Antioxidative and Antidiabetic Activity and Phytochemicals Analysis of Some Selected Sudanese Traditional Medicinal Plants

BY

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Submitted in fulfillment of the academic requirements for MSc degree in Biochemistry, School of Life Sciences, University of KwaZulu-Natal (Westville campus), Durban 4000, South Africa

As the candidate's supervisor I have approved this thesis/dissertation for submission.

Signed: [redacted] Name: **Prof. MS Islam** Date: **December, 2021**

PREFACE

The content presented in this dissertation is the results of the candidate's research. It was conducted at the Department of Biochemistry, School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville Campus, Durban, South Africa, from June 2019 to November 2021 under the supervision of Prof. Md. Shahidul Islam has not been submitted in any form to any other university to award a degree or diploma. The text has appropriately recognized when other people's work has been included in the text through a reference.

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Candidate: Almahi Idris Mohamed

ABSTRACT

This study was conducted to evaluate the antioxidant and anti-diabetic properties of selected traditional Sudanese medicinal plants (*Cyperus rotundus, Nauclea latifolia, and Hibiscus sabdariffa*) using *in vitro, ex vivo,* and *in silico* experimental models. The crude extracts (ethyl acetate, ethanol, and aqueous) were screened *in vitro* for their antioxidant activities using ferric-reducing antioxidant power (FRAP), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and nitric oxide radical (NO) scavenging activities, as well as their carbohydrate digesting enzyme inhibitory activities for antidiabetic evaluation. Subsequently, the extracts were subjected to Gas Chromatography-Mass Spectrometry (GC-MS) analysis to elucidate their possible bioactive compounds. Additionally, *ex vivo* studies was conducted to investigate their capability to promote muscle glucose uptake and suppress glucose absorption in the intestine as well as to analyze antioxidative effects in iron–induced oxidative stress in hepatic tissue. Molecular docking was carried out to determine the probable enzymes' inhibitory mode of action by ligands identified through GC-MS. This study indicates that these traditional Sudanese medicinal plants have remarkable antioxidant and antidiabetic activities, which may help to ameliorate oxidative stress and diabetes. Therefore, these plants may be considered a natural source of bioactive compounds beneficial for human health, particularly for managing diabetes and oxidative stress-related metabolic disorders.

DECLARATION 1 – PLAGIARISM

I, Almahi Idris Mohamed, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other persons' writing unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

   a. their words have been re-written, but the general information attributed to them has been referenced.

   b. where their exact words have been used, then their writing has been placed in italics and inside quotation marks and referenced.

5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed

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DECLARATION 2 - PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication).

I Almahi Idris Mohamed declared that all the publications included in this thesis was designed and performed all the experiments in the following articles with guidance from my supervisor. The co-authors contributed partly in the work.

PUBLICATIONS FROM THE THESIS

Published/accepted

1. **Mohamed, A. I.**, Beseni, B. K., Msomi, N. Z., Salau, V. F., Erukainure, O. L., Aljoundi, A., & Islam, M. S. (2021). The antioxidant and antidiabetic potentials of polyphenolic-rich extracts of *Cyperus rotundus* (Linn.). **Journal of Biomolecular Structure and Dynamics** [2021 Impact Factor 3.392, ePub ahead of print]. https://doi.org/10.1080/07391102.2021.1967197

Prospective publications from this thesis

1. **Mohamed, A. I.,** Beseni, B. K., Msomi, N. Z., Salau, V. F., Erukainure, O. L., Aljoundi, A., & Islam, M. S. The Antioxidant and Antidiabetic Activities of Promising Polyphenolic Compounds from the Fruit of *Nauclea latifolia* (Smith)- *An Invitro and Insilico Study*. [In preperation]
2. **Mohamed, A.I.**, Salau, V. F., and Islam, M. S. *Hibiscus sabdariffa* (Linn). polyphenolic-rich extract improves antioxidant activities and modulates key enzymes linked to Type 2 diabetes in Fe^{2+} induced oxidative hepatic injuries. [In preperation]

Presentations at conferences

1. **Mohamed, A. I.**, and Islam, M. S. The antioxidant and antidiabetic potentials of polyphenolic-rich extracts of *Cyperus rotundus*(Linn.). **College of Agriculture, Engineering, and Sciences Postgraduate Research & Innovation Symposium (PRIS),** 9-10 December 2021, University of KwaZulu-Natal, Durban, South Africa.

DEDICATION

I learned all the words and broke them up to make a beautiful word, my parents:

To my mother, Ayesha Ahmed Idris

To my Father, Idris Mohamed Idris

And because you are my world, I have on this earth what makes life worth living.

For you again more than ever.

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My deepest gratitude is extended to my family, particularly my brothers and sisters, for their understanding, prayers, and unwavering support.

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LIST OF ABBREVIATIONS

WHO	World Health Organisation
TCA	Tricarboxylic acid
T1D	Type 1 diabetes
T2D	Type 2 diabetes
SGLT2	Sodium/glucose co-transporter 2
SGLT2	Sodium glucose-transporters inhibitors
ROS	Reactive oxygen species
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PKC	Protein kinase C
O2.-	Superoxide anion
OH.	Hydroxyl radical
$ONOO^-$	Peroxynitrite
MDA	Malondialdehyde
NAD^+	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
H_2O_2	Hydrogen peroxide
$FeCl_3$	Iron (III) chloride
FRAP	Ferric reducing antioxidant power
GDM	Gestational diabetes mellitus
DPPH	2,2'-diphenyl-1-picrylhydrazyl
DM	Diabetes mellitus

DNS	Dinitrosalicylic acid
DETAPAC	Diethylenetriaminepentaacetic acid
CVD	Cardiovascular disease
AGEs	Advanced glycation end products
NIDDM	Non insulin dependent diabetes mellitus
GFAT	Glutamine:fructose-6 phosphate amidotransferase
DPP	Dipeptidyl peptidase
TBARS	Thiobarbituric acid reactive substances

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Diabetes is a serious epidemic that is rapidly spreading worldwide (Unnikrishnan *et al.*, 2017). The incidence of non-communicable disorders is rising at an alarming rate in all over the globe. Every year, over 18 million people worldwide die from cardiovascular disease, with diabetes and hypertension being key risk factors (Gan, 2015). In 2019, an estimated 463 million persons worldwide had diabetes **Figure 1.1**, from 108 million cases recorded in 1980, thus representing a 46.2% increase in the adult population. The number is expected to rise to 578 million by 2030 and 700 million by 2045 (IDF, 2019; Saeedi *et al.*, 2019). As a result of its complications, one death every 8 seconds occurs from diabetes and is responsible for 11.3 % of all fatalities among adult (20–79 year-old age range) individuals worldwide (IDF, 2019). Global diabetes health spending has been predicted to reach $760 billion in 2019 (IDF, 2019). However, diabetic patients are at risk of having various significant and life-threatening conditions, resulting in a higher demand for medical care, poor living standards, and excessive stress on communities (Piette *et al.*, 2004). This unprecedented financial, health, and economic burden of diabetes contributes to the current development challenges in Africa (Mbanya *et al.*, 2003). Numerous pharmacological agents such as metformin, biguanides, and thiazolidinediones have been developed to manage T2D. Despite their far-reaching therapeutic benefits, such pharmacological agents have several drawbacks, including inaccessibility, exorbitant pricing, and several inherent undesirable side effects (Esposito *et al.*, 2009; Yancy *et al.*, 2018; Kenny *et al.*, 2019).

Due to the high cost and limited availability of contemporary treatments for many populations in developing countries, alternative methods to modern pharmacotherapy for diabetes are continuously required (Payyappallimana, 2010; Pereira *et al.*, 2016). Plants are widely utilized throughout the African continent, with up to 90% of the population in some areas relying only on plants as a primary source of medication to treat various ailments such as diabetes (Hostettmann *et al.*, 2003). The need for more agents to treat hyperglycemia and associated consequences has created an opportunity to explore traditional antidiabetic treatments (Gray *et al.*, 1998; Rani, 2021).

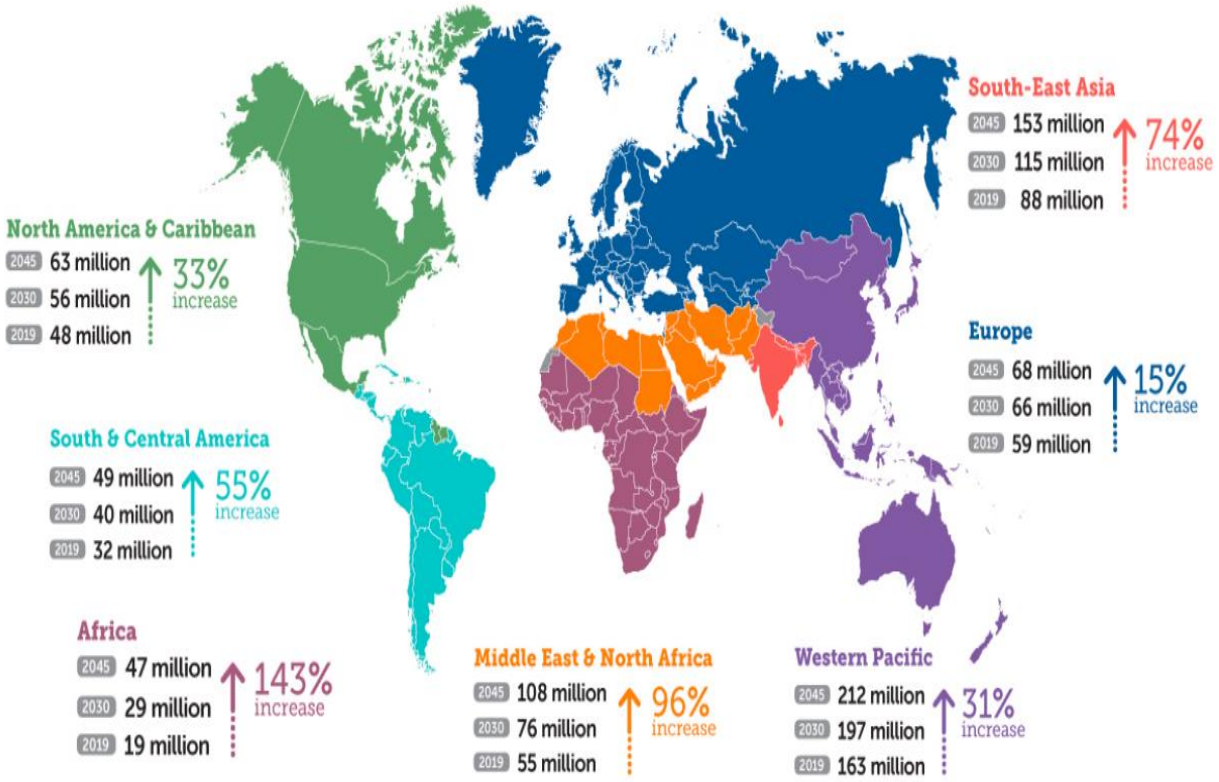

Figure 1.1: Diabetic statistics worldwide (Copied without permission from IDF Atlas, 9th edition 2019)

1.2 Diabetes mellitus (DM)

Diabetes mellitus (DM) is a chronic disease related to hyperglycemia which occurs due to inadequate insulin production and inadequate sensitivity of cells to insulin action (Devi *et al.*, 2019). This happens either as a result of the pancreatic failure to secrete insulin as seen in type 1 diabetes or decreased capability of the pancreas to secrete insulin as well as the inability of insulin to work (insulin resistance) as seen in type 2 diabetes (IDF, 2016; Seino *et al.*, 2010). The lack of insulin activity in target tissues is the cause of abnormalities in carbohydrates, lipid, and protein metabolism in diabetic condition (Dronavalli *et al.*, 2008). Polyuria, polydipsia, and weight loss are common signs of severe chronic hyperglycemia as well as diabetes (Koenig *et al.*, 2004). DM is usually associated with glucotoxicity, lipotoxicity, elevated inflammation/oxidative stress, and endoplasmic reticulum-induced stress, all of which cause apoptotic or necrotic cell death in pancreatic cells (Jadhav, 2013). These factors also contribute to the development of comorbidities such as retinopathy, nephropathy, neuropathy, and heart disease (CVD) (Tripathi *et al.*, 2006). Over half of diabetic patients suffer from these problems, linked to increased morbidity and death (Islam, 2011), and the prevalence of diabetes continues to grow across the globe (Beagley *et al.*, 2014).

1.3 Types of diabetes

Diabetes generally has been classified into few main categories based on their etiology and clinical presentations. Type 1 diabetes (T1D), type 2 diabetes (T2D), gestational diabetes mellitus (GDM), and other specific types of diabetes mellitus (WHO,1999; Collares *et al.*, 2013).

1.3.1 Type 1 diabetes (T1D)

T1D is identified as an asymptomatic condition influenced by autoantibodies, genes, and environmental circumstances, which appears in childhood-onset (Smyth *et al.*, 2008). It is also assumed to be caused by autoimmune disease syndrome, which damages the insulin-producing cells or pancreatic β-cells (Bluestone *et al.*, 2010; Smyth *et al.*, 2008). The variations in gene expression between different population groups could partially explain the distinguished variation in the incidence of T1D among ethnic groups. Studies have indicated that the environment is an essential factor associated with the prevalence of the disease. (Onkamo *et al.*, 1999).T1DM is also triggered by autoimmune-destruction of pancreatic β-cells, necessitating exogenous insulin for the rest of the life (Eizirik *et al.*, 2009). This condition is influenced by a complex interaction between invading or resident macrophages and T-cells, which start releasing chemokines and cytokines in the islet microenvironment and eventual apoptotic β-cell death (Gonzalez-duque *et al.*, 2018). This interaction is influenced by genetic history, age, and environmental factors, including viral infections and food habits (Sofia *et al.*, 2018). T1D doubles in frequency in children every 25 years, resulting in an average loss of 11–12 years of life duration (Sofia *et al.*, 2018).

Currently, no treatment methods are available to prevent or treat T1D (Greenbaum *et al.*, 2018). Furthermore, T1D therapy is difficult and time-consuming (Lingensmith, 2005). T1D treatment and management deteriorate during adolescence, increasing the possibility of complications and growing health care expenses (Johnson *et al.*, 1992; Wagner *et al.*, 2021). T1D management necessitates the close coordination of a complex treatment plan. Insulin delivery, life style modifications, physical exercise, regular blood glucose testing, and meal planning, particularly carbohydrate counting, are tasks that patients and their families must manage and organize all the times (Mcknight *et al.*, 2008).

1.3.2 Type 2 diabetes (T2D)

T2D is identified by partial inability of insulin secretion and insufficient inability of insulin action also called insulin resistnace (Withers *et al.*, 1998; Ling, 2020). It is also distinguished from T1D by insulin resistance occurring from an impairment in the function of pancreatic β -cells. Inadequate insulin production can develop due to the pancreatic β – cells' inability to compete with demand (Rachdaoui, 2020). T2D is quite frequent in older individuals, although it is becoming more prevalent in children and young adults as obesity, physical inactivity, and poor nutrition become more prevalent (Hegazi *et al.*, 2015). The reasons for T2D are unknown (Sutherland *et al.*, 2017); however, there is a clear relationship between obesity, growing age, ethnicity, and family history with diabetes. In addition to multi-gene predisposition and other environmental factors, all these are risk factors of T2D (Lambert *et al.*, 2016; Yamamoto *et al.*, 2018). Genetic history and environmental factors influence T2D epidemiology. Genetic variables impact exposure to an obesogenic environment characterized by sedentary behavior and an unhealthy diet (West *et al.*, 2011).

T2D is prevalent worldwide and estimates more than 90% of all cases of diabetes types (Arredondo *et al.*, 2017). Aging population, economic development, and urbanization contribute to T2D and its complications. Furthermore, many other factors encourage the high-scale widespread diabetes, such as physical inactivity, unhealthy eating habits, and multiple complications that result from auto-oxidation of glucose and the production of free radicals in the bloodstream (Oyebode *et al.*, 2020). The guidelines for managing T2D include practicing a healthy lifestyle, eating healthy meals, regular physical exercise, quitting smoking, checking up on the blood glucose level regularly, and maintaining a healthy body weight (Nam *et al.*, 2011). Oral treatment of T2D is

generally started with metformin as a first-line drug when lifestyle changes are insufficient to manage blood glucose level (Genuth, 2015).

1.3.3 Gestational diabetes (GDM)

Gestational diabetes is a specific case during pregnancy as a transient disorder and is distinguished by hyperglycemia and its diverse symptoms (Ananthakrishnan *et al.*, 2020). Risk factors for GDM include age (the older a woman has an increased risk of GDM), overweight or obesity during pregnancy, family background with diabetes, and high blood glucose throughout pregnancy (Catalano *et al.*, 2006).

1.3.4 Other types of diabetes mellitus

These are classified into two classes:

Class (A) Diabetes with known genetic disorders: many specific genetic anomalies have been reported as developing diabetes mellitus due to recent advances in genetic engineering. They are classified into

(i) Genetic abnormalities correlated with the pancreatic β-cell operations; and

(ii) genetic irregularities related to insulin response processes (Fajans, 1990; Collares *et al.*, 2013).

Class (B) Diabetes Different types associated with other diseases and disorders: certain illnesses, syndromes, and conditions may be followed by diabetes developments, historically called secondary diabetes, which includes pancreatic diabetes illnesses, an endocrine condition, liver disease, medications use, vulnerability to chemical ingredients, viral infections, and many genetic abnormalities (Seino *et al.*, 2010).

1.4 Diabetes prevalence in Africa

In Africa, the spread of diabetes affects an estimated 19.4 million persons aged (20 to 79 years), resulting in a regional prevalence of 3.9%, considered the lowermost prevalent worldwide (IDF, 2019). However, diabetes has the highest incidence (8.8%) among individuals aged 65 to 69 years old in Africa. The lack of information and awareness of diabetes among African people, including untrained local medical staff, weak health care infrastructures, and poverty, has increased the incidence of diabetes (Ahmed *et al.*, 2001). Furthermore, modern lifestyles and civilization have changed the African population's living style. These are considered the principal circumstances of the growing rate of diabetes in societies. Economically, there is a lack of data on the economic impact of diabetes in Africa, with limited research in the diabetes field. Predictions suggest that Africa will experience the highest growth in diabetes and complications (Lipsky *et al.*, 2015). Statistics have shown that by 2025, the number of people living with diabetes in Africa will reach 300 million. As a result, diabetes causes 10% of all hospitalizations and deaths (Ahmed *et al.*, 2001; Walicka *et al.*, 2021). In 2019, total health expenditures related to diabetes in Africa amounted to $ 9.5 billion. However, diabetes-related annual health expenditures are expected to reach $ 12.7 billion and $17.4 billion in 2030 and 2045, respectively (IDF, 2019).

1.5 Diabetes status in Sudan

Up until the late 70s in the last century, diabetes was considered a rare disease in Sudan. However, at present, it is deemed to be regarded as a significant health problem, especially in Northern Sudan (Ahmed, 2003), with about 19% compared to 2.5% in the rural areas (Elmadhoun *et al.*, 2016). Like in other developing countries, the rate of uncontrolled diabetes is becoming significant in Sudanese people with T2D (85%) (Noor *et al.*, 2016). In 2015, IDF reported that 7.7% of the adult

population of Sudan were affected by diabetes mellitus, and this increasing prevalence has reached an alarming point (IDF, 2015). According to IDF Diabetes Atlas (9th edition), by 2045, Sudan's diabetes status will rank as a top 10 country in the African region, particularly in the adult population (20-79 years old) (IDF, 2019). Nonetheless, Sudan has the highest diabetes prevalence among adults (20- 70 years) compared to the middle east and north Africa (22.1%) (IDF, 2019).

1.6 Diabetes and oxidative stress

Oxidative stress is a common term indicating the imbalance of reactive oxygen molecules and the ability of the biological system to restore the predictable harmful result. Oxidative stress develops when the body's antioxidants fail to counteract free radicals (Poljsak et al., 2013). The principle by which free radicals inroad the body involves reducing antioxidant production and extreme production of free radicals (Maritim *et al.,* 2003). Hyperglycemia has been linked directly to the generation of free radicals through various oxidative pathways, including the polyol, advanced glycated end products, hexosamine, and protein kinase activation C (Vanessa Fiorentino *et al.,* 2013). Excessive generation of free radicals can lead to damages in lipids, proteins, and DNA which progresses diabetes mellitus and development of its complications (Johansen *et al.,* 2005).

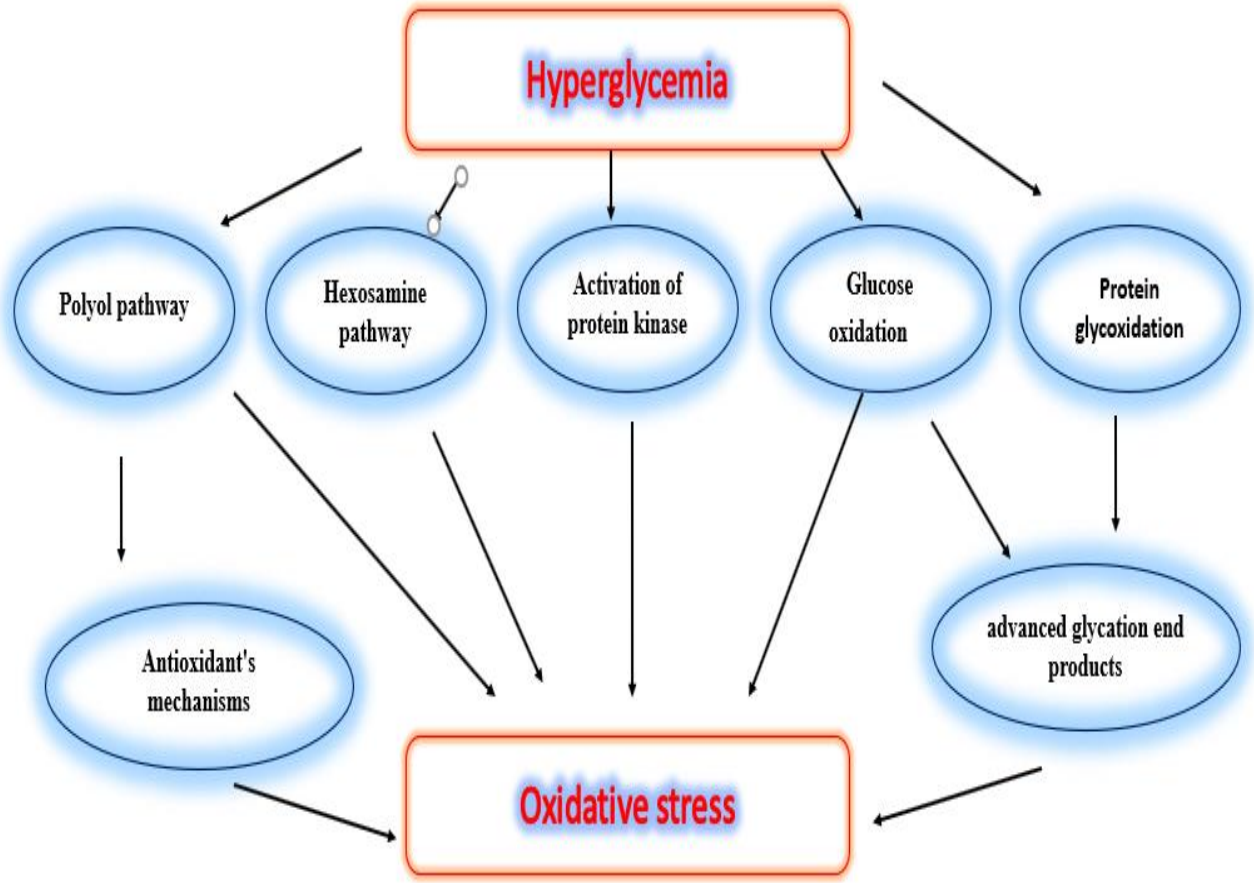

Figure 1.2: Pathways leading to oxidative stress.

1.6.1 Polyol pathway

The polyol pathway is a two-step metabolic process that involves reducing glucose to sorbitol and then converting it to fructose. It is one of the most intriguing potential pathways for determining, at least in part, diabetes hyperglycemia's cellular toxicity (Lorenzi, 2007). When intracellular glucose levels increase, the polyol pathway of glucose metabolism becomes active. The first and rate-limiting enzyme in the process, aldose reductase (AR), uses NADPH as a cofactor to convert glucose to sorbitol, which is subsequently converted to fructose sorbitol dehydrogenase, which

employs NAD^+ as a cofactor **Figure 1.2** (Szwergold *et al.*, 1990; Physiol *et al.*, 2021). Therefore, regulation of the polyol pathway can trigger and amplify various processes of cellular damage by changing intracellular tonicity, producing AGEs precursors, and exposing cells to oxidative stress, perhaps through reduced antioxidant defenses and production of oxidant species (Dagher *et al.*, 2004).

1.6.2 Advanced glycation end products

Advanced glycation end-products (AGEs) are diverse and complicated substances associated with diabetic consequences **Figure 1.2**. One of the pathogenic mechanisms of hyperglycemia-induced oxidative damage has been identified as increased production of AGEs (Vistoli *et al.*, 2013). It results from non-enzymatic glycosylation of proteins and lipids that occurs in hyperglycemic conditions due to prolonged glucose exposure. Enzymatic activities, cellular processes, molecular conformation, and receptor recognition are all affected by the presence of AGEs (Goldin *et al.*, 2006). AGEs reduce Endothelium-derived NOs bioavailability and activity (Hogan *et al.*, 1992; Guizoni *et al.*, 2020). This impact of AGEs on NO may be implicated in atherogenesis because NO suppresses several processes that lead to atherosclerosis, including leukocyte adherence to the artery wall, vascular smooth muscle development, and platelet adhesion and aggregation (Bucala *et al.*, 1991; Zhang *et al.*, 2020).

1.2.3 Hexosamine pathways

The hexosamine biosynthetic pathway has been implicated in developing insulin resistance and diabetic vascular problems (Medicine, 2000). Hyperglycemia-induced production of transforming growth factor- β (TGF- β1), a prosclerotic cytokine, was shown to be causally involved in the development of diabetic nephropathy (Medicine, 2000). The rate-limiting enzyme of this pathway is glutamine: fructose-6-phosphate-amidotransferase (GFAT), an enzyme that catalyzes the

amidation of fructose-6-phosphate to glucosamine-6-phosphate (GlucN-6-P) in this pathway. Increased activity of GFAT has been implicated in the progression of insulin resistance and the development of diabetic complications such as diabetic nephropathy (Marshall *et al.*, 1991; Zibrova *et al.*, 2017; Ohiagu *et al.*, 2021).

1.6.4 Protein kinase C

Protein kinase C (PKC) is an essential enzyme in cell growth, hypertrophy control, and signal transmission in the heart (Anna *et al.*, 1990; Marrocco *et al.*, 2019). Recent research has found that hyperglycemia-induced activation of PKC and elevated diacylglycerol levels are linked to various vascular retinal diseases and cardiovascular tissues problems (Das *et al.*, 2007). PKC is persistently activated in diabetes and non-diabetic insulin resistance due to high intracellular lipid diacylglycerol, an intermediate in glucose and fat metabolism (Schooneman *et al.*, 2013).

1.7 Diabetes complications

Chronic hyperglycemia develops in people living with long-term diabetes. Untreated chronic hyperglycemia leads to the development of diabetic complications (Mukhopadhyay *et al.*, 2019). Free radicals have been highlighted as underlying pathways correlating various mechanisms for diabetes complications (Beagley *et al.*, 2014). However, the high risk of complications may be disabling or even life-threatening. The possible complications include:

1.7.1 Microvascular complications

Microvascular complications are a group of illnesses that affect small blood vessels among people with diabetes. Retinopathy, nephropathy, and neuropathy are the most common consequences, which can have a disastrous impact on human life if not early diagnosed or neglected (Lilly *et al.*, 2006).

1.7.1.1 Diabetic retinopathy

Diabetic retinopathy is a severe diabetic microvascular complication and a primary cause of blindness and visual impairment (Lilly *et al.*, 2006). Several reports have linked visual impairment induced by diabetic retinopathy to poor survivability. This is frequently associated with cardiovascular disease (Hong *et al.,* 2020). The peripheral retina, the macular, or both can be affected by diabetic retinopathy (Markan *et al.,* 2020). In both T1D and T2D diabetes, the impact of diabetic retinopathy and its accompanying clinical characteristics is fundamentally comparable (Vogt *et al.,* 1992; Hong *et al.*, 2020). The age at which a person first develops diabetes has also been a determinant in developing chronic complications (Henricsson *et al.,* 1997; Dart, 2021). Studies have shown that patients with diabetes onset in puberty (up to diabetes duration of 20 years) have a significantly higher risk of diabetic microvascular complications, including diabetic retinopathy than those with diabetes onset before puberty. One possible explanation is hormonal changes during puberty (Vogt *et al.,* 1992; Tommerdahl *et al.,* 2021).

1.7.1.2 Diabetic nephropathy

Diabetic neuropathy impacts approximately two-thirds of all people with diabetes. It is caused by chronic hyperglycemia, which destroys the nerves and blood vessels, as well as other risk conditions, including being older, getting obese, and experiencing a peripheral vascular disease. Peripheral neuropathy is characterized by altered sensations, which generally begin in the lower extremities and progress upward (Mutua, 2021). Individuals with diabetic neuropathy may have a complete sensory loss, paresthesias, numbness, and a loss of temperature feeling (Motiwala, 2021). This probably leads to severe complications like unclear, failure of physical activities, and amputations. The most common condition of diabetic neuropathy is diabetic food, where an

inflamed ulcer develops as an injury that the patient is unconscious of due to sensory loss (WHO, 2020).

1.7.1.3 Diabetic neuropathy

Diabetic neuropathy is a prevalent diabetic complication that can have severe consequences in terms of morbidity and death (Girach *et al.*, 2006). The main risk factor of diabetic neuropathy is chronic hyperglycemia, resulting in severe complications such as cellular damage of neurons, vascular endothelial cells, glial cells, and macrophage activation. The most symptoms of neuropathy are pain, paresthesia, and loss of sensation (Schäfers, 2001; Callaghan *et al.*, 2012). Oxidative stress, advanced glycation end products, polyol pathway flow, and protein kinase C activation; all correlate to microvascular condition and nerve dysfunction in diabetic neuropathy (Baynes *et al.*, 1999; Vlassara *et al.*, 2002; Shayesteh *et al.*, 2017). Diabetic neuropathy has a different-faceted development of diabetes that could be controlled symptomatically with a line-up of medications. However, complementary drugs such as clonidine, midodrine, dihydroergotamine, or caffeine have also been proven effective(Duby *et al.*, 2004).

1.7.2 Macrovascular complications

Diabetes and its consequences have been linked to long-term impairment and organ damage in various body systems. The fundamental cause for the development of macrovascular illnesses has been identified as atherosclerosis, which results in narrowing artery walls (Ighodaro*et al.*, 2017). As a result, cardiovascular diseases (CVDs) constitute a significant cause of death in people with T2D and account for the vast majority of healthcare expenditure (Fowler, 2008). Congestive heart failure, peripheral vascular disease, myocardial infarction, and coronary artery disease are examples of these conditions (Ighodaro*et al.*, 2017). Individuals with T2D are more vulnerable to CVD (Fowler, 2008).

Atherosclerosis is triggered by chronic inflammation and oxidative damage to the peripheral artery wall or coronary vascular system. This is due to the buildup of oxidized lipids in the endothelial walls of the arteries, which causes the lumen to shrink (Fowler, 2008). This is accompanied by monocyte penetration across the artery wall, where they differentiate into macrophages, producing foam cells when exposed to oxidized lipids. T-lymphocytes are attracted to foam cells because they boost macrophage growth. T-lymphocytes then cause the smooth muscle of the artery walls to proliferate, attracting collagen and causing acute vascular infarcts (Ighodaro *et al.*, 2017).

1.8 Pharmacological management of T2D.

Changes in lifestyle, dietary modification, and frequent physical activity are usually recommended in conjunction with pharmacological treatments to attain a reasonable glycemic control. Various oral anti-diabetic drugs with multiple modes of action are currently available (Beagley *et al.*, 2014). They include α- glucosidase inhibitors, biguanides, sulfonylureas, thiazolidinediones, sodium-glucose co-transporters (SGLT2) inhibitors, and dipeptidyl peptidase (DPP) IV inhibitors (Souto *et al.*, 2013). **Figure 1.3** shows the various categories of antidiabetic agents and their target organs.

