Ethyl acetate (H:E) (9:1), six (6) spots in H: E (7:3). Seven (7) spots were observed in Chloroform: Ethyl acetate (9:1) (Plate II). TLC of GKHE developed in C: E (9:1) sprayed with Lieberman-Buchard, Ferric chloride, Bontragers, Aluminium chloride and Dragendorff`s reagents showed various spots: Brown, blue, pink, yellow, black and yellow fluorescence spots for Lieberman-Buchard, Ferric chloride and Aluminium chloride spray reagents (Plate III). No coloured sports were observed for TLC plates sprayed with Bontrager`s and Dragendorff`s reagents (Plates not shown).



A

Plate II: TLC profile of *G. kola* seed n-hexane extract in Chloroform:Ethyl acetate (9:1) sprayed with *p*-Anisaldehyde/H_2SO_4

(A) Chromatogram of *G. kola* seed n-hexane extract on precoated silica plate developed in C: E (9:1) sprayed with *p*-Anisaldehyde/H_2SO_4. Showed 7 spots with R_f values for 1-VII as 0.19, 0.31, 0.40, 0.60, 0.75, 0.84, 0.93 respectively.

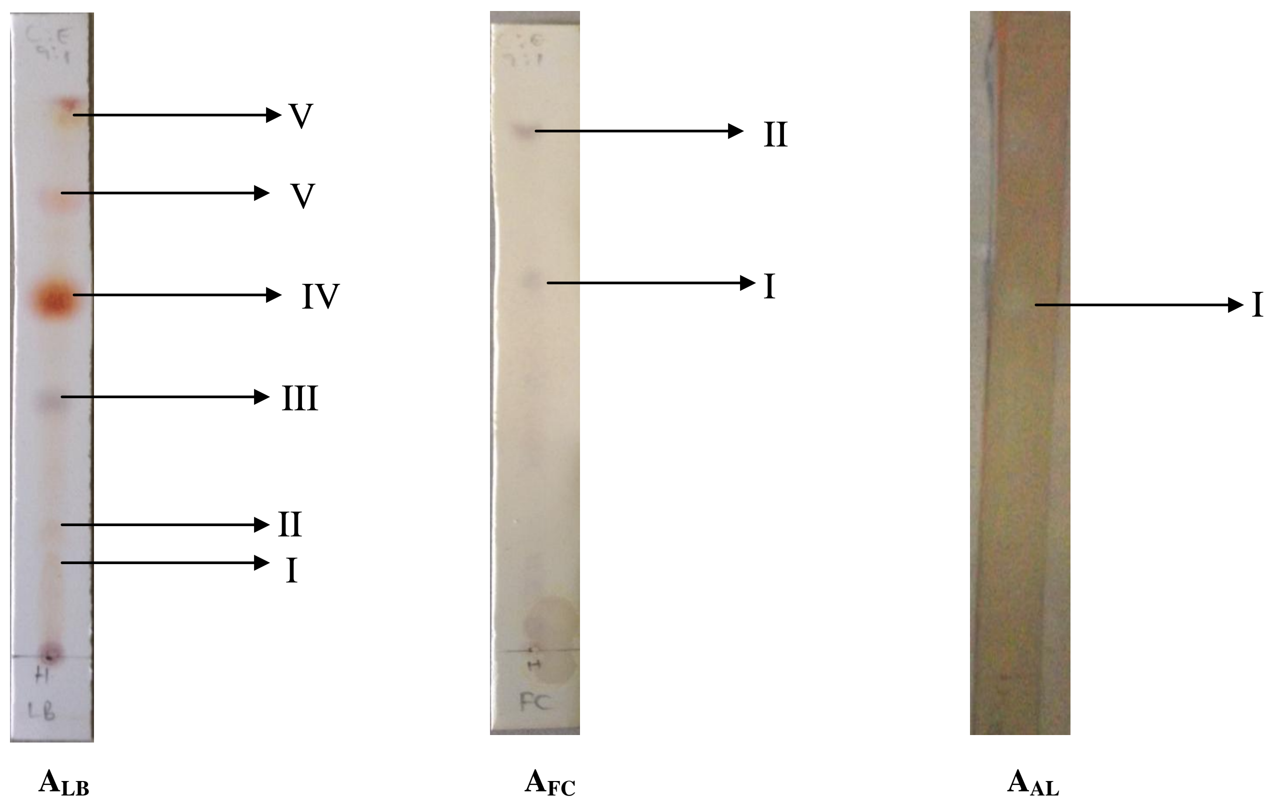


Plate III: TLC profile of *G. kola* seed n-hexane extract in Chloroform:Ethyl acetate (9:1) sprayed with specific detecting reagent

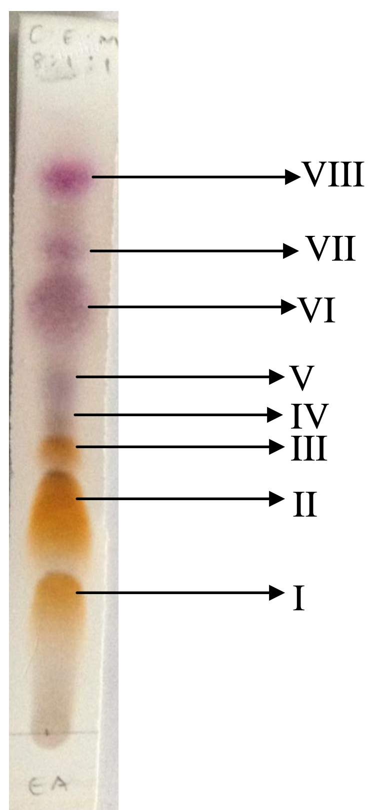
(A_{LB}) Chromatogram of *G. kola* seed n-hexane extract on precoated silica plate developed in C: E (9:1) sprayed with Lieberman-Buchard reagent and heated at 110ºC for about 2 minutes. Showed 6 coloured spots with R_f values for I-VI as 0.19, 0.31, 0.60, 0.75, 0.84, 0.93 respectively.

(A_{FC}) Chromatogram of *G. kola* seed n-hexane extract on precoated silica plate developed in C: E (9:1) sprayed with Ferric chloride reagent. Showed 2 coloured spots with R_f values for 1-II as 0.75 and 0.93.

(A_{AL}) Chromatogram of *G. kola* seed n-hexane extract on precoated silica plate developed in C: E (9:1) sprayed with Aluminium chloride reagent. Showed 1 yellow flourescence spot with R_f values for I-II as 0.75 and 0.93.

4.3.2 TLC profile of *Garcinia kola* ethyl acetate extract

Garcinia kola Ethyl actate Extract (GKEE) was noted to have three (3) spots in Hexane: Ethyl acetate (H:E) (7:3), five (5) spots in Chloroform: Ethyl acetate (C:E) (4: 1), six (6) spots in C:E (1:1). Eight (8) spots were observed in Chloroform: Ethyl acetate: Methanol (8:1:1) (Plate IV). TLC of GKEE developed in Chloroform: Ethyl acetate: Methanol (8:1:1) sprayed with Lieberman-Buchard, Ferric chloride, Bontragers, Aluminium chloride and Dragendorff`s reagents showed various spots: brown, pink, black and yellow fluorescence spots for Lieberman-Buchard, Ferric chloride and Aluminium chloride spray reagents (Plate V). No coloured spots were observed for TLC plates sprayed with Bontrager`s and Dragendorf`s reagents (Plates not shown).



B

Plate IV: TLC profile of *G. kola* seed ethyl acetate extract in Chloroform:Ethyl acetate:Methanol (8:1:1) sprayed with *p*-Anisaldehyde/H_2SO_4

(B) Chromatogram of *G. kola* seed ethyl acetate extract on precoated silica plate developed in C:E:M (8:1:1) sprayed with *p*-Anisaldehyde/H_2SO_4. Showed 8 spots with R_f values for I-VIII as 0.23, 0.40, 0.45, 0.50, 0.55, 0.70, 0.76 and 0.88 respectively.

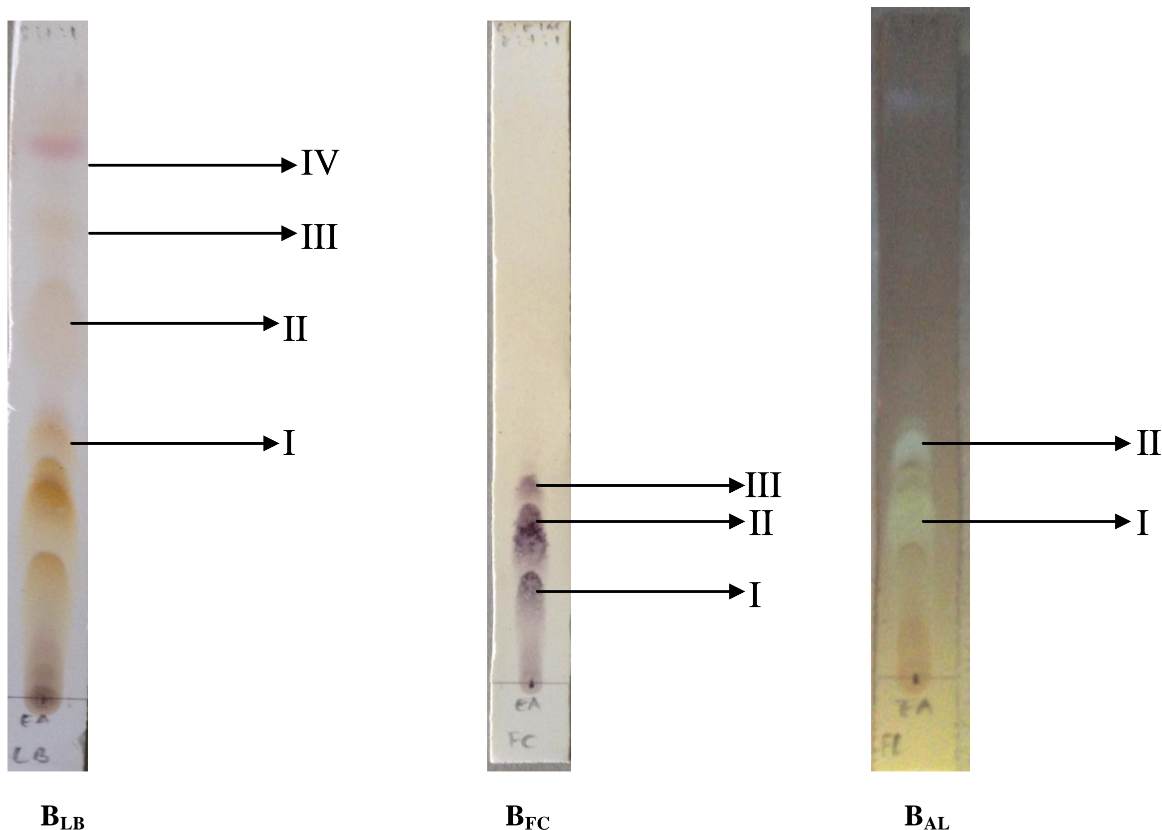


Plate V: TLC profile of *G. kola* seed ethyl acetate extract in Chloroform:Ethyl acetate:Methanol (8:1:1) sprayed with specific reagents

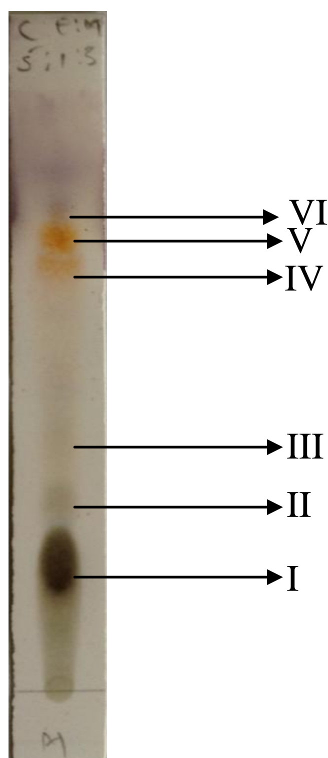
(B_{LB}) Chromatogram of *G. kola* seed ethyl acetate extract on precoated silica plate developed in C:E:M (8:1:1) sprayed with Lieberman-Buchard reagent and heated at 110ºC for 2 minutes. Showed 4 coloured spots with R_f values for I-IV as 0.50, 0.70, 0.76 and 0.88 respectively.

(B_{FC}) Chromatogram of *G. kola* seed ethyl acetate extract on precoated silica plate developed in C:E:M (8:1:1) sprayed with Ferric chloride reagent. Showed 3 coloured spots with R_f values for I-III as 0.23, 0.40 and 0.45 respectively.

(B_{AL}) Chromatogram of *G. kola* seed ethyl acetate extract on precoated silica plate developed in C:E:M (8:1:1) sprayed with Aluminium chloride reagent. Showed 3 flourescens spots with R_f values for I-II as 0.40 and 0.45.

4.3.3 TLC profile of *Garcinia kola* methanol extract

Garcinia kola Methanol Extract (GKME) was noted to have four (4) spots in Chloroform: Ethyl acetate: Methanol (C:E:M) (6:3:1), six (6) spots in C:E:M (5:3:2), five (5) spots in C:E:M (5:1:3) (Plate VI). TLC of GKME developed in Chloroform: Ethyl acetate: Methanol (5:1:3) visualized with Lieberman-Buchard, Ferric chloride, Bontragers, Aluminium trichloride and Dragendorff`s reagents showed various spots: blue, yellow, pink, black and yellow fluorescence bands for Lieberman-Buchard, Ferric chloride and Aluminium chloride spray reagents (Plate VII). No coloured spots were observed for TLC plates sprayed with Bontrager`s and Dragendorff`s reagents (Plates not shown).



C

Plate VI: TLC profile of *G. kola* seed methanol extract in Chloroform:Ethyl acetate:Methanol (5:1:3) sprayed with *p*-Anisaldehyde/H_2SO_4

(C) Chromatogram of *G. kola* seed methanol extract on precoated silica plate developed in C:E:M (5:1:3) sprayed with *p*-Anisaldehyde/H_2SO_4. Showed 6 spots with R_f values for I-V as 0.27, 0.34, 0.71, 0.78 and 0.88 respectively.

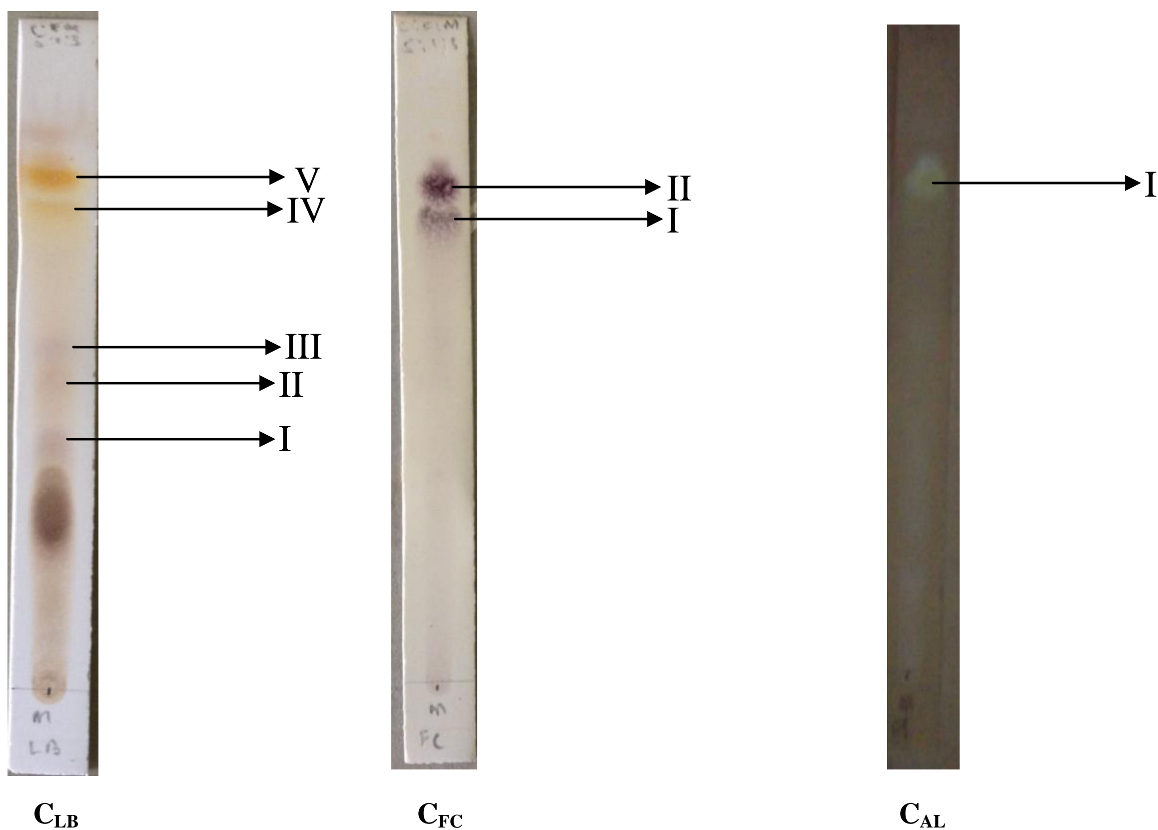


Plate VII: TLC profile of *G. kola* seed methanol extract in Chloroform:Ethyl acetate:Methanol (5:1:3) sprayed with specific reagents

(C_{LB}) Chromatogram of *G. kola* seed methanol extract on precoated silica plate developed in C:E:M (5:1:3) sprayed with Lieberman-Buchard reagent. Showed 5 spots with R_f values for I-V as 0.55, 0.61, 0.83, 0.89 and 0.97 respectively.

(C_{FC}) Chromatogram of *G. kola* seed methanol extract on precoated silica plate developed in C:E:M (5:1:3) sprayed with Ferric chloride reagent. Showed 2 spots with R_f values for I-II as 0.71 and 0.78.

(C_{AL}) Chromatogram of *G. kola* seed methanol extract on precoated silica plate developed in C:E:M (5:1:3) sprayed with Aluminium chloride reagent. Showed 1 flourescence spot with R_f values for I as 0.78.

4.4 Crude Enzyme Yield

Porcine pancrease (8 g) and 9 g of rat intestine used for the enzymes extraction gave 40 ml and 35 ml yield of crude enzymes. Crude α-amylase was reddish in color while crude intestinal α-glucosidase was faint green.

4.5 Crude Enzyme Activity

Each of the crude enzymes source was assayed for activity. The results are as shown in table 4.3.