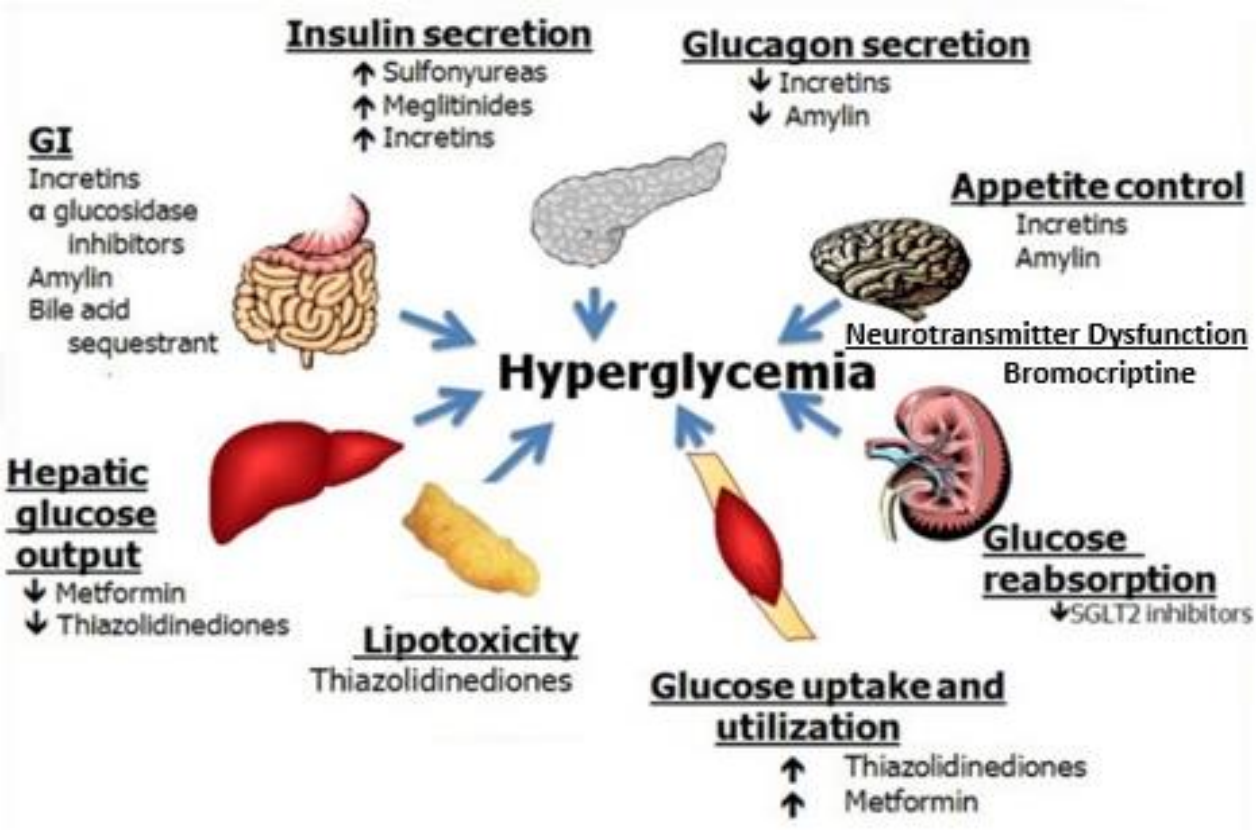

Figure 1.3: Site of action and different mechanisms of the therapeutic drugs of T2D (Copied without permission from Evans, 2016).

1.8.1 Sulfonylureas

Sulfonylureas (SUs) are a type of anti-hyperglycemic medicine mainly used to treat T2D by lowering blood glucose levels (Prato, 2007). SUs stimulate pancreatic beta cells, which leads to an increase in insulin secretion (Eliasson, 1996; Canpolat *et al.,* 2020). Also, there are two kinds of sulfonylurea subunits, SUR1, which is found on pancreatic beta cells and in the brain, and SUR2, which is located in cardiac, skeletal, and smooth muscles. Individual sulfonylurea medicines have a varying affinity for these receptors, which might explain some sulfonylureas' adverse effects, specifically regarding the cardiovascular problems of these insulin secretagogues (Proks *et al.*, 2002). Sulfonylureas also induce hepatic gluconeogenesis and increase the development and

sensitivity of insulin receptors in other tissues such as adipocytes. Hypoglycemia seems to be the most common and severe side effect associated with SU treatment, attributable to the commencement of insulin secretion even when glucose levels are below the normal range for average insulin production produced by physiologic glucose (Sola *et al.*, 2015).

1.8.2 α-glucosidase Inhibitors

The α-glucosidase inhibitors **(**AGIs) are competitive inhibitors of pancreatic α-amylase and intestinal brush border α-glucosidase, leading to delayed absorption of ingested polysaccharides, oligosaccharides, and disaccharides (Kwaku *et al.*, 2010). AGIs such as acarbose are active membrane bond inhibitors of the small intestine and hydrolyze oligosaccharides, trisaccharides, and disaccharides. Other monosaccharides delay postprandial glucose uptake (Laar, 2008). Many studies on acarbose, an α-glucosidase inhibitor, have shown that acarbose is associated with reducing a possible risk of cardiovascular disease according to the reduction of oxidative stress resulting from postprandial hyperglycemia. But the gastrointestinal tolerability has limited their uses (Moses *et al.*, 2015). However, AGIs are also absolutely safe and non-toxic medications. They mostly have gastrointestinal side effects, including flatulence and diarrhea *(*Godbout *et al.*, 2007).

1.8.3 Thiazolidinediones

The insulin-sensitizing thiazolidinediones (TZDs), specific ligands of the nuclear transcription factor peroxisome-proliferator–activated receptor g (PPARg), are the first medicines to target the critical problem of insulin resistance in T2D (Yki-j *et al.*, 2004; Ciavarella *et al.*, 2020). TZDs stimulate insulin sensitivity in the body by gene arrangement (Krentz *et al.*, 2005). These substances improve glucose uptake in the muscle tissue through glucose transporter-4 (GLUT 4) and minimize gluconeogenesis levels in the liver. Furthermore, this category of drugs could be used to treat people with non-diabetic insulin resistance (Yki-j *et al.*, 2004). Clinical investigations

have indicated that TZDs lower plasma glucose and insulin levels and ameliorate certain lipid metabolic anomalies, consistent with animal research. Treatment of T2D patients with TZDs reduces blood glucose, and insulin levels increase peripheral glucose uptake and lower triglyceride levels in these model systems (Saltiel *et al.,* 2000; Shamshoum *et al.,* 2020). The common side effects of anti-diabetic thiazolidinediones (TZDs) on subsidizing to heart failure, gain weight, Oedema, idiosyncratic hepatotoxicity, and fluid retention (Nanjan *et al.,* 2018; Riess *et al.,* 2020)

1.8.4 Biguanides

The two most essential biguanides, metformin, and phenformin, were first developed as oral glucose-lowering medicines in 1957 to treat non-insulin-dependent diabetic Mellitus (NIDDM) (Osadebe *et al.,* 2015). Metformin enhances insulin-mediated glucose absorption and oxidative metabolism in peripheral tissues. Metformin also improves the intestine's glucose consumption, mainly through nonoxidative metabolism (Zilov *et al.,* 2020). It is an antihyperglycemic medication since it does not produce clinical hypoglycemia. It hinders weight gain, fights hypertriglyceridemia, and has vasoprotective effects. Thus, it is an effective therapy for insulin-resistant NIDDM patients who are overweight (Bosi, 2009). diarrhea, abdominal puffiness, and flatulence were the most symptoms reported in the metformin group (Norman *et al.,* 2007), and headaches, dizziness, and hair loss have also been documented as side effects (Ladson *et al.,* 2011).

1.8.5 Dipeptidyl Peptidase-IV (DPP-IV) inhibitors

DPP-IV inhibitors inhibit DPP-IV, which prevents the breakdown of glucagon-like peptide-1, thereby improving their activity (Hinnen *et al.,* 2006). In 2006, the FDA recommended Sitagliptin as an alternative to nutrition and exercise in T2DM-patients (Moses *et al.,* 2015). Sitagliptin, vidagliptin, saxagliptin, alogliptin, and linagliptin are examples of antidiabetic medicines in this family (Osadebe *et al.,* 2015). The most common side effects of the Dipeptidyl Peptidase-IV

Inhibitors family with known anti-diabetic treatments are hypoglycemia, weight gain, edema and GI problems, pharyngitis, headaches, and acute pancreatitis (Gupta *et al.*, 2009).

1.8.6 Sodium/glucose co-transporter 2 (SGLT2) inhibitors

This anti-diabetic medication category lowers blood glucose levels by inhibiting SGLT2 and preventing glucose reabsorption in the proximal renal tissues (Gallo *et al.*, 2015). They have been demonstrated to have remarkable cardiorenal advantages in large-scale clinical studies for treating patients with T2D with either existing cardiovascular disease or numerous cardiovascular risk factors (Erondu *et al.*, 2017). Examples of medications in this category include canagliflozin and dapagliflozin. Due to their capability to lower blood glucose levels without requiring insulin, these medications are frequently thought safe for people with chronic T2D (Kurosaki *et al.*, 2013). They reduce the work pressure on the pancreatic β-cells. They also lead to weight loss and lower your blood pressure. They have, however, been linked to vaginal and urinary infections, which can result in urosepsis, pyelonephritis, and genital mycosis (Chaudhury *et al.*, 2017).

1.9 Importance of medicinal plants in the treatment and management of T2D

According to the World Health Organization (WHO), about 80% of the population in Sub-Saharan Africa relies on herbal medicine for their primary healthcare needs (WHO, 2013). A substantial number of contemporary western pharmaceutical therapeutic agents are currently being employed to manage several conditions that originate from plants (Wolfender *et al.,* 2015). Globally, there has been a renewed interest in research on phyto-compound based pharmacological agents with clinical benefits. (Witters, 2001; Yuan *et al.,* 2016). Their beneficial bioactivities have been linked to their inherent phytochemical constituents termed secondary metabolites. Secondary metabolites vary among species and serve different functions, including allelopathy, photo protectivity, and

deterrence of herbivory/pests/microbial infections (Sari *et al.*, 2017). Noteworthy of these secondary metabolites are the polyphenols, alkaloids, flavonoids, and saponins with proven therapeutic effects in humans (Pandey *et al.*, 2014).

Several medicinal plants have been used as herbal drugs. Hence, they play a significant role as alternative medications due to decreased side effects and low cost. The benefit of medicinal plants in treating T2D has been reported in several reports (Kavishankar, 2011; Mohamed *et al.*, 2021). The antidiabetic properties of traditional plants have been linked to their rich phytochemical constituents (Mohamed *et al.* , et al., 2021).

1.10 Traditional treatment of diabetes in Sudan

Sudan is mainly characterized by the most diversified climates as well as biological, ecological, and environmental features, making it a differentiated natural reservoir of rich flora and varied plant species. The Sudanese flora encompasses 3137 recognized species belonging to 170 plant families and 1,280 genera of flowering plants (Halid *et al.*, 2012). **Table 1.1** shows some information about the traditionally utilized plants for treating diabetes in Sudan.

Table 1.1: Traditionally utilized plants for treating diabetes in Sudan

Plant species	Family	Common name	Part used	Reference
Acacia albida	Fabaceae	Haraz	Root bark	(Gaber *et al.*, 2013)
Acacia nilotica	Fabaceae	Garad	Pods, bark	(Ahmed *et al*., 2005)
Acacia senegal	Fabaceae	Hashab	Fruit	(Yagi *et al.*, 2018)
Aloe sinkatana	Aloaceae	Sabar	Leaves	(Yagi *et al.*, 2018)
Allium cepa	Liliaceae	Basal	Bulb	(Qureshi et al., 2018)
Ambrosia maritima	Asteraceae	Damesisa	Leaves	(Yagi *et al*., 2013)
Ammi visnaga	Apiaceae	Bizrat al khalla	Fruit	(Yagi *et al.*, 2018)
Balanites aegyptiaca	Balanitaceae	Laloub	Fruit	(Gaber *et al.*, 2013)
Bauhinia rufescens	Caesalpiniaceae	Kulkul	Leves	(Yagi *et al.*, 2018)
Capparis decidua	Capparaceae	Tundub	Stem	(Alrasheid *et al.*, 2018)
Cicer arientinum	Fabaceae	Humus	Seeds	(Abdulwehab *et al.*, 2015)
Cinnanomum verum	Lauraceae	Gerfa	Stem bark	(Yagi *et al.*, 2013)
Citrullus colocynthis	Cucurbitaceae	Hundal	Seeds	(Saad *et al.*, 2017)
Cyperus rotundus	Cyperaceae	Sieda	Rhizome	(Ahmed *et al.*, 2019)
Eucalyptus globulus	Myrtaceae	El kafour	Leaves	Houacine *et al.*, 2012
Foeniculum vulgare	Apiaceae	Shamar	Fruit	Anitha *et al*., 2014
Geigeria alata	Asteraceae	Al Gadad	Roots	(Yagi *et al.*, 2018)
Guiera senegalensis	Combretaceae	Ghubeish	Leaves	(Yagi *et al.*, 2018)
Kigelia africana	Bignoniaceae	Um Shutour	Fruits	(Musa Sulieman, 2011)
Lupinus termis	Papilionaceae	Turmus	Fruits	(Yagi *et al.*, 2018)
Mitragyna inremis	Rubiaceae	Um Gatto	Stem bark	(Yagi *et al.*, 2018)
Momordica balsamina	Cucurbitaceae	Abu el Efain	Leaves	(Yagi *et al.*, 2018)
Nauclea latifolia	Rubiaceae	Karmadoda	Leaves	Ahmed *et al*., 2008
Hibiscus sabdariffa	Malvaceae	Karkadi	Flowers	(Ali *et al.*, 2005)

1.11 The rationale of the study

The present significant growth in diabetes rates worldwide can be ascribed to unhealthy lifestyles, industrialization, and aging (My *et al.*, 2004; Tabatabaei-Malazy *et al.*, 2021). The prevalence of diabetes has continued to increase, causing high mortality rates across the globe (IDF, 2019). The World Health Organization (WHO) has stated that preventing diabetes and its consequences is a big challenge for the future. However, for universal health to be achieved, the optimal and sensible use of traditional and natural indigenous remedies is encouraged (IDF, 2015).

Even though modern medicine has resulted in the development of modern pharmaceuticals such as insulin, biguanides, sulfonylureas, and thiazolidinediones, there have been concerns about the side effects of most of these drugs as well as the cost of treatment, especially in developing and under-developed countries (Rani, 2021). Therefore, there is a need for continuous research on alternative antidiabetic agents with fewer or no side effects and cost-effectiveness (Fokoun *et al.*, 2021). Besides searching for potential antidiabetic plants, plant species that have been historically used to treat diabetes must be evaluated to find novel bioactive compounds that may be considered for developing novel antidiabetic drugs (Mohamed *et al.*, 2021).

1.12 Research problem

Traditional medicine is used by a substantial segment of the Sudanese people to manage their primary healthcare requirements. It is part of their life systems in addition to being accessible and inexpensive. Traditional medicine is sometimes the only kind of healthcare available to the public in many countries' regions. It is paramount for researchers to investigate the novel compounds responsible for these plants' therapeutic activities.

Aim of the study

To investigate the antioxidant and antidiabetic activities of some selected traditional Sudanese medicinal plants, namely *Cyperus rotundus, Nauclea latifolia* and *Hibiscus sabdariffa L.*, using *in vitro*, *in silico* and *ex vivo* models.

Objectives of the study

- To screen the ethyl acetate, ethanol, and aqueous extracts of the three plant species for phytochemical constituents.
- To investigate the total phenolic content of the plant extract *in vitro*
- To investigate the antioxidant activities of the plant extract *in vitro* and *ex vivo*
- To investigate the *in vitro* antidiabetic activities of the extract by determining their inhibitory effect on α-amylase and α-glucosidase activities.
- By evaluating the effect of the extracts on intestinal glucose absorption in isolated rat jejunum *ex vivo*.
- By evaluating the effect of the extracts on glucose uptake in isolated rat psoas muscle *ex vivo.*
- To dock the phytochemical compounds of the extract with relevant target proteins *in silico*
- To predict the bioactivities and toxicity properties of the phytochemical compounds of the extract *in silico.*

CHAPTER TWO

MATERIAL AND METHODS

2.1 Chemicals and reagents

Sodium chloride, calcium chloride di-hydrate, ferric chloride, magnesium chloride, ferrous sulphate heptahydrate, magnesium sulphate, sodium phosphate, ascorbic acid, dinitrosalicylic acid (DNS), sulphuric acid, trichloroacetic acid, thiobarbituric acid, mono-basic sodium phosphate, sodium hydrogen carbonate, sodium nitroprusside, sodium hydroxide, absolute ethanol, ethyl acetate, Folin ciocalteau reagent, sodium salt, Griess reagent, hydrogen peroxide, starch, reduced glutathione, methanol were purchased from Merck Chemical Company, Durban, South Africa. 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), glucopyranoside (pNPG), 6-hydroxydopamine, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), α-glucosidase, porcine pancreatic amylase, ammonium molybdate tetrahydrate, diethylenetriaminepentaacetic acid (DETAPAC), acarbose, di-basic sodium phosphate, gallic acid, p-nitrophenyl-α-D- 3-(N-morpholino) propane sulfonic acid (MOPS), p-nitrophenol, potassium ferricyanide, sodium acetate, glucose were bought from Associated Chemical Enterprise (ACE), South Africa.

2.2 Equipment

Synergy HTX Multi-mode Reader (BioTek Instruments Inc., USA); Shimadzu UV mini 1240 spectrophotometer (Shimadzu Corporation, Kyoto, Japan); Labmax Plenno (Labtest Inc., Lagoa Santa, Brazil); Buchi Rotavapor II (Buchi, Switzerland); Nuair CO_2 incubator (USA); Ultra Turrax Tube Drive Work Station Homogenizer (IKA-Works, Staufenim Breisgau, Germany); Electron

incubation shaker (InfrostHT, Switzerland) and Hettich Mikro 200 Microcentrifuge (Hettich Lab Technology, Tuttlingen, Germany) were used in this study.

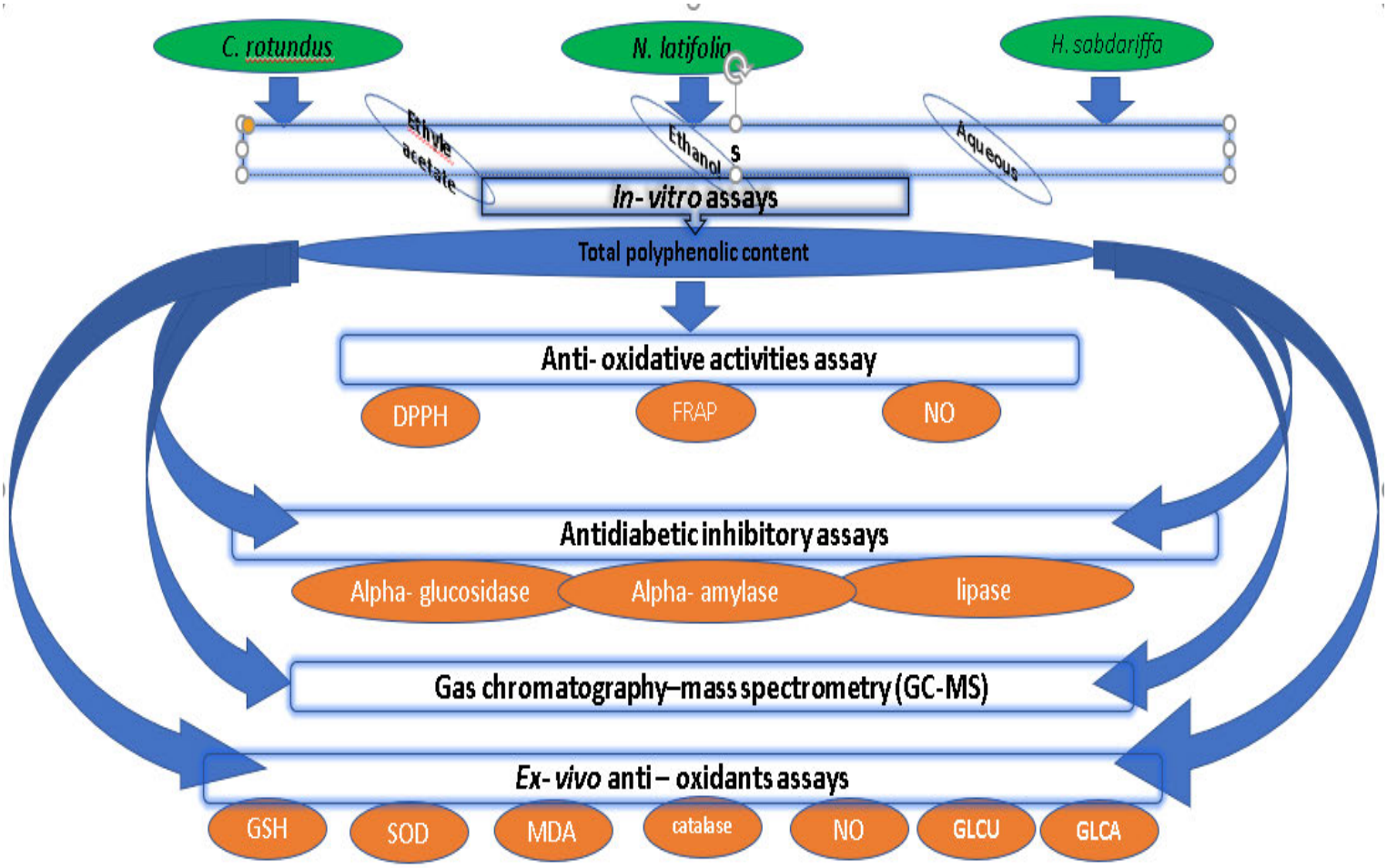

Figure 2.1: Experimental overview

2.3 Plant materials

2.3.1 Preparation of extracts

The aqueous extract was prepared by simple maceration of dry powdered plant material (250 g) in distilled water maintained at room temperature for 24 h. The extract was filtered and freeze-dried. The ethanolic and ethyl acetate extracts were prepared by soaking the dry powdered plant material

(250 g) in 70% ethanol and ethyl acetate, respectively, at room temperature for 6 days. The extracts were decanted, filtered under vacuum, concentrated in a rotary evaporator, and freeze-dried.

2.3.2 Total phenolic content

The total phenolic content of the different plant extracts was determined by using the Folin-Ciocalteu's phenol reagent according to Humadi and Istudor (2009). 50 µL of each extract (240 µg/mL) was diluted with distilled water (450 µL) before Folin-Ciocalteu reagent (125 µL) was added and left to stand for 10 min in the dark. Then 100 µL of 7% sodium carbonate solution was added and appropriately made up to a final volume of 1000 µL with distilled water, after which the mixture was allowed to stand for 30 min in the dark. The absorbance was measured at 750 nm, and the total phenolic content was extrapolated from a gallic acid standard curve.

2.4 *In vitro* anti-oxidative activities assay

2.4.1 Free radical scavenging activity

The free radical scavenging activity of the extract was determined by using 2,2'-diphenyl-1-picrylhydrazyl (DPPH) as a free radical (Ak & Gülçin, 2008). Equal volumes of 0.2% DPPH in methanol and different concentrations (15 µg/mL to 240 µg/mL) of the various plant extracts or standard were prepared and incubated in the dark at room temperature for 30 minutes. The absorbance was measured at 490 nm. The blank sample containedg the same amount of methanol and DPPH solution and was measured as the control (Eun *et al.*, 2003). Ascorbic acid was used as a standard. The experiment was carried out in triplicate. Results were calculated using the following formula:

$$\%\ \text{DPPH radical scavenging} = \frac{(\text{Absorbance of control} - \text{Absorbance of test Sample})}{(\text{Absorbance Of control})} \times 100$$

2.4.2 Ferric cyanide (Fe^{3+}) reducing antioxidant power (FRAP)

The total reducing power of the extracts was measured using the FRAP method of Oyaizu (Oyaizu, 1986) with slight modifications. To perform this assay, 1 mL of each extract (15–240 µg/mL) was incubated with 1 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 1% potassium ferricyanide at 50°C for 30 min. Thereafter, 1 mL of 10% trichloroacetic acid was used to acidify the reaction mixtures. After the acidification, 1 mL of the sample was mixed with 1 mL of distilled water and 200 µL of 0.1% $FeCl_3$. The absorbance of the resulting solution was read at 700 nm in a spectrophotometer. The sample's absorbance is proportional to the extracts' reduction capability (Gülçin *et al.*, 2004). Results were reported as a percentage of the absorbance of the sample to the absorbance of gallic acid as expressed below;

$$\%\ \text{FRAP radical scavenging} = \frac{(\ \text{Absorbance of test Sample})}{(\text{Absorbance Of control})} \times 100$$

2.4.3 Nitric oxide (NO) radical inhibitory activity

At physiological pH, sodium nitroprusside could create much nitric oxide (NO), reacting with oxygen to form nitrite ions. This capability forms the basis of this test (Kurian *et al.*, 2010). The test was performed by incubating 500 µL of 10 mM sodium nitroprusside in sodium phosphate buffer (pH 7.4) and 500 µL of crude extract/fractions at various concentrations (15-240 µg/mL) for 2 hours at 37°C. The reaction mixture was then given a 500 µL dose of Griess reagent. The absorbance was measured at 546 nm to detect a chromophore formed by the reaction of nitrite with sulphanilamide. By comparing the absorbance of a prepared control to the percentage inhibition of NO released, the percentage inhibition of NO emitted was computed (10 mM sodium nitroprusside in phosphate buffer). The experiment was repeated three times, and the plant's scavenging capacity was calculated using the formula below:

$$\%\ \text{scavenging activity} = \frac{(\text{Absorbance of control} - \text{Absorbance of test Sample})}{(\text{Absorbance Of control})} \times 100$$

2.5 *In vitro* anti-diabetic inhibitory assay

2.5.1 α-glucosidase inhibitory activity

The α-glucosidase inhibitory was assayed according to a previously described method (Ademiluyi & Oboh, 2013) with slight modifications. Briefly, 100µl of 1 u/mg α-glucosidase solution was added to different concentrations of plant extract or acarbose (15-240 µg/mL) and incubated at 37°C for 15 min. Then 50µl of pNPG solution (5 mM) in 100 mM phosphate buffer (PH 6.8) was added, and the mixture was further incubated at 37°C for 30 min. The absorbance of the released nitrophenol was measured at 405 nm using a Synergy HTX Multi-mode Reader. The α-glucosidase was carried out in triplicate, and the activities were calculated by using the following formula:

$$\%\ \text{Inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of test Sample})}{(\text{Absorbance Of control})} \times 100$$

2.5.2 α-Amylase inhibitory activity

The α-amylase inhibitory technique described by Shai *et al.* (Shai *et al.*, 2010) was used to determine the α-amylase inhibitory activity with slight modifications. A volume of 250 µL of each fraction or acarbose at different concentrations (15-240 µg/mL) was incubated with 500 µL of porcine pancreatic amylase (2 U/mL) in phosphate buffer (100 mM, pH 6.8) at 37°C for 20 minutes. Thereafter, 250 µL of 1% starch dissolved in 100 mM phosphate buffer (pH 6.8) was further added to the reaction mixture and incubated at 37°C for 1 hour. Dinitrosalicylate color reagent (1 mL) was then added and boiled for 10 minutes. The absorbance of the resulting mixture was read at 540 nm, and the inhibitory activity was expressed as a percentage of control without

the inhibitors. The inhibitory activities of the fractions on the α-glucosidase and α-amylase were calculated by using the following formula:

$$\% \text{ Inhibition } = \frac{(\text{Absorbance of control } - \text{ Absorbance of test Sample})}{(\text{Absorbance Of control})} \times 100$$

2.6 *In vitro* glucose uptake and diffusion

2.6.1 Glucose uptake/ transport by yeast cells

The effect of the extracts and fractions on glucose uptake/transport by yeast cells was carried out according to previously established protocol (Nirupama *et al.*, 2014). Different extracts and fractions were dissolved in 1 mL of distilled water containing 25 mM glucose. The resulting solution was incubated for 10 mins at 37°C. Thereafter, 100µL of 1% yeast suspension was added, vortexed, and incubated for 60 mins at 37°C. The glucose concentration of the solution was determined with the dinitro salicylic acid method (DNS) and calculated by the formula:

$$\% \text{ uptake } = \frac{(\text{Absorbance of control } - \text{ Absorbance of test Sample})}{(\text{Absorbance Of control})} \times 100$$

2.7 Gas chromatography-mass spectrometric analysis (GC-MS)

The solvents (aqueous, ethanol, and ethyl acetate) of the different extractions were subjected to Gas Chromatography-Mass Spectrometric (GC-MS) analysis using Agilent technology 6890 series G.C. coupled with (an Agilent) 5973 Mass Selective Detector and driven by Agilent Chemstation software. The conditions were set according to previous reports (Erukainure *et al.*,

2018). Briefly, a HP-5MS capillary column (30 m × 0.25 mm I.D., 0.25-µm film thickness, 5% phenylmethylsiloxane) was used. Ultra-pure helium was utilized as the carrier gas at a flow rate of 1.0 mL min−1 and a linear velocity of 37 cm s^{-1}. The injector temperature was set at 250°C. The oven temperature was programmed to 280°C from 60°C at the rate of 10°C min^{-1} with a hold time of 3 min. One microliter (1 µL) injection of the samples was made in splitless mode with a split ratio of 20:1. The mass spectrometer was operated in the electron ionization mode at 70 eV, while the electron multiplier voltage at 1,859 V. The ion source temperature was 230°C, and the quadrupole temperature, 150°C. The solvent delay was set at 4 min, with a scan range of 50–70 amu. Direct comparison of the retention times and mass spectral data in the NIST library identified the compounds.

2.8 *Ex- vivo-* Studies

2.8.1 Animals

Fifteen male Sprague-Dawley rats were obtained from the University of Kwazulu-Natal Biomedical Research Unit (BRU), Westville Campus, Durban, South Africa. The rats were euthanized with halothane, and the psoas muscle, whole gastrointestinal tract (GIT), and liver were collected for glucose uptake, glucose absorption, and oxidative stress investigations. All animal protocols were maintained according to the criteria of the Animal Ethics Research Committee of the University of KwaZulu-Natal in Durban, South Africa. (Ethical approval number AREC/038/019D)

2.8.2 Preparation of tissue homogenates

A 0.5 g of each freshly excised tissue (liver) was weighed and finely minced. Using an electronic homogenizer, the finely minced tissues were homogenized in 5 mL of 50 mM ice-

cold homogenization buffer (sodium phosphate buffer with 10% Triton X-100, pH 7.4). After homogenization, the mixture was placed into 2 mL microcentrifuge tubes and centrifuged at 15000 rpm for 15 minutes in a chilled microcentrifuge set at 4°C (Beckman Coulter, Inc. Microfuge 20 series centrifuge). For further analysis, the supernatant was collected into 2 mL Eppendorf microtubes and preserved at -80°C.

2.8.3 Induction of oxidative stress and treatment of tissues

The induction of oxidative stress on the liver was performed according to an earlier described method (Oboh *et al.*, 2013; Erukainure *et al.*, 2017). Briefly, 30 µL volume of pro-oxidant (15 mM $FeSO_4$) was incubated with 100 µL different sample and gallic acid (standard) concentrations (15-240 µg/mL) and tissue homogenate at 37°C in 5% CO_2 for 30 minutes. A reaction with no extract and the pro-oxidant was the positive (normal) control, whereas the reaction mixture with only the tissue and pro-oxidant served as the negative (untreated) control. As stated below, the incubated samples were tested for oxidative and proinflammatory indicators**.**

(a) Determination of lipid peroxidation

In screw cap test tubes containing 50 µL of sample/MDA standards, 50 µL of 8.1 percent SDS solution was added, followed by 187.5 µL of 20 percent acetic acid solution, 500 µL of 0.25 % TBA solution, and 212.5 µL of MiliQ water. The contents were thoroughly mixed by repeated inversion of the covered tubes (Shinohara *et al.*, 2000; Chowdhury *et al.*, 2002). This mixture was then heated for 1 hour in a water bath at 95°C before cooling to room temperature. Then 200 µL of cooled reaction mixture was pipetted in a 96 well plate and the absorbance was measured at 532 nm. The MDA concentrations were calculated from a plotted MDA standard curve.