Table 4.3 Crude Enzyme Activity for α-amylase and α-glucosidase

S/No.	Crude Enzymes	Enzyme activity (U/ml)
1.	α-amylase	2.93 ± 0.41
2.	α-glucosidase	0.0037 ± 0.0003

Values represent means and SD of triplicate tests

4.6 Inhibitory Effect of *G. kola* Extracts on α-Amylase

n-Hexane extract inhibited α-amylase activity in a biphasic concentration-response manner following a bell shaped curve inhibitory activity evident from visual examination of the experimental data. The maximum inhibitory effect, 61.73 % was obtained at a concentration of 2.5 mg/ml, while the least inhibitory effect, 19.66 % was observed at 15 mg/ml (Fig. 4.1). Lower and higher concentrations led to adecrease ininhibition activity of n-hexane extract. An IC_{50} value of 4.89mg/ml was obtained for n-Hexane extract (Table 4.4).

Ethyl acetate extract had the highest percentage inhibitory effect, 37.4 5% at a concentration of 2.5 mg/ml. The least inhibitory effect, 4.89 % was recorded at 15 mg/ml of the extract (Fig. 4.1). The pattern of inhibition was neither concentration dependent nor concentration inverse dependent. The IC_{50} value was calculated to be 3.44 mg/ml (Table 4.4).

The highest inhibitory effect (46.82 %) for methanol extract was observed at 0.32 mg/ml followed by 33.79 % at 2.5 mg/ml, while the lowest inhibitory effect, 4.69 % was observed at 5mg/ml. No inhibition (NI) was observed at 10 mg/ml and 15 mg/ml concentrations of the extract (Fig. 4.1). The IC_{50} value, 0.78 mg/ml was obtained for the same extract (Table 4.4).

Percentage inhibition by the different extracts revealed n-hexane extract to have the highest % inhibition, while ethyl acetate had the least % inhibition (Table 4.5). Percentage inihibition of α-amylase by combined extracts showed that at 2.5 mg/ml *G. kola* seeds had the highest inhibitory effect of 44.32 % and the least inhibitory effect of 2.71 % at 10 mg/ml (Table 4.6).

Table 4.4 IC_{50} (mg/ml) Values for α-amylase Treated with Different Extracts

S/No.	Extracts	α-amylase IC_{50}
1.	n-Hexane	4.89±4.62
2.	Ethyl acetate	3.44±3.46
3.	Methanol	0.78±0.32

Values are expressed as means and SD of triplicate tests (n=3)

Table 4.5 Percentage Inhibition of α-amylase by Different Extracts

S/No.	Extracts (mg/ml)	% inhibition Means
1.	n-Hexane (4.89)	27.92
2.	Ethyl acetate (3.44)	16.42
3.	Methanol (0.78)	26.35

Values are expressed as means of concentration treatments and % inhibition means of triplicate tests (n=3).

Table 4.6 Percentage Inhibition of α-amylase by Different Concentrations of Combined Extracts

S/No.	Concentrations of extracts (mg/ml)	% inhibition Means
1.	15.00	18.76
2.	10.00	2.71
3.	5.00	15.87
4.	2.50	44.32
5.	1.25	24.01
6.	0.63	19.07
7.	0.32	30.56

Values are expressed as % inhibition means of triplicate tests (n=3) for all extract combined in the experiment.

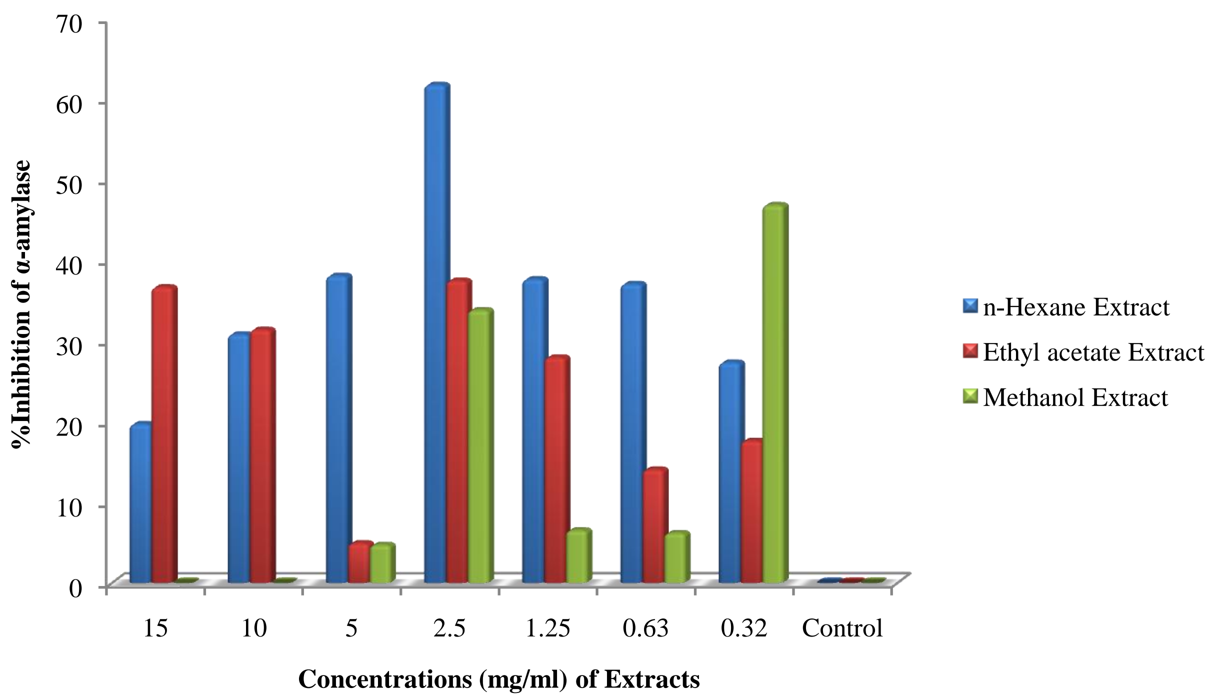


Fig. 4.1 Inhibitory effect of crude extracts from *G. kola* on α-amylase

Values are expressed as % means of triplicate tests (n=3)

4.7 Inhibitory Effect of *G. kola* Extracts on α-Glucosidase

n-Hexane and methanol extracts inhibited α-glucosidase in a concentration-dependent manner (Fig. 4.2). Only methanol extract showed direct concentration inhibition while n-hexane extract showed inverse concentration dependent inhibitory activity. The IC_{50} value for n-hexane extract was calculated to be 10.29 mg/ml and 2.67 mg/ml for methnol extracts (Table 4.7).

Ethyl acetate extract showed a biphasic (*U*-shaped) concentration response pattern for the inhibition of α-glucosidase activity. At 2.5 mg/ml, the highest inhibitory effect of 49.11 % and a least inhibitory effect of 9.80 % at 0.63 mg/ml were observed in ethyl acetate extract (Fig 4.2). Lower and higher concentrations led to a decrease in inhibitory activity of the extract. The extract had an IC_{50} value of 1.68 mg/ml (Table 4.7).

Percentage inhibition of α-glucosidase by the different extracts revealed methanol extract to have the highest % inhibition, while ethyl acetate had the least % inhibition (Table 4.8). Percentage inihibition of α-glucosidase by different concentrations of combined extracts showed that at 5 mg/ml *G. kola* seeds had the highest inhibitory effect of 42.28 % and the least inhibitory effect of 29.54 % at 1.25 mg/ml (Table 4.9).

Table 4.7 IC_{50} (mg/ml) Values for α-glucosidase Treated with Different Extracts

S/No.	Extracts	α-glucosidase IC_{50}
1.	n-Hexane	10.29±4.08
2.	Ethyl acetate	1.68±1.27
3.	Methanol	2.67±0.74

Values are expressed as means and SD of triplicate tests (n=3)

Table 4.8 Percentage Inhibition of α-glucosidase Using Different Extracts

S/No.	Extracts (mg/ml)	% inhibition Means
1.	n-Hexane (10.29)	15.32
2.	Ethyl acetate (1.68)	14.05
3.	Methanol (2.67)	30.38

Values are expressed as means of concentration treatments and % inhibition means of triplicate tests (n=3).

Table 4.9 Table 4.6 Percentage Inhibition of α-glucosidase by Different Concentrations of Combined Extracts

S/No.	Concentrations of extracts (mg/ml)	% inhibition Means
1.	10.00	35.02
2.	5.00	42.28
3.	2.50	41.48
4.	1.25	29.54
5.	0.63	30.65

Values are expressed as % inhibition means of triplicate tests (n=3) for all extract combined in the experiment.

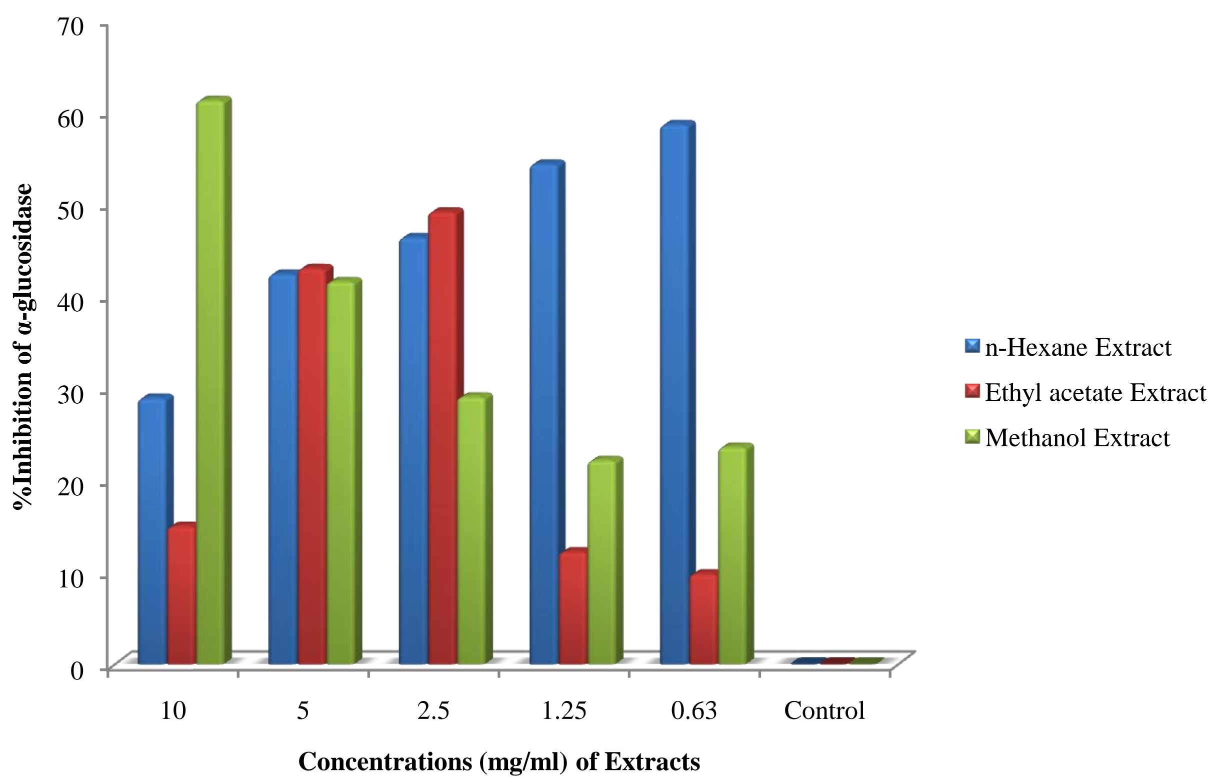


Fig. 4.2 Inhibitory effects of crude extracts from *G. kola* on α-glucosidase

Values are expressed as %means of triplicate tests (n=3)

4.8 Column Chromatography of *G. kola* Ethyl acetate Extract

Column chromatography of *G. kola* ethyl acetate extract (GKEE) yielded 82 fractions of 20 ml each which were pooled together based on their TLC profiles to obtain six (6) combined fractions coded (A-F) (Appendix K). Fraction B (142mg) was purified by preparative TLC (PTLC). A coloreless amorphous substance weighing 23mg after PTLC soluble in chloroform and ethyl acetate was obtained and labeled ZAAK. The TLC profile of ZAAK developed in chloroform: Ethyl acetate (9:1) visualized with *p*-Anisaldehyde/H_2SO_4 and heated at 110ºC for 2 minutes gave single spot with R_f = 0.72 (Plate VIII).

D

Plate VIII: Chromatogram of compound ZAAK developed in C:E (9:1) visualized with *p*-Anisaldehyde/H_2SO_4.

(D) Chromatogram of compound ZAAK developed in C:E (9:1) visualized with *p*-Anisaldehyde/ H_2SO_4. Showed a single spot/band with R_f =0.72.

4.9 Physico-Chemical Studies on ZAAK

The isolate, ZAAK had a melting point of 47ºC − 54ºC and tested negative to Ferric chloride and Lieberman-Buchard spray reagents.

4.10 Fourier Transformed-InfraRed (FT-IR) Spectroscopy

The FT-IR spectra of the isolated compound ZAAK revealed a total of 14 major bands (KBr), (Fig. 4.3). Absorbance was observed at 3424.73 cm^{-1} indicating a hydroxyl (-OH) stretching vibration. Bands at 2933.83 cm^{-1} and 2872.10 cm^{-1} were indicative of C-H strectching vibrations occurring in the molecule.

Frequencies corresponding to carbonyl (C=O) stretching vibrations were recorded at 1731.17 cm^{-1} and 1608.69 cm^{-1}. The C-O strectching vibrations were observed at bands 1040.63 cm^{-1} and 1175.65 cm^{-1}.

While vibrations corresponding to –OH bending (914.29 cm^{-1}) and C-H bending (1452.45 cm^{-1}, 1373.36 cm^{-1} and 740.69 cm^{-1}) were observed in the molecule of compound ZAAK.

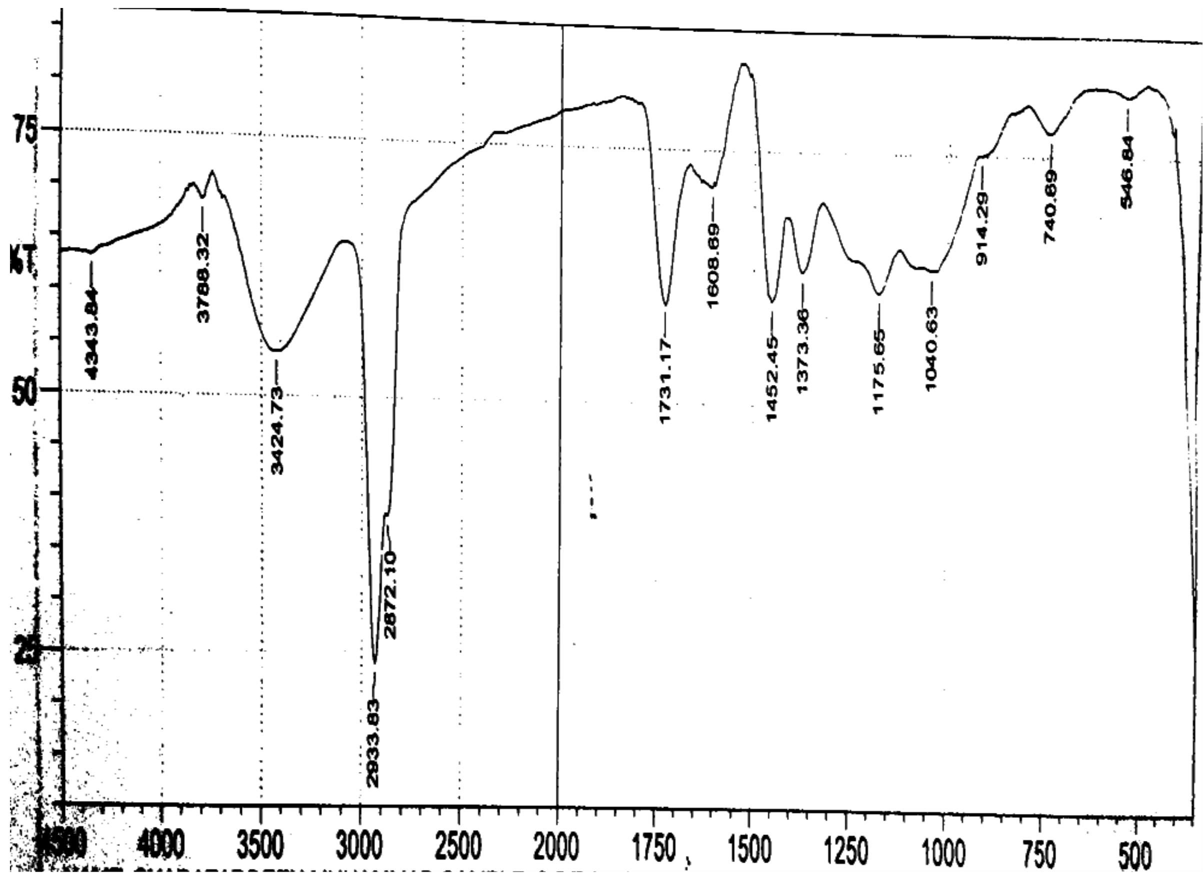


Fig. 4.3: FourierTransformed Infrared spectra of compound ZAAK

4.11 Gas Chromatography-Mass Spectrophotometry

Total Ion Chromatogram (TIC) of the isolate, ZAAK revealed three major peaks corresponding to peaks 8, 12 and 13 at retention time (t_R) 17.99, 20.83 and 21.08 minutes (Fig. 4.4). These peaks were subsequently coded $ZAAK_1$, $ZAAK_2$ and $ZAAK_3$ respectively.

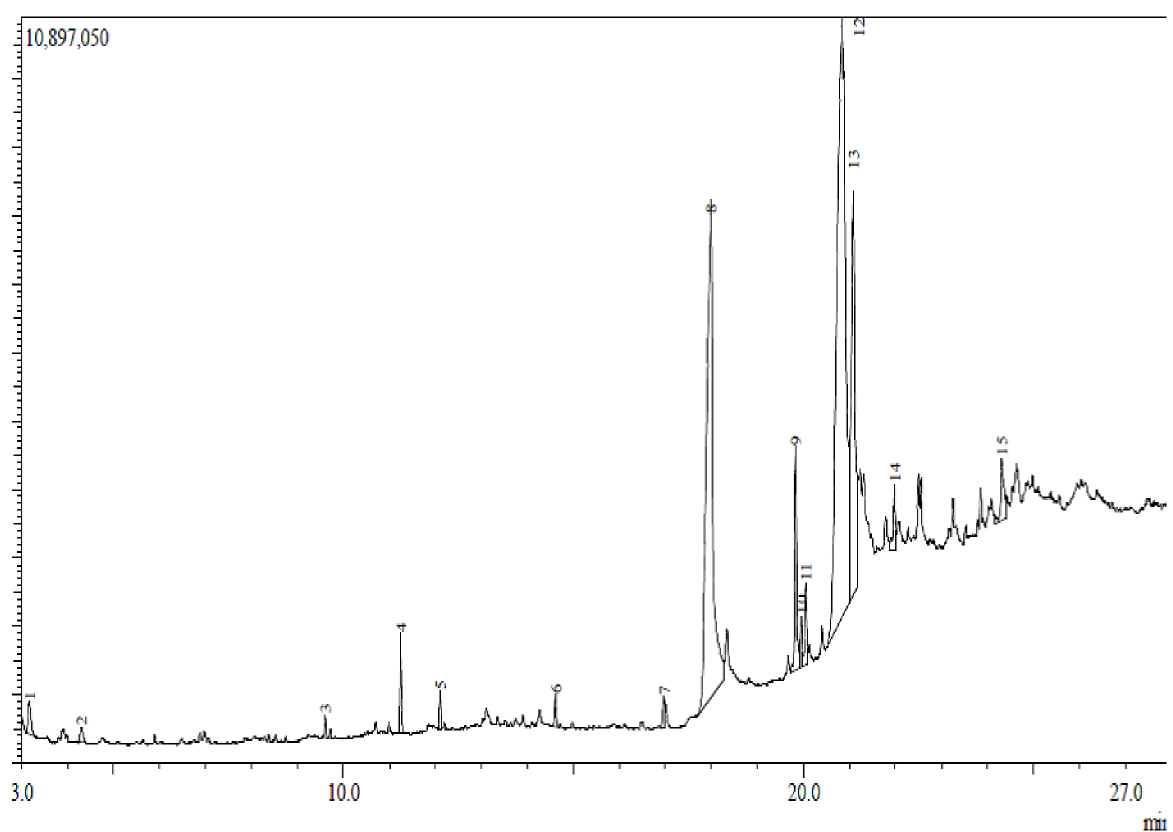


Fig. 4.4: Total Ion Chromatogram of ZAAK

The mass spectrum of $ZAAK_1$ showed a molecular ion peak $[M]^{+\bullet}$ at m/z 256. Prominent fragment ions were observed at m/z 41, 55, 57, 60 and 73 with m/z 43 having the highest relative abundance being the base peak. Characteristic peaks of other fragment ions at m/z 59, 71, 85, 99, 113, 115, 127, 129, 143,157, 171, 185, 199, 213 and 227 were also observed. Several other ion peaks were also observed (Fig. 4.5). $ZAAK_1$ contributed 27.09 % to the TIC. Schematic representation of the fragmentation pattern of $ZAAK_1$ is shown in (Fig. 4.6).

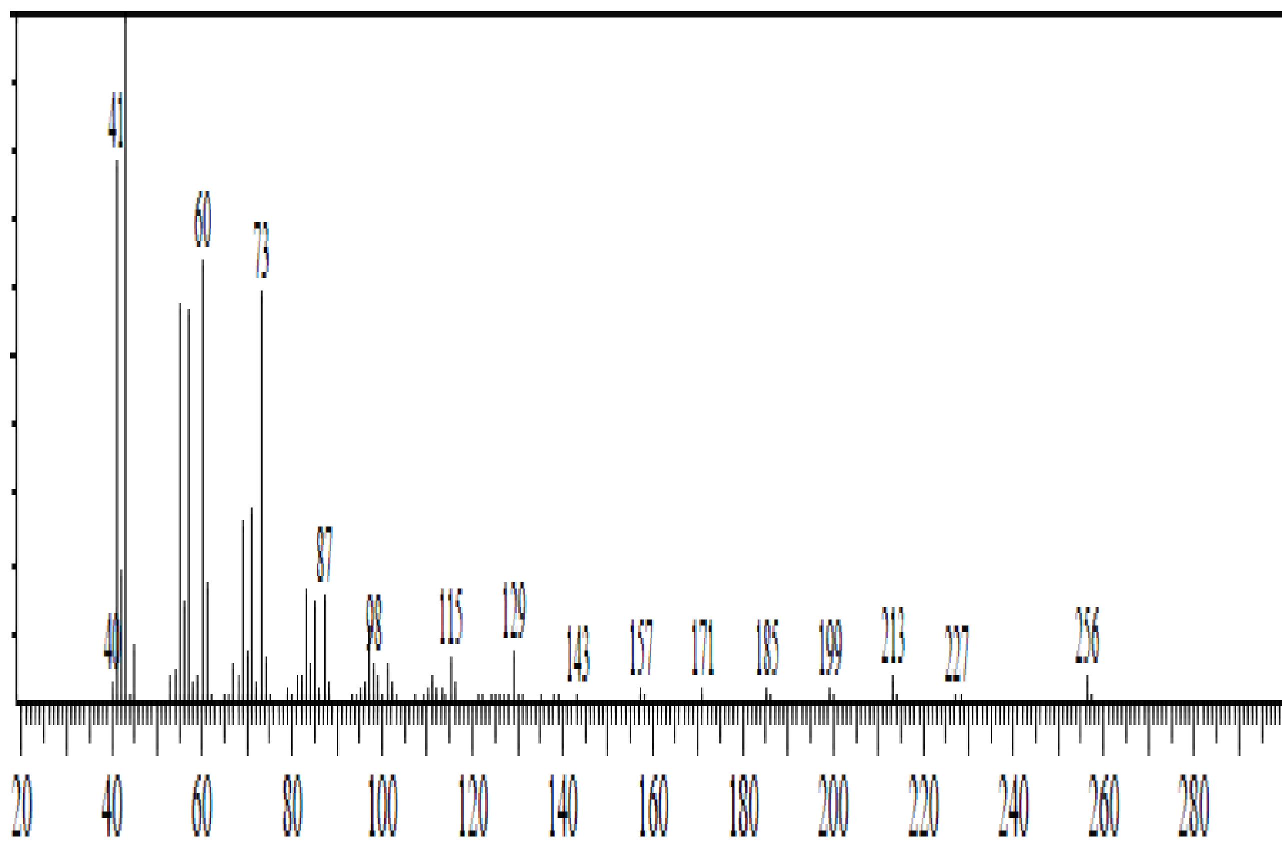


Fig. 4.5: Mass spectra of $ZAAK_1$

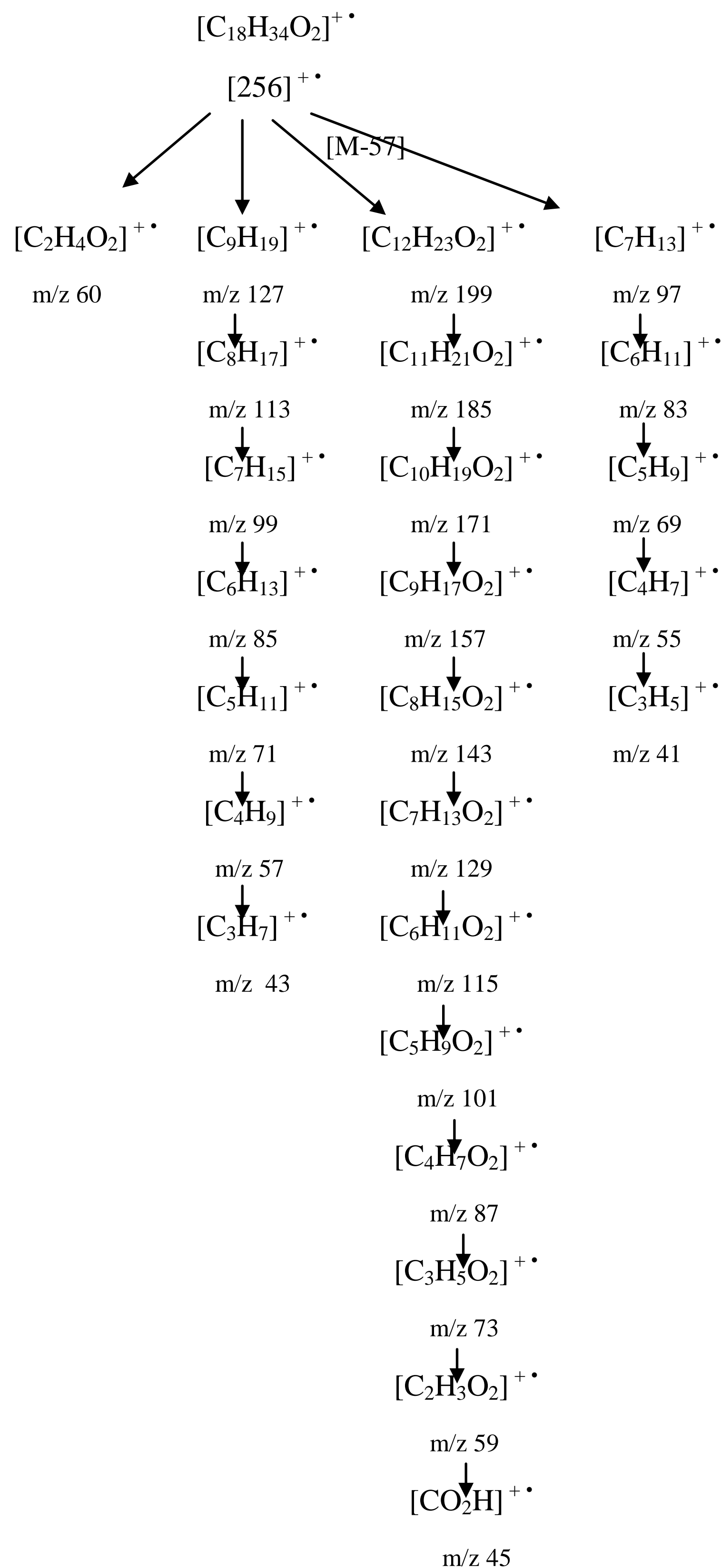


Fig.4.6 Schematic representation of some fragmentation patterns of $ZAAK_1$

Mass spectra analysis of $ZAAK_2$ revealed a molecular ion peak $[M]^{+\bullet}$ at m/z 282. Major peaks at m/z 41, 43, 67, 69, 83 and 97 were observed. The base peak was at m/z 55 with other fragment ions at m/z 125, 111, 85, 73, 71, 70, 59, 57 and 45 (Fig. 4.7). The percentage area peak was recorded as 45.33 % which was the highest among the peaks. Schematic representation of some fragmentation pattern is shown in (Fig. 4.8).

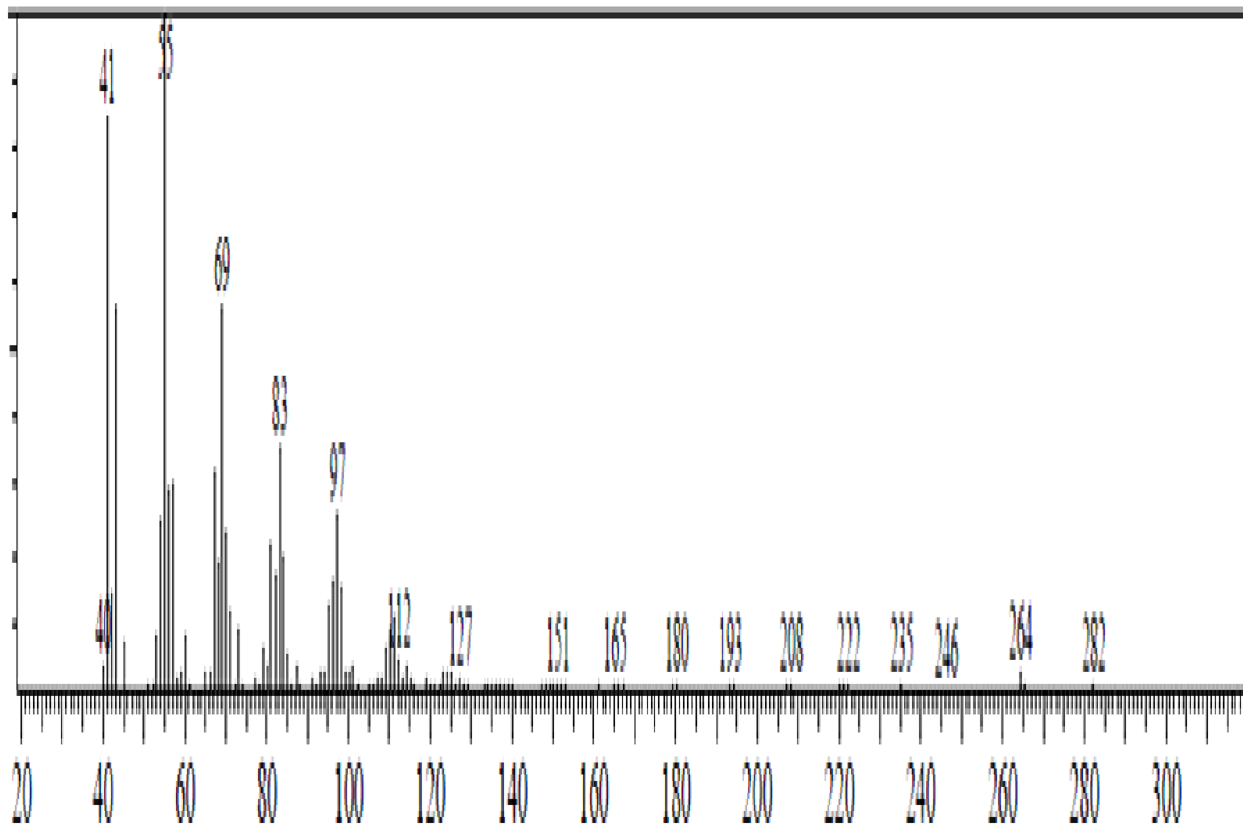


Fig. 4.7: Mass spectra of $ZAAK_2$

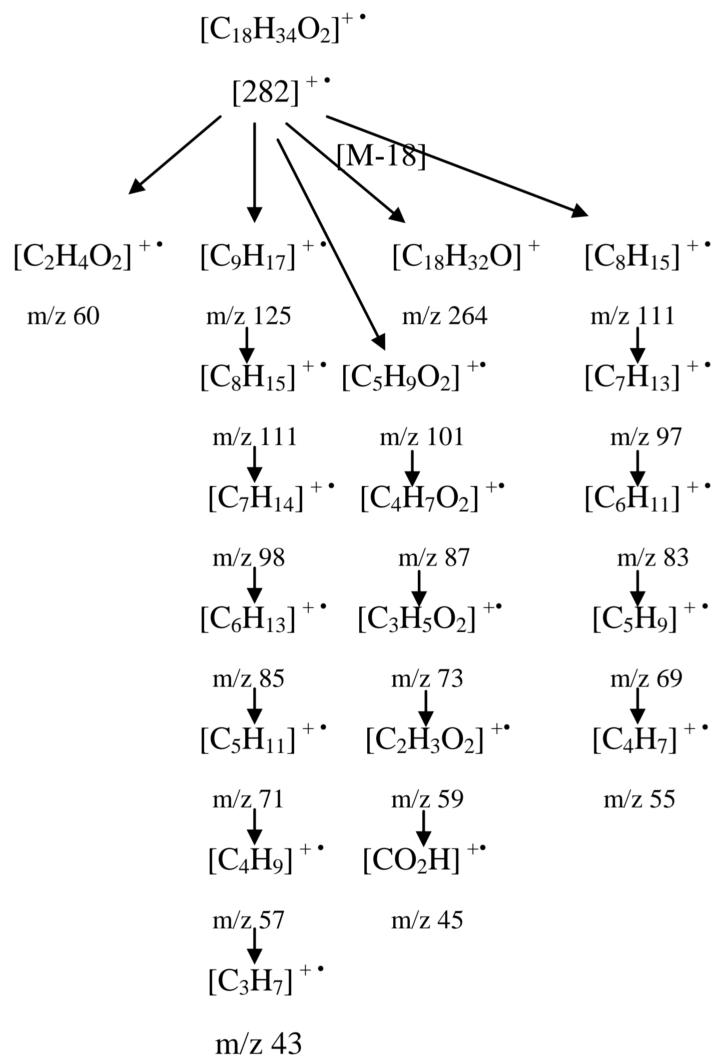


Fig. 4.8: Schematic representation of some fragmentation patterns of $ZAAK_2$

Mass spectra analysis of $ZAAK_3$ revealed a molecular ion peak $[M]^{+\bullet}$ at m/z 372. The peak corresponding to $ZAAK_3$ from the TIC was observed to have the least percentage area peak of 14.17 %. $ZAAK_3$ revealed intense ion peaks at m/z 43, 55, 56, 57, 60 69, 71, 73 and 97 with m/z 41 as the base peak. Other important fragment ions were observed and recorded at m/z 45, 57, 59, 85, 87, 99, 101, 113, 115, 127, 129 and 143 (Fig. 4.9). The schematic representation of some important $ZAAK_3$ fragmentation pattern is shown in (Fig. 4.10).