(b) Determination of reduced glutathione (GSH) concentration

The reduced glutathione concentrations of tissue samples were determined according to Ellman's procedure (Ellman, 1984). Briefly, all samples were first precipitated with 10% TCA (300 L TCA was added to 300 L of each sample) and then centrifuged for 10 minutes at room temperature (25°C) at 2000 rpm. Thereafter, 80 µL of the resultant supernatant, 40 µL of 0.5 mM DTNB, and 200 µL of 0.2 M sodium phosphate buffer (pH 7.8) were placed in 96 micro-well plates. After 15 minutes of incubation at 25 °C, the absorbance of this reaction mixture was measured at 415 nm.

(c) Determination of superoxide dismutase (SOD) activity

At room temperature (25°C), 170 L of 0.1 mM DETAPAC solution was added to a 96-well plate, followed by 15 L of diluted sample or SOD assay buffer (blank) (Gee *et al.*, 1989). Then 15 µL of 1.6 mM 6-HD solution was then added, and the mixture was immediately mixed by lightly tapping the plate on all four sides. A multi-plate absorbance reader was used to record absorbance at 492 nm for 5 minutes at 1-minute intervals.

(d) Determination of catalase activity

The samples were evaluated using a previously established procedure based on detecting reduced absorbance of test samples caused by H_2O_2 breakdown (Aebi, 1984). After mixing 10 µL of samples with 340 µL of 50 mM sodium phosphate buffer (pH 7.0), 150 µL of 2 M H_2O_2 were added to the mixture. The absorbance was measured at 240 nm for three minutes at one-minute intervals.

2.8.4 *Ex-vivo* antidiabetic activity Assay

(a) Determination of glucose absorption in isolated rat small intestine

A previously reported technique was used to investigate the effect of the crude extracts/fractions on intestinal glucose absorption (Erukainure *et al.*, 2017). The jejunal segments of the freshly

harvested small intestine were cut into smaller pieces of 5 cm. The inner jejunal lumen was inverted and cleaned with 2 mL of Krebs buffer (118 mM NaCl, 5 mM KCl, 1.328 mM $CaCl_2 \cdot 2H_2O$, 1.2 mM KH_2PO4, 1.2 mM $MgSO_4$, and 25 mM $NaHCO_3$) by an antiseptic syringe. Thereafter, each piece was incubated with different concentrations of the crude extracts/fractions, 8 mL of kerb buffer containing 11.1 mL of glucose for 2 h at 5% CO_2, 95% oxygen, and 37°C in a Steri-Cult CO_2 incubator (Labotec, South Africa). Then 1 mL of the mixture was taken before the incubation and after the 2 h incubation period, and the amount of glucose in the mixture was measured using an Automated Chemistry Analyzer (Labmax Plenno, Lago Santa, Brazil). The small intestinal glucose absorption was calculated using the following formula:

$$\text{Glucose absorption/cm of rat jejunum} = \frac{(\text{GC1} - \text{GC2})}{(\text{length of jejunum (cm)})}$$

Where GC1 and GC2 are glucose concentrations (mg/dL) before and after incubation, respectively.

(b) Glucose uptake in the isolated rat psoas muscle

The impact of crude extracts/fractions on muscle glucose uptake was investigated using the procedure previously described (Chukwuma & Islam, 2015). A 0.5 g freshly harvested psoas muscle was incubated in 8 mL of Kreb's buffer solution, including increasing concentrations of extracts (30, 60, 120, and 240 g/mL) and 11.1 mM glucose with or without insulin (50 mU/L) for 1 hour in a CO_2 incubator at 5% CO_2 and 95% oxygen at 37°C. The glucose concentration was then determined by collecting 2 mL of an aliquot from each sample mixture before incubation and after 2 h incubation period. It was measured using an Automated Chemistry Analyzer (Labmax Plenno, Lago Santa, Brazil). Muscle glucose uptake was expressed using the following formula.:

$$\text{Muscle glucose uptake/g} = \frac{(\text{GC1} - \text{GC2})}{(\text{Weight of muscle tissue (g)})}$$

Where GC1 and GC2 are glucose concentrations (mg/dL) before and after incubation, respectively.

2.9 *In silico* analysis

2.9.1 Molecular modeling

Computer-guided docking experiments were carried out using Molecular Operating Environment software version 10 (Chemical Computing Group, Montreal, Canada). According to GC/MS results, seven ligands were selected, representing the significant plant's compounds found in the *N. latifolia*, *C. rotundus*, and *H. sabdariffa*. X-ray crystal structure of the α glycosides (3WY1)(Shen *et al.*, 2015) and α-amylase (6GXV) (Agirre *et al.*, 2019) proteins models were downloaded from the Protein Data Bank website (https://www.rcsb.org/) (Berman *et al.*, 2002). The active ligands were prepared by hydrogens addition, partial charges calculation, and energy minimization using Force Field MMFF94x. In addition, the preparation of proteins was performed by omitting the repeating chains, water molecules, and surfactants. MOE Quick Prep functionality was used for correcting structural issues, 3D protonation, and calculation of partial charges. The default procedure in the MOE Dock protocol was utilized to detect the best binding poses of the studied ligands, using triangle matcher as placement method and London dG as the primary scoring function. An extra refinement step was set to rigid receptor method with GBVI/WSA dG scoring function to retain poses with the highest hydrophobic, ionic, and hydrogen-bond interactions with the protein. The output database comprised the scores of ligand enzyme complexes in kcal/mol. Then, the resulting docking poses were visually examined with BIOVIA Discovery Studio, and interactions with binding pocket residues were studied. Poses fitting into the binding pocket with the top scores and showing useful ligand enzyme contacts were selected.

2.9.2 Evaluation of ADME (absorption, distribution, metabolism, and excretion) properties for identified compounds

The identified significant compounds from the selected traditional medicinal plants, *C. rotundus*, *N. latifolia*, and *H. sabdariffa* were used to compute their physicochemical descriptors and predict ADME parameters. The Swiss ADME and Pre ADME were used to calculate molecular weight, topological polar surface area, solubility in water, absorption, lipophilicity, num. rotatable bonds, H-bond acceptors, H-bond donors, and toxicity for the rat and hERG_inhibition (Daina, Michielin and Zoete, 2017;Abdelli *et al.*, 2021).

2.10 Statistical analysis

One-way analysis of variance (ANOVA) was used in establishing the statistical significance and set at $p < 0.05$. Data were presented as mean ± SEM/SD. All statistical analyses were carried out using IBM SPSS for Windows, version 27.0 (SPSS Inc., Chicago, IL, USA).

CHAPTER THREE

THE ANTIDIABETIC AND ANTIOXIDANTS ACTIVITIES OF *CYPERUS ROTUNDUS* (LINN) *IN VITRO* AND *IN SILICO*

3.1 Cyperus rotundus (Linn)

Cyperus rotundus (purple nutsedge; Cyperaceae family) is a noxious plant that is found worldwide in tropical as well as subtropical climates. *C. rotundus* is a slender, erect perennial sedge with a fibrous root structure. Its short subterranean roots, called rhizomes, are initially white, fleshy, and covered with scaly, modified leaves but eventually become brown and woody (Al-Snafi, 2016). When a rhizome reaches the surface, it may inflate into a tiny, spherical structure known as a basal bulb, from which shoots, roots, and new rhizomes emerge (Huang, 1999; Bajpay *et al.*, 2018a). The tubers range in length from 1 to 3.5 cm and are white and juicy while young before becoming brown and hard (Elnour, 2021).

Figure 3.1: *Cyperus rotundus* copied from (Hema Nidugala, 2016) without permission

3.1.2 Traditional and folkloric uses

Cyperus rotundus was traditionally used to treat gastrointestinal tract spasms, stomach ailments, nausea, vomiting, intestinal parasites, food poisoning, indigestion, and bowel irritation (Talukdar *et al.*, 2011). Additionally, it was utilized to redeem fevers, wounds, bruises, and carbuncles, malaria, cough, bronchitis, renal and vesical calculi, urinary tenesmus, amenorrhoea, dysmenorrhoea, insufficient lactation, memory loss, insect bites, dysuria, bronchitis, infertility, cervical cancer, and menstrual disorders (Sivapalan, 2013). Moreover, the rhizomes of *C. rotundus* have been employed in producing perfumes, spices, and Ayurvedic treatments in the middle east, Africa, China, and India for hundreds of years back (Sharma *et al.*, 2007). The plant's leaves were

widely employed as a flavoring element in Asian nutrients for a long time. Its seeds are still used in pickles, curries, and a variety of bread items today, among other things (Jabier *et al.*, 2008).

3.1.3 Biological and pharmacological activities

Cyperus rotundus has numerous biological and pharmacological activities which have been well reported. However, the dominant biological activities of the plant are antimicrobial action (Kilani-Jaziri *et al.*, 2011; Anand *et al.*, 2012). The antimicrobial activities of the methanol, chloroform, and ethyl acetate extracts (Ahmad *et al.*, 2012; Aeganathan *et al.*, 2015), ethyl alcohol extract (Mehta *et al.*, 2013), ethanolic extract (Vijisaral & Subramanian, 2013) and essential oil (Eltayeib & Ismaeel, 2014) have been documented. Additionally, there are other activities of the plant that has been reported, such as antidiabetic (Raut & Gaikwad, 2014), antioxidants (Mohamed *et al.*, 2021), antidiarrheal (Shamkuwar *et al.*, 2012), and antiinflammatory (Sghaier *et al.*, 2011).

3.1.4 Phytochemistry

Cyperus rotundus has been documented to contain a wide range of flavonoids such as (visnagin, khellin, ammiol, isorhamnetin, and tricin), tannins, quinones, saponins, alkaloids glycosides, and furochromones (Huang, 1999;Bajpay *et al.*, 2018b). The plants have also been reported to consist of essential oils and terpenoids, including monoterpenes and sesquiterpenes with different skeletons such as patchoulane, rotundane, eudesmane, guaiane, cadinane, and caryophyllene types (Sivapalan *et al.*, 2012; Chandratre *et al.*, 2011).

3.2 The antioxidant and antidiabetic potentials of polyphenolic-rich extracts of *Cyperus rotundus* (Linn.)

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Preface: This article examined the antioxidant and antidiabetic potential of *cyperus rotundus* by employing *in vitro* and *in silico* experimental models. **It has been published in the Journal of Biomolecular Structure and Dynamics. (Mohamed, A. I., Beseni, B. K., Msomi, N. Z., Salau, V. F., Erukainure, O. L., Aljoundi, A., & Islam, M. S. (2021). The antioxidant and antidiabetic potentials of polyphenolic-rich extracts of Cyperus rotundus (Linn.). Journal of Biomolecular Structure and Dynamics, 1–13)**. https://doi.org/10.1080/07391102.2021.1967197

3.2.1 Abstract

In this study, the rhizome of *Cyperus rotundus* L was investigated for its antioxidant and antidiabetic effects using *in vitro* and *in silico* experimental models. Its crude extracts (ethyl acetate, ethanol, and aqueous) were screened *in vitro* for their antioxidant activity using ferric-reducing antioxidant power (FRAP) and 1,1-diphenyl-2-picrylhydrazyl (DPPH), as well as their inhibitory effect on α-glucosidase enzyme. Subsequently, the extracts were subjected to Gas Chromatography-Mass Spectrometry (GC-MS) analysis to elucidate their possible bioactive compounds. Furthermore, computational molecular docking of selected phenolic compounds was conducted to determine their mode of α-glucosidase inhibitory activity. The aqueous extract displayed the highest level of total phenolic content and significantly higher scavenging activity in both FRAP and DPPH assays compared to ethyl acetate and ethanol extracts. In FRAP and DPPH assays, IC_{50} values of aqueous extract were 448.626 µg/mL and 418.74 µg/mL, respectively. Aqueous extract further presented higher α-glucosidase inhibitory activity with an IC_{50} value of 383.75 µg/mL. GC-MS analysis revealed the presence of the following phenolic compounds: 4-methyl-2-(2,4,4-trimethylpentan-2-yl) phenol, Phenol,2-methyl-4-(1,1,3,3-tetramethylbutyl)- and 1-ethoxy-2-isopropylbenzene. Molecular docking study revealed 1-ethoxy-2-isopropylbenzene formed two hydrogen bonds with the interacting residues in the active site of α-glucosidase enzyme. Furthermore, 4-methyl-2-(2,4,4-trimethylpentan-2-yl) phenol had the lowest binding energy inferring the best affinity for α-glucosidase active site. These results suggest the possible antioxidant and antidiabetic potential of *Cyperus rotundus*.

Keywords: Antioxidant; Antidiabetic; *Cyperus rotundus* L.; Molecular docking

3.2.2 Introduction

Diabetes mellitus (DM) is a complex metabolic disorder distinguishably characterized by hyperglycemia (Miranda *et al.*, 2007; Tai *et al.* , 2015). The two most prevalent types of diabetes are type 1 diabetes (T1D) and type 2 diabetes (T2D) (Seino *et al.*, 2010). The former occurs due to insufficient insulin secretion resulting from autoimmune disruption of β-cells while the latter is attributed to a host of factors that result in diminished insulin secretion, insulin resistance, or both which leads to persistent the production of reactive oxygen species (ROS), which leads to oxidative stress that further progresses T2D (U Asmat et al., 2016). chronic hyperglycemia leads to the over production of reactive oxygen species (ROS) which leads to oxidative stress, thus progression of T2D (Asmat *et al.,* 2016). Uncontrolled T2D manifests as associated diabetic complications such as retinopathy, neuropathy, nephropathy, and other cardiovascular conditions (Al-rawi, 2012; Seino *et al.*, 2010). Progression of T2D and the onset of its complications can be delayed by maintaining homeostatic blood glucose level via pharmacological and non-pharmacological strategies. These include oral medication integrated with regulated dietary control and physical activity programs (Marín *eal.*, 2019).

Oral pharmacological antidiabetic drugs such as sulfonylureas, biguanides, α-glucosidase inhibitors, and thiazolidinediones are among the first-line intervention strategies for the management of diabetes (Joshi *et al.*, 2015). Their modes of action range from inhibition of carbohydrate digestion, retardation of intestinal glucose absorption to stimulation of insulin production from pancreatic β-cells (Hannan *et al.,* 2012; Defronzo, 2000; Inzucchi, 2002). Despite their far-reaching and widely accepted use, these oral antidiabetic agents have multiple undesirable side effects such as weight loss/gain, hypoglycemic shock, stomach upset, renal damage and liver failure (Maruthur *et al.,* 2016; Kalsi *et al.,* 2015). These drawbacks have prompted continuous

search by scientists across the globe for alternative sources for the development of novel antidiabetic agents (Osadebe *et al.*, 2014). Traditional medicinal plants have thus emerged as a prominent reservoir of potent bioactive compounds such as the polyphenols, with multiple beneficial health effects (Asif, 2015).

Polyphenols are a very diverse group of biomolecules that are widely researched on due to their numerous health benefits (Rasouli *et al.*, 2017). Previous studies have reported their potent antioxidant, insulin mimetic/secretagogue and carbohydrate digestive enzyme inhibitory activities (Salinas *et al.*, 2020;Ibrahim *et al.*, 2018; Silva *et al.*. Consistent ingestion of these beneficial dietary polyphenols within the diet has been shown to delay the pathogenesis of several degenerative conditions, including diabetes (Rio *et al.*, 2010). Plants with high contents of polyphenols are globally being sought after as they can aid in the development of various novel therapeutic agents (Cushnie *et al.*, 2017). One of such plant is *C. rotundus* which has been documented to be high in polyphenols (O. Lawal *et al.*, 2015).

C. rotundus, commonly known as nutgrass and indigenous to Africa, southern Asia and Europe belong to the family of *Cyperaceae* and contain 109 genera with about 5,500 species (Patel *et al.*, 2010). *The* rhizomes and tubers of *C. rotundus* have been widely used traditionally in Asian countries to treat bowel and stomach discomforts (Masood et al., 2015). The plant has also been used as a herbal remedy for diarrhea, diabetes, pyresis, inflammation, malaria, blood disorders, and spasm (Kamala et al., 2018) Studies have reported the pharmacological benefits of this valuable herb such as- antibacterial, antidiabetic, antidiarrheal, anti-inflammatory, antioxidant, antipruritic, antimaterial, diaphoretic, and digestive influence in many previous studies (Jain *at al.*, 2016; Ju *et al.*, 2016;Yagi *et al.*, 2016;Singh *et al.*, 2012). Studies have reported the enhanced inhibitory effect of *C. rotundus* on advanced glycation end product (AGE) formation and protein

oxidation, as well as delaying the onset of diabetes-related complications (Raut *et al.*, 2006; Lawal *et al.*, 2009).

The present study was conducted to examine the antioxidant and antidiabetic effects of sequential extracts of *Cyperus rotundus* L *in vitro*. This study further examined the mode of its anti-diabetic potential by investigating the molecular interaction of its identified phenolic compounds with α-glucosidase enzyme using *in silico* experimental model.

3.2.3 Material and methods

Please refer to sections **2.4, 2.5, 2.6, and 2.9 of chapter 2** for detailed methodology.

3.2.4 Results

Total polyphenols content

The total phenolic content of the different *C. rotundus* extracts are shown in **Figure 3.2.1**. The aqueous extract of C. *rotundus* significantly ($P<0.05$) had the highest amount of total polyphenols (23.72 ± 0.87 mg/g GAE). This was followed by the ethanolic extract (15.00 ± 0.14 mg/g GAE), while the ethyl acetate extract (08.78 ± 0.08 mg/g GAE) had significantly ($P<0.05$) the lowest total polyphenol content compared to all other **Figure 3.2.1**. The number of total polyphenols followed the polarity trend of the solvents in which it was most abundant in the aqueous extract and least in the ethyl acetate extract.

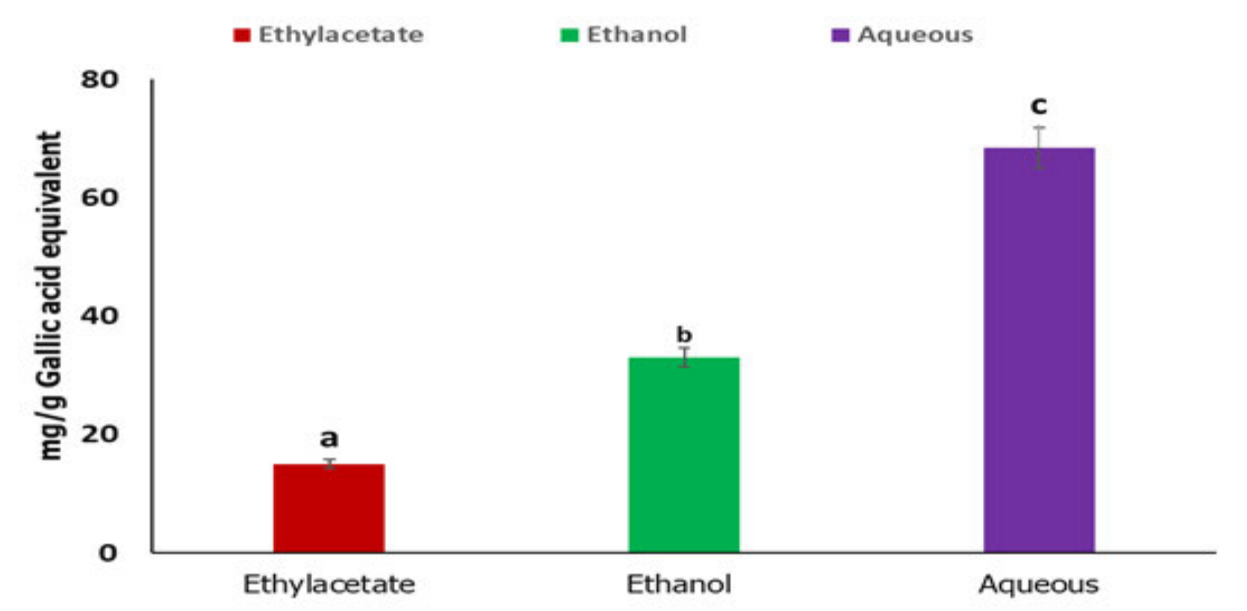
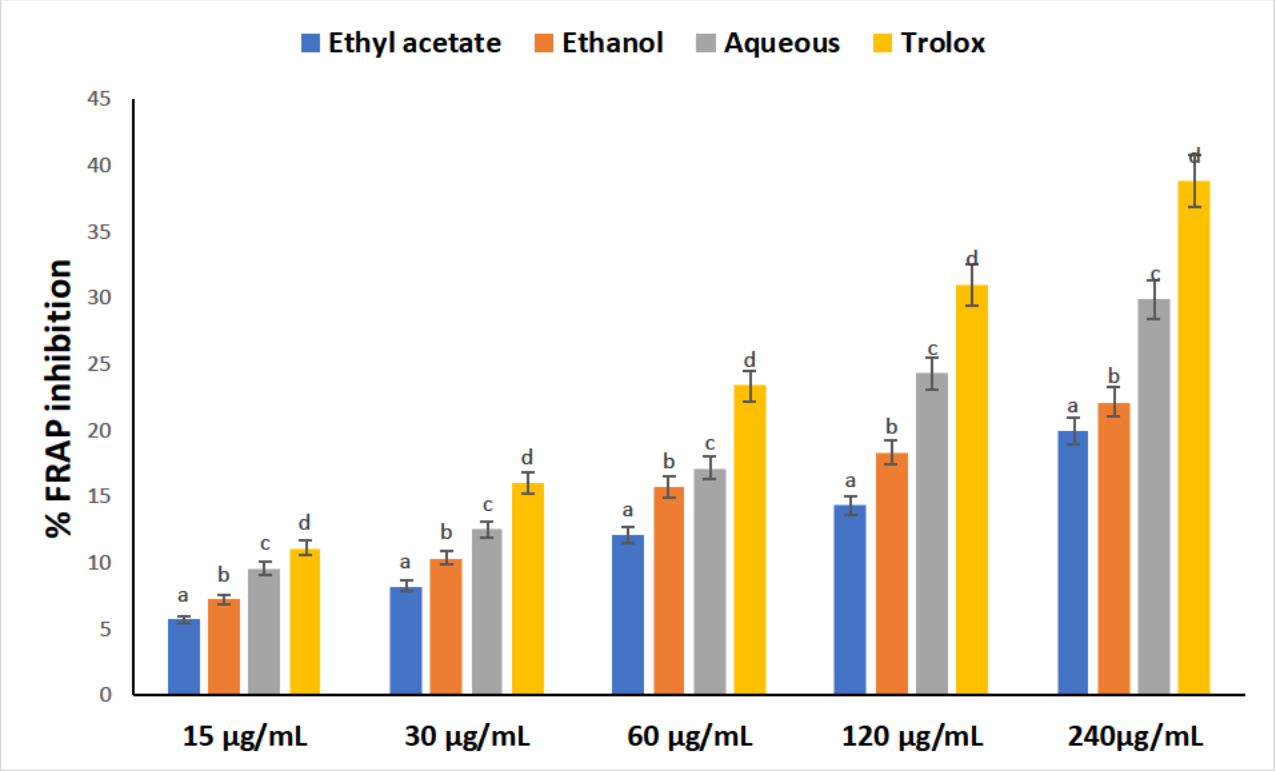


Figure 3.2.1: The total phenolic content of the different dry plant extracts of *C.rotundus* represented as gallic acid equivalents (GAE mg/g). Results are expressed as mean ± SD of triplicate determinations. [a-c]Different superscripts letters over the bars represent statistical significance of difference ($p<0.05$, Tukey's-HSD multiple range post hoc test).

The antioxidant capabilities of *C. rotundus*

The antioxidant potential of the different *C. rotundus* extracts were determined using the DPPH radical scavenging assay and ferric ion reducing power assay (FRAP), as shown in **Figure 3.2.2a** and **Figure 3.2.2b**, respectively. The different extracts of the rhizome of *C. rotundus* exhibited dose-dependently reduced capacity and radical scavenging activity in the FRAP and DPPH scavenging assays. For both assays, the aqueous extract demonstrated significantly ($p<0.05$) higher inhibitory activity compared to ethyl acetate and ethanol extracts. However, the extracts did not surpass the inhibitory activity of the positive control. According to DPPH IC_{50} values, the aqueous extract scored significantly ($p<0.05$) lower (IC_{50}= 418.74 mg/mL) than Trolox (IC_{50}= 364.89

mg/mL). In FRAP assay aqueous extract had counted (IC_{50} value = (448.626 mg/mL), Trolox (315.345 mg/mL) as shown in **Table 3.2.1**.



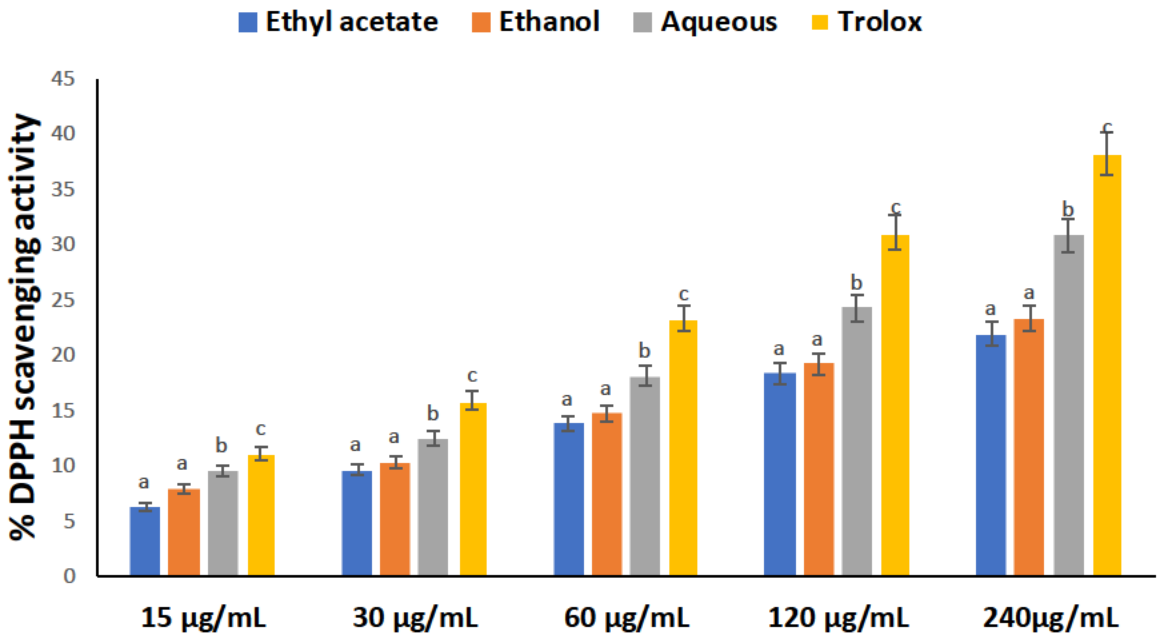


Figure 3.2.2: (a) Free radical antioxidant power or FRAP and (b) DPPH radical scavenging activities of increasing concentrations of different rhizome extracts of *C. rotundus*. Data presented as mean± standard deviation. [a-d] Different superscripts letters above the bars represent statistical significance of difference ($p<0.05$, Tukey's-HSD multiple range post hoc test).

Table 3.2.1: IC_{50} values of biological activities exhibited by *C. rotundus* on different assayed parameters.

Biological activities	**Ethyl acetate**	**Ethanol**	**Aqueous**	**Trolox**	**Acarbose**
	(µg/mL)				
FRAP	777.09	678.68	448.62	315.34	-
DPPH	496.81	458.19	418.74	364.89	-
α- glucosidase	500.16	429.65	383.75	-	332.81

Values are expressed as µg/mL. - = not applicable; FRAP, free radical antioxidant power; DPPH,

Assay for α-glucosidase inhibitory activity

The α-glucosidase inhibitory activity of *C. rotundus* extracts is shown in **Figure 3.2.3**. The extracts inhibited α-glucosidase in a dose-dependent manner. The aqueous extract with an IC_{50} value of 383.75g/mL showed significantly ($p<0.05$) higher inhibitory activity than the ethyl acetate and ethanol extracts. However, the extracts' inhibitory activity was significantly ($p<0.05$) lower than the standard drug, acarbose, which had an IC_{50} value of 332.81g/mL.

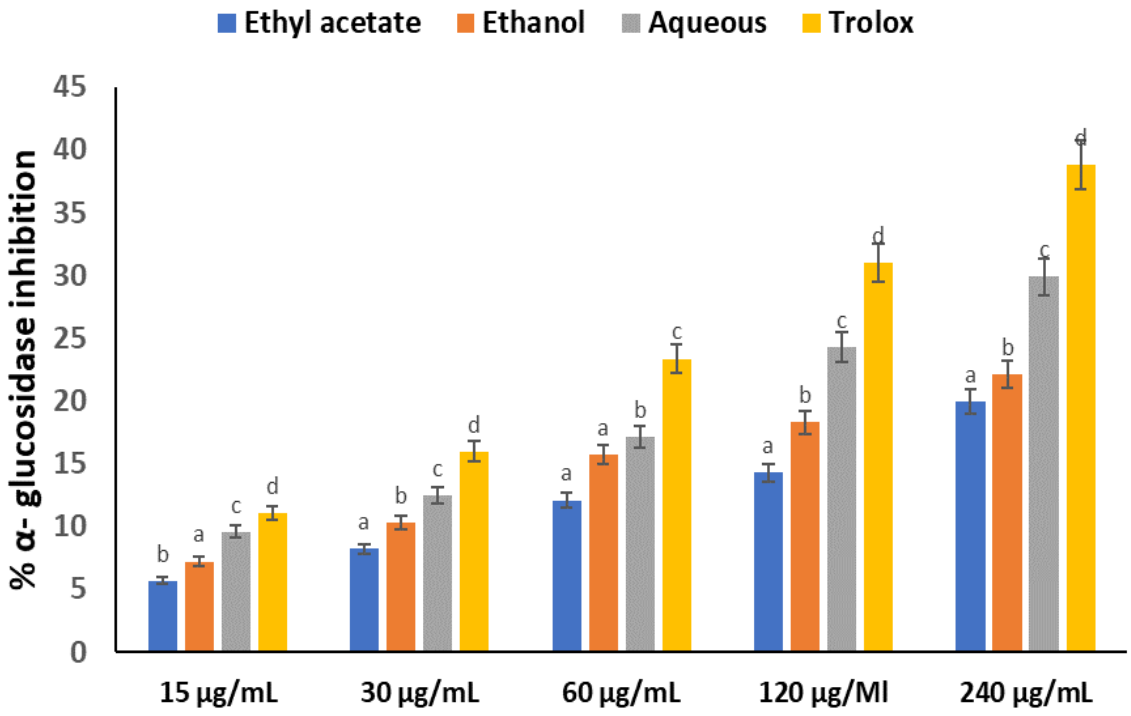


Figure 3.2.3: The α- glucosidase inhibitory activities of increasing concentrations of different rhizome extracts of *C. rotundus* represented as mean± standard deviation. $^{a-d}$Different superscripts letters above the bars represent statistical significance of difference ($p<0.05$, Tukey's-HSD multiple range post hoc test).

GC-MS analysis

GC-MS analysis of *C. rotundus* Aqueous extract which outperformed the other extracts revealed the presence of a phenolic compounds namely as; Phenol, 2-methyl-4-(1,1,3,3-tetramethylbutyl)-,4-(3,5-di-tert-butyl-4-hydroxyphenyl)butyl acrylate, 4-methyl-2-(2,4,4-trimethylpentan-2-yl)phenol, 2-methyl-4-(2,3,3-trimethylbutan-2-yl)phenol, 1-ethoxy-2-isopropylbenzene, 2-methoxy-1,3,4-trimethylbenzene, (4-isopropylphenyl)methanol, 4-isopropyl-3-methylphenol, 2-ethyl-4,5-dimethylphenol, 2-isopropyl-5-methylphenol, 5-isopropyl-2-methylphenol, 1,3-di-tert-butyl-2-methoxy-5-methylbenzene. Furthermore, the compound that exhibited the highest abundance was 4-methyl-2-(2,4,4-trimethylpentan-2-yl) phenol, while the compound shown to have the lowest abundance was 1,3-di-tert-butyl-2-methoxy-5-methylbenzene as shown in **Figure 3.2.4 and Table 3.2.2**.