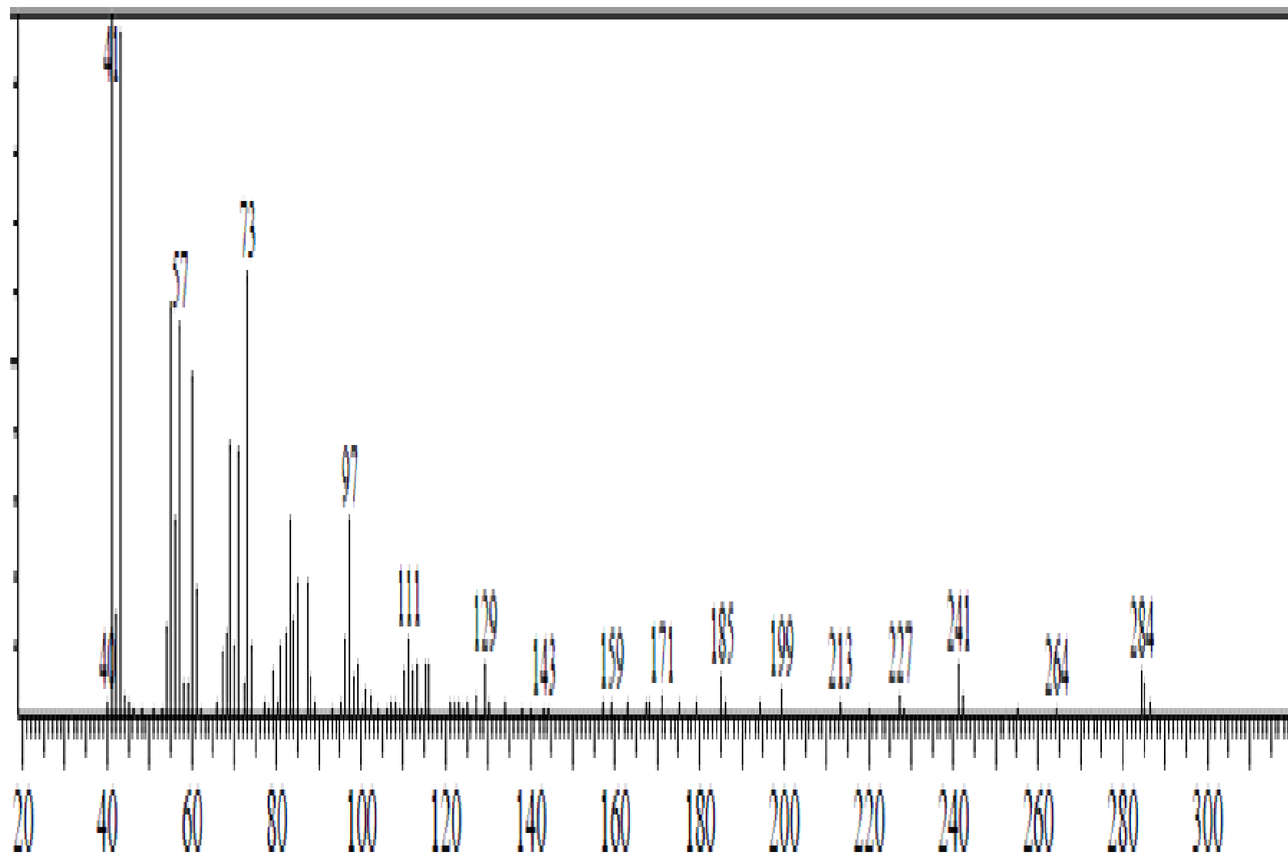


Fig. 4.9: Mass spectra of $ZAAK_3$

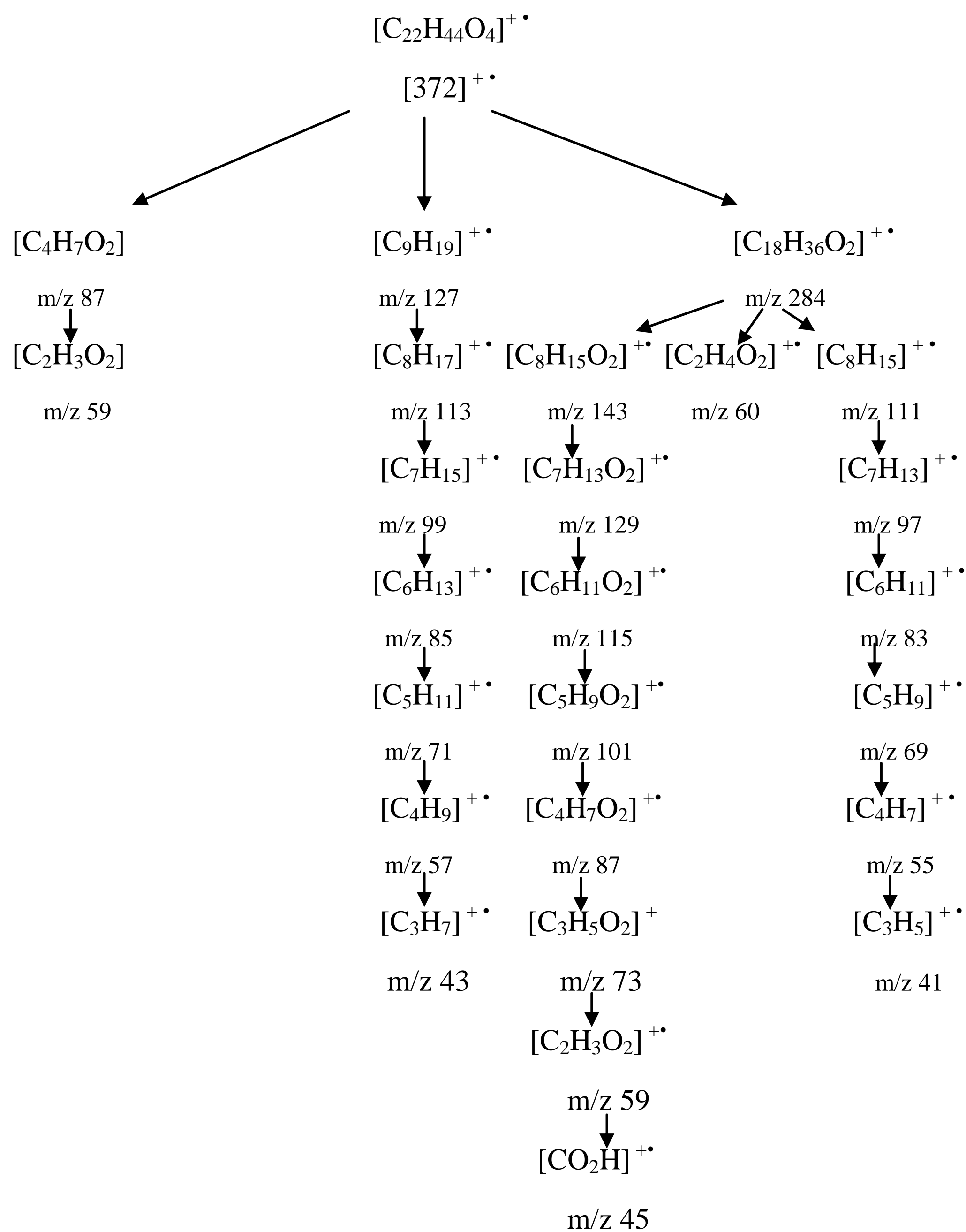


Fig. 4.10: Schematic representation of some fragmentation patterns of $ZAAK_3$

CHAPTER FIVE

5.0 DISCUSSION

Powdered *G. kola* seeds were extracted with n-hexane, ethyl acetate and methanol solvents in order of increasing polarity in a soxhlet apparatus with the aim of separating its components on the basis of polarity. The result of this study revealed that methanol had the highest yield which was followed by ethyl acetate and then n-hexane (Table 4.1). These could be attributed to the pability of highly polar solvents to attract more of the phytochemical constituents present in a plant material.

Preliminary Phytochemical screening of the extracts from *G. kola* seeds in this study revealed the presence of steroids/triterpenes, phenolics, alkaloids and cardiac glycosides in all the extracts (Table 4.2). These findings agree with the report of Adegboye *et al.,* (2008) on the presence of these compounds in the extract. The presence of phenolic compounds in the n-hexane extract may be due to the ability of non-polar solvents to attract low molecular weight phenolics which can be in trace amount. Previous reports from other works showed the presence of proteins (Esimone *et al.,* 2007), combined and free anthraquinones (Adejumo *et al.,* 2011) in extracts from *G. kola* seeds. However, in this study, proteins, combined and free anthraquinones were absent. This could be attributed to the difference in geographical locations of the plant source and/environmental effects. Geographical locations and environmental effects (time and period of plant collection) have been known to affect the accumulation and diversity of plant active constituents (Evans, 2002; Mendonca-Filho, 2006). Flavonoid, saponin, phlobatannin and coumarin were also present (Table 4.2). These phytochemicals are known to play important roles in the bioactivity of medicinal plants. The

medicinal values of medicinal plants actually lie in their phytochemical constituents and as such produce a definite or marked physiological actions on the human body.

Thin layer chromatographic analysis of n-hexane, ethyl acetate and methanol extracts from *G. kola* seeds in four different solvent systems in different ratios gave various degrees of separations. The chromatogram of n-hexane extract in Chloroform: Ethyl acetate (9:1) solvent system had a clear separation with 7 bands (Plate II). For ethyl acetate extracts, Chloroform: Ethyl acetate: Methanol (8:1:1) solvent system gave the best separation with 8 visible bands (Plate IV) while methanol extract gave a separation with 5 bands in Chloroform: Ethyl acetate: Methanol (5:1:3) solvent system (Plate VI). Hence, chloroform, ethyl acetate and methanol solvents were found to be good solvent combinations for TLC, especially in the separation of polar components in extracts from *G. kola* seeds. The successful separation of bio-molecules by chromatographic technique depends upon suitable solvent system which needs an ideal range of partition coeffiecient (k) for each target compounds (Ito, 2005).

Chemical screening is performed to target isolation of new or useful type of constituent having potential pharmacological activities. These procedures have been found to enable recognition of known metabolites in plant extracts in the earliest stages of separation and thus economically very important (Patra *et al.,* 2012). In the present study, screening for identification of specific types of compounds confirmed the presence 3 types of compounds *viz:* steroids/triterpenes, phenolic compounds and flavonoids in all the extracts (Plates III, V and VII). The presence of these compounds support the use of this plant part in African traditional medicine and the basis for the reported antidiabetic property as well as an array of

biological activities attributed to it. However, flavonoid was not detected in preliminary phytochemical screening for the n-hexane extract which may be due to their presence in minute quantity. Alkaloids and anthraquinones were confirmed absent in all the extracts implying a false positive result for alkaloids in the preliminary phytochemical test. Thin layer chromatographic analysis is a simple and cheap method for detection of plant active constituents due to its good selectivity and sensitivity of detection providing convincing results (Patra *et al.,* 2012) hence considered a reliable technique for qualitative phytochemical screening of plant active constituents. In this study, phenolic compounds, steroids/triterpenes and flavonoids were observed to be the most abundant bioactive constituents in *G. kola* seeds.

The most challenging goal in the management of type II diabetes mellitus is to achieve blood glucose levels as close to normal as physiologically possible (Tiwari and Rao, 2002). In the present study, n-hexane extract inhibited α-amylase activity across all the concentrations tested (Fig. 4.1) but had the highest IC_{50} 4.89±4.62 compared to ethyl acetate and methanol extracts, therefore, considered a weak inhibitor of α-amylase. At 2.5 mg/ml, n-hexane and ethyl acetate extracts recorded the highest inhibitory effects and the inhibition was strong for n-hexane extract at this concentration which is undesirable of α-amylase inhibitor (Fig. 4.1). In order for hypoglycemic agents to elicit their pharmacological effects and prevent the side effects experienced with the use of synthetic drugs, medicinal plants should be mild inhibitors of α-amylase (Kwon *et al.,* 2007). n-Hexane extract demonstrated a biphasic concentration response pattern indicating lower or higher concentrations of the extract will lead to decrease in inhibitory activity. The least IC_{50} of 0.78±0.32 mg/ml obtained was observed with methanol extract making it a stronger inhibitor of α-amylase and its inhibitory effect was second to n-hexane extract (Table 4.5). Synthetic inhibitors or natural inhibitors of plant origin with lower

IC_{50} value have been considered stronger inhibitors as strong inhibitors are active at low concentrations and thus have low IC_{50} values and vice-versa. However, the IC_{50} values did not reflect on the inhibitory effect of the extract. This could be due to the complex biomolecules and/other proteins that are present in the crude enzyme interfering with the activity of the inhibitor on the enzyme.

The extracts did not show a concentration-dependent inhibitory effect as observed in most reported literatures suggestive of the crude enzyme used in this study. The inhibition of α-amylase activity is not unconnected to the abundance of steroids/triterpenes and phenolic compounds which may be acting individually or in synergy. Triterpenes and their esters such as β-amyrin palmitat have been reported to have inhibitory effect on carbohydrate hydrolyzing enzymes (Martinez *et al.,* 2012). Also plant phenolics such as flavonoids have often been reported to be strong inhibitors of α-amylase (El-Kaissi and Sheerbeni, 2011) which might explain the lower IC_{50} value of the methanol extract.

Nevertheless, the inhibitory action of *G.kola* seeds on α-amylase activity agreed with some earlier reports where *Telfairia occidentalis* inhibited saliva α-amylase activity and the inhibition of pancreatic α-amylase activity by red and white ginger (Oboh *et al.,* 2010) grapefruits and orange peels (Oboh and Ademosun, 2011; Oboh and Ademosun, 2011a), chilli pepper and bitter leaf (Oboh *et al.,* 2011; Saliu *et al.,* 2012).

The n-Hexane extract showed a concentration-dependent inhibition on α-glucosidase activity with an IC_{50} of 10.29±4.08 mg/ml which was invariably high, hence a weak inhibitor of α-glucosidase. The inhibition was inverse concentration dependent. Methanol extract demonstrated a direct concentration-dependent inhibition (Fig. 4.2). This pattern of inhibition

exhibited by n-hexane and methanol extracts could be attributed to the different classes of compounds present in the extracts exerting their effects on α-glucosidase independently via different mechanisms. The extract had the highest inhibitory effect (Table 4.8) although with an IC_{50} of 2.67±0.74 mg/ml. The ethyl acetate extract was considered a strong inhibitor owing to its lower IC_{50} 1.68±1.27 mg/ml which can be attributed to abundance of flavonoids in the extract; however it was not concentration dependent. Visual examination of the experimental data in this study showed that ethyl acetate ectract demonstrated a biphasic inhibitory pattern which may be due tobinding of the extract in low affinity state. This is in agreement with previous reports that phytochemical from plants are strong inhibitors of α-glucosidase (Kwon *et al.,* 2007) and can be potentially used as an effective therapy for postprandial hyperglycemia with minimal side effects. However, the inhibition was not concentration dependent suggestive of interference from other macro molecules present alongside the enzyme. The presence of steroids/triterpenes, phenolic compounds and flavonoids in *G. kola* seeds, acting individually or in synergy could be responsible for this activity. Phenolic compounds derived from red cabbage, strawberries and raspberries have been reported to act as inhibitors of α-glucosidase (Mc Dougall *et al.,* 2005). The result obtained from this study agreed with reports on the inhibitory potentials of commonly used medicinal plants, herbs and spices in Latin America against key enzymes relevant to hyperglycemia and common constituents from some traditional Chinese medicine used for diabetes mellitus (Ye *et al.,* 2010). With postprandial hyperglycemia being the earliest metabolic abnormality detectable, modulation of this rise in blood glucose is an important tool in the management of diabetic patient (Lebovitz, 1998).

Column chromatography of GKEE yielded 82 fractions which were pooled together based on their TLC profiles to give 6 combined fractions coded (A-F). Fractions B was subjected to further purification by PTLC which lead to the isolation of ZAAK (23 mg).

Information from the FT-IR spectra (Fig. 4.3) revealed strong bands at 2933.83 cm^{-1} and 2872.10 cm^{-1} indicating an asymmetrical methylene ($-CH_2$) and symmetrical methyl (-CH_3) stretching vibrations. The strong band at 2933.83 cm^{-1} is indicative of the presence of an aliphatic compound (John, 2000). The symmetrical bending vibration involving the in-phase bending of methyl C-H was occurring at 1373.36 cm^{-1} while the asymmetrical out-of-phase bending of methyl C-H bonds occurred at 1452.45 cm^{-1}. The band resulting from C-H bending of methylene rocking vibrations in which all of the methylene groups rock in phase, occurred at 740.69 cm^{-1} which is indicative of a staright chain hydrocarbon of seven or more carbon atoms (John, 2000; Silverstein *et al.,* 2005). Presence of absorption band at 1040.63 cm^{-1} showing symmetrical C-O-C stretching vibration was indicative of an alkyl ether group. Carbonyl (C=O) stretching vibration for dimerized aliphatic carboxylic acids was observed at 1608.69 cm^{-1}. A shift in this band from 1720-1680 cm^{-1} to a lower frequency was observed which could be due to intermolecular hydrogen bonding occurring in the molecule. Hydrogen bonding reduces the frequencies of the carbonyl stretching absorption which could be intermolecular or intramolecular in nature (Anderson *et al.,* 2004).

Hydroxyl (-OH) bending vibration is an important characteristic band in the spectra of dimeric carboxylic acid resulting from the out-of-plane bending of the bonded –OH (Siverstein *et al.,* 2005). Hence, the absorption at 914.29 cm^{-1} is indicative of dimerized carboxylic acid. A broad band for –OH stretching vibration was observed at a higher

frequency, 3424.73 cm^{-1} suggesting the presence of an isolated –OH group that maybe resulting from stearic hinderance (John, 2000). Carbonyl (C=O) stretching band for esters was observed at 1731.17 cm^{-1} indicating a saturated aliphatic ester. An asymmetrical C-C(=O)-O stretching band at 1175.65 cm^{-1} was evident in spectrum. Therefore, this result is confirmatory of the presence of fatty acids and and an ester in ZAAK.

The mass spectra of $ZAAK_1$ showed a molecular ion $[M]^{+\bullet}$ at m/z 256. The molecular ion peak represents the intact molecule and also gives the exact molecular weight of the compound (Halilu, *et al.,* 2013). Ion peak at m/z 45 indicates loss of $C_{15}H_{31}$ resulting from β-bond cleavage, chaterristics of carboxylic acids. An intense ion peak at m/z 60 (Fig. 4.5) that may have resulted from the intramolecular γ-hydrogen transfer to the ionized carbonyl oxygen via the hexacyclic transition state (McLafferty rearrangement) followed by $C\alpha-C_\beta$ bond cleavage (Dinh-Nguyen, *et al.,* 1961) is diagnostic for straight chain carboxylic acids. In straight chain monocarboxylic acids, the most characteristic and sometimes the base peak is m/z 60 (Silverstein, *et al.,* 2005). Diagnostic peaks at m/z 43, 57, 71, 85, 99, 113 and 127 are suggestive of alkyl ion fragment resulting from C−C bond cleavage. Oxygen containing fragment ions observed at m/z 45, 59, 73, 87, 101, 115, 129, 143, 157, 171, 185, 199, 213 and 227 can be attributed to the formation of hydrocarbon clusters at an interval of 14 mass units giving by C_nH_{2n}-$_1O_2$ (Burdi, *et al.,* 2007). In long chain carboxylic acids, the spectrum consist of two series of peaks resulting from cleavage at each C−C bond with retention of charge either on the oxygen containing fragment or on the alkyl fragment (Silverstein, *et al.,* 2005). These ions together with a molecular ion at m/z 256 are suggestive of $C_{16}H_{32}O_2$ molecular formula called 1-pentadecanecarbxylic acid (palmitic acid). This data is in agreement with the

library hit and reported literatures (McLafferty and Turecek, 1993; Hernandez-Medel, *et al.,* 2009).

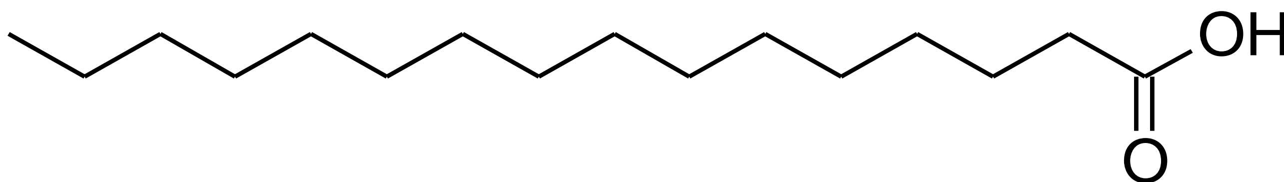


Fig. 5.1 Structure of 1-pentadecanecarboxylic acid (palmitic acid)

The fragment ion at m/z 264 for $ZAAK_2$ (Fig. 4.8) is associated with the loss of 18 amu $[M-18]^+$ which is equivalent to mass of water molecule. The loss of water molecule suggests the presence of a hydroxyl (−OH) group. A rearrangement of hydrogen accompanied fragmentation, which is evident from the even-numbered fragment ions such as those at m/z 264, 222 and 180 (Fig. 4.7) arising from an even-numbered molecular ion (Silverstein, *et al.,* 2005). The peaks at m/z 60 and m/z 45 are indicative of McLafferty rearrangement and β-bond cleavage, characteristic of carboxylic acids. Ion peaks at m/z 41, 55, 69, 83 and 97 could be attributed to the loss of 14 amu equivalent to $-CH_2$ resembling the series of hydrocarbon clusters at interval of 14 mass units. The diference of 13 amu between homologous fragmentation ions at m/z 111, C_{11} and m/z 98, C_{12} (Fig. 4.8) can be associated to the presence of a double bond indicating Δ_{11} unsaturation. Other diagnostic peaks could be associated with the cleavage of the C−C bond giving alkyl ions and oxygen containing ions (Fig. 4.4), a common feature of aliphatic carboxylic acids. This fragmentation pattern suggests the presence of an unsaturated fatty acid (18:1) with $C_{18}H_{34}O_2$ molecular formula called (Z)-11-

octadecenoic acid (cis-Vaccenic acid). This is in agreement with reported literatures (Silverstein, *et al.,* 2005; Hernandez-Medel, *et al.,* 2009; Kilulya *et al.,* 2011).

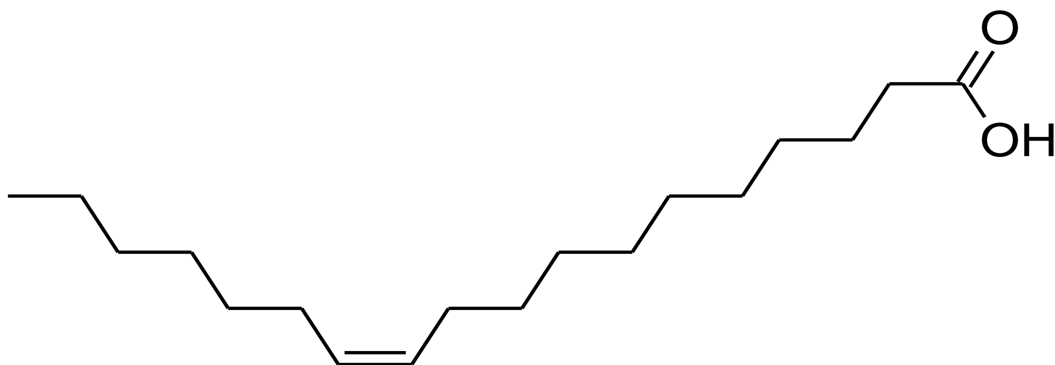


Fig. 5.2 Structure of (Z)-11-Octadecenoic acid (cis-Vaccenic acid)

The intensity of a molecular ion peak depends on the stability of the molecular ion, for, if substituent's that have favorable modes of cleavage are present, the molecular ion peak will be less intense and the fragment peaks relatively more intense (Silverstein, *et al.,* 2005) which might have accounted for the less intense molecular ion peak of $ZAAK_3$. Characteristic fragment ion at m/z 60 could be associated with possible rearrangement and fragmentation of the acid portion of the molecule i.e. McLafferty rearrangement (McLafferty, 1959). Fragment ion at m/z 284 suggests loss of 89 amu (Fig. 4.10) equivalent to $C_4H_9O_2$. Fragmentation pattern for esters of straight chain carboxylic acids can be described in the same terms as for the free acid (Silverstein, *et al.,* 2005). This may be responsible for the fragment ions at m/z 143, 129, 115, 101, 87, 73, 59, suggesting oxygen containing ions and alkyl fragment ions at m/z 41, 57, 69, 83, 97 and 111 (Fig. 4.5). Other fragment ions such as those at m/z 87 and 59 (Fig. 4.5) could be associated with aliphatic ether. Therefore, this mass ionization pattern indicates a 372 molecular mass compound of $C_{22}H_{44}O_4$ formula, suggesting an octadecanoic

acid, 2-(2-hydroxyethoxy) ethyl ester (aqua cera). These mass features were consistent with computer data and reported literatures (Burdi *et al.,* 2007; Oduje *et al.,* 2015; McLafferty and Turecek, 1993).

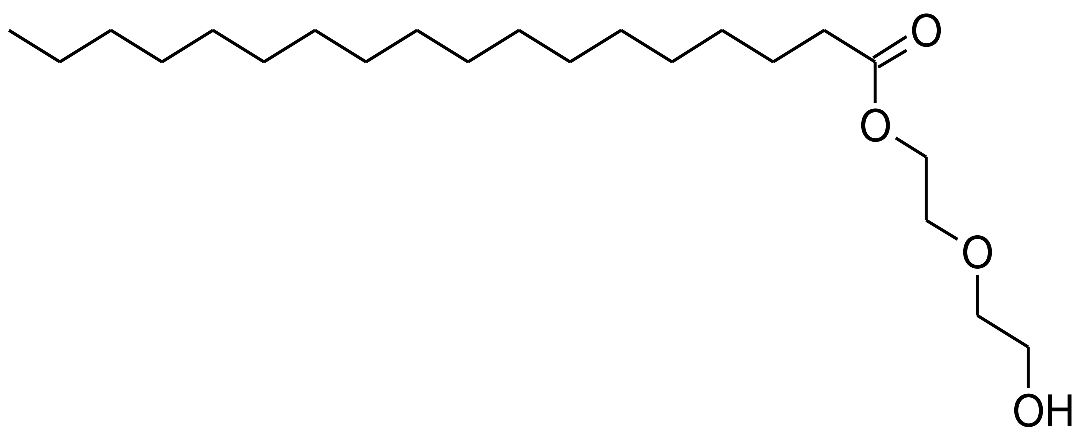


Fig. 5.3 Structure of Octadecanoic acid, 2-(2-hydroxyethoxy) ethyl ester (Aqua cera)

However, it is not clear if these fatty acids have contributing effect on the inhibition of α- amylase and α-glucosidase as observed in this study. Although seed oil such as those of *Mormordica charantia* that is highly rich in fatty acid (stearic acid) have been reported to have inhibitory effect on α- amylase and α-glucosidase, hence its antidiabetic property (Ahmad *et al.,* 2012). Also, palmitic acid has been shown to have hypoglycemic activity (Thode *et al.,* 1989).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

Several studies have demonstrated the antidiabetic property of *G.kola* seed extracts but no previous report have shown the biochemical pathway by which these extracts exert their effects and the type of compounds that maybe responsible for the activity. Hence, in this study, the extracts from *G.kola* (Clusiaceae) seeds were evaluated for their inhibitory effect on glycosidase with a view of characterizing the bioactive constituents and isolating bioactive compounds from it. The powdered seed was extracted with solvents in order of increasing polarity in a soxhlet apparatus and methanol was discovered to have the highest yield. Preliminary phytochemical screening showed the presence of steroids/triterpenes, phenolic compounds, alkaloids and cardiac glycosides in all three extracts. Proteins, free and combined anthraquinones were absent in all the extracts while flavonoids and coumarins were present in the polar solvents and phlobatannins were present in ethyl acetate extract only. Thin layer chromatographic analysis visualized with specific reagents confirmed the presence of steroids/triterpenes, phenolic compounds and flavonoids in all the extracts while alkaloids and anthraquinones were absent.

The extracts demonstrated good inhibitory effect on both enzymes with methanol and ethyl acetate extract being strong inhibitors of α-amylase and α-glucosidase respectively. n-Hexane extract was a weak inhibitor of both enzymes. Column chromatographic investigations of the ethyl acetate extract lead to the isolation of ZAAK which was ellucidated

using FT-IR Spectroscopy and GC-MS analysis. IR spectra revealed the presence of carboxylic acids and a carboxylic acid ester in ZAAK. The Total ion chromatogram of ZAAK revealed the presence of three major peaks which were subsequently coded $ZAAK_1$ $ZAAK_2$ and $ZAAK_3$. The fragmentation pattern of the mass spectra compared with computer data and reported literatures identified $ZAAK_1$ $ZAAK_2$ and $ZAAK_3$ as 1-pentadecanecarboxylic acid, (Z)-11-Octadecenoic acid and octadecanoic acid, 2-(2-hydroxyethoxy) ethyl ester respectively.

6.2 Conclusion

On the basis of qualitative phytochemical screening of *G. kola* seeds, the most abundant bioactive constituents were steroids/triterpenes, phenolic compounds and flavonoids. The extracts demonstrated potent α- amylase and α-glucosidase inhibitory activity *in vitro*. The inhibition of these enzymes in the digestive tract is one of the possible pathways by which *G. kola* elicit its antidiabetic properties. These characteristics is attributed to the contributions of bioactive constituents such as steroids/triterpenes, phenolic compounds and particularly flavonoids which were in abundance in the extracts and which maybe acting individually or in synergy.

The isolated compound ZAAK was identified to be a mixture of fatty acids and fatty acid ester, and not a single pure compound. This is the first report on these compounds from the seeds of *G. kola*.

6.3 Recommendations

The results indicate that n-hexane, ethyl acetate and methanol extracts of *G. kola* seeds demonstrated potent inhibitory activity. Therefore, exploiting the dual advantage of these seeds having α-glucosidase and pancreatic α-amylase inhibitory action could prove to be an effective treatment for diabetes mellitus. But the *in vitro* inhibitory activity does not always relate to the corresponding *in vivo* activity. Thus proof of concept needs to be demonstrated in pre-clinical animal studies. Further attempts at isolation, purification and testing of the isolated compounds for carbohydrate hydrolyzing enzyme inhibitory activity holds a lot of prospects. There is also the need to exploit the isolated compounds further as they may be useful in the pharmaceutical industry. Determination of enzyme kinetics in the presence of the extract or isolated compound is required. Furthermore, an extensive investigation is required to demonstrate the effect of *G. kola* seeds as an antidiabetic agent by studying its effect on other pathways such as gluconeogenic and glycerogenolysis pathways.

REFERENCES

Adaramoye, O. A. and Adeyemi, E. O. (2006). Hypoglycaemic and Hypolipidaemic Effects Of Fractions from Kolaviron, A Biflavonoid Complex from *Garcinia Kola* in Streptozotocin-Induced Diabetes Mellitus Rats. *Journal of Pharmacy and Pharmacology*, **58** (1): 121-128.

Adebukunola, O. A, Bernice O. A., Adebayo, K. A., Olayinka, O. A., Elsie, S., and Kehinde, O. (2010). Efficacy of *Garcinia kola* 0.5% Aqueous Eye Drops in Patients with Primary Open-Angle Glaucoma or Ocular Hypertension. *Middle East African Journal of Ophthalmology*, **17** (1): 88–93.

Adegboye, M. F., Akinpelu, D. A. and Okoh, A. I. (2008). The Bioactive and Phytochemical properties of *Garcinia kola* (Heckel) Seed Extract on Some Pathogens. *African Journal of Biotechnology,* **7** (21): 3934-3938.

Adejumo, O. E., Ayoola, M. D., Kolapo, A. L., Orimoyegun V. O. and Olatunji, P. O. (2011). Antisickling Activities of Extracts of Leaf, Seed and Seed Pod of *Garcinia kola* Heckel. *African Journal of Pharmacy and Pharmacology,* **5**(1): 48-52.

Adesina, S. K., Gbile, Z. O. and Odukoya, O. A. (1995). *Survey of indigenous plants of West Africa with special emphasis on medicinal plants and issues associated with management.* The United Nations Programme on Natural Resources in Africa. 2nd (ed.). Pp.84–85.

Adesuyi, A. O. Elumm, I. K. Adaramola, F. B. and Nwokocha A. G. M. (2012). Nutritional and Phytochemical Screening of *Garcinia kola. Advance Journal of Food Science and Technology,* **4** (1): 9-14.

Ahmad, Z., Zamhuri, K. F., Yaacob, A., Siong, C. H., Selvarajah, M., Ismail A., and Hakim, M. N. (2012). *In Vitro* Anti-diabetic Activities and Chemical Analysis of Polypeptide-k and Oil Isolated from Seeds of *Momordica charantia* (Bitter Gourd). *Molecules*, **17**: 9631-9640.

Akinloye, O.A., Oshilaja, R.T., Okelanfa O.A., Akinloye, D.I and Idowu, O.M.O (2013). Hypoglyceamic Activity of *Alstonia boonei* Stem Bark Extract in Mice. *Agriculture and Biology Journal of North America*, **4** (1): 1-5.

Akpantah, A. O., Oremosu, A. A., Noronha, C. C., Ekanem, T. B. and Okanlawon, A. O. (2005). Effects of *Garcinia kola* Seed Extract on Ovulation, Oestrous Cycle and Foetal Development in Cyclic Female Sprague - Dawley Rats. *Nigerian Journal of Physiological Sciences,* **20** (1, 2): 58-62.

Akram, M. (2013). Diabetes Mellitus Type II: Treatment Strategies and Options: A Review. *Journal of Diabetes & Metabolism,* **4** (9): 1-9

Alade, A. and Ani, R. E. (1990). Protective Effects of *Garcinia kola* Seed Extract against Paracetamol-Induced Hepatotoxicity in Rats. *Journal of ethnopharmacology,* **29** (2): 207–211.

Anderson,R.J., Bendell, D.J. and Groundwater, P.W. (2004). Organic Spectroscopic Analysis. Royal society of chemistry, Cambridge, United Kingdom. Pp. 35-38

Aniszewski, T. (2007). Alkaloids—Secrets of Life. In: *Alkaloid Chemistry, Biological Significance, Applications and Ecological Role*. Netherlands, Elsevier Science: Amsterdam. Pp. 334

Ansari, W. H., Rahman, W., Barraclough, D., Maynard, R. and Scheinmann, F. (1976). Biflavanoids and a Flavanone-Chromone from the Leaves of *Garcinia dulcis* (Roxb.) Kurz, *Journal of Chemical Society,* **1** (13): 1458-1463.

Aplin, R. T., Blasdale, J. K. C., Halsall, T. G., Hornby, G. M. J. (1967). The Isolation of Cycloartenol and 24-Methylenecycloartenol From False Kola Nuts (*Garcinia kola* Heckel). *Journal of Chemical Society.* Pp. 246-248.

Asai, F. T., H. Tanaka, T. Iinuma, M. (1995). A Xanthone from pericarps of *Garcinia mangostana*. *Phytochemistry,* **39**(4): 943-944.

Asano, J., Chiba, K., Tada, M., Yoshi, T. (1996). Cytotoxic Xanthones from *Garcinia hanburyi*. *Phytochemistry*, **41**: 815–820.

Asawalam, E. F., Emosairue, V., Ekeleme, F., and Wokocha, R. C. (2007). Insecticidal Effects of Powdered Parts of Eight Nigerian Plant Species against Maize Weevil *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionideo). *Electronic journal of environmental, agriculture and food chemistry (EJEAFC)*, **6** (11): 2526-2533.

Attele, A.S., Zhou, Y.P., Xie, J.T.,Wu, J.A., Zhang, L., Dey, L., Pugh,W., Rue, P.A., Polonsky, K.S., Yuan, C.S., 2002. Antidiabetic effects of *Panax ginseng* berry extract and the identification of an effective component. *Diabetes,* **51**: 1851–1858

Ayensu, E. S. (1978). *Medicinal Plants of West Africa*. Reference Publication Inc. Algonac, Michigan. P. 162.

Bennett, R. N. and Wallsgrove, R. M. (1994). Secondary Metabolites in Plant Defense Mechanisms. *Phytotherapy Resources*. **127**: 617-633.

Bernfield, P. (1955). Amylases, α and β. In: S. P. Colowick, and N. O. Kaplan, (Eds). *Methods in Enzymology*, United Kingdom, Academic press Inc. Ltd., London, 1: 149-255

Bojsen, K., Yu, S., Kragh, K. M. and Marcussen, J. (1999). A Group of α-1,4-glucan Lyases And Their Genes From The Red Alga *Gracilariopsis lemaneiformis*: Purification, Cloning And Heterologous Expression. *Biochemistry and Biophysics Acta*, **14** (30): 396–402.

Bouquet, A. (1969). *Féticheurs et Médecines Traditionnelles du Congo (Brazzaville)*; Office de la Recherche Scientifique et Technique Outre-Mer O.R.S.T.O.M.: Paris, France. pp. 132–133.

Braide, V. B. (1991). Antihepatotoxic Biochemical Effects of Kolaviron, A Biflavonoid of *Garcinia kola* seeds. *Phytotherapy Resources,* **5**: 35-37.

Brayer, G. D., Sidhu, G., Maurus, R., Rydberg, E. H., Braun, C., Wang, Y., Nguyen, N. T., Overall, C. M., Withers, S. G. (2000). Sub-site Mapping of The Human Pancreatic Alpha-Amylase Active Site Through Structural, Kinetic and Mutagenesis Techniques. *Biochemistry-US*, **39**: 4778-4791.