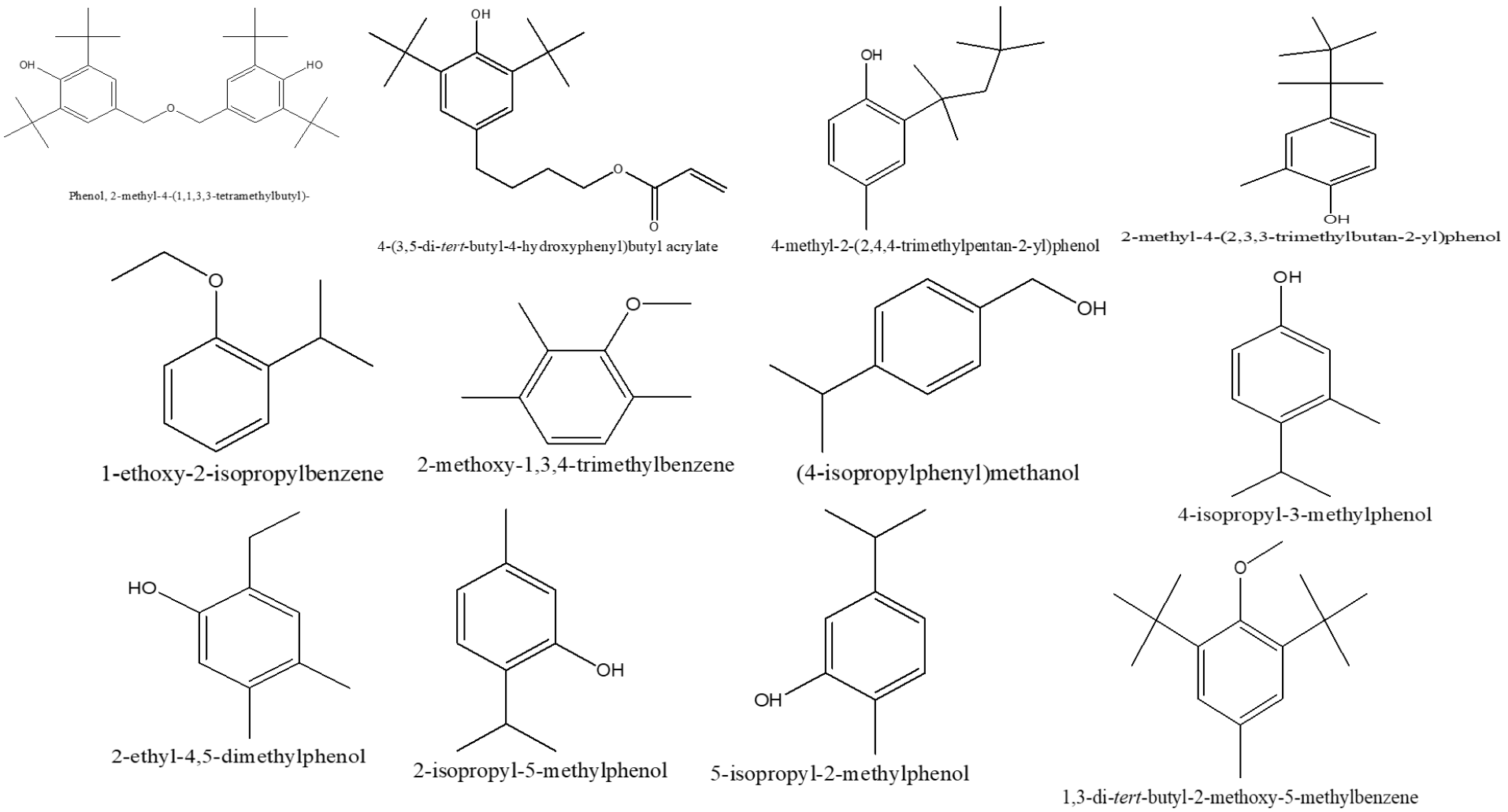


Figure 3.2.4: Chemical structures of GC-MS identified phenolic compounds in aqueos extract of *C. rotundus*

Table 3.2.2: Identified compounds of aqueous extract of rhizome of *C. rotundus* by GC-MS

Phenol name	**Formula**	**Mol weight (g/mol)**	**R Time (min)**	**Area%**	**Ret. Index**
Phenol,2-methyl-4-(1,1,3,3-tetramethylbutyl)-	$C_{30}H_{46}O_3$	454	14.675	0.34	3391
4-(3,5-di-tert-butyl-4-hydroxyphenyl) butyl acrylate	$C_{21}H_{32}O_3$	332	17.417	0.54	2422
4-methyl-2-(2,4,4-trimethylpentan-2-yl) phenol	$C_{15}H_{24}O$	220	14.675	0.34	1654
2-methyl-4-(2,3,3-trimethylbutan-2-yl) phenol	$C_{15}H_{24}O$	220	14.675	0.34	1654
1-ethoxy-2-isopropylbenzene	$C_{11}H_{16}O$	164	14.675	0.34	1217
2-methoxy-1,3,4-trimethylbenzene	$C_{10}H_{14}O$	150	9.252	0.05	1209
(4-isopropylphenyl) methanol	$C_{10}H_{14}O$	150	9.252	0.05	1284
4-isopropyl-3-methylphenol	$C_{10}H_{14}O$	150	7.786	0.10	1262
2-ethyl-4,5-dimethylphenol	$C_{10}H_{14}O$	150	7.786	0.10	1340
2-isopropyl-5-methylphenol	$C_{10}H_{14}O$	150	7.786	0.10	1262
5-isopropyl-2-methylphenol	$C_{10}H_{14}O$	150	7.786	0.10	1262
1,3-di-tert-butyl-2-methoxy-5-methylbenzene	$C_{16}H_{26}O$	234	17.417	0.54	1637

Molecular Docking with α-glucosidase

The possible binding affinities of selected phenolic compounds on the targeted interior sections of α-glucosidase were assessed using computational molecular docking tools and summarized in **Figure 3.2.5a.** The targeted binding sites of the α-glucosidase that can initiate the impeccable inhibition of *C. rotundus* nine compounds as antidiabetic effecters **Figures 3.2.5b and 3.2.5c**. In other words, *C. rotundus* compounds' stabilization at the site of interaction enhances its affinity and binding propensity, which is essential in antidiabetic resistive conditions. The targeted binding site of α-glucosidase reveals active site residues' increased involvement in stabilizing the extracted compounds' inhibition. Additionally, the interaction of residues involved in the catalytic site with nine compounds was studied to understand ligand-residue interactions Figur. Hydrogen bond network is essential in the binding of these extracted inhibitors. Compound 1 to compound 9 formed a direct hydrogen bond with active site residues, respectively Lys534, Arg520, Lie523, Phe522, Ala536, Met567, Ala780, Gly533, Ser521, and Leu286. Noticing this, two significant hydrogen bonds are formed in the compound 5 complex involving Met567 and Ala780. The arrangement of both the inhibitors within the active site residues has been shown in **Figures 3.2.5a-3.2.5c**, respectively.

ADME (absorption, distribution, metabolism, and excretion) properties

The ADME profiles of identified phenolic compounds that scored high binding energy are shown in **Table 3.2.4**. The results indicate that these compounds are not toxic to rats and hERG inhibition for assessing potential cardiotoxicity, painless, and high gatrointestinal absorption. I addition, most of these compounds were shown to be completely soluble or moderately soluble in water. These results show that these compounds are unlikely to produce complications with oral bioavailability,

demonstrating the compound's potential value in developing drugs with excellent therapeutic qualities.

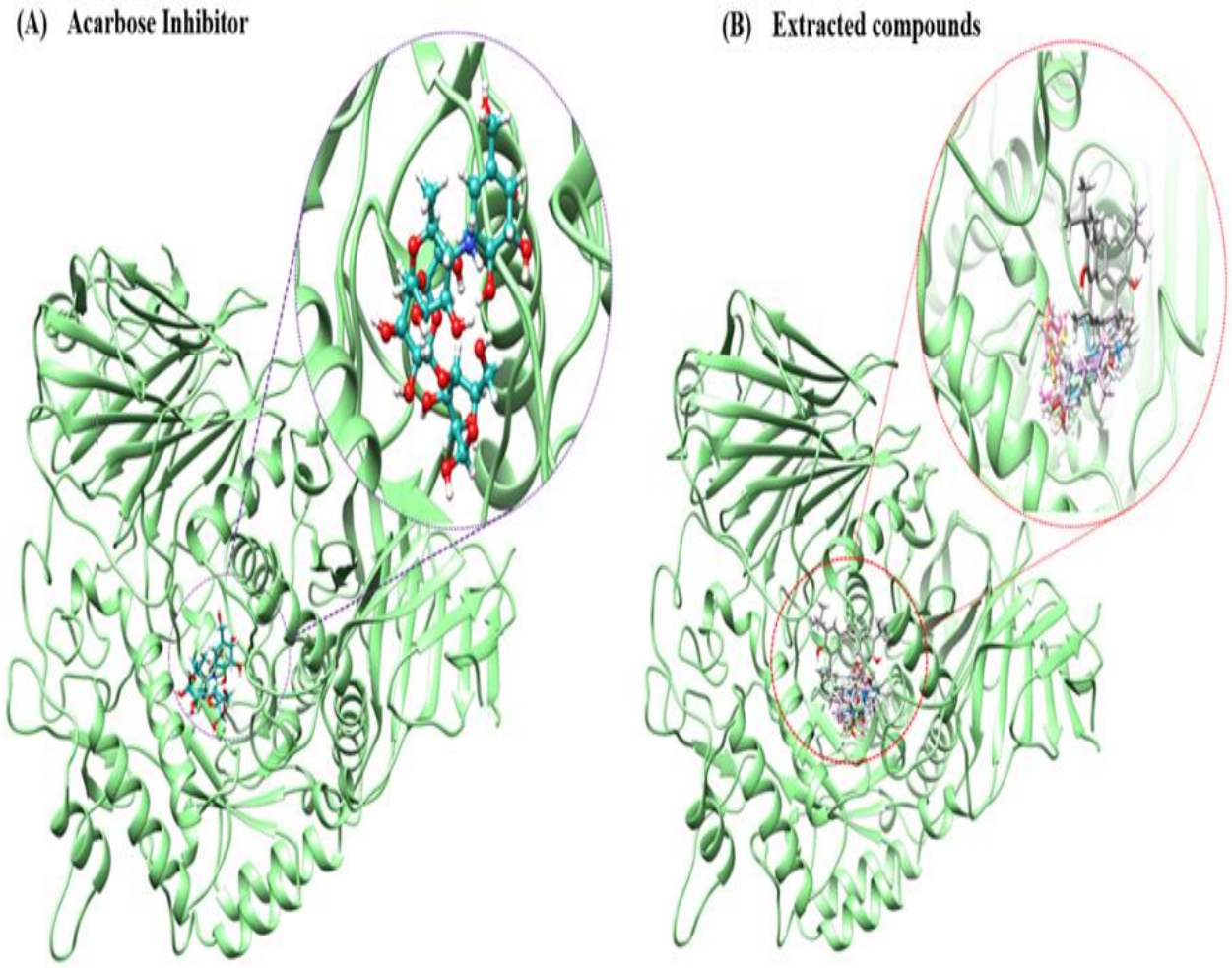
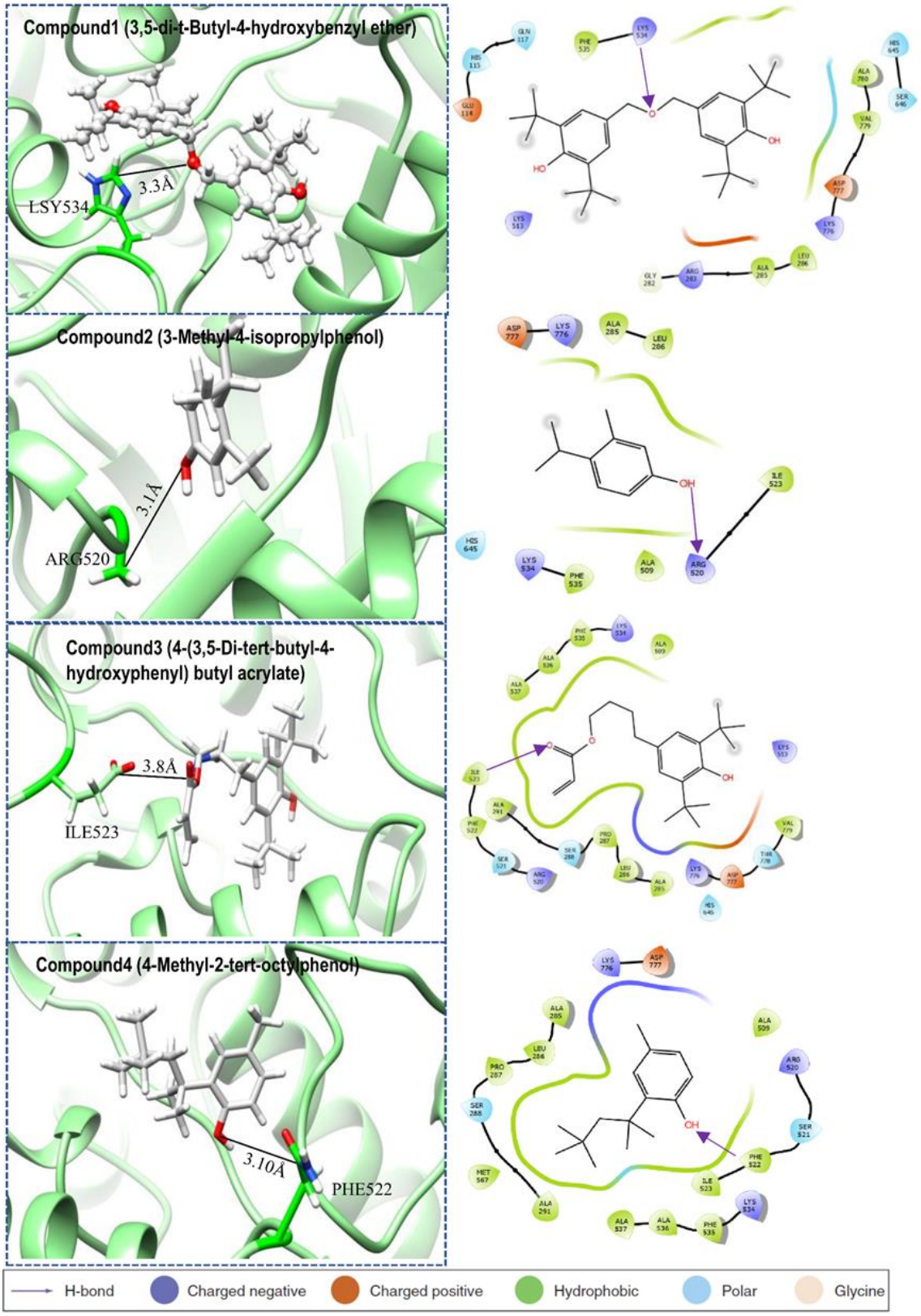
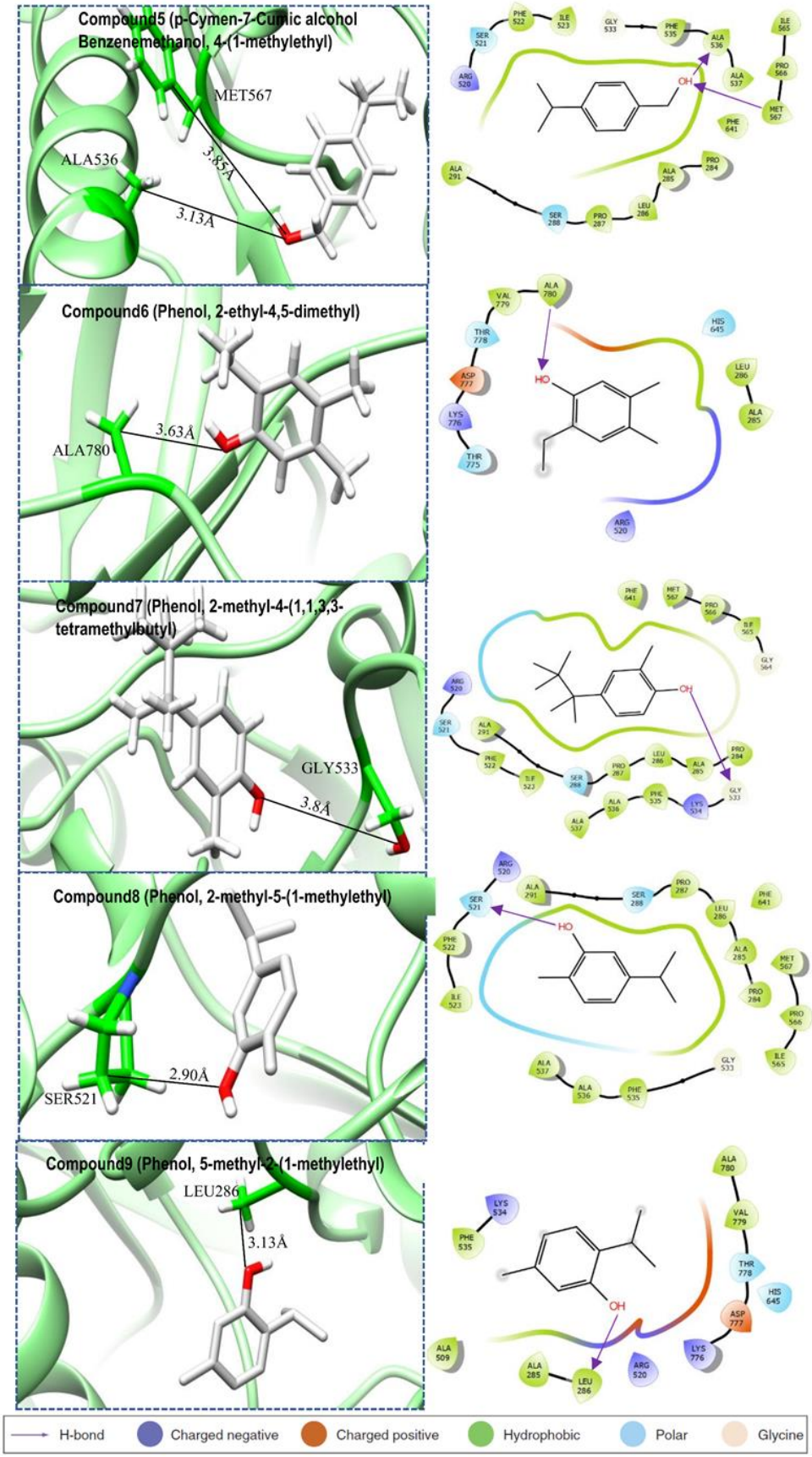


Figure 3.2.5: Structure view of human α-glucosidase bonded with acarbose inhibitor (A) and *C. rotundus* isolated nine compounds (B) via molecular docking at N-terminal domain. A close view of the docked compounds superimposed at the binding site.


Compound1 (3,5-di-t-Butyl-4-hydroxybenzyl ether)
3.3Å
LSY534
Compound2 (3-Methyl-4-isopropylphenol)
3.1Å
ARG520
Compound3 (4-(3,5-Di-tert-butyl-4-hydroxyphenyl) butyl acrylate)
3.8Å
ILE523
Compound4 (4-Methyl-2-tert-octylphenol)
3.10Å
PHE522
H-bond
Charged negative
Charged positive
Hydrophobic
Polar
Glycine


Compound5 (p-Cymen-7-Cumic alcohol
Benzenemethanol, 4-(1-methylethyl)
MET567
ALA536
3.85Å
3.13Å
Compound6 (Phenol, 2-ethyl-4,5-dimethyl)
3.63Å
ALA780
Compound7 (Phenol, 2-methyl-4-(1,1,3,3-
tetramethylbutyl)
GLY533
3.8Å
Compound8 (Phenol, 2-methyl-5-(1-methylethyl)
2.90Å
SER521
Compound9 (Phenol, 5-methyl-2-(1-methylethyl)
LEU286
3.13Å
H-bond
Charged negative
Charged positive
Hydrophobic
Polar
Glycine

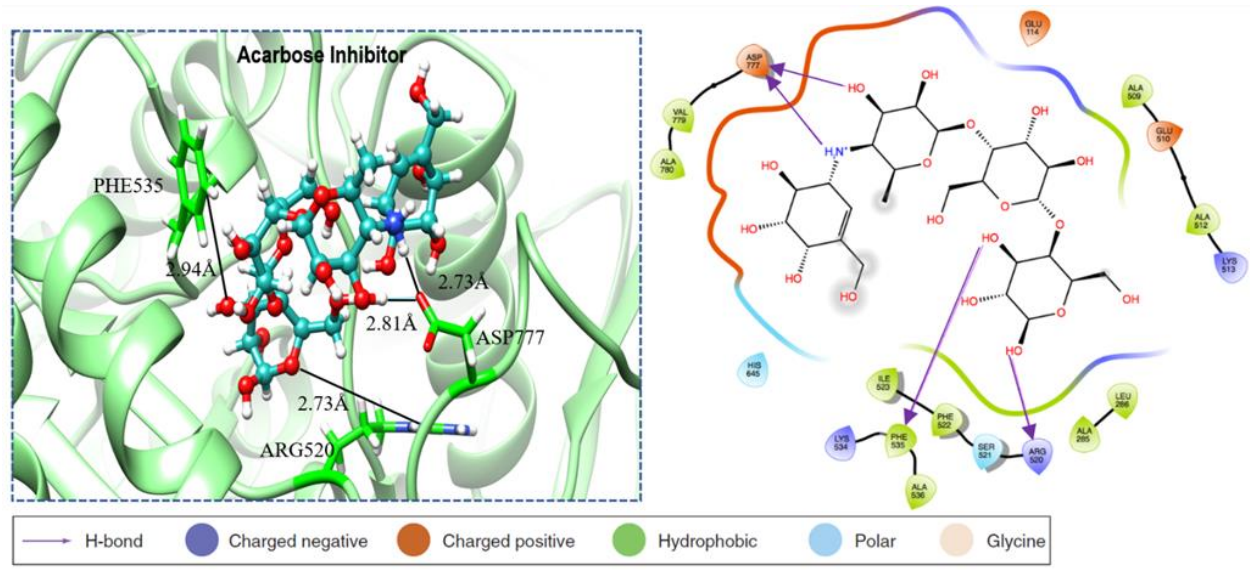


Figure 3.2.5C: The key interactions between the active site residues of human α-glucosidase enzyme and the Acarbose inhibitor.

Table 3.2.3: Molecular docking results and binding free energy (kcal/mol) of *Cyperus rotundus* extracted compounds with the N-terminal domain of human α-glucosidase

Compound	**Binding energy (Kcal/mol)**	**Number of Hydrogen bonds**	**Interacting residue of the α- glucosidase**	**Hydrogen Bond distance (Å)**
Acarbose	-6.652	4	ASP 7771H-don 22.0%	2.73
			ASP 7771H-don 32.0%	2.81
			PHE 535 1H-don 43.0%ARG 5201H-don 26.0%	2.94
			LYS[a] 534 1H-acc 46.8%	2.73
Phenol,2-methyl-4-(1,1,3,3-tetramethylbutyl)-	-6.79	1		3.31
4-(3,5-di-tert-butyl-4-hydroxyphenyl) butyl acrylate	-4.68	1	ARG[b] 520 1H-don 33.2%	3.15
4-methyl-2-(2,4,4-trimethylpentan-2-yl) phenol	-7.07	1	ILE[c] 523 1H-acc 24.7%	3.8
2-methyl-4-(2,3,3-trimethylbutan-2-yl) phenol	-5.18	1	PHE[d] 522 1H-acc 13.2%	3.10
1-ethoxy-2-isopropylbenzene	-6.11	2	ALA[e] 536 1H-don 22.0%	3.13
			MET[f] 567 1H-acc 43.0%	3.85

2-methoxy-1,3,4-trimethylbenzene	-4.92	1	ALA[e] 780 1H-acc 59.0%	3.63
(4-isopropylphenyl) methanol	-4.62	1	GLY[g] 533 1H-don 72.1%	3.8
4-isopropyl-3-methylphenol	-5.85	1	SER[h] 521 1H-don 47.5%	2.90
2-ethyl-4,5-dimethylphenol	-4.31	1	LEU[i] 286 1H-don 41.4%	3.13

[a] = Lysine, [b] = Arginine, [c] = Isoleucine, [d] = phenylalanine, [e] = Alanine, [f] = methionine, [g] = Glycine, [h] = Serine, [I] = Leucine

Table 3.2.4: ADME properties for selected identified phenolic compounds

COMPOUND	**MW g/mol**	**TPSA (Å2)**	**SOL in water**	**ABS**	**NRBs**	**MLOGP**	**HBAs**	**HBDs**	**Lipinski's violations**	**PAINS alert**	**TOX**	
											RAT	**hERG inhibition**
Phenol,2-methyl-4-(1,1,3,3-tetramethylbutyl)-	220.35	20.23	Moderately soluble	High	3	4.12	1	1	0	0	neg	Low- risk
4-(3,5-di-tert-butyl-4-hydroxyphenyl) butyl acrylate	332.48	46.53	Moderately soluble	High	9	4.36	3	1	1	0	neg	Med-risk
4-methyl-2-(2,4,4-	220.35	20.23	Moderately soluble	High	3	4.12	1	1	0	0	neg	Low- risk

trimethylpentan-2-yl) phenol												
2-methyl-4-(2,3,3-trimethylbutan-2-yl) phenol	206.32	20.23	Moderately soluble	High	2	3.87	1	1	0	0	neg	Low- risk
1-ethoxy-2-isopropylbenzene	164.24	9.23	Soluble	High	3	3.05	1	0	0	0	neg	Med- risk
2-methoxy-1,3,4-trimethylbenzene	150.22	9.23	Soluble	High	1	2.76	1	0	0	0	neg	Med- risk
(4-isopropylphenyl) methanol	471.67	32.70	Poorly soluble	High	7	5.07	3	1	1	0	neg	Low- risk
4-isopropyl-3-methylphenol	150.22	20.23	Soluble	High	1	2.76	1	1	0	0	neg	Low- risk
2-ethyl-4,5-dimethylphenol	150.22	20.23	Soluble	High	1	2.76	1	1	0	0	neg	Low- risk

*MW= molecular weight * TPSA= Topological Polar Surface Area *SOL= solubility *ABS= absorption * MLOGP = Lipophilicity

*NRBs=Num. rotatable bonds *HBAs= H-bond acceptors * HBDs= H-bond donors *TOX= toxicity

3.2.5 Discussion

As the prevalence of diabetes continues to rise globally, researchers are rigorously investigating several avenues to develop novel antidiabetic therapeutic agents. Traditional medicinal plants have thus emerged as a promising reservoir of undiscovered pharmacological agent templates that may help combat several ailments, including diabetes (Kasole *et al.*, 2019). Plants such as *C. rotundus* used in folklore medicine are being investigated for their therapeutic benefits (Bajpay *et al.*, 2018a). Previous studies on *C. rotundus* have revealed several biological active compounds that successfully manage many chronic diseases, including diabetes (Seebun *et al.*, 2012). The therapeutic activity of traditional medicinal plants stems from a vast group of compounds known as secondary metabolites which are categorized according to their structures and sizes. The most prominent plant secondary metabolites include saponins, alkaloids, terpenes, tannins, and phenolics. *C. rotundus* was shown to have high phenolic content in the present study, particularly in the aqueous extract **Figure 3.2.1**. The trend of the total phenolic compounds in *C. rotundus* followed the polarity of the extracts which suggests that the plant's phenolic compounds are mostly polar in nature. This was similarly reported in a study by (Dirar *et al.*, 2018) in which polar extracts containing higher polyphenolic constituencies. This corroborates the GC-MS identified compounds shown in **Table 3.2.2** and **Figure 3.2.4**. These compounds have been reported for a wide variety of biological properties, including antioxidant, antidiabetic, antibacterial, antiviral, anti-inflammatory, and the potential to reduce the risk of coronary heart disease activities (Diseases *et al.*, 2015; Ezzat 2013; Taherkhorsand *et al.*,2018; Shahidi *et al.*, 2018; Tohma *et al.* 2019). The high amount of phenolic compounds present in *C. rotundus* may also indicate its potency to provide extrinsic supplementary antioxidants. Phenolic compounds are good

antioxidant compounds as they are able to readily donate delocalized π electrons from their ring structures (Terra *et al.* 2009; Nagulendran *et al.* 2007)

The potential of the *C. rotundus* extracts to act as antioxidants was investigated *in vitro*, using the FRAP and DPPH scavenging assays (Arabshahi-Delouee *et al.,* 2007). Interestingly, there was a similar trend in the total phenolic compound quantification and the studied antioxidant activities of the extracts **Figures. 3.2.2a** and **3.2.2b**. The aqueous extract, which had the highest total phenolic compounds, outperformed both the ethyl-acetate and ethanol extracts. This insinuates that the antioxidant capacity of the extracts is directly proportional to the total phenolic content. (Jung *et al.*,Liang *et al.* 2010). Despite this enhanced antioxidant ability, the aqueous extract did not surpass vitamin C (positive control). This may have resulted from the masking effect of active compounds in the crude extracts by those without antioxidant capabilities. The differences between the extracts in their antioxidant activities may be due to the different qualitative and quantitative compositions of their phenolics and other phytochemicals. The polarity of the extractants also varies (Nile *et al.*, 2017a). Several studies have reported the higher reduction capacity of the aqueous extracts from the plant *C. rotundus* (Safriani *et al.,* 2016). Previous studies documented that *C. rotundus* with elevated levels and potent antioxidant compounds contribute significantly in ameliorating disorders caused by oxidative stress, including diabetes mellitus (Hussein *et al.,* 2020; Jivad *et al.*,2014).

Alpha-glucosidase is an essential carbohydrate digesting enzyme found in the small intestine's brush border interface, where it catalyzes the breakdown of terminal α(1→4) glycosidic bonds (Sinha *et al.*, 2015). This is a critical step that precedes the absorption of cleaved glucose molecules into the bloodstream, where they are subsequently transported throughout the body. Therefore, compounds that can inhibit the function of alpha-glucosidase retard absorption of glucose into the

bloodstream and reduce postprandial hyperglycemic spikes (Barrett *et al.,* 2011). This is particularly beneficial in people with diabetes who have poorly regulated glucose level control due to aberrant function of glucose mechanistic metabolic machinery (Smith *et al.*, 2015). It has been shown that such compounds may delay or postpone the development and progression of T2D and its associated complications (Ørskov Ipsen *et al.,* 2020). Thus, the inhibitory activity of *C. rotundus* on α-glucosidase **Figure 3.2.3** indicates its antidiabetic potentials. This activity is consistent with previous studies suggesting the enhanced α-glucosidase inhibitory activity of *C. rotundus* rhizome extracts (Hanh *et al.,* 2013). Despite its impressive activity, the aqueous extract failed to outperform the inhibitor activity of acarbose as depicted by the latter.

Previous reports by (Kajaria, 2013) postulate that the α-glucosidase inhibitory activity by *C. rotundus* may be due to the effect of some of its polyphenolic compounds. These compounds are known to form stable interactions with various moieties in the hydrophobic pocket of the catalytic active site of α-glucosidase. There interactions are mainly hydrophobic interactions or hydrogen bonds. Further inquest into the likely binding mechanism of the identified polyphenolic compounds from the most active *C. rotundus* extract (aqueous extract) were subjected to computer aided molecular docking. Although the results indicate slight differences in binding affinities between the extracted compounds (compounds 1 to 9) with α-glucosidase and acarbose, almost a similar negative value with favorable binding affinities were observed **Table 3.2.3** The results indicate that there are slight differences between the extracted compounds from compounds 1 to 9 with their α-glucosidase complexes, with almost a similar negative value showing favorable binding in **Table 3.2.3**. The result indicates that the nine-compound inhibition is energetically promising. The compounds 4-methyl-2-(2,4,4-trimethylpentan-2-yl) phenol, Phenol,2-methyl-4-(1,1,3,3-tetramethylbutyl)- and 1-ethoxy-2-isopropylbenzene exhibited a strong binding affinity

towards the active site of protein with binding energy of -6.79, -7.07 and -6.11 kcal/mol respectively. In addition, Compound 1-ethoxy-2-isopropylbenzene displayed two strong hydrogens bonds, thus could be explored, and exploited to achieve a drug like candidate. The molecular interactions of the identified phenolic compounds with α-glucosidase **Table 3.2.3**, and **Figures. 3.2.5a** and **3.2.5b** further indicates the antidiabetic potentials of *C. rotundus*. These calculated energies provide comprehensive molecular-level evidence that could help a drug design by creating better ligand binding. This also confirms the molecular interaction of the compounds with α-glucosidase **Table 3.2.3**. Several molecular docking and simulated screening experiments have shown that phenolic compounds could significantly inhibit the α- glucosidase enzyme (Rasouli, Hosseini-ghazvini, *et al.*, 2017). According to Lipinski's rule (Franc et al. 1997), generally, an orally active drug should have no more than one of the following requirements violated: (a) There should be no more than 5 hydrogen bond donors (HBDs), (b) A maximum of 10 hydrogen bond acceptors (HBAs), (c) A molecular weight (MW) less than 500 D. (d) A (milogP) not exceeding 5. Consequently, the identified phenolic compounds have a favorable pharmacokinetic profile and showed great promise as therapeutic candidates development templates **Table 3.2.4**.

3.2.6 Conclusion

The present study revealed significant antioxidant and antidiabetic properties of *C. rotundus* sequential extracts (ethyl acetate, ethanol, and aqueous) as depicted by their ability to scavenge free radicals and inhibit α-glucosidase activity. The aqueous extract demonstrated the most potent efficacy as an antidiabetic agent. Additionally, molecular docking results were strongly aligned with the significant antidiabetic property of the plant. Since the phenolic compound 1-ethoxy-2-

isopropylbenzene, has two strong hydrogen bonds, it may be researched and potentially used as an anti-diabetic medication. However, further scientific validation *ex vivo* and *in vivo* should be conducted to confirm the reported *in vitro* results to support its therapeutic applications.

Conflict of interests

The authors declare no conflict of interests

Acknowledgments

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CHAPTER FOUR

ANTIOXIDANTS AND ANTIDIABETIC ACTIVITIES OF THE MEDICINAL PLANT *NAUCLEA LATIFOLIA (SMITH) IN VITRO* AND *IN SILICO*

4.1 *Nauclea latifolia* (Smith)

Recently, the plant *N. latifolia* (family: Rubiaceae) has been categorized under the name Sarcocephalus latifolius (smith) E.A. Bruce, which is a subspecies of the plant *N. latifolia* (Boucherle et al., 2016). Its Shrubs or tiny trees with drooping branches that grow to a height of 33 meters. The bark is grey or brown, and the leaves are opposite and generally oblong, with ovate stipules that are persistent (Gidado et al., 2005). Flowers that are white and scented. Fruit is globose or ovoid in shape, reddish brown in color, pitted, and marked with pentagonal scars from the flower. Numerous seeds are encased in a sweet and palatable eating pulp(Gidado et al., 2005; Abdel-Rahman, 2019).

Figure 4.1: *Nauclea latifolia (Smith)* copied from (Abdel-Rahman, 2019) without his permission

4.1.2 Traditional and folkloric uses

According to the previous research, *Nauclea latifolia* stem bark, leaves, roots, and fruit are all used in folk medicine to cure T2D (Mohammed et al., 2014). Furthermore, this plant has been utilized in Cameron to cure fever, yellow fever, malaria, epilepsy, anxiety, and agitation. It is also used to treat other ailments (Wanda et al., 2015). In Nigeria, *N. latifolia* has been applied as a treatment of hypertension, diarrhea, TB, dysentery, and other ailments (Maitera et al., 2011b). In Sudan and other African nations utilize maceration of the barks and fruit to treat tape worms, cough, and stomach colics, while boiling water extract of the roots is used to treat diarrhea (Alamin et al., 2015a; Oyedemi & Afolayan, 2011).

4.1.3 Biological and pharmacological activities

A study on guinea pig found that an aqueous extract of *N. latifolia* reduced rectal temperature and showed analgesic, antidiabetic, and hepatoprotective effects. Previous research has also shown that the plant's leaves and bark have antibacterial and antiplasmodial properties. On various animal models of epilepsy, *N. latifolia* exhibited anticonvulsant, antipyretic, and antiepileptic effects. Two further investigations demonstrated that *N. latifolia* extracts had anti-diabetic properties.

4.1.4 Phytochemical investigation

Some chemical analysis of the infusion of *N. latifolia* roots revealed that it contains various secondary metabolites such as alkaloids, anthraquinones, flavonoids, glycosides, phenols, saponins, and tannins, but no bufadienolides, which were previously thought to be present (Bum et al., 2009; Mittal et al., 2011). Analyses of the plant's phytochemical constituents have shown the existence of angustine, nauflone, cadambine, indole alkaloids, strictosidine lactaum gluco alkaloid, saponins, and flourides, among other things. Additionally, the plant has a high concentration of total polyphenol as well as a significant quantity of protein (Nicolas Cyrille et al., 2011). Its antihyperglycemic activity has been reported in animal trial using the roots and leaves of *N. latifolia* (Saidu et al., 2012; Alaribe et al., 2020).

4.2 The Antioxidant and Antidiabetic Activities of Promising Polyphenolic Compounds from the Fruit of *Nauclea latifolia (Smith)- An Invitro and Insilico Study*

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4.2.1 Abstract

The current study assessed the antioxidant and antidiabetic properties of *Nauclea latifolia* fruit invitro and insilico. The aqueous infusion of the fruit was analysed for its in vitro antioxidant capacity using ferric-reducing antioxidant power (FRAP), nitric oxide (NO) and 1,1-diphenyl-2-picrylhydrazyl (DPPH). Its inhibitory effect on carbohydrate digestive enzymes was also determined. The phytoconstituents of the fruit were docked with carbohydrate digestive enzymes (α-amylase and α-glucosidase) and the compounds were further evaluated for ADME parameters. The total phenolic content of *N. Latifolia* was 44.56 ± 0.78 mg/g GAE. *N latifolia* aqueous extract exhibited remarkable ferric reducing power (IC_{50} value of 5.58 µg/mL) when compared to the control Trolox (IC_{50} value of 6.42 µg/mL). The extract displayed definite capability in inhibiting nitric oxide production than its radical scavenging effect on DPPH with IC_{50} values of 5.04 and 9.10 µg/mL, respectively. Aqueous extract further exhibited comparable inhibitory activity of the carbohydrate digestive enzymes compared to the standard drug Acarbose. Moreover, the extract significantly increased glucose uptake in yeast in a dose dependant manner. *N. latifolia* aqueous fruit compound profiling with GCMS revealed several phenolic compounds with 2-mercaptophenol, exhibiting the highest abundance. The *in silico* study revealed that compounds 7,8-dihydroxy-4-methylcoumarin and 4-methoxy-2-pent-3-en-2-ylphenol, showed higher binding activity against α-amylase (-28.8651 kcal/mol), and α-glycosidase (-31.0689 kcal/mol), respectively. Furthermore, the ADME properties of the compounds revealed oral bioavailability, pharmacokinetics, and toxicity properties. These results suggest the possible antioxidant and antidiabetic potential *N. Latifolia.*

Keywords: Antioxidant, Antidiabetic, *Nauclea latifolia*, GC-MS, Molecular docking, ADME properties

4.2.2 Introduction

According to the World Health Organization (WHO), about 80% of the population in Sub-Saharan Africa relies on herbal medicine for their primary healthcare needs (WHO, 2013). A substantial number of contemporary western pharmaceutical therapeutic agents are currently being employed to manage several conditions that originate from plants (Wolfender *et al.,* 2015). Globally, there has been a renewed interest into research on Phyto-compound based pharmacological agents with clinical benefits (Witters, 2001; Yuan *et al.*, 2016). Their beneficial bioactivities have been linked to their inherent phytochemical constituents termed secondary metabolites. Secondary metabolites vary among species and serve different functions, including allelopathy, photo protection and deterrence of herbivory/pests/microbial infections (Sari *et al.,* 2017). Noteworthy secondary metabolites with proven therapeutic effects in humans belong to polyphenols, alkaloids, flavonoids, and saponins (Pandey *et al.,* 2014). Several reports have documented the use of traditional medicinal plants against diseases such as cancer, malaria, microbial infections diabetes mellitus (DM) (Farnsworth *et al.*, 2010; Atanasov *et al.*, 2015).

Diabetes mellitus (DM) is a chronic disorder characterized by hyperglycemia arising from impaired insulin production and the insensitivity of cells to its action (Devi *et al.,* 2019). The former occurs due to dysfunctional pancreatic β-cells, while the latter is attributed to systemic tissue insulin resistance, and both are typical of type 2 diabetes (T2D) (IDF, 2016). Chronic hyperglycemia resulting from diabetic-linked aberrant lipid and glucose metabolism is implicated in the exacerbation of oxidative stress (Bajaj *et al.*, 2012). Hyperglycemia induces excessive reactive oxygen species (ROS) production via the mitochondria electron chain. Increased reactive oxygen species production crumbles the body's antioxidant defense system leading to oxidative stress (Penckofer *et al.*, 2002; Aversa *et al.*, 2016). These ROS, such as superoxide radical anion

and hydroxyl radicals cause lipid disruption, DNA and protein modifications. (Ceriello *et al.*, 2004; Reddy *et al.*, 2009; Touyz, 2012). Uncontrolled diabetes leads to diabetes complications such as neuropathy, hepatotoxicity, and cardiomyopathy (Maritim *et al.*, 2003; Mahmoud, 2014; Shahrajabian *et al.*, 2019). Proper glycemic control thus plays a critical role in the management of diabetes and its complications (Olofinsan *et al.*, 2021).

Numerous pharmacological agents have been developed for the management of T2D. Despite their far-reaching therapeutic benefits, such pharmacological agents have several drawbacks, including inaccessibility, exorbitant cost, and several inherent undesirable side effects (Esposito *et al.*, 2009; Yancy *et al.*, 2018; Kenny *et al.*, 2019). However, there is a promising role of medicinal plants and their bioactive compounds, such as polyphenols which have been proven as favorable alternatives in treating chronic diseases like diabetes and mitigating oxidative stress (Teodoro, 2019). They exert an influence of multimodality, which is mild and distributed through multiple enzyme targets and thus reduces many interrelated diseases in a concerted manner (Veerapur *et al.*, 2010). One of these medicinal plants is *Nauclea latifolia*, prescribed to manage diabetes mellitus (Length, 2005; Ademola *et al.*, 2014; Onyekwere *et al.*, 2014).

Recently, *N. latifolia* (family: Rubiaceae) has been reclassified under the synonym *Sarcocephalus latifolius* (smith) E.A. Bruce (Boucherle et al., 2016). It is a small tree that exist in tropical regions, especially in Africa and Asia. The plant's popularity in many African communities stems from its purported potency against different ailments. It has been reported for its use against malaria, hypertension (Akubue *et al.*, 1982; Abbah *et al.*, 2010a; Maitera *et al.*, 2011); cough, gonorrhea, stomach disorders, dysentery, ulcers, and liver ailments (Traore-Keita *et al.*, 2000; Abbah *et al.*, 2010b; Odeniyi *et al.*, 2020). Its aqueous leaf extract was reported to possess antidiabetic properties (Length, 2005; Alamin *et al.*, 2015b).

The present study was conducted to evaluate the antioxidant and antidiabetic properties of the fruit of *N. latifolia*, using *in vitro* and *in silico* experimental models.

4.2.3 Materials and methods

Please refer to sections **2.4, 2.5, 2.6, and 2.9 of chapter 2** for detailed methodology.

4.2.4 Results

Total phenol contents

As shown in **Table 4.2.1** *N. latifolia* aqueous extract's total polyphenol content was 44.56 ± 0.78mg/g GAE.

Table 4.2.1: Percentage yield and total phenolic content of the *N. latifolia* aqueous extract.

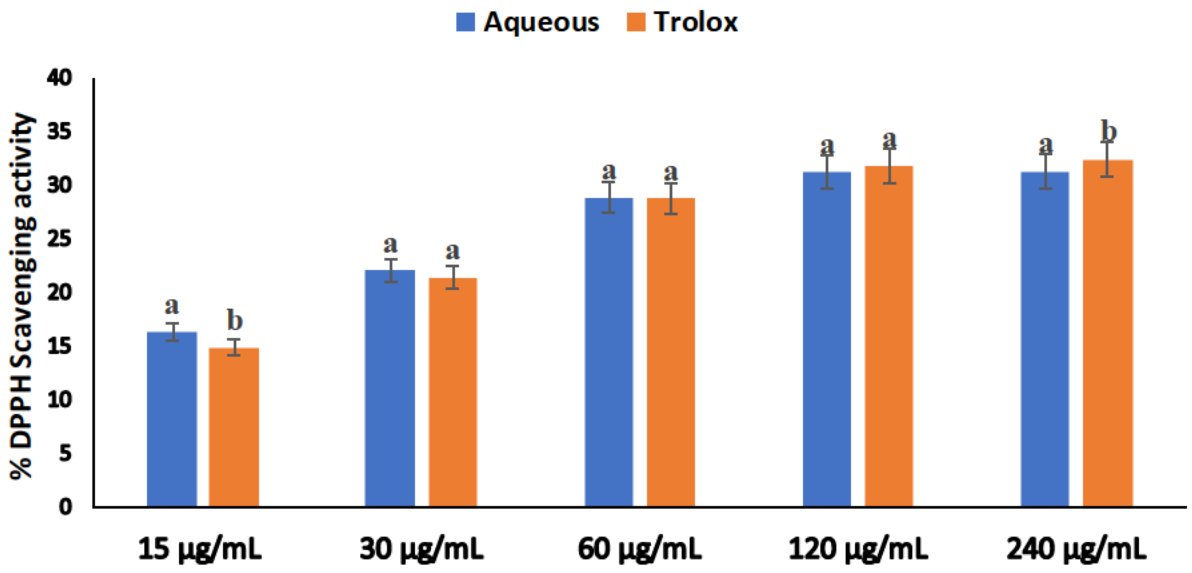
Plant extract	%Yield	Color	Texture	Total phenolic (mg/g GAE)
Aqueous	22	Dark brown	Fine powder	44.56 ± 0.781841

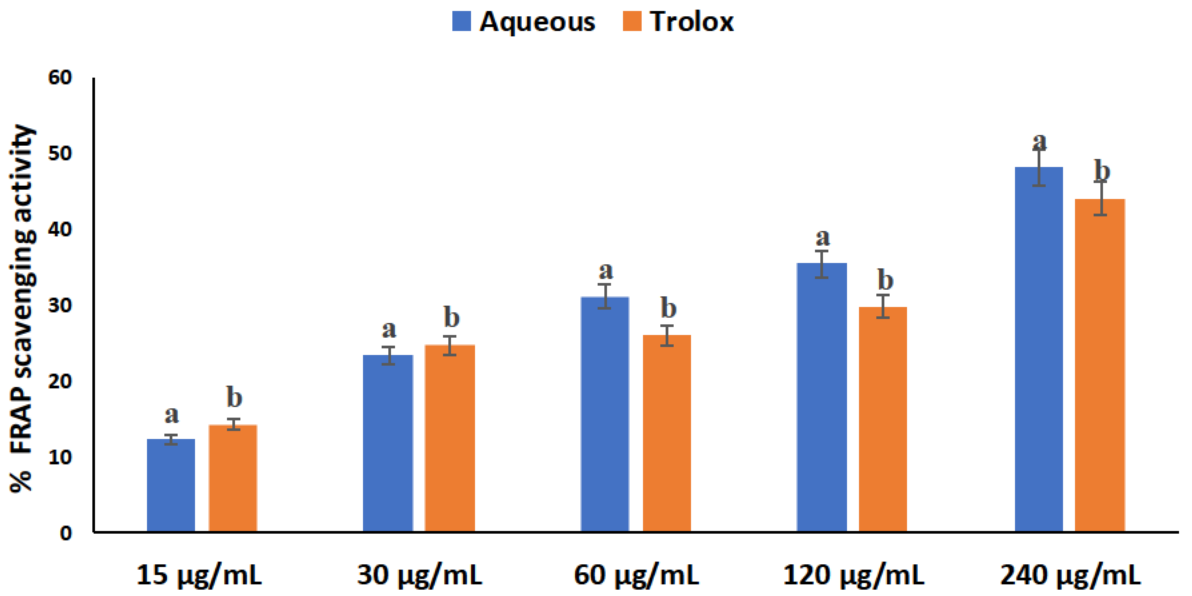
Results are presented as mean ± SD of triplicate determinations. GAE = Gallic acid equivalent

Antioxidants scavenging potential activities of N. latifolia

The DPPH radical scavenging, ferric ion reducing power (FRAP), and Nitric oxide (NO) radical inhibition assays were used to determine the antioxidant scavenging potential of *N. latifolia*, aqueous extract as shown in **Figure 4.2.1a-4.2.1c**, respectively. The extract's activities were concentration-dependent in all the assays performed. It exhibited better capabilities to inhibit NO production than its DPPH scavenging activities with IC_{50} values of 5.04 and 9.10 µg/mL, respectively **Table 4.2.2**. The extract significantly ($p<0.05$) had higher ferric ion reducing power

than the positive control (Trolox) at concentrations higher than 60 µg/mL. The IC_{50} for FRAP for the aqueous extract and Trolox were 5.58 and 6.42 µg/mL, respectively **Table 4.4.2.**





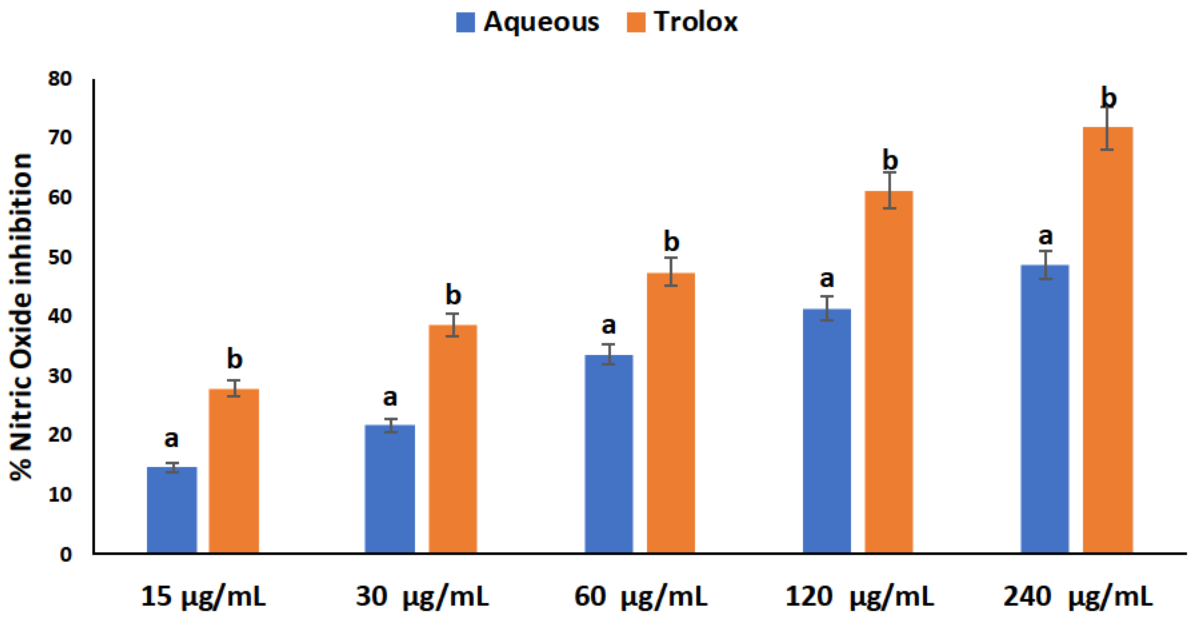


Figure 4.2.1: (a) DPPH scavenging activity of *N. latifolia* aqueous extract, (b) Ferric reducing antioxidant power (FRAP) activity of *N. latifolia* aqueous extract and(c) Nitric Oxide (NO) inhibition of *N. latifolia* aqueous extract. Data are presented as mean ± SEM. Values with a different letter [ab] above the bars for a given concentration are significantly different from each other ($p < 0.05$, Tukey's HSD-multiple range post hoc test, IBM SPSS for Windows).

Carbohydrate enzymes inhibitory activities

As shown in **Figure 4.2.2a**, the α-glucosidase inhibitory activity of the *N. latifolia* aqueous extract was significantly ($P < 0.05$) higher than the standard drug acarbose activity at 60 – 240 µg/mL. The low IC_{50} value of the aqueous extract (6.94 µg/mL) shows it has better inhibitory activity than acarbose, having an IC_{50} value of 8.32 µg/mL **Table 4.2.2**. The extract showed comparable α-amylase inhibitory activities to acarbose **Figure 4.2.2b** with IC_{50} values of 5.8 µg/mL and 4.20 µg/mL, respectively, as displayed in **Table 4.2.2**.

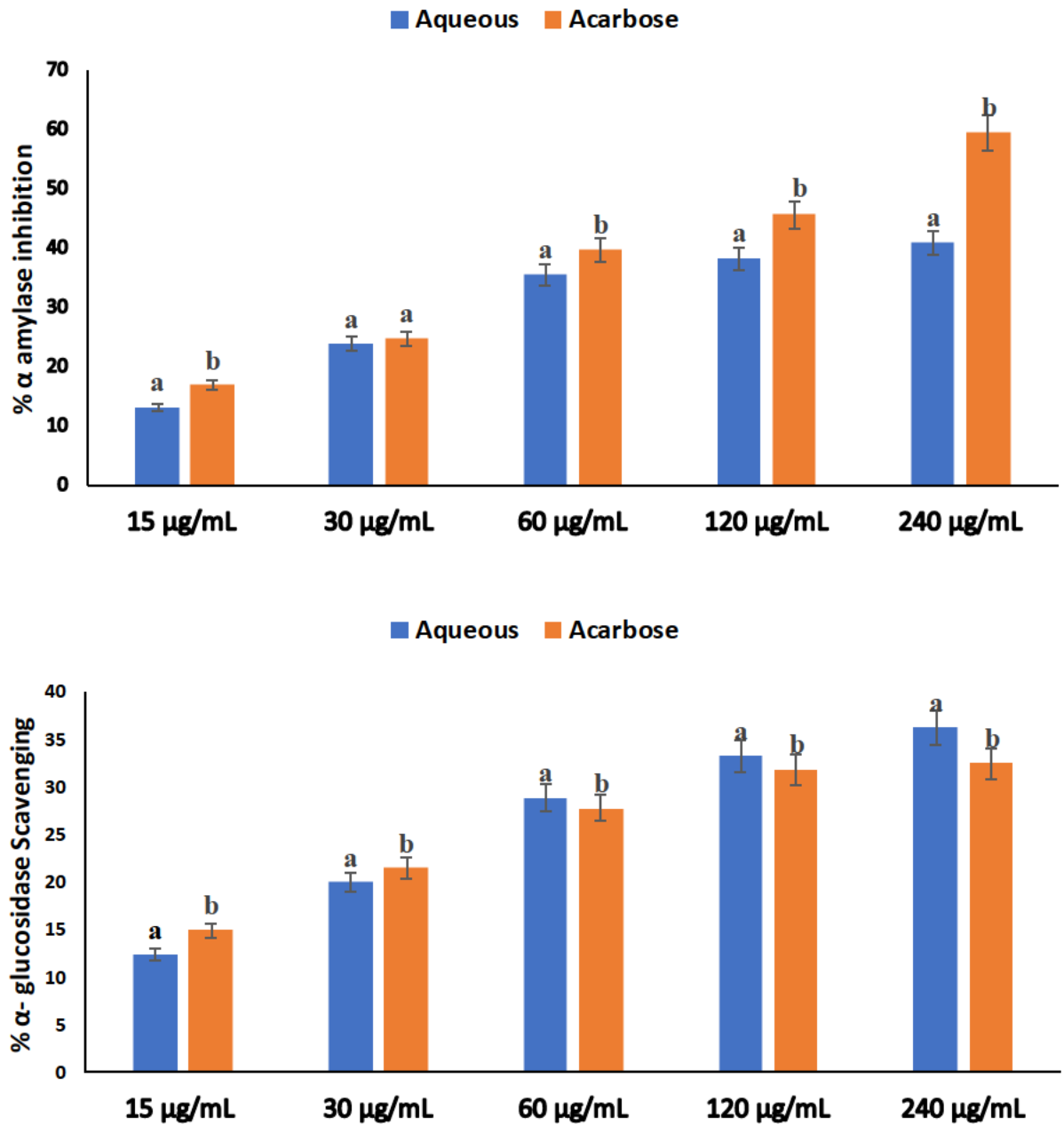


Figure 4.2.2: (a) α-glucosidase inhibitory activity of *N. latifolia* aqueous extract and (b) α-amylase inhibitory activity of *N. latifolia* aqueous extract. Data are presented as mean ± SEM. Values with the different letters [ab] above the bars for a given concentration are significantly different from each other ($p < 0.05$, Tukey's HSD-multiple range post hoc test, IBM SPSS for Windows).

Glucose uptake/ transport by yeast cells

As displayed in **Figure 4.2.3**, there was a consistent significant increase in the glucose uptake by the yeast cells and metformin (standard) with increasing concentration. The standard has better activity than the extract.

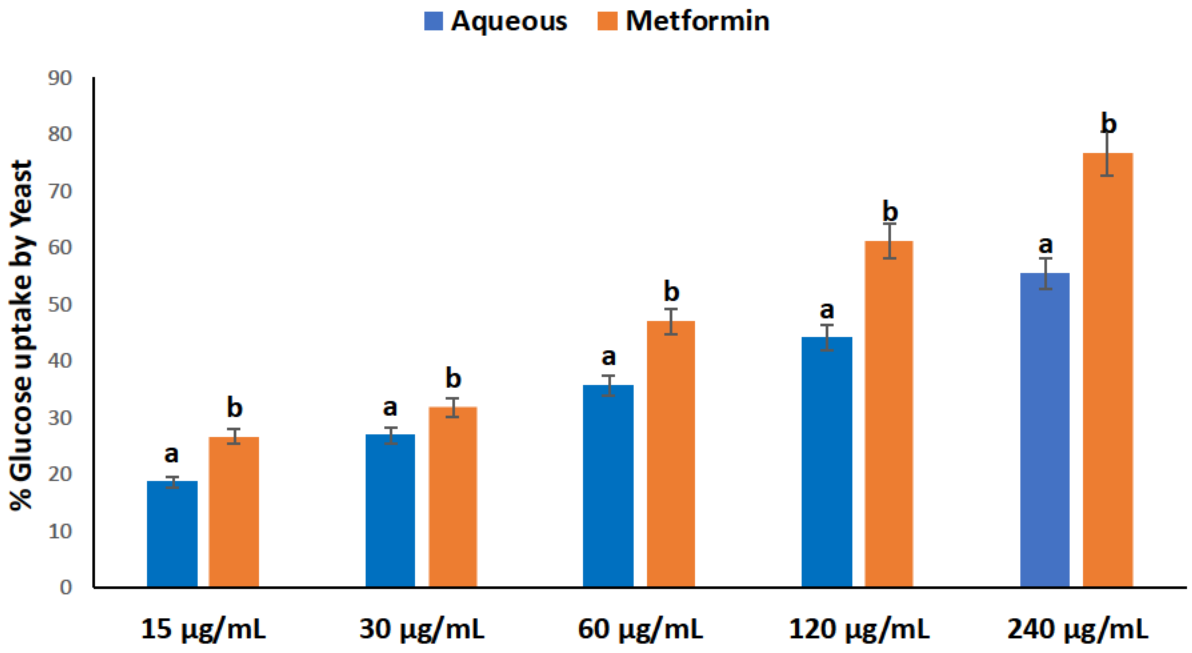


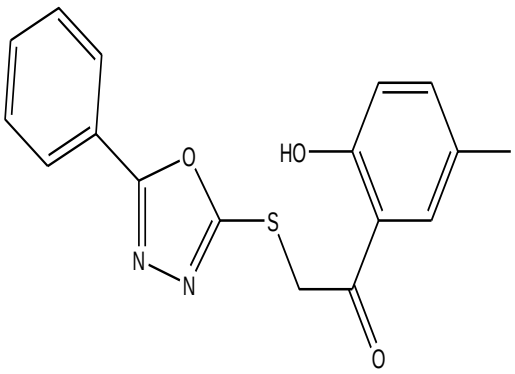
Figure 4.2.3: Effect of *N. latifolia* aqueous extract on glucose uptake in yeast cells. Data are presented as mean ± SEM. Values with the different letters[ab] above the bars for a given concentration are significantly different from each other ($p < 0.05$, Tukey's HSD-multiple range post hoc test, IBM SPSS for Windows).

Table 4.2.2: IC_{50} values of biological activities exhibited by *N. latifolia* on different antioxidant and antidiabetic parameters

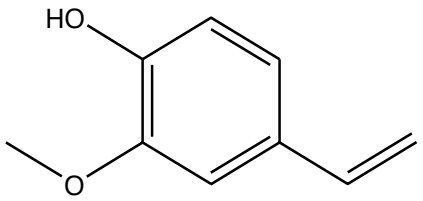
Biological activities	Aqueous	Trolox	Acarbose	Metformin
DPPH	9.1	8.3	-	-
FRAP	5.58	6.42	-	-
NO	5.04	3.04		-
Alpha glucosidase	6.94	-	8.32	-
Alpha amylase	5.80	-	4.20	-
Glucose uptake/yeast	4.52	-	-	3.10

GC-MS analysis

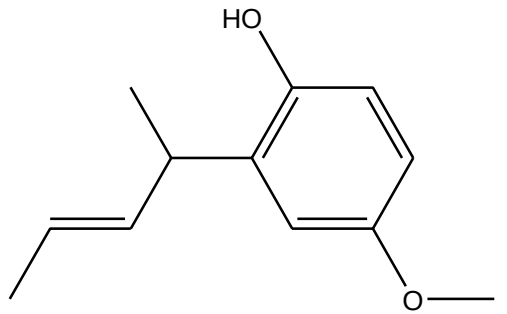
GC-MS analysis of *N. latifolia* aqueous fruit revealed the presence of several phenolic compounds, namely: 1-(2-hydroxy-5-methylphenyl) ethanone, 2 methoxy-4-vinylphenol, 4-methoxy-2-pent-3-en-2-ylphenol, 7,8-dihydroxy-4-methylcoumarin, 2-(2-penten-4-yl)-4-methoxy. Furthermore, the compound that exhibited the highest abundance were 4-methoxy-2-pent-3-en-2-ylphenol and 2-(2-penten-4-yl)-4-methoxy, while the compound with the lowest abundance was 2-Methoxy-4-vinylphenol as shown in **Figure 4.2.4** and **Table 4.2.3**.



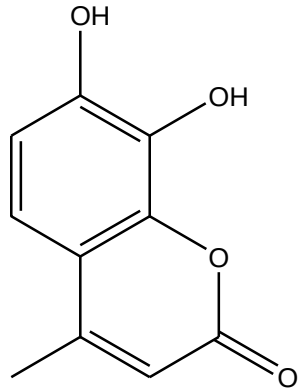
2-(5-phenyl-1,3,4-oxadiazol-2-ylthio)-1-(2-hydroxy-5-methylphenyl)ethanone



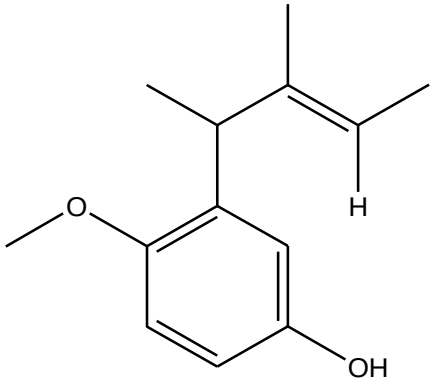
2-methoxy-4-vinylphenol



4-methoxy-2-((*E*)-pent-3-en-2-yl)phenol



7,8-dihydroxy-4-methyl-2*H*-chromen-2-one



2-(2-penten-4-yl)-4-methoxy

Figure 4.2.4: The structures of polyphenolic compounds identified in *N. latifolia* aqueous fruit extract

Table 4.2.3: Polyphenolic compounds identified in *N. latifolia* extract via GCMS analysis**.**

Phenol name	**Formula**	**Mol weight (g/mol)**	**R. Time (min)**	**Area%**	**Ret index**
2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150	9.525	0.21	1293
4-methoxy-2-pent-3-en-2-ylphenol	$C_{12}H_{16}O_2$	192	15.340	0.61	1545
7,8-dihydroxy-4-methylcoumarin	$C_{10}H_8O_4$	192	15.342	0.61	1905
1-(2-hydroxy-5-methylphenyl) ethanone	$C_9H_{10}O_2$	150	9.524	0.21	1363
2-(2-penten-4-yl)-4-methoxy	$C_{12}H_{16}O_2$	192	15.340	0.61	1545

C***ompounds Dynamic Conformational Stability and Fluctuations***

MD simulations were carried out to investigate the inhibition performance and interactions of the potential compounds with α-glucosidase and α-amylase protein targets. The validation of systems stability is essential to trace disrupted motions and avoid artifacts that may arise during the simulation. In this study, Root-Mean-Square Deviation (RMSD) was calculated to measure the systems' stability during the 20ns simulations **Figures 4.2.5a and 4.2.5b**. The recorded average RMSD values for all frames of α-glucosidase bonded compounds systems, 1-(2-hydroxy-5-methylphenyl) ethanone, 2-methoxy-4-vinylphenol, 4-methoxy-2-pent-3-en-2-ylphenol, 7,8-dihydroxy-4-methyl coumarin, 2-(2-penten-4-yl)-4-methoxy and Acarbose were 1.09 Å, 1.12 Å, 1.07 Å, 1.26 Å, 1.16 Å and 1.29 Å respectively. Whereas the average values for the α-amylase bonded compounds systems were 1.11 Å, 1.14 Å, 1.05 Å, 1.43 Å, 1.21 Å, and 1.74 Å, respectively.