Braun, C. and Brayer, G. D. (1995). Mechanism-Based Inhibition Of Yeast α-Glucosidase and Human Pancreatic α-amylase By A New Class of Inhibitors 2-Deoxy-2,2- Difluoro-α-Glycosides. *Journal of Biology and Chemistry,* **270** (45): 26778-26781.

Breitmaier, E. (2008). Terpenes: Flavors, Fragrances, Pharmaca, Pheromones, 1st (ed.). Wiley-VCH Verlag GmbH and Co. KGaA, Germany. Pp. 1-4

Burdi, D. K., Samejo, M. Q., Bhanger, M. I., and Khan, K. M. (2007). Fatty Acid Composition of *Abies pindrow* (West Himalayan Fir). *Pakistani Journal of Pharmaceutical Sciences*, **20**: 15-19.

Butler, M. S. (2004). The Role of Natural Product Chemistry in Drug Discovery. *Journal of Natural Products,* **67** (12): 2141– 2153.

Butters, T. D., Dwek, R. A. and Platt, F. M. (2003). Therapeutic Applications of Imino Sugars in Lysosomal Storage Disorders. *Current Top Medicinal Chem*istry, **3**: 561–574.

Cardona, M. L., Fern´andez, M. I., Pedro, J. R., Seoane, E. and Vidal, R. (1986). Additional New Xanthones and Xanthonolignoids from *Hypericum canariensis*, *Journal of Natural Products*, **49** (1): 95–100.

Carpenter I., Mc Garry E. J. And Scheimann F. (1971). Extractives from Guttiferae. Part XXI. The Isolation and Structure of Nine Coumarins From The Bark Of *Mammea Africana* G. Don *Journal of Chemical Society,* **22**: 3783-3789.

Chiba, S. (1997). *Handbook of Amylase and Related Enzymes* (Eds.), Pergamon Press, Oxford. Pp. 104–105, 109–116.

Cotterhill, P. J., Scheinmann, F., Stenhouse, T. A. (1978). Extractives from Guttiferae: Kolaflavanone, A New Biflavanone from the Nuts of *Garcinia kola*. *Journal of Chemical Society,* **1**: 532-538.

Cragg, G. M., Newman, D. J. and Snader, K. M. (1997). Natural Products in Drug Discovery and Development. *Journal of natural products,* **60** (1): 52-60.

Cuesta-Rubio, O., Frontana-Uribe, B. A., Ramirez-Apan, T., and Cardenas, J. (2002). *Naturforschung Teil C*, **57**: 372–378.

Dakanali, M., and Theodorakis, E. A. (Ed.) (2011). Polyprenylated Phloroglucinols and Xanthones. Biomimetic Organic Synthesis. Wiley-VCH Verlag GmbH & Co. KGaA. Pp. 433-467.

Dale, I. R., and Greenway, P. J. (1961). Kenya Trees and Shrubs. Nairobi. Buchanan's Kenya Estates Ltd, in association with Hatchards, London. Pp. 5-14

Davies, G. J., Gloster, T. M. and Henrissat, B. (2005). Recent Structural Insights into the Expanding World of Carbohydrate-Active Enzymes. *Current Opinion in Structural Biol*ogy, **15**: 637–645.

DeFronzo, R. A. (2004). Pathogenesis of Type 2 Diabetes Mellitus. *Medicinal Clinic of North America,* **88**: 787-835, doi: 10.1016/j.mcna.2004.04.013

DeMelo, E. B., Gomes, A. D. and Carvalho, I. (2006). Alpha- And Beta-Glucosidase Inhibitors: Chemical Structure and Biological Activity. *Tetrahedron*. **62**: 10277–10302.

Dewick, P. M. (2002). *Medicinal natural products: a biosynthetic approach*, 2nd edn. Wiley and Sons Ltd, Chichester, United Kingdom. Pp. 167-168

Dey, B., Mitra, A., Katakam, P. and Singla, R. K. (2014). Exploration of Natural Enzyme Inhibitors with Hypoglycemic Potentials amongst *Eucalyptus* Spp. by *in vitro* Assays. *World Journal of Diabetes,* **5** (2): 209-218.

Dinh-Nguyen, N. G., Ryhage, R., Stallberg-Stenhagen, S. and Stenhagen, E. (1961) *Arkademicia Kemistry***, 18**: 393–399.

Dixon, R. A., Dey, P. M. and Lamb, C. J. (1983). Phytoalexins: Enzymology and Molecular Biology. *Advance Enzymology*, **55**: 1-69.

Dwek, R. A. and Butters T. D. (2002). Targeting Glycosylation as a Therapeutic Approach. *Nattional Review on Drug Discovery,* **1** (1): 65-75.

Ekene, E. N. and Erhirhie, E. O. (2014). *Garcinia kola*: A review of its ethnomedicinal, chemical and pharmacological properties. *International Journal of Current Research Review*, **6** (11): 1-7.

Eldridge, G. R., Vervoot, H. C., Lee, C.M., Cremin, P.A., Williams, C. T., Hart, M.S., Goering, M.G., O'Niel-Johnson, M., and Zheng, L. (2002). High-Throughput Method for the Production and Analysis of Large Natural Product Libraries for Drug Discovery. *Analytical chemistry,* **74**: 3963-3971.

El-Kaissi, S., Sherbeeni, S. (2011). Pharmacological Management of Type 2 Diabetes Mellitus: An Update. *Current Diabetes Review*, **7** (6): 392-405.

Ernawati, D. W. and Yusnelti, A. (2014). Isolation of Antibacterial Compounds from *Garcinia Cf Cymosa*. *Journal of Natural Sciences Research*, **4** (4): 23-25.

Esimone, C. O., Nwafor, S. V., Okoli, C. O., Chah, K. F., Uzuegbu, D. B., Chibundu, C., Eche, M. A., and Adikwu, M. U. (2002). *In Vivo* Evaluation of Interaction between Aqueous Seed Extract of *Garcinia kola* Heckel and Ciprofloxacin Hydrochloride. *American Journal of Therapeutics,* **9** (4): 275-280.

Esimone, C. O., Adikwe, M. U., Nworu, C. S., Okoye, F. B. C., Odimegwu, D. C. (2007). Adaptogenic Potentaps of *Camellia Sinensis* leaves, *Garcinia kola* and *Kola nitida* Seeds. *Science Resources Essays*, **2** (70): 232-237.

Esomonu, U. G., El-Taalu, A. B., Anuka, J. A., Ndodo, N. D., Salim, M. A and Atiku, M. K. (2005). Effect of Ingestion of Ethanol Extract of *Garcinia kola* Seed on Erythrocytes in Wistar Rats. *Nigerian Journal of Physiological Sciences,* **20** (1, 2): 30-32.

Evans W. C. (2002). *Trease and Evans Pharmacognosy*. 15th (ed.) Saunders Publishers, an imprint of Elsevier science Ltd. India. Pp. 42-44, 221-229, 246-249, 304-306, 331-332, 391-393.

Evans W. C. (2009). *Trease and Evans Pharmacognosy*. 16th (ed.) Saunders Publishers, an imprint of Elsevier science Ltd. India. P. 333.

Frandsen, T. P., Svensson, B. (1998). Plant α-Glucosidases of the Glucoside Hydrolase Family 31: Molecular Properties, Substrate Specificity, Reaction Mechanism, and Comparison with Family Members Of Different Origin. *Plant Molecular Biology*, **37**: 1–13.

Fukuyama, Y., Kuwayama, A., and Minami, H. (1997). *Chemistry and Pharmacy Bulleting*, **45**: 947–949.

Funke, I., and Melzing, M. F., (2006). Traditionally used plants in diabetes therapy - phytotherapeutics as inhibitors of *a*-amylase activity. *Review Brasilia Farmacognosia*, **16**: 1-5.

Gabriel, F. I. and Emmanuel, O. O. (2011). Pharmacological Evaluation of *Garcinia kola* Nut for Antitrichomonal Activity. *International Journal of Pharmacy and Biological Sciences,* **2** (2): 264-269.

Geissman, T. A. (1963). Flavonoid Compounds, Tannins, Lignins and Related Compounds. In: M. Florkin, and E.H. Stotz (Eds). *Pyrrole Pigments, Isoprenoid Compounds and Phenolic Plant Constituents*. Elsevier, New York, USA. P. 265

Gennaro, A. R., (2000). *Remington- The Science and Practice of Pharmacy.* 20th (ed.). Lippincott Williams and Wilkins, Maryland, U.S.A. **1**: 606-609.

Gershenzon, J. and Croteau, R. (1991). Terpenoids. Rosenthal, G.A. and Berenbaum, M.R. (Eds). *In Herbivores their interaction with secondary plant metabolites,* Volume I, 2nd ed., The chemical participants, San Diego, Academic press. Pp. 165-219.

Grover, J.K., Yadav, S., Vats, V., 2002. Medicinal plants of India with anti-diabetic potential. *Journal of Ethnopharmacology,* **81**: 81–100.

Guillermo, D. and Victor, N. (1999). Two Antimicrobial Alkaloids from *Bocconia arborea*. *Journal of Ethanopharmacology,* **66**: 223-226.

Gustafsson, M. H. G., Bittrich, V. and Stevens, P. F. (2002) *International Journal of Plant Science*, **163**: 1045–1054.

Guthikonda, R. N., Cama, L. D., Quesada, M., Woods, M.F., Salzman, T.N. and Christensen, B. G. (1987). Modification of Natural Products to Improve Their Biological Properties. *Pure and applied chemistry*, **59** (3): 455-458.

Halilu, M. E., October, N., Balogun, M., Musa K. Y and Abubakar, M. S (2013). Isolation and Characterization of Triterpenes from Petroleum Ether and Ethyl acetate Extracts of Stem Bark of *Parinari curatellifolia* Planch Ex. Benth *(Chrysobalanaceae)*. *Chemistry and Materials Research*, **3** (9): 100-107.

Hammer, K. A., Carson, C. F. and Riley, T. V. (1999). Antimicrobial Activity of Essential Oils and Other Plant Extracts. *Journal of Applied Microbiology*, **86** (6): 985.

Han, Q. B., Yang, N. Y., Tian, H. L., Qiao, C. F., Song, J. Z., Chang, D. C., Chen, S. L., Luo, K. Q. and Xu, H. X. (2008). Xanthones with Growth Inhibition Against HeLa cells from *Garcinia xipshuanbannaensis*, *Phytochemistry*, **69**: 2187-2192.

Harbourne, J. B. (1988). *Phytochemical Methods, A guide to Modern Techniques of Plant Analysis,* 2nd ed., Chapman and Hall, New York, USA. Pp. 4-193.

Hashimoto, Y., Ishizone, H., Suganuma, M., Ogura, M., Nakatsu, K., Yoshioka, H. (1983). Periandrin I, A Sweet Triterpene-Glycoside from *Periandra dulcis*. *Phyto-chemistry,* **22**: 259–264.

Hemshekhar, M., Sunitha, K., Santhosh, M. S., Devaraja, S., Kemparaju, K., Vishwanath, B. S., Niranjana, S. R. and Girish, K. S. (2011). An Overview on Genus *Garcinia*: Phytochemical and Therapeutical Aspects. *Phytochemistry Revview*, **10**: 325-351.

Henrissat, B. (1991). A Classification of Glycosyl Hydrolases Based on Amino-Acid sequence Similarities. *Biochemistry Journal,* **280**: 309–316.

Henrissat, B. and Bairoch, A. (1993). New Families in The Classification of Glycosyl Hydrolases Based on Amino-Acid-Sequence Similarities. *Biochemistry Journal* **293**: 781–788.

Hernández-Medel, R. M., Méndez-Olivares, R., Solís-Fuentes, J. A., and Méndez-Ventura, L. M. (2009). Characterization and Biological Activity of Fatty Acids from *Picramnia Polyantha* Fruits**.** *Review Latinoamerica Quím,* **37** (3): 262-269.

Hostettmann, K. and Wolfende, J. L. (2004). Application of Liquid Chromatography/UV/MS and Liquid Chromatography/NMR for The On-Line Identification Of Plant Meatbolites. In: C. Tringali (Eds.) *Bioactive Compounds from Natural Sources Isolation, Characterization and Biological Properties*. e-Library, Taylor and Francis. Pp. 32-68

Hussain, R. A., Owegby, A. G., Parimoo, A. G., Waterman, P. G. (1982). Kolanone, A Novel Polyisoprenylated Benzophenone with Antimicrobial Properties from the Fruit of *Garcinia kola. Planta Medica,* **44**: 78-81.

Hutchinson, J. (1969). *Evolution and Phylogeny of Flowering Plants*. New York Academic press. P. 175

Ishiguro, K., Nagata, S., Oku, H. and Masae, Y. (2002). Bisxanthones from *Hypericum japonicum*: Inhibitors of PAF-Induced Hypotension, *Planta Medica*, **68** (3): 258–261

Ito Y. (2005). Golden Rules and Pitfalls in Selecting Optimum Conditions for High-Speed Counter Current Chromatography. *Journal of Chromatography,* **1065**: 145-168

Iulek, J., Franco, O. L., Silva, M., Slivinski, C. T., Bloch, J. C., Rigden, D. J. and Sám, F. G. (2000). Purification, Biochemical Characterisation and Partial Primary Structure of a New α-Amylase Inhibitor from *Secale cereale* (rye). *International Journal of Biochemistry and Cell Biology,* **32**: 1195-1204.

Iwu, M. M. (1982). *Traditional Igbo Medicine*. Institute of African studies, University of Nigeria, Nsukka, Nigeria. Pp. 104-110.

Iwu, M. M. (1985). Anti-hepatotoxic Constituents of *Garcinia kola* seeds. *Experientia,* **41**: 699-700.

Iwu, M. M. (1989). Food for Medicine. In: M. Iwu, (Eds.) *Dietary Plants and Masticatories as Sources of Biologically Active Substances.* Ife Press, University of Ife, Nigeria. Pp. 303–10.

Iwu, M. M. (1993). *Handbook of African medicinal plants*.CRC Press, Boca Raton, FL., USA. pp. 32-38.

Iwu, M. M., Igboko, O. A., Okunji, C. O. and Tempesta, M. S. (1990). Antidiabetic and Aldose Reductase Activities of Biflavanones of *Garcinia kola*. *Journal of Pharmacy and Pharmacology,* **42**: 290-292.

Iwuji, T. C. and Herbert, U. (2012). Semen Characteristics and Libido of Rabbit Bucks Fed Diets Containing *Garcinia kola* Seed Meal. *Rabbit Genetics,* **2** (1): 10-14.

Jackson, B., Locksley, H. D., Scheinmann, F., and Wolstenholme, W. A. (1967). *Tetrahedron Letter,* 787.

Janecek, S., Svensson, B., Henrissat, B. (1997). Domain Evolution in the α-Amylase Family. Journal of Molecular Evolution, **45**: 322-331.

Jayaprakasha, G. K., Negi, P. S. and Jena, B. S. (2006). Antioxidative And Antimutagenic Activities of the Extracts from the Rinds of *Garcinia pedunculata*. *Innovative Food Science and Emerging Technologies*, **7** (3): 246–250.

Jianhong Y., Shichao H., Shucai L., Ronghong Z., Aihua P. and Lijuan C. (2013). *in vitro* and *in vivo* Antiangiogenic Activity of Caged Polyprenylated Xanthones Isolated from *Garcinia hanburyi* Hook. f. *Molecules*, **18**: 15305-15313.

John C. (2000). Interpretation of Infrared Spectra, a Practical Approach. In: R. A. Meyers (Eds.), *Encyclopedia of Analytical Chemistry*. Chichester, John Wiley & Sons Ltd. Pp. 10815–10837.

Juliana, A., Moctar, S. and Christophe, K. (2006). *Garcinia kola* Heckel. Forest & Landscape Denmark. P. 17

Kakjing, D. F., Mary, O. U. and Nkoli, L. N. (2014). Anti-pyretic Activity of *Garcinia kola* Seed Extract. *European Journal of Medicinal Plants,* **4** (5): 511-521.

Kandra, L. (2003). α-Amylases of Medical and Industrial Importance. *Journal of Molecular Structure,* **667**: 487- 498.

Karanjgaokar, C. G., Nair, P. M., Venkataraman, K. (1996). Coloring Matters of *Garcinia morella*-(VI): Morellic, Isomorellic, and Gambogic Acids. *Tetrahedron Letter*, **7**: 687–691.

Kathirvel A., Prabhakar T., Vinoth K., Sadasivam S., and Thayumanavan B. (2012). Identification of α-Glucosidase Inhibitors from *Psidium guajava* Leaves and *Syzygium cumini* Linn. Seeds. *International Journal of Pharmaceutical Sciences and Research*, **3** (2): 316-322.

Kijjoa A. and Vieira, L. M. M. (2009). Triterpenes from the Plants of the Family Clusiaceae (Guttiferae): Chemistry and Biological Activities. In: G. Brahmachari, (Eds.). *Natural Products: Chemistry, Biochemistry and Pharmacolog*. UK, Alpha Science International Ltd., Oxford. Pp. 711-715

Kilulya, K.F., Msagati, T.A.M., Mamba, B.B., Ngila, J.C., and Bush, T. (2011). Imidazolium Ionic Liquids As Dissolving Solvents for Chemical-Grade Cellulose in The Determination of Fatty Acids Using Gas Chromatography-Mass Spectrophotometry. *BioResources* **6** (3): 3272-3288.

Kim, Y. M., Jeong, Y. K., Wang, M. H., Lee, W. Y. and Rhee, H. I. (2005). Inhibitory Effects of Pine Bark Extract on Alpha-Glucosidase Activity And Postprandial Hyperglycemia. *Nutrition* **21**: 756-761.

Kimura, A. 2000. *Trends in Glycoscience and Glycotechnology*, **12**: 373–380.

Kinghorn AD (1992). Plants as Sources Of Medicinally and Pharmaceutically Important Compounds. In: H. N. Nigg, and D. Seigler (Eds.), *Phytochemical Resources for Medicine and Agriculture*. New York, Plenum Press. Pp. 75-95

Kinghorn, A. D., and Soejarto, D. D. (2002). Discovery of Terpenoid and Phenolic Sweetneers from Plants. *Pure and Applied Chemistry*, **74** (7): 1169-1179.

Knentz, A. J. and Nattras, M. (1991). Diabetic Ketoacidosis, Nonketotic Hyperosmolar Coma and Lactic Acidosis. In: J.C. Pickup, and G. Williams, *Handbook of diabetes*. United Kingdom, Blackwell Science, Oxford. 2: 479-494

Koehn, F. E. and Carter, G. T. (2005). The Evolving Role of Natural Products in Drug Discovery. *Nature Reviews Drug Discovery,* **4** (3): 206– 220.

Koehn, F. K. and Carter, G. T. (2005). The Evolving Role of Natural Products in Drug Discovery. *Nature Reviews Drug Discovery*, **4**: 206-220.

Kokate, C. K. (2003). *Practical Pharmacognosy*. Vallabh Prakashan, New Delhi, India. 4: 107-111.

Korfmacher, W. A. (2005). Principles and Applications of LC-MS in New Drug Discovery. *Drug Discovery Today*, 10 (20): 1357-1367.

Kosela, S. L. H., Hu, T. R., Hanafi M. and Sim, K. Y. (2000). Dulxhanthones F-H, Three New Pyranoxanthones from *Garcinia dulci*. *Journal of Natural Product*, **63**: 406-407.

Kumar, P. J. and Clark, M. (2002). *Textbook of Clinical Medicine*. Saunders, London. Pp. 1099–1121.

Kuriki, T., Imanaka, T. (1999). The Concept of the α-amylase Family: Structural Similarity and Common Catalytic Mechanism. *Journal of Bioscience and Bioengineering*, **87**: 557-565.

Kwon, Y. I., Apostolidis, E., Kim, Y. C., Shetty, K. (2007). Health Benefits of Traditional Corn, Beans and Pumpkin: In Vitro Studies for Hyperglycemia and Hypertension Management. *Journal of Medicinal Food,* **10**: 266-275.

Lebovitz, H. E. (1998). Alpha-Glucosidase Inhibitors as Agents in the Treatment of Diabetes. *Diabetes Review,* **6** (2): 132-145.

Likhitwitayawuid K, Chanmahasathien W, Ruangrungsi N, Krungkrai J. (1998). Xanthones with Antimalarial Activity from *Garcinia dulcis*. *Planta Medica*, **64** (3): 281-282.

Lin, L. J., Lin, L. Z., Pezzuto, J. M., Cordell, G. A. (1993). Isogambogic Acid and Isomorellinol from *Garcinia hanburyi*. *Magnetic Resonance in Chemistry*, **31**: 340–347.

Lossow, W. J., Migliorini, R. H., Brot, N., and Chaikoff, I. L. (1964). Effect of Total Exclusion of The Exocrine Pancreas in the Rat Upon in Vitro Esterification of C14 –Labeled Cholesterol By the Intestine and Upon Lymphatic Absorption of C14 – Labeled Cholesterol. *Journal of Lipid Research,* **5**: 198-202.

Muller, W. E., Singer, A., Wonnemann, M., Hafner, U., Rolli, M., and Sch¨afer, C. (1998). *Pharmacopsychiatry*, **31** (1): 16–21.

Maarel, V. D. M. J. E. C., Veen, B., Uitdehaag, J. C. M., Leemhuis, H. and Dijkhuizen, L. (2002). Properties and Applications Of Starch-Converting Enzymes of the α-Amylase family. *Journal of Biotechnology*, **94**: 137-155.

Mabberley, D. J. (1987). *Plant-Book: A Portable Dictionary of the Higher Plants*. Cambridge University Press, Cambridge. Pp. 87-94

Magadula, J. J. (2012). Bioactive Mammae-Type Coumarins and Benzophenones from Two Clusiaceae Plants. *Journal of pharmacy and Science innovation*, **1** (5): 31-33.

Mahabusarakum W, Wiriyachitra P and Taylor W.C. (1987). Chemical Constituents of *Garcinia mangostana*. *Journal of Natural Products*, **50**: 474 - 478.

Marderosian, D. A., Beutler, J. A. (2002). *The Review of Natural Products*. 2nd (ed.). Facts and Comparisons; Seattle, WA, USA. Pp. 13–43.

Martinez, A., Conde, E., Moure, A., Dominguez, H., Estevez, R. J. ((2012). Protective Effect against Oxygen Reactive Species and Skin Fibroblast Stimulation of *Couroupita guianensis* Leaf Extracts. *Natural product research*, **26**: 314-22,

Mathew, O. W. and Blessing C. D. (2007). Hepatoprotective Effects of *Garcinia kola* Seed against Hepatotoxicity Induced by Carbon Tetrachloride in Rats. *Biokemistri,* **19** (1): 17-21.

Mazid, M., Khan, T. A., and Mohammad, F. (2011). Role of Secondary Metabolites in Defense Mechanisms of Plants. *Biology and Medicine,* **3** (2): 232-249,

McChestney, J. D., Venkataraman, S. K., and Henri, J. K. (2007). Plant Natural Products: Back To Future Or Into Extinction? *Phytochemistry*, **68**: 2015-2022.

McCue, P., Kwon, Y. and Shetty, K., (2005). Antidiabetic and antihypertensive potential of sprouted and solid-state bioprocessed soybean. *Asia Pacific Journal of Clinical Nutrition* **14**: 145-52.

Mccue, P. and Shetty, K. (2004). Inhibitory Effects of Rosmarinic Acid Extracts on Porcine Pancreatic Amylase *in vitro*. *Asia Pacific Journal of Clinical Nutrition,* **13** (1): 101–106.

McDougall, G. J., Shpiro, F., Dobson, P., Smith, P., Blake, A., and Stewart D. (2005). Different Polyphenolic Components of Soft Fruits Inhibit Alpha-Amylase and Alpha-Glucosidase. *Journal of Agricultural and Food Chemistry,* **53**: 2760-6.

McLafferty, F. W. (1959). Mass spectrometric analysis. Molecular rearrangements. *Analytical Chemistry,* **31** (1): 82-87.

McLafferty, F. W., and Turecek, F. (1993). *Interpretation of Mass Spectra*. 4th (ed.) University Science Books, California, USA pp. 226-236, 252-258

Mennini, T. and Gobbi, M. (2004). The Antidepressant Mechanism of *Hypericum perforatum. Life Science,* **75**: 1021–1027.

Mesembe, O. E., Asuquo, O. R., Fischer, V. A., Udoaffah, G. U., Mfem, C. C. and Kalu, N. N. (2013). Influence of Long-term Ingestion of *Garcinia Kola* Seed Diet on Sperm Count, Sperm Motility, and Fertility in the Wistar Rat. *Journal of Health, Medicine and Nursing,* **1**: 20-22.

Mitra, A., Dewanjee, D., Dey, B. (2012). Mechanistic Studies Of Lifestyle Interventions In Type 2 Diabetes. *World Journal of Diabetes,* **3**: 201-207. DOI: 10.4239/wjd.v3.i12.201.

Nakai, H., Okuyama, M., Kim, Y., Saburi, W., Wongchawalit, J., Mori, H., Chiba, S.and Kimura, A. (2005). Molecular analysis of ***α***-glucosidase belonging to GH-family 31. *Biologia, Bratislava*, **60** (16): 131—135.

Namdeo, A. G., Mahadik, K. R., and Kadam, S. S. (2006). Antimalarial Drug-*Artemisia annua*. *Pharmacognosy Magazine*, **2** (6): 106-111.

Negi, J. S., Bisht, V. K., Singh, P., Rawat, M. S. M. and Joshi, G. P. (2013). Naturally Occurring Xanthones: Chemistry and Biology. *Journal of Applied Chemistry, 9*.

Newman, D. J., and Cragg, G. M. (2007). Natural Products as Source of New Drugs over the Last 25 Years. *Journal of natural products*, **70**: 461-477.

Nguyen, H. D., Trinh, B. T. D. and Nguyen, L. H. D. (2011). Gutttiferones Q-S, Cytotoxic Polyisoprenylated Benzophenones from the pericarp of Garcinia cochinchinensis. *Phytochemistry letter*, **4**: 129-133.

Nkengfack, A. E., Mkounga, P., Meyer, M., Fomum, Z. T. and Bodo, B. (2002). Globulixanthones C, D and E: Three Prenylated Xanthones with Antimicrobial Properties from the Root Bark of *Symphonia globulifera*. *Phytochemistry*, **61** (2): 181–187.

Nwangwa, E. K. (2012). Effects of *Garcinia kola* on the Lipid Profile of Alloxan-Induced Diabetic Wistar Rats. *British Journal of Pharmacology and Toxicology,* **3** (2): 39-42.

Oboh, G., Ademosun, A. O. (2011). Phenolic Extracts from Grapefruit Peels (*Citrus paradisii*) Inhibit Key Enzymes Linked With Type-2 Diabetes And Hypertension. *Journal of Food Biochemistry*, **35** (6): 1703-1709.

Oboh, G., Ademiluyi, A. O., Faloye, Y. M. (2011). Effect of Combination on the Antioxidant and Inhibitory Properties of Tropical Pepper Varieties Against α-amylase and α-glucosidase Activities *in-vitro*. *Journal of Medicinal Food*, **14** (10): 1152 - 1158.

Oboh, G., Ademosun, A. O. (2011a). Phenolic Rich Extracts from Orange Peels (Citrus Sinensis) Inhibit Key Enzymes Linked to Non-Insulin Dependent Diabetes Mellitus (NIDDM) and Hypertension. *La Rivista Italiana Delle Sostanze Grasse*, **88**: 16-23.

Oboh, G., Akinyemi, A. J., Ademiluyi, A. O., Adefegha, S. A. (2010). Inhibitory Effects of Aqueous Extract of Two Varieties of Ginger on Some Key Enzymes Linked to Type-2 Diabetes *in vitro*. *Journal of Food and Nutritional Research*, **49** (1): 14-20.

Oduje, A. A., Awode, A., Edah, A. and Sagay, I. (2015). Characterization and Phytochemical Screening of n-Hexane Oil Extract from *Cissus* aralioides Seeds. *International Journal of Scientific & Engineering Research*, **6** (1): 112-116

Ofokansi, K. C., Mbanefo, A. N., Ofokansi, M. N and Esimone, C. O. (2008). Antibacterial Interaction of Crude Methanol Extract of *Garcinia kola* Seed with Gatifloxacin. *Tropical Journal of Pharmaceutical Research,* **7** (4): 1159-1165.

Okuyama, M., Okuno, A., Shimizu, N., Mori, H., Kimura, A. and Chiba, S. (2001). *European Journal of Biochemistry*, **268**: 2270–2280.

Olviera, C.M.A., Porto, A.L.M, Biurich, V. and Marsaioli, A.J (1999). Two Prenylated Benzophenones from Floral Resins of Three *Clusia* Species. *Phytochem*istry, **50**: 1073-1079.

Onasanwo, S. A., Singh, N., Olaleye, S. B. and Palit, G. (2011). Anti-Ulcerogenic And Proton Pump (H^+, K^+ ATpase) Inhibitory Activity of Kolaviron from *Garcinia kola* Heckel in Rodents. *Indian Journal of Experimental Biology*, **49** (6): 461-468.

Otor, J. U., Abdulkadir, U. and Abu, M. A. (2001). Some Biological Activities of *Garcinia kola* in Growing Rats. *Veterinary archive,* **71** (5): 287-297.

Panthong, K., Hutadilok-Towatana, N. and Panthong, A. (2009). Cowaxanthone F, A New Tetraoxygenated Xanthone, and Other Anti-Inflammatory and Antioxidant Compounds from *Garcinia cowa*, *Canadian Journal of Chemistry*, **87**: 1636-1640.

Paquin, R., Chapdelaine, P., Dube, J. Y., Tremblay, R. R. (1984). Similar Biochemical Properties of Human Seminal Plasma and Epididymal Alpha-1, 4-Glucosidase. *Journal of Andrology,* **5**: 277–282.

Patra, J. K., Gouda, S., Sahoo, S. K., Thatoi H. N. (2012). Chromatography Separation, ^1H NMR Analysis and Bioautography Screening of Methanol Extract of *Excoecaria agallocha* L. from Bhitarkanika, Orissa, India. *Asian Pacific Journal of Tropical Biomedicine*. Pp. S50-S56.

Pauli, G. (2006). *Countercurrent Chromatography of Natural Products CCC*. Paper presented at the 4th international conference of countercurrent chromatography, Bethesda. MD. Pp. 3-5

Perry, L. and Metzger, J. (1980). Medicinal plants of East and Southeast Asia: Attributed Properties and Uses. Cambridge, Massachusetts, MIT Press. Pp. 4-48

Phillipson, J. D. (2001). Phytochemistry and Medicinal Plant. *Phytochemistry*, **56**: 237-243.

Phillipson, J. D. (2007). Phytochemistry and Medicinal Plant. *Phytochemistry*, **68**: 2960-2972.

Piccinelli, A. L., Cuesta-Rubio, O., China, M. B., Mahmood, N., Pagano, B., Pavone, M., Barone, V., and Rastrelli, L. (2005) *Tetrahedron*, **61**: 8206–8211.

Pinheiro, L., Nakamura, C. V., Dias Filho, B. P., Ferreira, A. G., Young, M. C. M. and GarciaCortez, D.A. (2003). Antibacterial Xanthones from *Kielmeyera variabilis* Mart. (Clusiaceae). *Memorias do Instituto Oswaldo Cruz*, **98** (4): 549–552.

Pirttila, T., Wilcock, G., Truyen, L. and Damaraju, C. V. (2004). Long-term Efficacy and Safety of Galantamine in Patients with Mild-To-Moderate Alzheimer's Disease: Multicenter Trial. *European Journal of Neurology*, **11** (11): 734– 741.

Pontiroli A. E. Calderara A., and Pozza G. (1994). Secondary Failure of Oral Hypoglycaemic Agents: Frequency, Possible Causes, and Management. *Diabetes Metabolism Reviews*, **10**: 31.

Rajagopal, R. D., Gurudutt, K. N., Mamatha, S., Mohan Rao, L. J. (2007). Guttiferic Acid, A Novel Rearrangement Product from Minor Chromenoxanthone Pigments of *Garcinia morella* Desr. *Magnetic Resonance in Chemistry*, **45**: 578–582.

Rang, H. P., Dale, M. M. and Ritters, J. M. (1991). The Endocrine Pancreas and the Control of Blood Glucose. In: B. Simmons, and S. Beasley. *Pharmacology*. United Kingdom, Longman group Ltd. Pp. 403–410

Ranilla, L.G., Kwon, Y.I., Apostolidis, E., Shetty, K. (2010). Phenolic Compounds, Antioxidant Activity and *in vitro* Inhibitory Potential against Key Enzymes Relevant for Hyperglycemia and Hypertension of Commonly Used Medicinal Plants, Herbs And Spices In Latin America. *Bioresources Technology,* **101** (12): 4676-4689.

Reddy, M. K., Heda, G. D. and Reddy, J. K. (1987). Purification and Characterization of α-Amylase from Rat Pancreatic Acinar Carcinoma-Comparison with Pancreatic α-Amylase. *Journal of Biological Chemistry*, **242**: 681-687.

Rempel, B. P. and Withers, S. G. (2008). Covalent Inhibitors of Glycosidases and their Applications in Biochemistry and Biology. *Glycobiology*, **18** (8): 570-586. doi:10.1093/glycob/cwn041

Rimmele, M., and Boos, W. (1994). *Trehalose-6-phosphatehydrolase* of *Escherichia coli*. *Journal of Bacteriology*, **176**: 5654–5664

Ritthiwigrom, T., Laphookhieo, S. and Payne, S.G. (2013). Chemical Constituents and Biological Activities of *Garcinia cowa* Roxb. *Maejo Internation Journal of Science and Technology,* **7** (2): 212-231.

Rolo, A. P., Palmeira, C. M. (2006) Diabetes and mitochondrial function: Role of hyperglycemia and oxidative stress. *Toxicology and Applied Pharmacology* **212**: 167-178

Rukachaisirikul, V., Ritthiwigrom, T., Pinsa, A., Sawangchote, P. and Taylor, W. C. (2003). Xanthones from the Stem Bark of *Garcinia nigrolineata*. *Phytochemistry*, **64**: 1149-1156.

Rukangira, E. (2009). *Medicinal Plants and Traditional Medicine In Africa: Constraints And Challenges.* Retrieved from http//www.p2pays.org/ref/40/39776.pdf179-184.

Russell, R. R. B. and Ferretti, J. J. (1990). *Journal of Genetics and Microbiology*, **136**: 803–810.

Rye, C. S. and Withers, S. G. (2000). Glycosidase Mechanisms. *Current Opinion in Chemical Biology,* **4**: 573–580.

Salim, A. A., Chin, Y. W. and Kinghorn, A. D. (2008). Drug Discovery from Plants. In: K. G. Ramawat, and J. M. Merillon, (Eds). *Bioactive Molecules and Medicinal Plants*. DOI: 10.1007 / 978-3-540-74603-4_1

Salisu, Y., Agunu, A., Abubakar, M.S. and Ibrahim, G. (2009). Hypoglycaemic Effects of *Acacia albida* del. (Mimosaceae) Methanol Root Bark Extract. *Nigerian Journal of Pharmaceutical Sciences*, **8** (1): 66 – 72.

Saliu, J. A., Ademiluyi, A. O., Akinyemi, A. J., Oboh, G. (2012). *In vitro* Antidiabetes and Antihypertension Properties of Phenolic Extracts from Bitter Leaf (*Vernonia amygdalina* del.). *Journal of Food Biochemistry*, **36** (5): 569-576.

Samuelsson, G. (2004). *Drugs of Natural Origin: a Textbook of Pharmacognosy*. Fifth Swedish Pharmaceutical Press, Stockholm, Sweden. 8-29

Sandhya, B., Thomas, S., Isabel, W., Shenbagarathai, R. (2006). Ethnomedicinal Plants Used by the Valiyan Community of Piranmallai Hills (Reserved Forest), Tamil Nadu, India. A Pilot Study. *African Journal of Traditional, Complementary and Alternative Medicine*, **3**: 101-14.

Schomburg, D., Salzmann, M. (1990). *Enzyme Handbook,* Springer-Verlag, Berlin, Germany. Pp. 1-25.

Schuster, B. G (2001). A New Integrated Program for Natural Products Development And The Value Of Ethnomedical Approach. *Journal of alternative and complementary medicine,* **7** (1): 61-72.

Sharma, A. K. (1993). *Diabetes Mellitus and its Complications; an Update*. Macmillan book publishers, New Delhi, India. Pp. 16-51

Shen, J. and Yang, J. S. (2006). Chemical Constituents from Fruit of *Garcinia cowa*, *Zhongguo Yaoxue Zazhi*, **41**: 660-661.

Shen, J. and Yang, J. S. (2007). A Novel Benzophenone from *Garcinia cowa*, *Acta Chimica Sinica*, **65**: 1675-1678.