The evolution of protein structure flexibility upon ligand binding is crucial in probing residue performance and their association with the ligand during MD simulation α-glucosidase and α-amylase enzymes bonded compounds residual fluctuations were evaluated using Root-Mean-Square Fluctuation (RMSF) algorithm to assess the effect of inhibitor binding towards the respective targets over 20ns simulations **Figures 4.2.6a and 4.2.6b.** The computed average atomic fluctuation of α-glucosidase bonded compounds 1-(2-hydroxy-5-methylphenyl) ethanone, 2 methoxy-4-vinylphenol, 4-methoxy-2-pent-3-en-2-ylphenol, 7,8-dihydroxy-4-methylcoumarin, 2-(2-penten-4-yl)-4-methoxy and Acarbose were 0.83 Å, 0.88 Å, 0.812 Å, 0.89 Å, 0.81Å and 1.33 Å respectively. Whereas the average atomic fluctuation values for the α-amylase bonded compounds systems were 0.865 Å, 0.88 Å, 0.76 Å, 0.89 Å, 0.78 Å, and 1.46 Å, respectively.

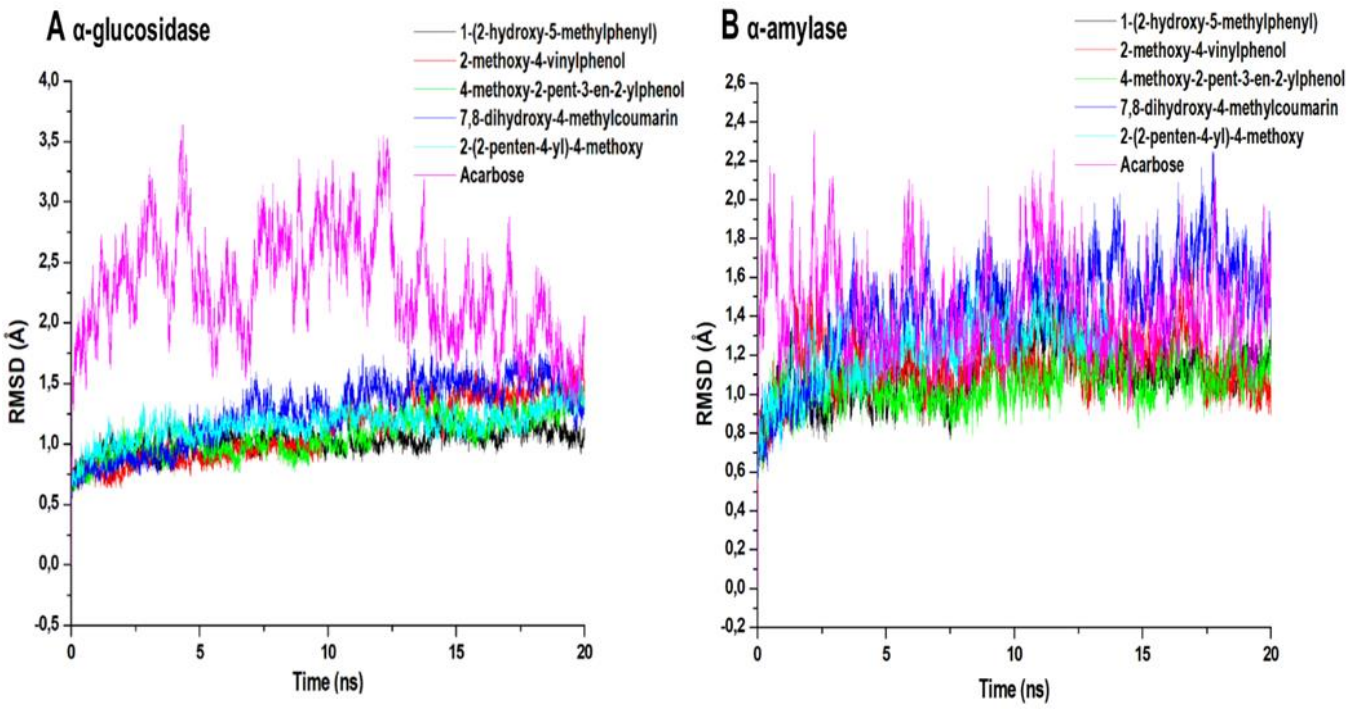
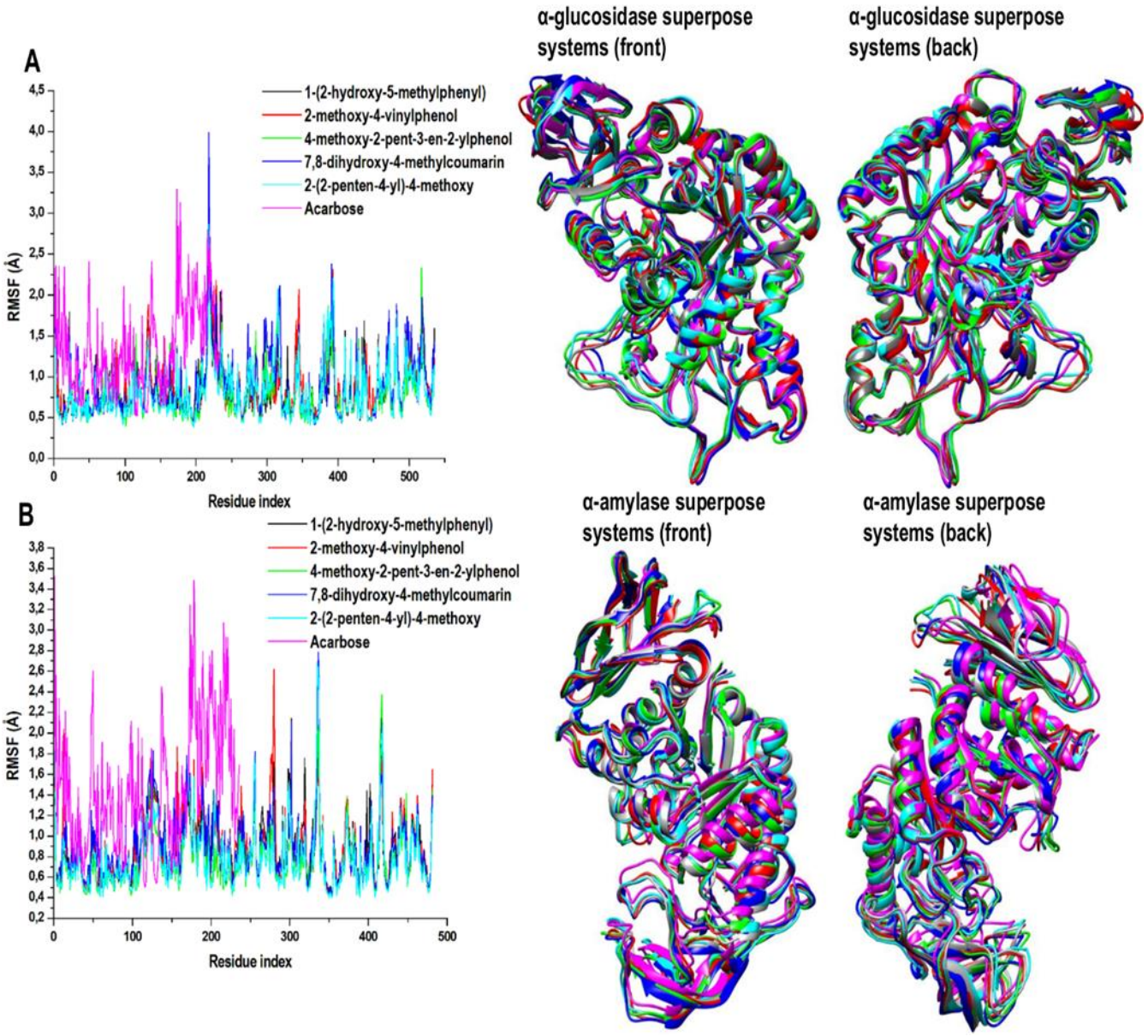


Figure 4.2.5a and 4.2.5b: Comparative C-α RMSD plots showing the degree of stability and convergence of the studied systems over the 20ns MD simulation time.



Figures 4.2.6a and 4.2.6b: The time evolution RMSF of each residue of the protein Cα atom over 20ns for the studied systems. Superposed of the crystal structures of the studied systems to show differences in fluctuations and conformational changes. Comparative Cα RMSF plot showing the degree of major flexibility of certain loops and helixes at the highest fluctuation during the simulation.

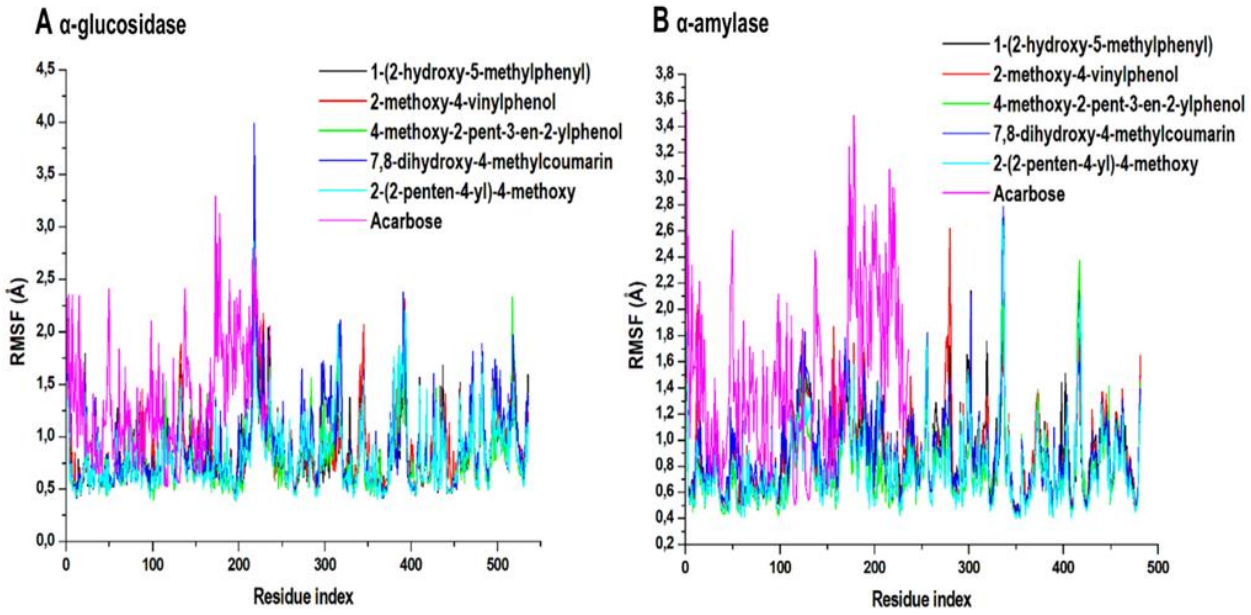


Figure 4.2.7a and 4.2.7b: The time evolution RMSF of each residue of the protein Cα atom over 20ns for the studied systems.

Binding free energy landscape of α-glucosidase and α-amylase enzymes bonded compounds systems

The MM/GBSA method is a standard technique to compute the free binding energy of small molecules to biological macromolecules (Genheden *et al.*, 2015). This approach was used to estimate the aggregate binding energy of five α-glucosidase and α-amylase enzyme bonded compounds complexes, as presented in **Tables 4.2.4** and **4.2.5** respectively**.** These calculated energies provide comprehensive molecular-level evidence that could be useful to design a drug by creating better ligand binding.

ADME parameters

The ADME parameters were used to determine the properties of the polyphenolic compound from *N. latifolia* aqueous extract, and the results are shown in **Table 4.2.6**. These compounds have passed the Lipinski bio model (Lipinski 2004). Generally, no more than one of these criteria should have been breached by an orally active drug: (a) There should not be more than 5 donors for hydrogen bonding (HBD); (b) not more than 10 acceptors for hydrogen bonding (HBAs), (c) not more than 500 for molecular weight (MW). (d) a maximum of (milogP) of 5. These compounds have also passed Jorgensen's bio-model (Jorgensen *et al.* 2000).

Table 4.2.4: MM/GBSA-based binding free energy profile of α-glucosidase enzyme bonded compounds

Systems	**Energy components (kcal/mol)**				
	ΔE_{vdw}	**ΔE_{ele}**	**ΔG_{gas}**	**ΔG_{sol}**	**ΔG_{bind}**
1-(2-hydroxy-5-methylphenyl)	-25.3692	-5.4943	-30.8635	13.3298	-17.5337
2 methoxy-4vinylphenol	-20.3163	-26.9826	-43.2989	20.9305	-22.3684
4-methoxy-2-pent-3-en-2-ylphenol	-30.2765	-16.8580	-47.1345	16.0656	-31.0689
7,8-dihydroxy-4-methylcoumarin	-26.4743	-33.5940	-60.0684	36.8102	-23.2581
2-(2-penten-4-yl)-4-methoxy	-29.6515	-11.9091	-41.5607	14.3408	-27.2199
Acarbose	-40.3782	-13.7763	-45.6213	29.5173	-33.0674

ΔEele = electrostatic energy; **ΔEvdw** = van der Waals energy; **ΔGbind** = total binding free energy; **ΔGsol** = solvation free energy **ΔG** = gas phase free energy.

Table 4.2.5: MM/GBSA-based binding free energy profile of α-amylase enzyme bonded compounds

Systems	**Energy components (kcal/mol)**				
	ΔE_{vdw}	**ΔE_{ele}**	**ΔG_{gas}**	**ΔG_{sol}**	**ΔG_{bind}**
1-(2-hydroxy-5-methylphenyl)	-22.3605	-8.4074	-30.7680	14.5011	-16.2668
2 methoxy-4vinylphenol	-22.9598	-18.8506	-41.8104	17.9506	-23.8598
4-methoxy-2-pent-3-en-2-ylphenol	-25.4615	-21.2898	-46.7513	17.8862	-28.8651
7,8-dihydroxy-4-methylcoumarin	-25.8651	-17.2227	-43.0879	21.4955	-21.5924
2-(2-penten-4-yl)-4-methoxy	-17.4202	-8.3363	-25.7565	12.5522	-13.2043
Acarbose	-42.3552	-12.2763	-54.6315	22.4873	-32.1442

ΔEele = electrostatic energy; **ΔEvdw** = van der Waals energy; **ΔGbind** = total binding free energy; **ΔGsol** = solvation free energy **ΔG** = gas phase free energy.

Table 4.2.6: ADME properties for phenolic compounds in *N. latifolia* aqueous extract

COMPOUNDS	MW g/mol	TPSA (Å2)	SOL in water	ABS	NRBs	MLOGP	HBAs	HBDs	Lipinski's violations	PAINS alert	TOX	
											RAT	hERG inhibition
2-methoxy-4-vinylphenol	150.17	29.46	Soluble	High	2	1.71	2	1	0	0	Neg	Low risk
4-methoxy-2-((E)-pent-3-en-2-yl)phenol	192.25	29.46	Soluble	High	3	2.58	2	1	0	0	Neg	Low risk
1-(2-hydroxy-5-methylphenyl)ethanone	150.17	37.30	Soluble	High	1	1.44	2	1	0	0	Neg	Low risk
7,8-dihydroxy-4-methylcoumarin	192.17	70.67	Soluble	High	0	0.76	4	2	0	0	Neg	Low risk
4-methoxy-2-pent-3-en-2-ylphenol	192.25	29.46	Soluble	High	3	2.58	2	1	0	0	Neg	Low risk

*MW= molecular weight * TPSA= Topological Polar Surface Area *SOL= solubility *ABS= absorption * MLOGP = Lipophilicity

*NRBs=Num. rotatable bonds *HBAs= H-bond acceptors * HBDs= H-bond donors *TOX= toxicity

4.2.5 Discussion

The prevalence of diabetes is rising at an alarming level worldwide every year (Farag *et al.*, 2011). According to the international diabetes federation (IDF) statistics in 2019, around 463 million persons were living with diabetes worldwide (I.D.F, 2019). In Africa, diabetes affects approximately 19.4 million people aged between 20 to 79 years (Thomas *et al.*, 2019). Its treatment expenditures are a severe economic burden for developing countries, especially in Africa, where the cost of diabetes medication is out-of-pocket expenses. The absence of a medical insurance system further heightens the burden (Lipsky *et al.*, 2015). Hence, the search for medicinal plants with bioactive compounds that can treat diabetes and its associated complications. In this study, we investigated the polyphenol phyto-constituents of aqueous extract of *N. latifolia* and determined their antioxidant activities and possible interactions with enzymes linked to diabetes.

The human body is vulnerable to various diseases linked to free radicals responsible for or detrimental to many illnesses such as diabetes and related complications (Lobo *et al.*, 2010). Antioxidants play an essential function in protecting the human body from the detrimental effects of free radicals (Sen & Chakraborty, 2011). Consequently, there has been an upsurge in research into prospective new sources of antioxidants due to their various therapeutic benefits such as anti-aging, anti-inflammatory, and anti-diabetic efficacies (Alan, 2013). Antioxidants exist naturally in medicinal plants, worthy of note are the plant polyphenolics with numerous biological functions (Kruawan *et al.*, 2006; Feumba *et al.*, 2020). Their potent antioxidant capabilities have been well reported (Heinonen, 2007; Fereidoon, 2010). Polyphenolics can scavenge a diverse range of reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as superoxides, hydroxyl, and peroxyl radicals peroxynitrous acid (Truong *et al.*, 2018). In the present study, *N. latifolia*

aqueous extract exhibited high phenolic content, as shown in **Table 4.2.1.** The extracts' polarity followed the pattern of total phenolic compounds in *N. latifolia*, suggesting that the plant's phenolic compounds are mainly polar in nature (Adenike & Samuel, 2019). Some phenolic compounds were identified from the GC-MS analysis of *N. latifolia* aqueous extract **Figure 4.2.4** and **Table 4.2.3**. Most of these compounds have been documented with various biological activities, such 2-methoxy-4-vinylphenol and 7,8-dihydroxy-4-methyl-2H-chromen-2-one with antibacterial and antioxidant activities (Shareef *et al.,* 2016; Kancheva *et al.,* 2010) and 1-(4-hydroxy-2-methylphenyl)ethenone with anti-inflammatory and antidiabetic activities (Muneeb *et al.,* 2018). Accordingly, *N. latifolia* is composed of diverse natural phenolic compounds with different structural characteristics and significant radical scavenging activities (Cai *et al.,* 2006). These results, thus suggest that structural differences in hydroxylation, glycosylation, and methoxylation might be related to the variance in radical scavenging activity (Cassidy *et al.*, 2000).

It has been established that excessive free radical generation beyond the body's antioxidant capacity can result in oxidative stress associated with many pathologies such as diabetes (Bajaj *et al.,* 2012). In this regard, the therapeutic efficacy of plant and their phytochemicals in ameliorating the effect of oxidative radicals in diseases progression has been well documented (Olofinsan *et al.,* 2021). In this study, *N. latifolia* displayed antioxidant activities using DPPH, FRAP, and NO assays **Figures 4.2.1a-4.2.1c.** Interestingly, these biological properties may be attributed to the plant intrinsic phenolics **Table 4.2.3** and other phytochemical components. Phenolic compounds are the largest group of exogenous antioxidants, classified as 'chain-breaking antioxidants' and reported as free radicals scavengers (Gaikwad *et al.,* 2010). In support of this study, previous findings have indicated that plant extracts with high phenolic content exhibit corresponding strong antioxidant activity (Jung *et al.,* 2008).

Inhibition of α-glucosidase enzyme activity can delay carbohydrate digestion, thus reducing postprandial blood glucose rise in diabetic individuals (Apostolidis *et al.*, 2006). Various studies have suggested that alternative treatments involving medicinal plants are promising due to their minimal side effects than currently available drugs (Sciences, 2017; Mohammadi *et al.*, 2020). Our findings in this study showed that aqueous extract of *N. latifolia* inhibits α-amylase and α-glucosidase activities **Figures 4.2.2a-4.2.2b**. This observation could be associated with the polyphenolic compounds in *N. latifolia* extract since similar compounds have been reported to suppress key carbohydrate digestive enzymes related to T2D (Ademiluyi *et al.*, 2013). Moreover, the low fluctuation, increased stability and high binding affinity exhibited by the digestive enzymes with these compounds as revealed by MD simulation, further suggests promising inhibition results compared to the natural inhibitor Acarbose **Table 4.2.4** and **4.2.5**. The increased activity of these compounds may be associated with the compound's chemical structure, which has a methoxy group with electron-withdrawing and electron-donating capacities (Sharma *et al.*, 1997). Several molecular docking and simulation screening investigations have revealed that phenolic compounds can inhibit these carbohydrate digestive enzymes (Rasouli *et al.*, 2017; Choudhary *et al.*, 2020; Kato-schwartz *et al.*, 2020).

Impaired skeletal muscle glucose uptake has been described as one of the mechanisms sustaining chronic hyperglycemia in T2D (Satoh, 2014). Glucose uptake in yeast cell suspension has been utilized as an in vitro assay to demonstrate the corresponding ability of substances to increase cellular glucose utilization under physiological conditions (Erukainure *et al.*, 2019). *N. latifolia's* dose-dependent increase in glucose uptake by yeast cells **Figure 4.2.3** may portray its hypoglycemic potential in T2D management. Since phenolic compounds have been suggested to

modulate GLUT4 activity (Collins et al. 2018), similar compounds **Table 4.2.3** identified in this plant may have accounted for the observed yeast glucose uptake activity.

Furthermore, in **Table 4.2.6**, the ADME analysis of *N. latifolia* polyphenolic compounds revealed excellent oral bioavailability, pharmacokinetics, and toxicity properties. Additionally, the compounds do not elicit any adverse toxic effect at the *in silico* toxicity prediction. Thus, suggesting that this plant can be a source of lead compounds for drug development in T2D management.

4.2.6 Conclusion

These results indicate that *N. latifolia* fruit aqueous extract has remarkable free radical scavenging activity and carbohydrate digestive enzymes inhibitory activities that may help prevent the onset of numerous oxidative stress-related diseases, including diabetes. Therefore, *N. latifolia* fruits may be considered as a natural source of bioactive compounds beneficial for human health and importantly, diabetes management therapies. However, further scientific validation in *in vivo* experimental models should be conducted to corroborate the observed *in vitro* and *in silico* outcomes to validate its medical benefits.

CHAPTER FIVE

ANTIOXIDANT AND ANTI-DIABETIC ACTIVITIES *HIBISCUS SABDARIFFA* (LINN) *IN VITRO, EX VIVO,* AND *IN SILICO*

5.1 *Hibiscus sabdariffa*

Hibiscus sabdariffa L. is one of the hibiscus species belonging to the Malvaceae family. It is native in various environments of many countries and multiple regions (Daudu *et al.*, 2019). It is a 2-2.5 m tall annual or perennial plant or woody-based subshrub. It is also known as roselle, hibiscus, Jamaica sorrel or red sorrel in English, and karkadeh in Arabic (Ali *et al.*, 2005). The leaves are 8-15 cm long, deeply 3-5 lobed, and alternately placed on the stalks (Riaz & Chopra, 2018). The blooms are white to light yellow with a dark red mark at the base of each petal and a strong meaty calyx at the base. They become fleshy and brilliant red as the fruit grows (Riaz *et al.*, 2018).

Figure 5.1: *Hibiscus sabdariffa* copied from (Dennis, 2018) without permission

5.1.1 Traditional uses

In many countries such as India, West Africa, and Mexico, the infusions of the leaves and calyces have historically been used for their diuretic, choleretic, febrifugal, and hypotensive properties. It has also been used for lowering the viscosity of the blood and increasing intestinal peristalsis, among other things (Al-Snafi, 2018). In Sudan and Egypt, an infusion of "Karkade" calyces is also utilized to reduce the body's temperature (Salami *et al.*, 2020). The medicinal benefit of calyces is well known in North Africa to cure coughs, as well as to treat genital issues (Da-Costa-Rocha *et al.*, 2014).

5.1.2 Biological and pharmacological activities

Hibiscus sabdariffa is widely applied in many countries for treating various diseases (Shruthi *et al.*, 2016). According to previous studies, *H. sabdariffa* has antihypertensive, antiseptic, sedative, diuretic, digestive, purgative, emollient, demulcent, and astringent properties (Mohamed *et al.*, 2012). The calyces have been used to cure cardiovascular disease, hypertension, and leukemia (Al-Ansary *et al.*, 2016). Furthermore, It is also utilized to treat pyrexia and abscesses (Azevedo *et al.*, 2010). The plant flower has been used to treat cough and bronchitis (P. Singh et al., 2017). Their health benefits come from their high concentration of antioxidants and the fact that they are naturally colored foods (Azevedo *et al.*, 2010). The aqueous plant extract has been shown to have antibacterial action against a wide range of microorganisms (Puro *et al.*, 2014) (Shruthi *et al.*, 2016). Among other properties, the plants extracts demonstrated an excellent effect on lipid metabolism and anti-diabetic properties (Shruthi *et al.*, 2016;Bala *et al.*, 2015)

5.1.3 Phytochemistry

Roselle has a high concentration of anthocyanins and protocatechuic acid (Sinela *et al.*, 2017). The flavonoids gossypetine, hibiscetine, and sabdaretine have been identified in high concentrations in dried calyces (Salem *et al.*, 2014). Trace levels of myrtillin (delphinidin 3-monoglucoside), chrysanthenin (cyanidin 3-monoglucoside), and delphinidin have also been detected in the plant extract (Nazeer *et al.*, 2015). *H. sabdariffa* seeds are a substantial source of lipid-soluble antioxidants, notably tocopherol, which helps to protect against free radical damage (Khaki & Fathiazad, 2012). Intensive scientific research has been conducted on the color qualities of the calyceal extract of *H. sabdariffa,* which has a rich red pigmentation due to anthocyanins (Abdel-Shafi *et al.*, 2019).

5.2 *Hibiscus sabdariffa L.* polyphenolic-rich extract promotes muscle glucose uptake and inhibits intestinal glucose absorption with concomitant amelioration of Fe^{2+}-induced hepatic oxidative injury

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5.2.1 Abstract

In the present study, the antidiabetic effect of *Hibiscus sabdariffa* and its protective role against Fe^{2+}-induced oxidative hepatic injury were elucidated, using *in vitro, in silico* and *ex vivo* studies. Oxidative injury was induced in liver tissues by incubation of normal hepatic tissues with 0.1 mMolar ferrous sulphate ($FeSO_4$) and treated by co-incubation of various concentrations of the crude extracts (ethyl acetate, ethanol, and aqueous) of *H. sabdariffa* flowers for 30 min at 37°C. The ethanolic extract exhibited the highest scavenging activity in ferric-reducing antioxidant power (FRAP), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and nitric oxide (NO) assays with IC_{50} values of 2.8 µg/mL, 3.3 µg/mL and 9.2 µg/mL respectively when compared to ethyl acetate and aqueous extracts. The extracts significantly ($p<0.05$) inhibited α-glucosidase and α-amylase activities, with the ethanolic extract exhibiting the best activity. *H. sabdariffa* significantly ($p<0.05$) raised reduced glutathione (GSH) levels while simultaneously decreasing malondihaldehyde (MDA) and NO levels and increasing superoxide dismutase (SOD) and catalase activity in Fe^{2+} induced oxidative hepatic injuries. The plant extract suppressed the intestinal glucose absoption and enhanced muscle glucose uptake. The extract revealed the presence of several phenolic compounds when subjected to Gas Chromatography–Mass Spectroscopy (GC-MS) analysis which were docked with α-glucosidase and α- amylase. The molecular docking displayed the compound 4-(3,5-Di-tert-butyl-4-hydroxyphenyl)butyl acrylate strongly interacted with α-glucosidase and α-amylase and had the lowest free binding energy compared to other compounds and acarbose. These results suggest the *H. sabdariffa* has promising antioxidant and antidiabetic activity.

Keywords: Antioxidant; Antidiabetic; Enzyme inhibition; *H. sabdariffa* L.; Molecular docking

5.2.2 Introduction

The prevalence of diabetes is growing every year. In 2019, the number of individuals who lived with diabetes globally where around 463 million (Cho *et al.*, 2018) and the figure is expected toto increase to 642 million by 2040. (Colagiuri, 2010). However, this epidemic is associated with changes lifestyle, diet, gene, family history and some health problems (West *et al.*, 2011). Out of the common categories of diabetes, type 2 diabetes (T2D) is the most dominant and accounts for over 90% of the total number of all diabetes cases around the world (Inzucchi *et al.*, 2015). T2D is typified by hyperglycemia due to insufficient insulin secretion or insulin action (Cantley & Ashcroft, 2015), causing disruptions in protein, carbohydrate, and lipid metabolism (ul Haq *et al.*, 2020). Hyperglycemia leads to increased reactive oxygen species (ROS) generation, consequently causing an imbalance in the body's natural antioxidant defense mechanisms, thus, producing oxidative stress as T2D progresses (Burgos-Morón *et al.*, 2019; Chen *et al.*, 2018). Incessant hyperglycemia coupled with oxidative stress instigates diabetes associated vascular complications that possibly harm various vital organ systems such as cardiovascular diseases (Levinthal *et al.*, 1999; Guven *et al.*, 2006). The liver is one of the body organs that could be affected by hyperglycemia-induced oxidative stress resulting in hepatic injury (Mahmoud *et al.*, 2012; Ayepola *et al.*, 2013).

Different therapeutic drugs are currently available for the management of T2D. Despite their wide-ranging therapeutic benefits, many side effects have been attributed to this drugs as well as issues of inaccessibility and cost (Esposito *et al.*, 2009; Yancy *et al.*, 2018; Kenny *et al.*, 2019). Medicinal plants have been used from time immemorial for the management and treatment of T2D

(Ezuruike *et al.*, 2014). These plants are considered as natural reservoir of antioxidant with multiple health benefits including antidiabetes properties (Hussain *et al.*, 2015). Their inherent health benefits have been linked to the presence of numerous bioactive substances such as polyphenolic compounds (Dahiru *et al.*, 2008; Bourogaa *et al.*, 2013). Among such plants is *Hibiscus sabdariffa* L.

Hibiscus sabdariffa L. commonly known as roselle is one of the hibiscus species, belonging to the Malvaceae family. It is native to Africa and Asia and commonly grown in many countries as garden shrub (Daudu *et al.*, 2019). This plant is frequently utilized in various traditional medicine applications such as hypertension, heart disease, stomach pain as well as kidney and skin diseases (Kapoor *et al.*, 2021). In addition, previous studies revealed that *H.sabdaaiffa* has many benefits including antioxidants, antihyperlipidemic, and anti-diabetes properties (Lin *et al.*, 2005; Bala *et al.*, 2015). Furthermore, it has been documented that the plant is rich in phytochemical compounds such as polyphenols, which showed a potent action in lowering the hepatic lipid content and enhancing density lipoprotein LDL uptake in hepatocytes (Alam *et al.*, 2016).

The present study was conducted to characterize the phytochemical constituent of *H. sabdariffa* and investigate its effect on redox imbalance in Fe^{2+}-induced oxidative hepatic injury, muscle glucose uptake and intestinal absorption *ex-vivo*, as well as its effect on carbohydrate digestive enzymes activities *in vitro* and *in silico.*

5.2.3 Materials and methods

Please refer to sections **2.4 to 2.9 of chapter 2** for more detailed methodology.