Shen, J. and Yang, J. (2007). Chemical Constituents of Branch of *Garcinia cowa* Roxb, *Zhongcaoyao*, **38**: 993-994.

Shen, J., Tian, Z. and Yang, J. S. (2007). The Constituents from the Stems of *Garcinia cowa* Roxb. and Their Cytotoxic Activities, *Pharmazie*, **62**: 549-551.

Silverstein, R. M., Webster, F. X. and Kiemele, D. J. (2005). Spectrometric Identification of Organic Compounds, 7th (ed.). John Wiley and sons Inc., New Jersey, USA. Pp. 1-**71**: 72-110.

Sim, L., Quezada-Calvillo, R., Sterchi, E. E., Nichols, B. L., and Rose, D. R. (2008). Human Intestinal Maltase-Glucoamylase: Crystal Structure of the N-Terminal Catalytic Subunit And Basis Of Inhibition And Substrate Specificity. *Journal of Molecular Biology,* **375**: 782-92.

Sneader, W. (2005). *Drug Discovery: a History*. Wiley, Chichester, United Kingdom. Pp. 9-25

Sofowora, E. A. (2008). *Plants In African Traditional Medicine-An Overview.* In: W.C. Evans, (Eds.), *Trease and Evans Pharmacognosy* (15th ed.). India, Saunders an imprint of Elsevier. Pp. 488-496

Sofowora, E. A. (1993). Medicinal plants and traditional in Africa (2nd edition).Spectrum Book Ibadan, Nigeria. Pp. 261-265.

Sogaard, M., Abe, J., Martin-Eauclaire, M. F. and Svensson, B. (1993). *Carbohydrate Polymers,* **21**: 137-146.

Sordat-Diserens, I., Marston, A., Hamburger, M., Rogers, C. and Hostettmann, K. (1989). Novel Prenylated Xanthones from *Garcinia gerrardii*. *Helvetica Chimica Acta*, **72**: 1001-1007.

Spencer, P. E. and Jeremy, P. E (2008) Flavonoids: Modulators of Brain Function? *British Journal of Nutrition*, **99**: 60–77.

Stahl, E. (2005). *Thin Layer Chromatography: A Laboratory Handbook*. Springer India Private Limited, New Delhi, India pages: P. 1041.

Susksamrarrnetal, S., Suwannapoch, N., Ratananukul, P., Aroonlerk, N., Suksamrarn, A. (2002). Xanthones from the Green Fruit Hulls of *Garcinia mangostana*. *Journal of Natural Product*, **65** (5): 761-3.

Sultanbawa, M. U. S. (1973). Chemistry of the Guttiferae of Ceylon. *Journal of the National Science Council of Sri Lanka*, **1**: 123-165

Svensson, J., Ismail, A., Palva, E. T., Close, T. J. (2002). Dehydrins. In: K. B. Storey, and J. B. Storey, (Eds.) *Cell and Molecular Responses to Stress*. Netherlands, Elsevier Science, Amsterdam. Pp. 155–171

Tangphatsornruang, S., Naconsie, M., Thammarongtham, C. and Narangajavana J. (2005). Isolation and Characterization of an α-Amylase Gene in Cassava (*Manihot esculent*a). *Plant Physiology and Biochemistry*, **43**: 821-827.

Tebekeme, O. (2009). *In vitro* Antioxidant and Free Radical Scavenging Activities of *Garcinia kola* seeds. *Food and Chemical Toxicology*, **47** (10): 2620–2623.

Terashima, K., Y. Takaya and M. Niwa, (2002). Powerful Antioxidative Agents Based on Garcinoic Acid from *Garcinia kola*. *Bioorganic and Medicinal Chemistry*, **10** (5): 1619-1625.

Tewary, D. K., Bhardwaj, A. and Shanker, A. (2005). Pesticidal Activities In Five Medicinal Plants Collected From Mid Hills Of Western Himalayas Industrial Crops And Products, **22**: 241-247.

Thode, J., Pershadsingh, H. A., Ladenson, J.H., Hardy, R., and McDonald, J. M. (1989). Palmitic Acid Stimulates Glucose Incorporation in the Adipocyte By A Mechanism Likely Involving Intracellular Calcium. *Journal of Lipid Research*, **30**: 1299-1305.

Tiwari, A. K, Rao, J. M. (2002). Diabetes Mellitus And Multiple Therapeutics Approaches Of Phytochemicals: Present Status And Future Prospects. *Current Science*, **83** (1): 30-38.

Tomer, K., Singh, V., Sethiya, N. K., Singh, H. P., Kumar, M., Chandra, D. (2009). Isolation and Characterization of New Lanosteroid from Ethanolic Extracts Of *Eclipta alba* Linn. *Journal of Pharmacy Resources,* **2** (10): 1635-1637.

Truschelt, E., Frommer, W., Junge, B., Moiler, L., Schmidt, D. D., Wingender, W.A. (1981). *Chemical lnternational Edition England,* **20**: 744-761.

Uchida, R., Nasu, A., Tokutake, S., Kasal K., Tobe, K. and Yamaji, N. (1999). *Chemical and Pharmceutical Bulletin.* **47**: 187-193.

Udenze, E. C. C. Braide, V. B. Okwesilieze, C. N. and Akuodor, G. C. (2012). Pharmacological Effects of *Garcinia kola* Seed Powder on Blood Sugar, Lipid Profile and Atherogenic Index of Alloxan-induced Diabetes in Rats. *Pharmacologia*, **3** (12): 693-699.

Unigwe, C. R. and Nwakpu, P. E. (2009). Effect of Ingestion of *Garcinia kola* seed on Erythrocytes in Rabbits. *Continental Journal of Veterinary Sciences,* **3**: 7 – 10.

Van Der Heijden, R., Jacobs, D. I., Snoeijer, W., Hallard, D. and Verpoorte, R., (2004). The Catharanthus Alkaloids: Pharmacognosy and Biotechnology. *Current Medicinal Chemistry*, **11** (5): 607–628.

Vasella, A., Davies, G. J. and Bohm, M. (2002). Glycosidase Mechanisms. *Current Opinion in Chemical Biology,* **6**: 619–629.

Verpoorte, R. (1998). Chemodiversity and the Biological Role of Secondary Metabolites, Some Thoughts for Selecting Plant Material for Drug Development. *Proceedings of Phytochemical Society, Europe, Kluwer,* **43**: 11-24.

Visser, L. E. (1975). *Plantes Médicinales de la Côte d'Ivoire*; Mededelingen Landbouwhogeschool Wageningen: Wageningen, Netherlands. Pp. 54.

Wahyuni, F. S., Byrne, L. T., Dachriyanus, R. D., Jubahar, J., Lajis, N. H. and Sargent, M. V. (2004). A New Ring-reduced Tetraprenyltoluquinone and a Prenylated Xanthone from *Garcinia cowa*. *Australian Journal of Chemistry*, **57**: 223-226.

Webb, E. C., (1992). *Enzyme Nomenclature. Recommendation (1992) of the Nomenclature Committee of the International Union o Biochemistry and Molecular Biology.* Academic Press. Inc., San Diego, California, United States. Pp. 37-89

Weldegerima, B. (2009). Review On The Importance Of Documenting Ethnopharmacological Information On Medicinal Plants. *African journal of pharmacy and pharmacology*, **3** (9): 400-403.

Weng, J. R., Tsao, L. T., Wang, J. P., Wu, R. R., and Lin, C. N. (2004) *Journal of Natural Product*, **67**: 1796–1799.

Whitcomb, D. C. and Lowe, M. E. (2007). Human Pancreatic Digestive Enzymes. *Digestive Diseases Sciences,* **52**: 1-17.

WHO (2002). *Traditional Medicine Strategy*. A publication of World Health Organization Geneva. pp. 1-2, 14.

Wild, S. G., Roglic, A., Green, R. and King, H. (2004). Global Prevalence of Diabetes, Estimated For The Year 2000 and Projection for 2030. *Diabetes Care,* **27**: 1047-1054.

Winkelmann, K., Heilmann, J., Zerbe, O., Rali, T., and Sticher, O. (2001) *Helvetica Chimica Acta*, **84**: 3380–3392.

Wong, D. W. S. (1995). *Food Enzymes: Structure* and *Mechanism.* Chapman and Hall, New York, USA. Pp. 32-35.

Worthington V. (1993). Alpha Amylase. In: *Worthington Enzyme Manual*. Freehold, Worthington Biochemical Corporation. Pp. 36-41

Xu, G., Kan, W. L. T., Zhou, Y., Song, J.Z., Han, Q.B., Qiao, C.F., Cho, C.H., Rudd, J. A., Lin, G. and Xu, H.X. (2010). Cytotoxic Acylphloroglucinol Derivatives from the Twigs of *Garcinia cowa*, *Journal of Natural Prod*uct, **73**: 104-108.

Yamamoto, T., Unno, T., Watanabe, Y., Yamamoto, M., Okuyama, M., Mori, H., Chiba, S. and Kimura, A. (2004). *Biochemistry and Biophysics Acta,* **1700**: 189–198.

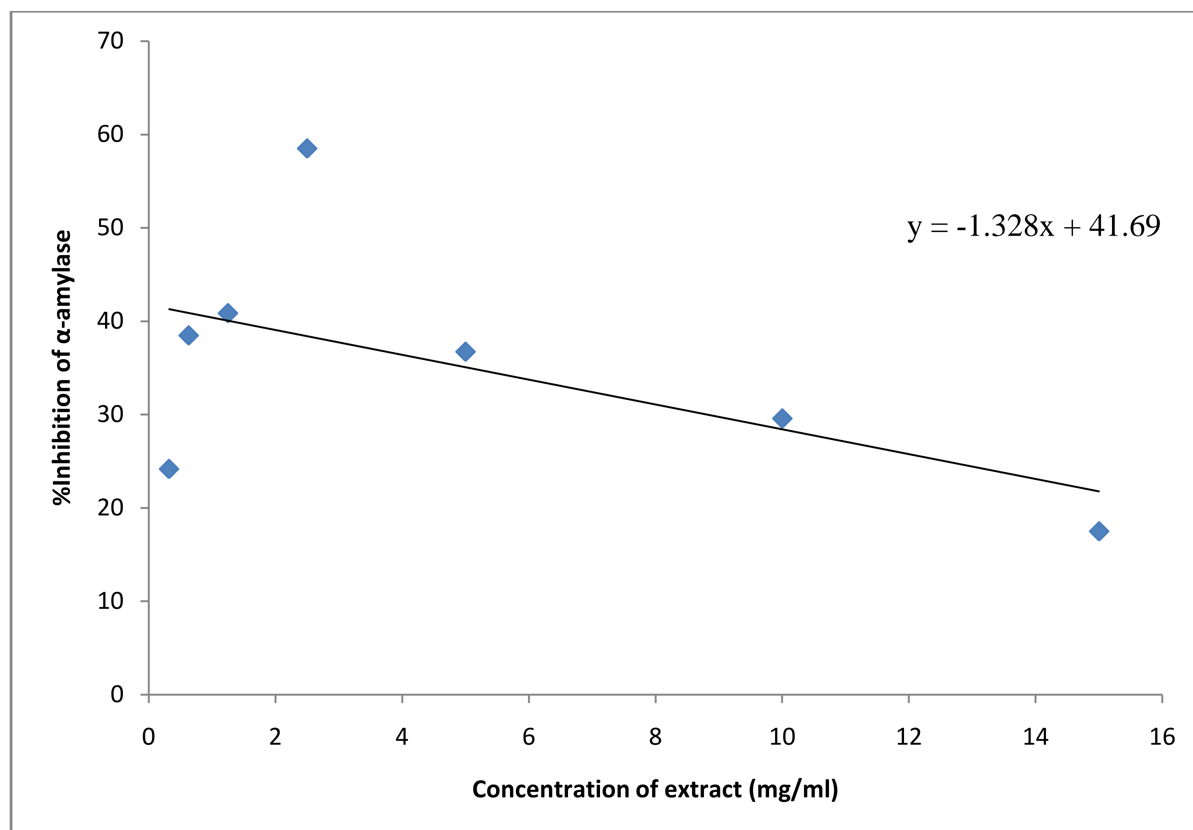
Ye, X., Song, C., Yuan, P., Mao, R. (2010). α-Glucosidase and α-Amylase Inhibitory Activity Of Common Constituents From Traditional Chinese Medicine Used For Diabetes Mellitus. *Chinese Journal of Natural Medicine*, **8**: 349-352.

Zakaria, H. M., Joseph, J. M., Julia, G., Pascal, R. (2012). Phytochemical and pharmacological investigations of *Garcina volkensii* Engl. *Spatula DD*, **2** (1): 1-7.

Zechel, D. L. and Withers, S. G. (2000). Glycosidase Mechanisms: Anatomy of a Finely Tuned Catalyst. *Access Chemical Res*ources, **33**: 11–18.

APPENDICES

APPENDIX A: Determination of IC_{50} values



$y = -1.3282x + 41.691$

where: y= highest percentage inhibition / 2

$= 58.50/2$

$= 29.25\%$

Therefore

$29.25 = -1.3282x + 41.691$

$1.3282x = 41.691 - 29.25$

$x = 12.441/1.3282$

$= 9.37$ mg/ml

APPENDIX B: Determination of α-glucosidase activity

$U/ml = \Delta A V_1 V_2 / M\varepsilon\ T\ V_3 V_4 \times df$

Where $\Delta A = 0.510$

$V_1 = 0.15$ ml

$V_2 = 2.15$ ml

$M\varepsilon = 18.3$

$T = 20$ min

$V_3 = 3$ ml

$V_4 = 0.1$ ml

$df = 2$

Therefore

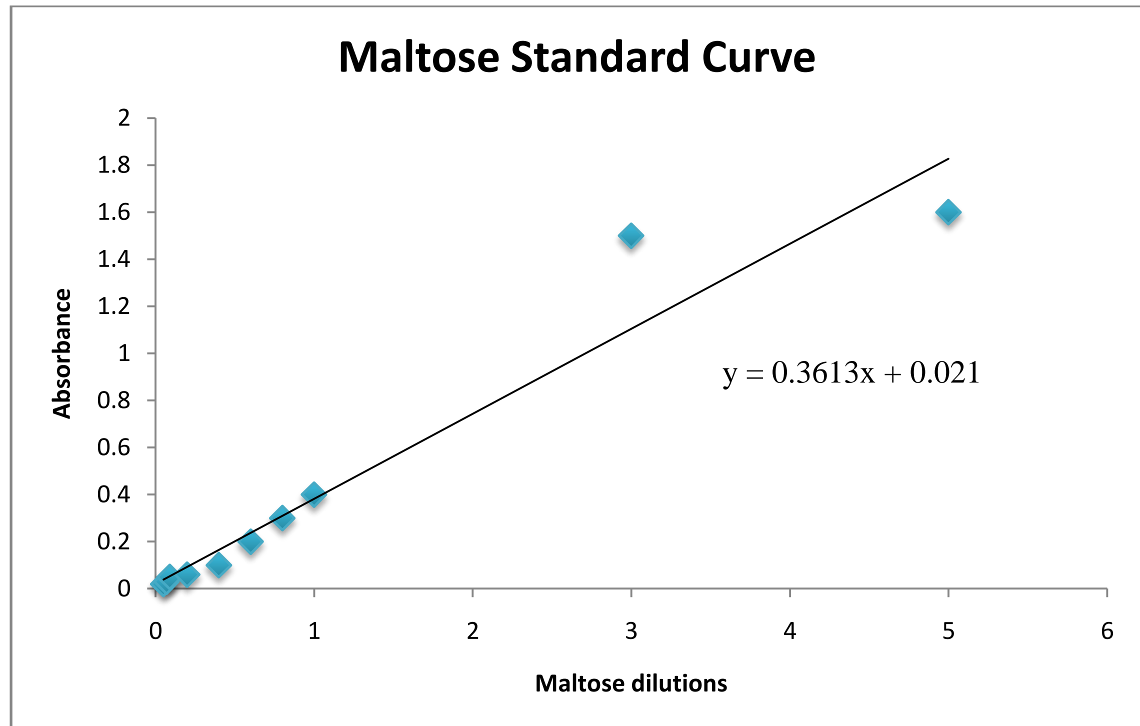
$U/ml = 0.510 \times 0.15 \times 2.15 / 18.3 \times 20 \times 3 \times 0.1$

$= 0.164475 / 109.8$

$= 0.0014 \times 2$

$= 0.0029$ U

APPENDIX C: Determination of maltose released



$y = 0.3613x + 0.021$

where

$y = \Delta A$

Therefore

$0.574 = 0.3613x + 0.021$

$-0.3613x = 0.021 - 0.574$

$x = -0.553 / -0.3613$

$= 1.53$ mg/ml

APPENDIX D: Determination of α-amylase activity

U/ml = maltose released / volume of enzyme used

= 1.53/ 0.5

= 3.06 U

APPENDIX E: Profile of Column Fractions (a summary)

Fractions	Code	Weight	Remark
A	A 1-11	75 mg	
	A 12-13		
B	B 27-29		
	B 30-36 recorded	113 mg	
	B_T after PTLC		
	B_T recorded ZAAK1		
	after second PTLC	23 mg	Elucidated by FT-IR and GC-MS
C	C 37-44	8 mg	
D	D 56-60	324 mg	
E	E 61-75	589 mg	
F	F 76-82		

APPENDIX F: Index of Laboratory Chemicals

1. Ferric Chloride Solution: 2.7g of ferric chloride salt dissolved in 100ml of 2M hydrochloric acid
2. Dragendorffs reagent: 0.11g of potassium iodide and 0.81g of bismuth sub nitrate were dissolved in 20ml of acetic acid and the volume was made up to 100ml with water.

3. Liebermann-Buchard reagent: 5ml of acetic anhydride was placed in an ice bath and to it, 5ml of concentrated sulphuric acid was added, the resulting mixture was added to 50ml ice cold absolute ethanol. This reagent was always freshly prepared before use.

4. Sulphuric acid spray reagent: 10ml of concentrated sulphuric acid was carefully added in 95ml of ethanol to obtain a 10% w/v of the acid in methanol.

5. *p*-Anisaldehyde/ sulphuric acid: 0.5ml of *p*-Anisaldehyde was dissolved in 50ml of glacial acetic acid and thereafter 1ml of concentrated sulphuric acid was carefully added and the mixture stirred to a homogenous mixture. This reagent was usually freshly prepared before use.