5.2.4. Results

Total polyphenolic contents:

As shown in **Figure 5.2.1,** the plant extracts revealed a high amount of total polyphenolic contents. The ethanolic exhibited an significantly higher level than ethyl acetate and aqueous.

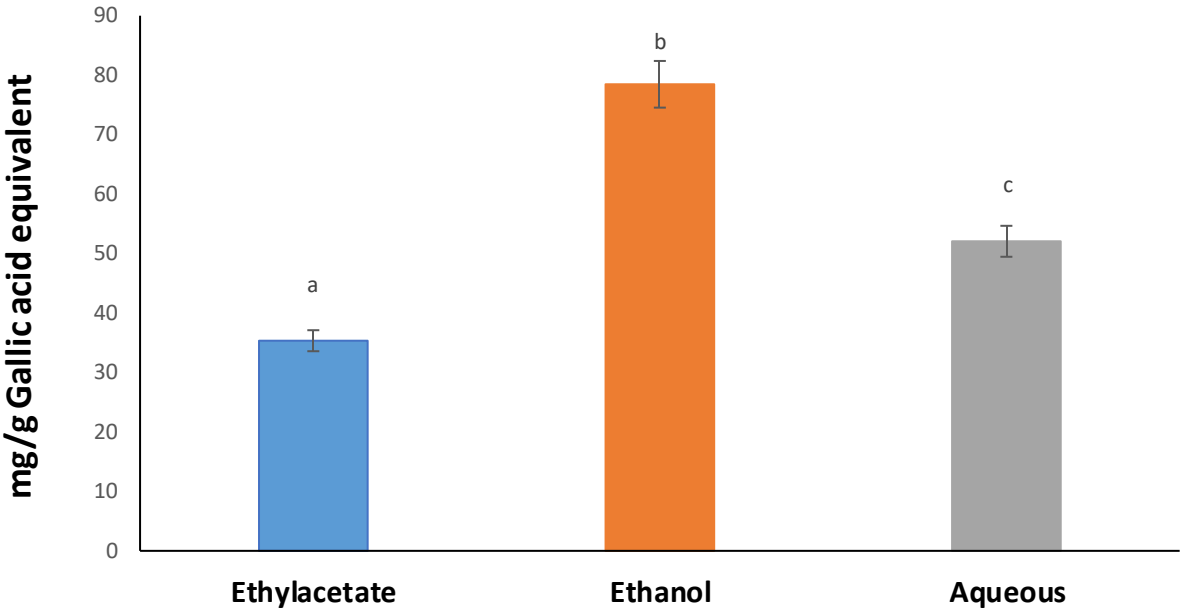
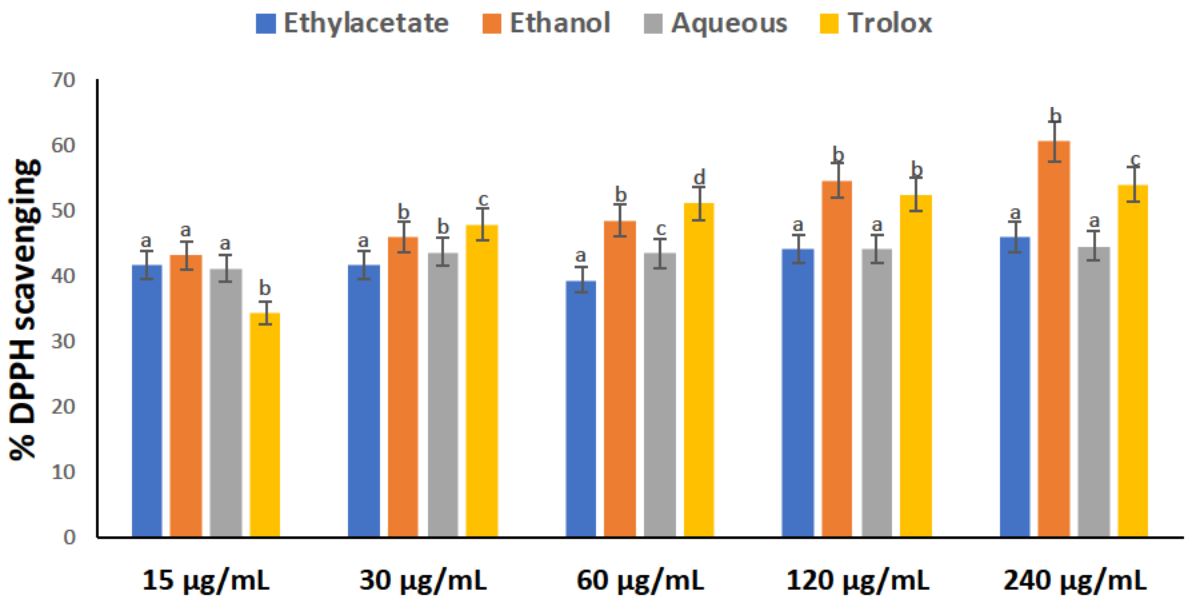


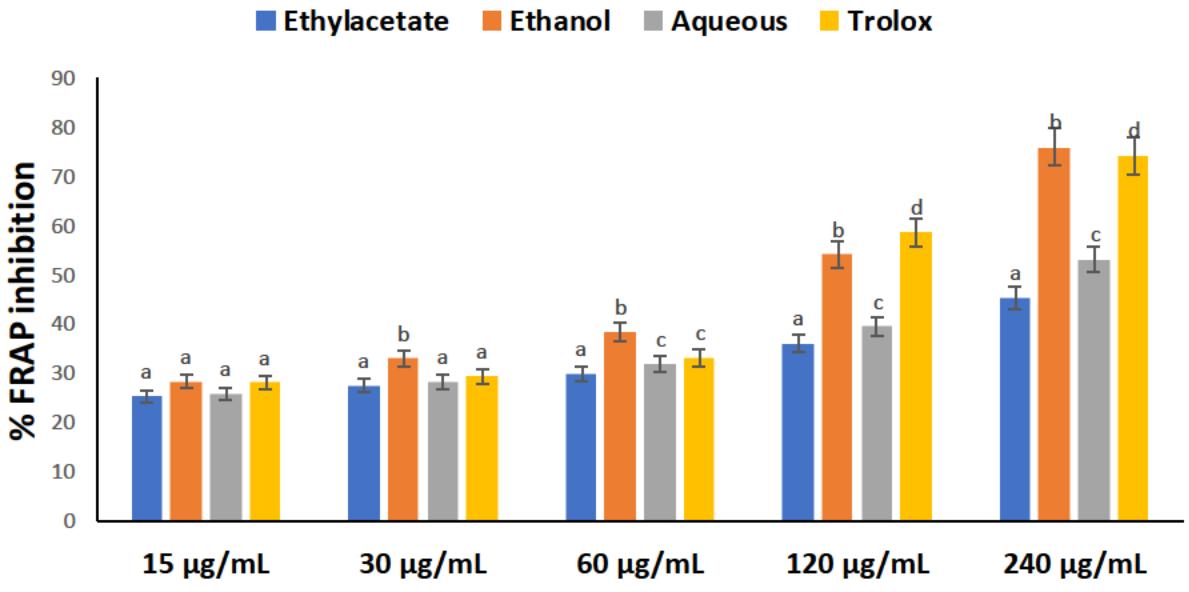
Figure 5.2.1: The total phenolic content of the different dry plant extracts of *H.sabdariffa* represented as gallic acid equivalents (GAE mg/g). Results are expressed as mean ± SD of triplicate determinations. [a-c]Different superscripts letters over the bars represent statistical significance of difference ($p<0.05$, Tukey's-HSD multiple range post hoc test).

Antioxidant scavenging activities of *H. sabdariffa*

As presented in **Figures 5.2.2 a-c**, free radical scavenging activity (DPPH), ferric ion reducing power (FRAP), and nitric oxide (NO) radical scavenging activity were utilized to determine the potential antioxidant power of the various extracts of *H. sabdariffa* respectively. The antioxidant

activities of the extracts were concentration-dependent in all the assays performed. The ethanolic extract significanly ($p<0.05$) scavenged DPPH radical, increased FRAP and inhibited NO radical more than other both the ethyl acette and aqueous extracts **(Figure 5.2.1 a-c)**. This compared favorably with the standard Trolox at IC_{50} values of 2.8 µg/mL, 3.3 µg/mL and 9.2 µg/mL respectively (**Table 5.2.1**).





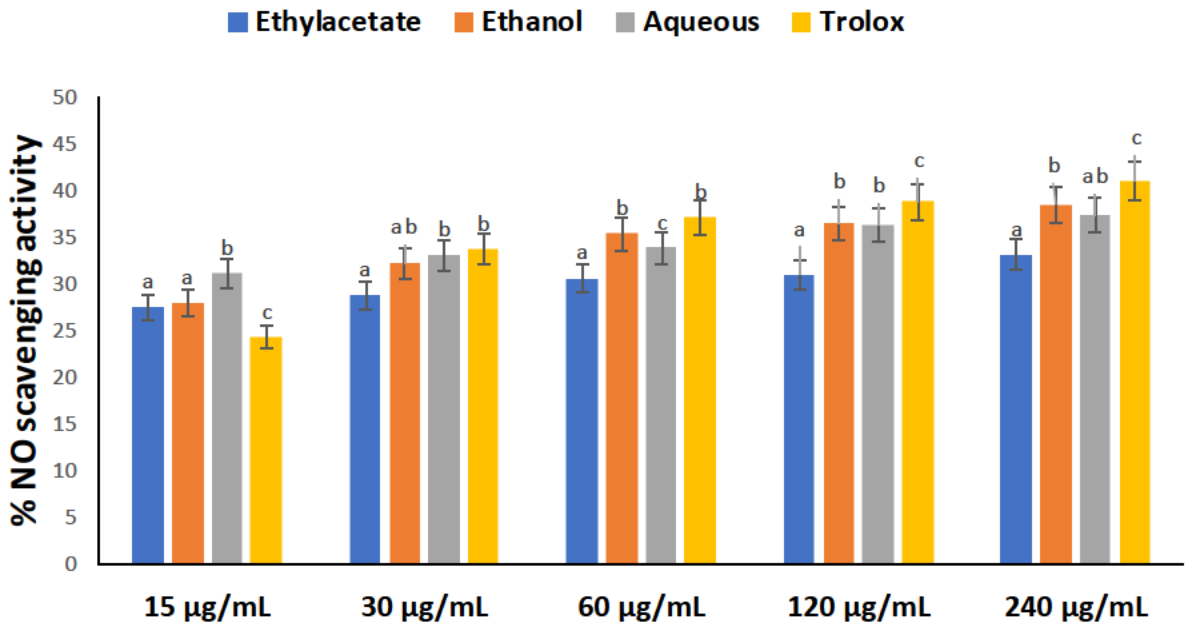


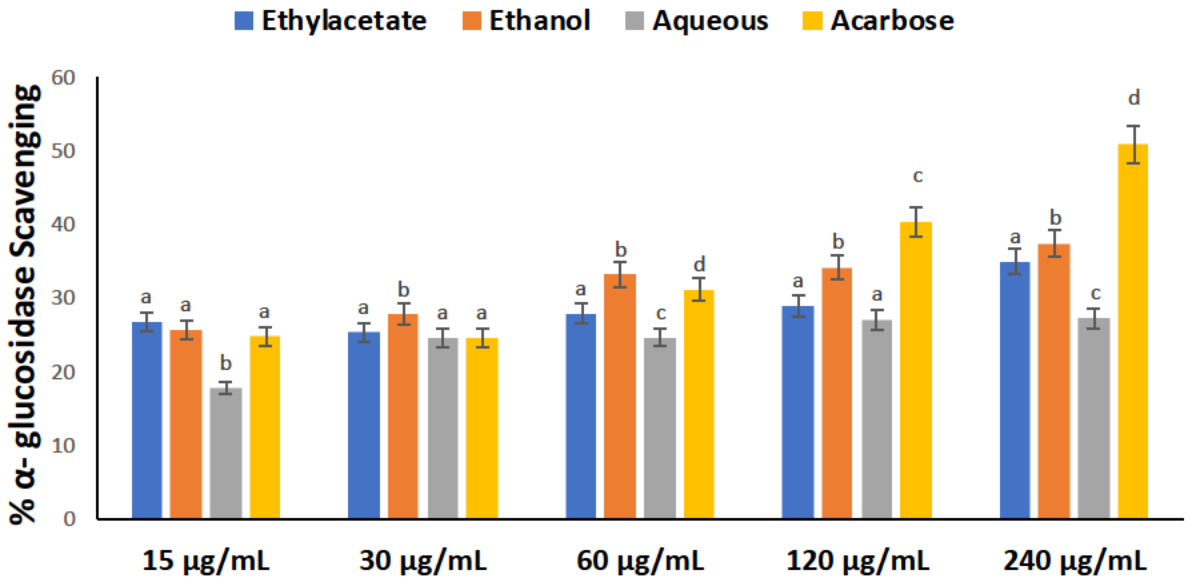
Figure 5.2.2: (a) DPPH scavenging activity (b) Ferric reducing antioxidant power (FRAP) and (c) Nitric oxide (NO) scavenging activity of *N. latifolia* extracts. Data are presented as mean ± SEM. Values with a different letter [ab] above the bars for a given concentration are significantly different from each other ($p < 0.05$, Tukey's HSD-multiple range post hoc test, IBM SPSS for Windows).

Table 5.2.1: IC_{50} values of biological activities exhibited by *H. sabdariffa* on different antioxidant and antidiabetic parameters

Biological activities	Ethyl acetate	Ethanol	Aqueous	Trolox	Acarbose
DPPH	9.8	2.8	11.9	3.4	-
FRAP	6.5	3.3	4.9	3.4	-
NO	17.6	9.2	12.9	6.8	
Alpha glucosidase	13.5	10.1	14.9	-	9.1
Alpha amylase	37.8	27.2	35.1		30.8

Carbohydrate enzymes inhibitory activities

As represented in **Figure 5.2.3a**, compared to ethylacetate and aqueous extracts, the ethanolic extract showed higher α-glucosidase inhibitory activities and this compared favourably with the standard, acarbose with IC_{50} values of 10.1µg/mL and 9.1 µg/mL (**Table 5.2.1**) respectively. All extracts showed excellent α-amylase inhibitory activities (**Figure 5.2.3b**) at increasing concentrations when compared with the standard. However, the ethanolic extract again showed a significantly ($p<0.05$) higher activity than acarbose with IC_{50} values of 27.2 µg/mL and 30.8 µg/mL respectively (**Table 5.2.1**).



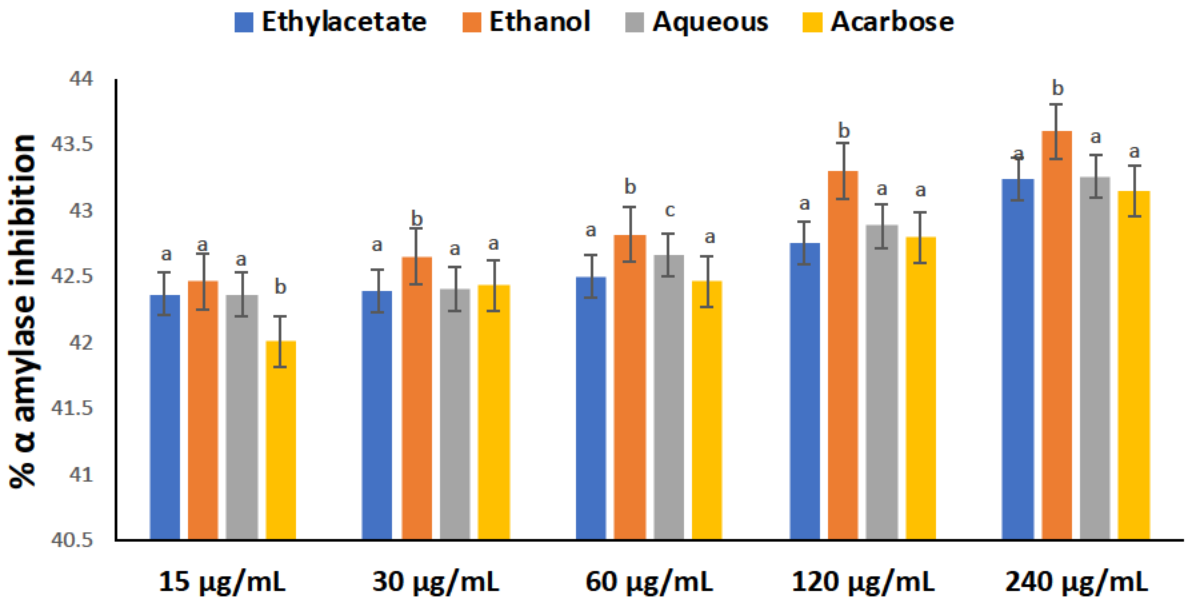


Figure 5.2.3: (a) α-glucosidase inhibitory activity of *N. latifolia* aqueous extract and (b) α-amylase inhibitory activity of *N. latifolia* aqueous extract. Data are presented as mean ± SEM. Values with the different letters [ab] above the bars for a given concentration are significantly different from each other ($p < 0.05$, Tukey's HSD-multiple range post hoc test, IBM SPSS for Windows).

Glucose absorption and uptake

Incubation of isolated rat jejunum with *H. sabdariffa* extracts led to significant ($p<0.05$) inhibition of intestinal glucose absorption, as shown in **Figure 5.2.4.** All plant extracts showed a dose-dependent activity at increasing concentrations. As displayed in **Figure 5.2.5**, *H. sabdariffa* extracts were able to significantly (($p<0.05$) facilitate muscle glucose uptake starting from 30 µg/mL to 240 µg/mL concentration. The glucose uptake was dose-dependently increased. However, the ethanolic extract showed the highest activity among all the extracts and almost leveled up with the standard insulin at the highest (240 µg/mL) concentration.

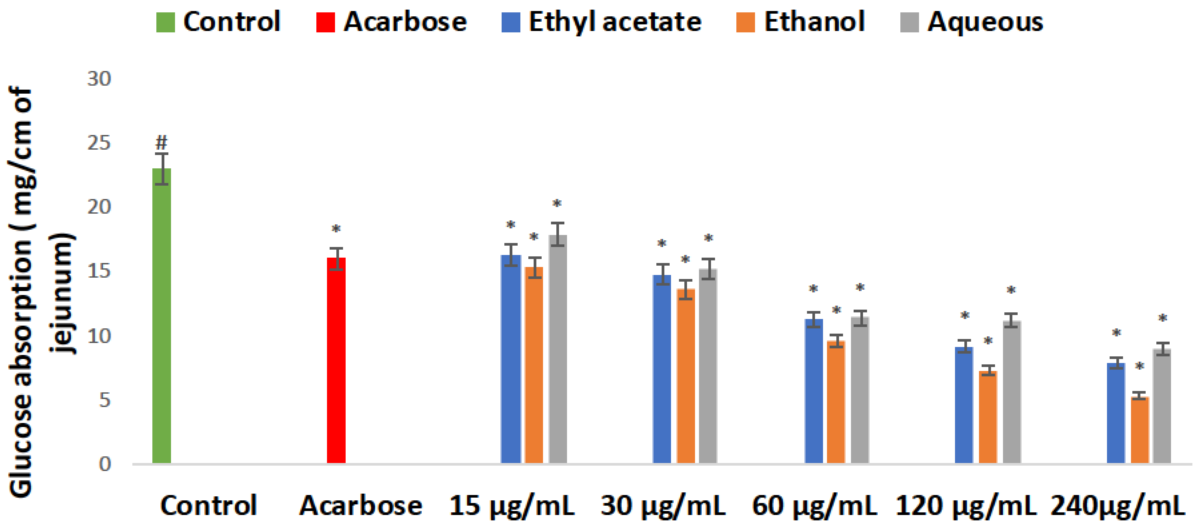


Figure 5.2.4: Effect of *H. sabdariffa* extracts on glucose absorption in isolated rat jejunum. Values = mean ± SD; n = 3. *Significantly different from acarbose sample and #Significantly different from control sample ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

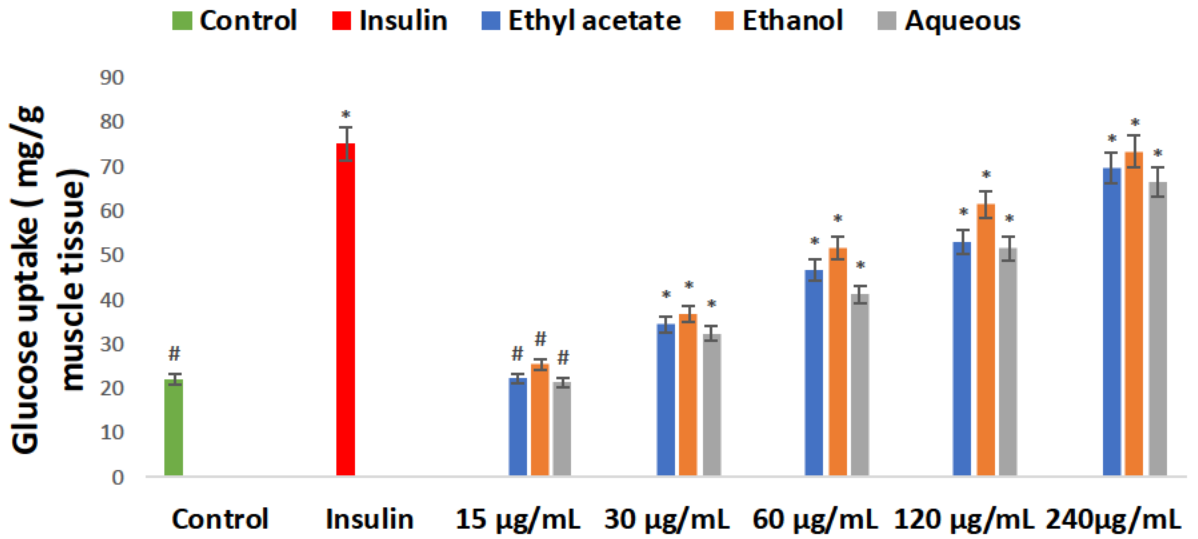


Figure 5.2.5: Effect of *H. sabdariffa* extracts on glucose uptake in rat psoas muscle. Values = mean ± SD; n = 3. *Significantly different from insulin sample and #Significantly different from control sample ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

Ex vivo antioxidant activities

As shown in **Figure 5.2.6a**, incubation of pancreatic tissues with FeSO4 significantly ($p<0.05$) reduced GSH levels. Hepatic GSH levels were significantly improved ($p<0.05$) after treatment with *H. sabdariffa* extracts when compared to gallic acid. The ethanolic extract had a higher activity than other extracs..

Figure 5.2.6b displayed that incubation with $FeSO_4$ decreased pancreatic SOD activity significantly ($p<0.05$). Treatment with *H. sabdariffa* extracts significantly ($p<0.05$) increased the tissue SOD levels at increasing concentrations. The ethanol extract achieved the highest activity than ethyl acetate and aqueous.

As shown in **Figure 5.2.6c**, there was a significant decrease in hepatic catalase activity of the untreated tissue. *H. sabdariffa* extracts significantly ($p<0.05$) increased catalase activities and compared favorably with the normal tissues. However, there was no significant ($p<0.05$) increase among the concentrations of the groups.

As shown in **Figure 5.2.6d**, there was a significant ($p<0.05$) rise in hepatic MDA levels after incubation with FeSO4. Following treatment with *H. sabdariffa*, the elevated levels were considerably ($p<0.05$) reduced to levels indistinguishable from those seen in normal tissues. However, ethanolic and aqueous extracts showed more potency in suppressing the MDA level.

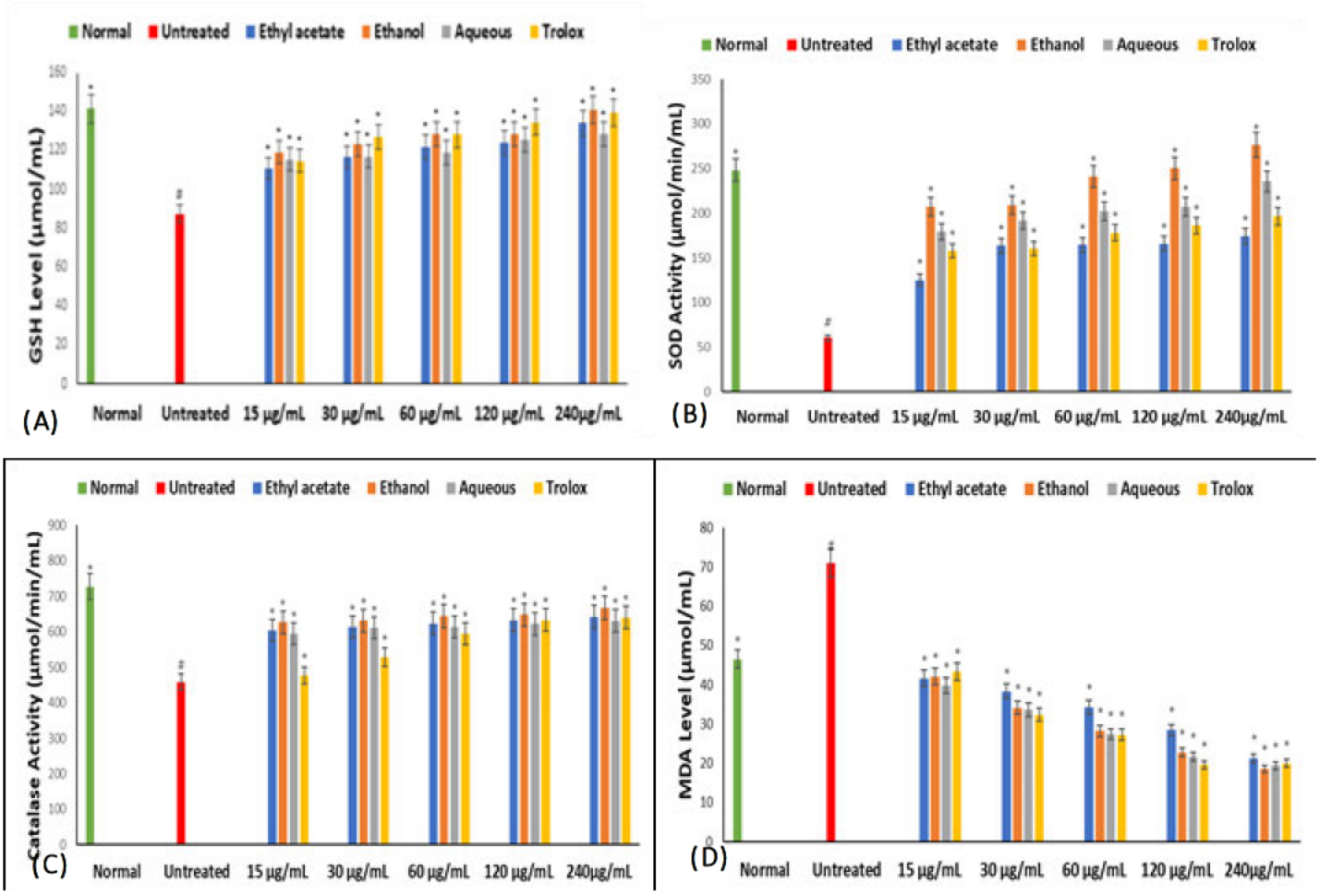


Figure 5.2.6: Effect of *H. sabdariffa* extracts on (a) GSH level, (b) SOD activity, (c) Catalase activity and (d) MDA levels in oxidative hepatic injury. Values = mean ± SD; n = 3.

*Significantly different from untreated sample and #Significantly different from normal sample ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

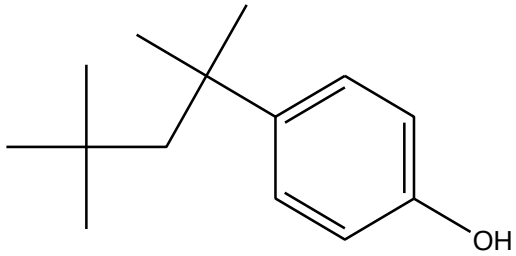
GCMS results

GC-MS analysis of *H. sabdariffa* extracts revealed the presence of several phenolic compounds, namely: p-(1,1,3,3-Tetramethylbutyl)phenol, p-(.alpha.,.alpha.-Dimethylpropyl)phenol, Menthol, trans-1,3,trans-1,4-, Phenol, p-(1,1,3,3-tetramethylbutyl)-, Phenol, 4,4'-(1,2-diethylethylene)di-,

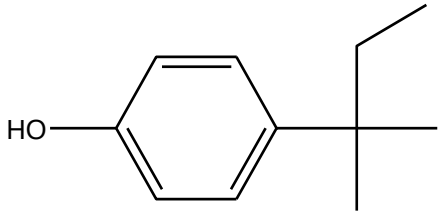
Phenol, 2-methyl-4-(1,1,3,3-tetramethylbutyl)-, Phenol, 2-(1,1-dimethylethyl)-6-methyl-, 2-Methyl-6-(1,1,3,3-tetramethylbutyl)phenol, 4-(3,5-Di-tert-butyl-4-hydroxyphenyl)butyl acrylate, b-(3,5-Di-tert-butyl-4-hydroxyphenyl)propionic acid hydrazide, 2,5-Dihydroxyphenol, 2-Methyl-4-hydroxyacetophenone. Furthermore, the compound that exhibited the highest abundance was 4-(3,5-Di-tert-butyl-4-hydroxyphenyl)butyl acrylate, while the compounds with a lowest abundance were p-(1,1,3,3-Tetramethylbutyl)phenol, p-(.alpha.,.alpha.-Dimethylpropyl)phenol as exhibited in **Figure 5.2.7** and **Table 5.2.2.**

Table 5.2.2: Identified compound in *H. sabdariffa* extracts by GCMS

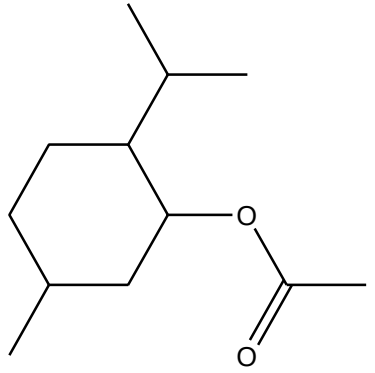
Phenol name	**Formula**	**Mol weight (g/mol)**	**R Time (min)**	**Area %**	**Ret. Index**
p-(1,1,3,3-Tetramethylbutyl)phenol	C14H22O	206	14.390	0.16	1541
p-(.alpha.,.alpha.-Dimethylpropyl)phenol	C11H16O	164	14.390	0.16	1327
Menthol, trans-1,3,trans-1,4-	C10H20O	156	18.435	4.69	1164
Phenol, p-(1,1,3,3-tetramethylbutyl)-	C14H22O	206	14.390	0.16	1541
Phenol, 4,4'-(1,2-diethylethylene)di-	C18H22O2	270	14.390	0.16	2277
Phenol, 2-methyl-4-(1,1,3,3-tetramethylbutyl)-	C15H24O	220	14.525	2.37	1654
Phenol, 2-(1,1-dimethylethyl)-6-methyl-	C11H16O	164	14.590	2.28	1341
2-Methyl-6-(1,1,3,3-tetramethylbutyl)phenol	C15H24O	220	14.995	2.94	1654
4-(3,5-Di-tert-butyl-4-hydroxyphenyl)butyl acrylate	C21H32O3	332	17.345	6.47	2422
b-(3,5-Di-tert-butyl-4-hydroxyphenyl)propionic acid hydrazide	C17H28N2O2	292	16.720	6.23	2510
2,5-Dihydroxyphenol	C6H6O3	126	10.995	0.68	1342
2-Methyl-4-hydroxyacetophenone	C9H10O2	150	9.520	0.84	1363



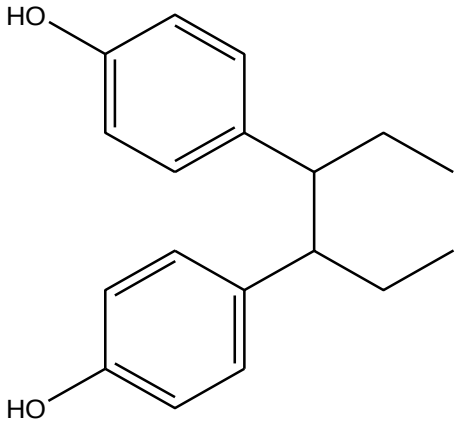
4-(2,4,4-trimethylpentan-2-yl)phenol



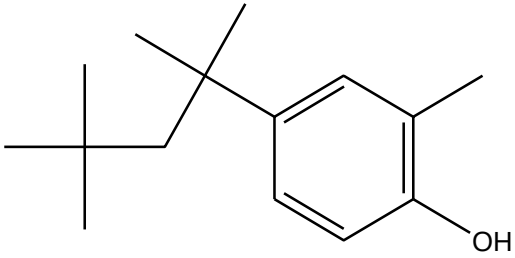
4-*tert*-pentylphenol



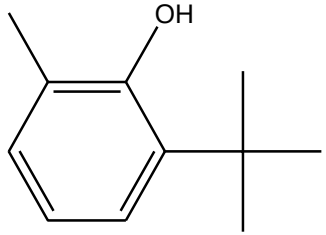
2-isopropyl-5-methylcyclohexyl acetate



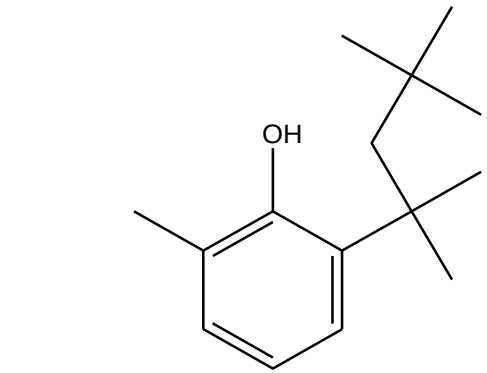
Phenol, 4,4'-(1,2-diethylethylene)di-



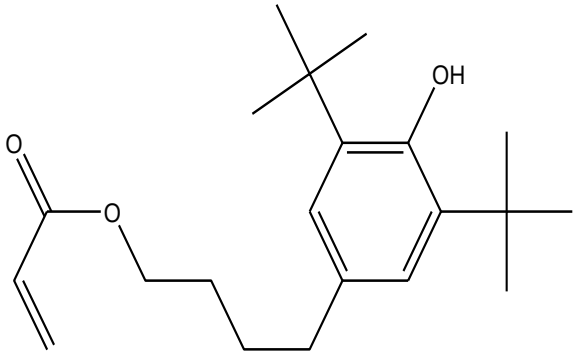
2-methyl-4-(2,4,4-trimethylpentan-2-yl)phenol



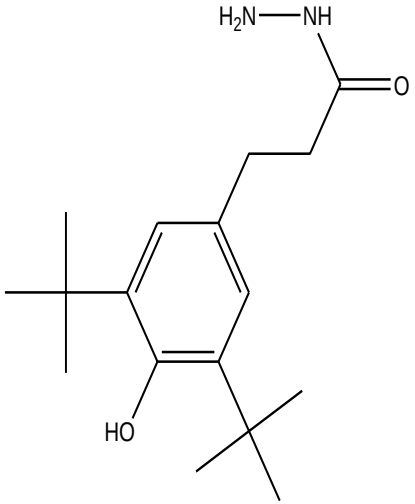
2-*tert*-butyl-6-methylphenol



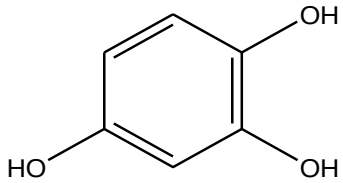
2-methyl-6-(2,4,4-trimethylpentan-2-yl)phenol



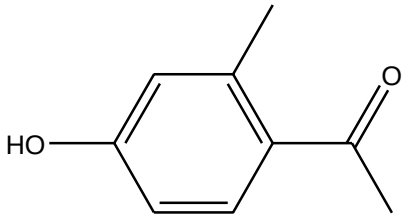
4-(3,5-di-*tert*-butyl-4-hydroxyphenyl)butyl acrylate



3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propanehydrazide



benzene-1,2,4-triol



1-(4-hydroxy-2-methylphenyl)ethanone

Figure 5.2.7: The structures of the polyphenolics identified from the extracts of *H. sabdariffa*

Molecular docking

A molecular docking study was carried out to determine the interaction between the phenolic metabolites from *H. sabdariffa* extracts and the target receptors on carbohydrate digestive enzymes, α- glucosidase and α- amylase. Their free energies binding are shown in **Table 5.2.3**. The best bnding energy based on the molecular interaction are represented in **Table 5.2.4**. The compound 4-(3,5-Di-tert-butyl-4-hydroxyphenyl)butyl acrylate was most frequently interacting with proteins. The docked compound 4-(3,5-Di-tert-butyl-4-hydroxyphenyl)butyl acrylate with α-glucosidase complex showed ionic interactions involving five residues common to Acarbose – α-glucosidase complex (ASP 202, ARG 202, HIS 105, PHE 166, ASP 202, ARG417, LYS 389, ASP 414, and PHE 417). However, Pi-Alkyl interaction was noted between the aromatic ring of PHE 166 and the carbonyl group of the compound 4-(3,5-Di-tert-butyl-4-hydroxyphenyl)butyl acrylate; this was peculiar, as PHE 166 was not involved in any ionic interaction the Acarbose – α-glucosidase complex. On the other hand, the docked compound 4-(3,5-Di-tert-butyl-4-hydroxyphenyl) butyl acrylate with α-amylase complex showed that the pentene group interacts with the ASP 229, PHE 261, and Pi-Alkyl interaction, which was noted between the aromatic ring of PHE 261. In contrast, the acarbose interacts with GLN 51, GLN 51, GLY 110and LYS 72. Furthermore, the compounds b-(3,5-Di-tert-butyl-4-hydroxyphenyl)propionic acid hydrazide showed higher biological activity against α- glucosidase (- 6.7 kcal/mol), and α- amylase (- 5.9 kcal/mol), respectively. The best poses are shown in **Figure 5.2.8**.

Table 5.2.3: Free binding energy interaction of phenolic metabolites of *H. sabdariffa* with the target proteins

Ligand	**Free energy of binding (kcal/mol) Target proteins**	
	α-glucosidase	**α-amylase**
1,2,4-Benzenetriol	-4.7	-4.0
4-Hydroxy-2-methylacetophenone	-4.8	-4.4
2-Methyl-6-tert-octylphenol	**-6.0**	-4.8
4-(3,5-Di-tert-butyl-4-hydroxyphenyl)butyl acrylate	**-7.7**	**-6.1**
3,5-Di-tert-butyl-4-hydroxyhydrocinnamic acid hydrazide	**-6.7**	**-5.9**
4-Tert-Amylphenol	-5.3	-4.2
4-Tert-Octylphenol	-5.8	-4.7
2-Tert-Butyl-6-methylphenol	-4.9	-4.3
2-Methyl-4-tert-octylphenol	**-6.3**	-5.0
Dihydrodiethylstilbestrol	-5.6	-4.9
Acarbose	**-6.3**	-5.9

Table 5.2.4: Summary of Molecular Operating Environment (MOE) docking results for the phenolic metabolites with target proteins

ligands	Protein	*Hydrogen bonds between atoms of ligands and amino acids of receptor*						S- score (binding energy) (kcal/mol)
		ligands	receptor				Energy	
		Atoms	Atoms	Residues	Type	Distance (Å)	(kcal/mol)	
4-(3,5-Di-tert-butyl-4-hydroxyphenyl)butyl acrylate	α-glucosidase	N 45	OD2	ASP 202	H-donor	3.24	-1.7	**-7.7**
		N 47	OD2	ASP 62	H-donor	3.15	-2.8	
		O 44	NH1	ARG 400	H-acceptor	3.82	-1.0	
		N 47	NE2	HIS 105	H-acceptor	3.13	-1.3	
		N 47	6- ring	PHE 166	H-pi	4.31	-1.0	
	α- amylase	O 51	N	ASP 229	H-donor	3.06	-1.0	**6.1**
				PHE 261	pi-H	3.94	-0.6	
					H-acceptor			
3,5-Di-tert-butyl-4-hydroxyhydrocinnamic acid hydrazide	α-glucosidase	O 51	OG1	THR 226	H-acceptor	3.01	-1.1	**-6.7**
		O 51	CA	GLY 228	H-acceptor	3.54		
	α- amylase	N 45	O	GLY 7	H-acceptor	3.04	-1.2	**-5.9**
		6-ring	CD1				-0.6	

2-Methyl-4-tert-octylphenol	α-glucosidase	O 39	OD1	ASP 202	H- donor	2.74	-3.9	**-6.3**
Acarbose	α-glucosidase	O2	OD2	ARG 417	H-donor	3.20	-2.0	**-6.3**
		O 3	NZ	LYS 389	H-acceptor	3.11	-3.5	
		O 6B	NZ	ASP 414	H-acceptor	3.29	-0.9	
		O5	ND2	HIS 105	H-acceptor	2.91	0.2	
	α- amylase	N4A	OE1	GLN 51	H-donor	3.09	-8.2	**5.9**
		O6B	O	GLN 51	H-donor	3.02	-1.0	
		O2	N	GLY 110	H-acceptor	3.23	-0.8	
		O6B	NZ	LYS 72	H-acceptor	3.04	-4.9	

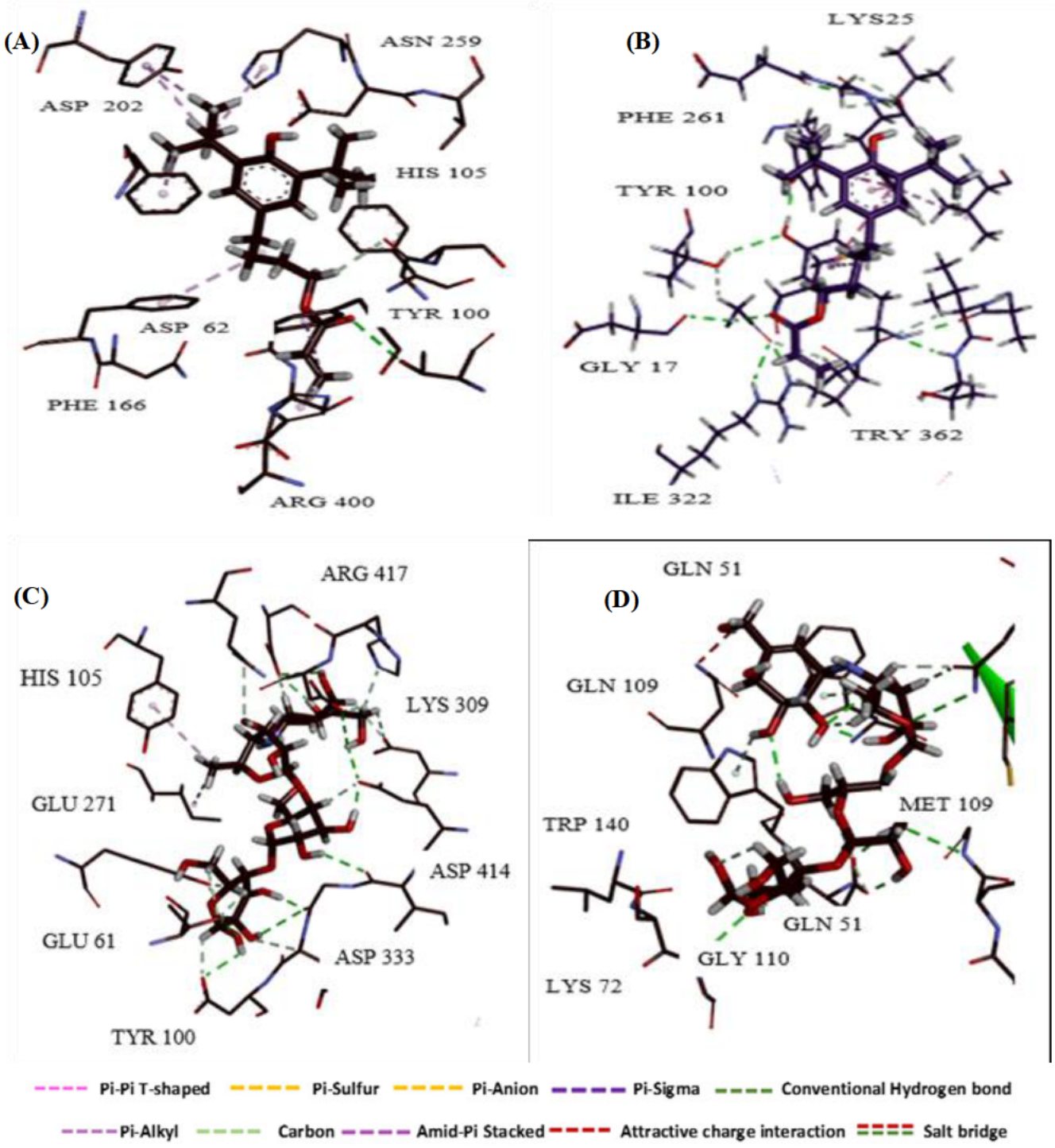


Figure 5.2.8: Inter-molecular interactions between (a) 4-(3,5-Di-tert-butyl-4-hydroxyphenyl) butyl acrylate - α glucosidase, (b) 4-(3,5-Di-tert-butyl-4-hydroxyphenyl) butyl acrylate - α - amylase, (c) acarbose – α -glucosidase, (d) acarbose – α- amylase.

5.2.5 Discussion

Hyperglycemia induces the generation of reactive oxygen species (ROS) which leads to oxidative stress and progression of T2D as well as development of its complications (Burgos-Morón *et al.*, 2019). Some cellular interactions lead to the generation of reactive oxygen species (ROS) and a rise in the amount of oxidative stress caused by iron, which is a transitional metal and powerful pro-oxidant (Rajpathak *et al.*, 2009). This may increase the chance of developing T2D as a result of the damage to the tissues such as the Liver (Robertson *et al.*, 2003; Folli *et al.*, 2011). In this study, the possible antidiabetic effect of *H. sabdariffa* and its antioxidant activities against Fe^{2+}-induced oxidative hepatic injury were investigated using *in vitro, ex vivo,* and *in silico* models.

Several research studies show a strong and positive link between antioxidant capacity and their phenolic component (Shan et al., 2005). This metabolic pathway found in plants plays a vital function in lowering the risk of acquiring certain illnesses such as diabetes (Lin et al., 2016). The high polyphenolic contents shown in **Figure 5.2.1** suggest that plant exhibited high antioxidant and anti-diabetic properties. According to the previous studies, phenolic compounds may help to prevent the increasing deterioration of pancreatic β-cell function caused by oxidative stress, hence lowering the development of T2D (Song *et al.*, 2005).

The human body has numerous strategies to combat ROS by creating antioxidants, either naturally generated inside the body or externally synthesized (Vijaya Lobo et al., 2010). Antioxidants have the ability to play a role in both preventing and healing damages induced by oxidative stress (Sczepanik et al., 2020). Phenolic and their involvement in protecting against oxidative stress in T2D have been extensively researched (Salau *et al.*, 2020). Accordingly, the antioxidant potency of *H. sabdariffa* extract against DPPH, FRAP, and NO assays **Figures 5.2.2. a-c** may be ascribed

to the inherent phenolics and other phytochemical properties of the plant. Phenolic compounds display antioxidant action through various mechanisms, including the donation of hydrogen atoms to free radicals, often resulting in breaking the cycle of ROS generation (Kumar *et al.*, 2014). Thus, our finding indicates that plant extracts with a high content of phenolic compounds had excellent antioxidant properties (Jung et al., 2008b).

α-glucosidase and α-amylase are critical enzymes of interest in controlling postprandial glucose levels in treating T2D (Telagari *et al.*, 2015). These enzymes are engaged in the hydrolysis of dietary starch into simple sugars such as glucose, increasing the systemic glucose concentration (Ahmad et al., 2021). The plant extracts' dose-dependent inhibition **Figures 5.2.3a-b** demonstrates their capacity to delay carbohydrate digestion, hence lowering the rate of glucose absorption. As a result, *H. sabdariffa's* anti-diabetic activities may be attributed to its ability to inhibit α-amylase α-glucosidase (Ademiluyi & Oboh, 2013a).

To further corroborate the antidiabetic potential of *H. sabdariffa*, the intestinal glucose absorption in rat jejunum and skeletal muscle glucose uptake were examined. Increased small intestine glucose absorption has been observed in T2D patients (Rayner *et al.*, 2002). It has previously been proposed that the proximal to the mid-small intestine (which includes portions of the duodenum and jejunum) is responsible for absorbing most dietary glucose (Chukwuma *et al.*, 2018). This modulation of the intestine's glucose-absorptive capability is accomplished by variations in the expression of the intestinal SGLT1 enzyme (Boles *et al.*, 2004). According to our results, in **Figure 5.2.4**, the intestinal glucose absorption was strongly inhibited dose-dependently with increasing concentration of *H. sabdariffa.* These results suggest the plant's capacity to decrease blood glucose levels.

Glucose is an essential energy source for muscle contraction, and maintaining a proper glucose metabolism is necessary to sustain a healthy life (Richter *et al.*, 2013). Thus, when the insulin level in T2D becomes high, specific tissues, such as skeletal muscle, develop insulin resistance (Nishida et al., 2020) and impairs glucose delivery into skeletal muscle (Premilovac *et al.*, 2019). In this study, the increased glucose uptake by the muscle on incubation with *H. sabdariffa* extracts (**Figure 5.2.5)** indicates that *H. sabdariffa* extracts can decrease hyperglycemia. This activity could be attributed to the presence of phenolic compounds which significantly promote glucose uptake (Nachar *et al.*, 2017). These compounds have been involved in controlling muscle glucose metabolism, particularly glucose transport, which is a rate-limiting step in the consumption of glucose in muscle (Egawa *et al.*, 2017).

GSH has been reported to be the first line of defense in the body's endogenous antioxidant system, with the most considerable quantity existing in the liver (Tiwari *et al.*, 2013). It is considered a sign of oxidative stress at the cellular level; the lower levels seen in the untreated tissues **Figure 5.2.6a** suggests the presence of oxidative stress due to the oxidation of Fe^{2+} to Fe^{3+} (Tiwari *et al.*, 2013). Several studies have linked a lower GSH level to liver injury as well as the development of T2D-related problems (Lu, 2020). The increased levels detected after treatment with *H. sabdariffa* extracts demonstrate an anti-oxidative protective capability of the plant.

The antioxidant activity of SOD in the prevention of oxidative cellular damage is well known. It catalyzes the dismutation of O_2^- radical to H_2O_2, which is then transformed into oxygen and water by catalase when it has completed its reaction. A further indication of oxidative damage is the considerable reduction in SOD and catalase activity. Decreased activities of SOD and catalase (**Figures 5.2.6b** and **c)** in the untreated tissues indicate the presence of oxidative injury after $FeSO_4$

treatment. Their increased activities in the *H. sabdariffa*-treated tissues clearly suggest the existence of the anti-oxidative capability of the plant.

Overproduction of (ROS) such as H_2O_2 and OH is a common characteristic of various living cells. This causes direct peroxidation of cells components like protein, lipid, and DNA, as well as membrane rupture, and eventually, cells collapse, which may be connected with cell death (Panda *et al.*, 2005). MDA levels have been found to be elevated in T2D and associated consequences (Saddala *et al.*, 2013). Increased level of MDA in hepatic tissues incubated with $FeSO_4$ suggests the existence of lipid peroxidation **(Figure 5.2.6d)**. Reduction in MDA levels by the plant extract which is also corroborated by elevated GSH level, SOD and catalase activities, thus suggest that *H. sabdariffa* extracts have strong antioxidant properties. These results also support the *in vitro* antioxidant activities of *H. sabdariffa* **(Figures 5.2.2. a-c)** and high polyphenolic content of the plant **(Figure 5.2.1)** may be responsible for its excellent antioxidant properties (Jung et al., 2008b).

In recent years, molecular docking has been employed successfully to examine the mechanism of natural products (Liu *et al.*, 2018). The findings of molecular docking are significant and have the potential to be utilized as a tool for pharmacophore models since the compounds are possibly located close to the active site pocket of the enzymes (Yan *et al.*, 2014). As found in the present study (**Figure 5.2.7, Tables 5.2.3** and **5.2.4)**, the compounds which gave the best docking score based on the binding free energy and H-bonding with its distance between the amino acid in the receptor and RMSD from the native ligand were 4-(3,5-Di-tert-butyl-4-hydroxyphenyl)butyl acrylate, followed by b-(3,5-Di-tert-butyl-4-hydroxyphenyl)propionic acid hydrazide. The result can be explained according to the chemical structures of compound 4-(3,5-Di-tert-butyl-4-hydroxyphenyl)butyl acrylate, which has a methoxy group with electron-withdrawing and

electron-donating properties(Sharma *et al.,* 1997). Besides, it contains a double bond, phenolic ring linked to side chain (pentene group) that increasing the binding of compound 4-(3,5-Di-tert-butyl-4-hydroxyphenyl)butyl acrylate to the receptor and produces better bioactivity against α-glucosidase and α-amylase (Jakobek, 2015; Morales-flores *et al.,* 2015). Thus, it is possible that the *H.sabdariffa* compounds could be used in drug discovery and in bioactivity targeting of α-glucosidase and α-amylase activity. Previous studies of molecular docking and simulation have proven that phenolic compounds could inhibit the carbohydrate digestive enzymes such as α-glucosidase and α- amylase (Rasouli, Hosseini-ghazvini, et al., 2017).

5.2.6 Conclusion

Taken together, the present study suggests the antidiabetic properties of *H. sabdariffa* and its protective role against oxidative hepatic injury. This is evident by the ability of the various extracts (ethyl acetate, ethanol, and aqueous) to significantly inhibit α-glucosidase and α-amylase activities, inhibit intestinal glucose absorption and facilitate muscle glucose uptake as well as mitigate oxidative stress in iron-induced hepatic oxidative injury. In comparison, the ethanolic extract showed the best activity among the extracts. Furthermore, molecular docking results revealed a high anti-diabetic property of the plant. However, further scientific *in vivo* validation is recommended to support these findings.

CHAPTER SIX

GENERAL DISCUSSION, CONCLUSION, AND RECOMMENDATIONS

6.1 General discussion

People living with diabetes globally have increased by more than 200% in the last two decades (Zimmet et al., 2014). It is becoming the most global health concern, with approximately 25% of the worldwide population (Arumugam et al., 2013). Furthermore, diabetes imposes a high economic cost on society. This burden is connected to societal healthcare expenditures associated with illness management (Skyler, 2000). Africa countries and other developing regions around the world are facing a larger part of this burden. In Africa, diabetes is funded with 7% of the overall local health expenditures (Pastakia et al., 2017). However, diabetes patients in developing countries are in danger of developing a variety of severe and life-threatening illnesses, resulting in the increased need for medical treatment, lower standards of living, and increased burden on society (Cho et al., 2018). A recent report from World Health Organization (WHO) revealed that around 80% of people living in Sub-Saharan Africa process herbal medicine for their medication purpose (WHO, 2013). As a result of the increased usage of the medicinal herb, numerous researches have been conducted to determine the beliefs about the use of medicinal herbs among people (Kamel et al., 2017). Hence, this study evaluated the antioxidant and anti-diabetic properties of selected traditional Sudanese medicinal plants (*Cyperus rotundus, Nauclea latifolia, and Hibiscus sabdariffa*) using *in vitro, ex vivo,* and *in silico* experimental models.

The first plant investigated in this plant was *Cyperus rotundus.* The crude extracts (ethyl acetate, ethanol, and aqueous) of the plant's rhizome were screened *in vitro* for total polyphenolic contents,

antioxidant scavenging, and carbohydrate digesting enzyme inhibitory activities. Molecular docking was carried out to determine the probable glucosidase inhibitory mode of action by ligands identified through GC-MS. The result revealed that the aqueous extract showed the highest activity among other extracts **(section 3.2)**. Moreover, it demonstrated significant antioxidant and antidiabetic properties of *C. rotundus* sequential extracts (ethyl acetate, ethanol, and aqueous) as depicted by their ability to scavenge free radicals and inhibit α-glucosidase activity. Additionally, molecular docking results were strongly aligned with the significant antidiabetic property of the plant. Since the phenolic compound 1-ethoxy-2-isopropylbenzene, has two strong hydrogen bonds, it may be researched and potentially used as an anti-diabetic medication.

The second plant was *Nauclea latifolia* the aqueous fruit extract was assayed *in vitro* for its polyphenolic contents, antioxidant, and carbohydrate digesting enzyme inhibitory activities. Furthermore, the dynamic molecular simulation was conducted to investigate the binding affinity phenolic compounds in aqueous extract on α-glucosidase as well as α-amylase **(section 4.2)**. The results indicate that *N. latifolia* fruit aqueous extract has remarkable free radical scavenging activity and carbohydrate digestive enzyme inhibitory activities that may help prevent the onset of numerous oxidative stress-related diseases, including diabetes.

Hibiscus sabdariffa was the last studied plant. The plant's flowers' crude extracts (ethyl acetate, ethanol, and aqueous) were evaluated *in vitro* for their polyphenolic contents, antioxidant scavenging, and carbohydrate digesting enzyme inhibitory properties. Its antioxidant effects *ex vivo* in hepatic tissue were confirmed by the extracts' capacity to increase GSH level, SOD and catalase activities while simultaneously decreasing MDA levels. The extracts also inhibited glucose absorption in the intestine and stimulated glucose uptake in rat psoas muscles. Molecular

docking was used to assess the likely α-glucosidase and α-amylase inhibitory mechanisms of ligands detected by GC-MS **(section 5.2).** According to the results, the ethanolic extract had the greatest activity among the other extracts. The results indicate the antidiabetic potential of *H. sabdariffa* and its antioxidative protective effect against oxidative hepatic injury as evidened by its ability to increase the activities of antioxidant enzymes. Furthermore, the molecular docking results were shown to be highly correlated with the plant's anti-diabetic properties.

6.2 Conclusion

This study indicates that the selected traditional Sudanese medicinal plants (*C. rotundus, N. latifolia and H. sabdariffa*) have a remarkable antioxidant activity that may help prevent the onset of oxidative stress and demonstrate the most potent efficacy as antidiabetic agents. This is evidenced by their free radical scavenging and carbohydrate digesting enzymes inhibitory capabilities. Additionally, *H. sabdariffa* inhibited intestinal glucose absorption, promoted muscle glucose uptake and protected against oxidative hepatic damage.

6.3 Recommendation

Therefore, these plants may be considered a natural source of bioactive compounds beneficial for human health and importantly, diabetes management therapies. However, further validation may be directed to the isolation, purification, and characterization of bioactive compounds in these plants. The outcome could provide a starting point for their possible therapeutic application. However, *in vivo* or animal studies followed by clinical studies are recommended to validate the results from *in vitro* and *ex vivo* studies.

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APPENDIX A: LABBORATORY ANIMAL TRAINING

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

15 September 2021

Mr Brian Kudakwashe Beseni (218088125)
School of Life Sciences
Westville

Dear Mr Beseni,

Protocol reference number: AREC/038/019D
Old Project title: Antioxidant, hypoglycaemic and hypolipidemic effects of Zn(II) poly-phenol complexes and indigenous plants from KwaZulu-Natal province, South Africa.

New Project title: Antioxidant, hypoglycaemic and hypolipidemic effects of selected African indigenous medicinal plants in a type 2 diabetes model of SD rats.

Approval – Amendment Application

With regard to your first amendment request received on 07 September 2021, the Animal Research Ethics Committee has accepted the documents submitted, and **FULL APPROVAL** for the protocol is granted.

We note your request to amend your study title and protocol by adding extracts from *Cyperus rotundus, Hauclea latifolia and Hibiscus sabdariffa*. We also note the addition of Idris Mohamed Almahi (219094524) as a researcher to your study.

Please note: There must be adherence to national and institutional COVID-19 regulations and guidelines at all times. Researchers will be personally responsible and liable for non-adherence to national regulations. If in doubt, please contact the Research Ethics Chair and/or the University Dean of Research for advice.

Any alteration/s to the approved research protocol, i.e. Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for 5 years.

The ethical clearance certificate is only valid for one year from the date of issue. Renewal for the study must be applied for before 14 September 2022 as a new application on RIG.

Please note: the study renewal in 2022 must be uploaded to the RIG online system as a new application.

Animal Research Ethics Committee (AREC)
Ms Karen Reinertsen (Administrator)
Westville Campus, Govan Mbeki Building
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Telephone: +27 (0) 31 260 8350 **Facsimile:** +27 (0) 31 260 4609 **Email:** animalethics@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

Attached to the Approval letter is a template of the Progress Report required at the end of the study or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health/wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

Dr Sanil D Singh, BVSc, MS, PhD
Chair: Animal Research Ethics Committee
/kr

cc Supervisor: Prof Shahidul Islam
cc BRU Manager: Dr Jaca

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APPENDIX B: PUBLICATION FROM THIS THESIS

JOURNAL OF BIOMOLECULAR STRUCTURE AND DYNAMICS
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The antioxidant and antidiabetic potentials of polyphenolic-rich extracts of *Cyperus rotundus* (Linn.)

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Communicated by Ramaswamy H. Sarma

ABSTRACT

In this study, the rhizome of *Cyperus rotundus* L was investigated for its antioxidant and antidiabetic effects using *in vitro* and *in silico* experimental models. Its crude extracts (ethyl acetate, ethanol and aqueous) were screened *in vitro* for their antioxidant activity using ferric-reducing antioxidant power (FRAP) and 1,1-diphenyl-2-picrylhydrazyl (DPPH), as well as their inhibitory effect on α-glucosidase enzyme. Subsequently, the extracts were subjected to Gas Chromatography-Mass Spectrometry (GC-MS) analysis to elucidate their possible bioactive compounds. Furthermore, computational molecular docking of selected phenolic compounds was conducted to determine their mode of α-glucosidase inhibitory activity. The aqueous extract displayed the highest level of total phenolic content and significantly higher scavenging activity in both FRAP and DPPH assays compared to ethyl acetate and ethanol extracts. In FRAP and DPPH assays, IC_{50} values of aqueous extract were 448.626 µg/mL and 418.74 µg/mL, respectively. Aqueous extract further presented higher α-glucosidase inhibitory activity with an IC_{50} value of 383.75 µg/mL. GC-MS analysis revealed the presence of the following phenolic compounds: 4-methyl-2-(2,4,4-trimethylpentan-2-yl) phenol, Phenol,2-methyl-4-(1,1,3,3-tetramethylbutyl)- and 1-ethoxy-2-isopropylbenzene. Molecular docking study revealed 1-ethoxy-2-isopropylbenzene formed two hydrogen bonds with the interacting residues in the active site of α-glucosidase enzyme. Furthermore, 4-methyl-2-(2,4,4-trimethylpentan-2-yl) phenol had the lowest binding energy inferring the best affinity for α-glucosidase active site. These results suggest the possible antioxidant and antidiabetic potential of *Cyperus rotundus*.

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KEYWORDS
Antioxidant; antidiabetic; *Cyperus rotundus* L.; molecular docking

1. Introduction

Diabetes mellitus (DM) is a complex metabolic disorder typically characterized by hyperglycemia (Miranda et al., 2007; Tai et al., 2015). The two most prevalent types of diabetes are type 1 diabetes (T1D) and type 2 diabetes (T2D) (Seino et al., 2010). The former occurs due to insufficient insulin secretion resulting from autoimmune disruption of pancreatic β-cells, while the latter is attributed to either partial pancreatic β-cells dysfuction followed by insulin secretion or insulin resistance, or both which leads to persistent hyperglycemia (Smith, 2010). Persistent hyperglycemia promotes the production of reactive oxygen species (ROS), which leads to oxidative stress that further progresses T2D (Asmat et al., 2016). medications integrated with dietary management and regular physical activities (Lean et al., 2019; Marín-Peñalver et al., 2016).

Oral pharmacological antidiabetic drugs such as sulfonylureas, biguanides, α-glucosidase inhibitors and thiazolidinediones are among the first-line intervention strategies for the management of diabetes (Joshi et al., 2015). Their modes of action range from inhibition of carbohydrate digestion, retardation of intestinal glucose absorption to stimulation of insulin production from pancreatic β-cells (Defronzo, 2000; Hannan et al., 2012; Inzucchi, 2002). Despite their far-reaching and widely accepted use, these oral antidiabetic agents have multiple undesirable side effects such as weight loss/gain, hypoglycemic shock, stomach upset, renal damage and liver failure (Kalsi et al., 2015; Maruthur et al., 2016). These