Investigation of the *in vitro* antidiabetic and neuroprotective effects of selected *Helichrysum* species against high monosaccharide-induced toxicity

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Declaration

I declare that: *Investigation of the in vitro antidiabetic and neuroprotective effects of selected Helichrysum species against high monosaccharide-induced toxicity* is my own work, that it has not been submitted for any degree or examination at any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

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Signature:

Date: May, 2022

Abstract

Chronic hyperglycaemia (glucotoxicity), a common complication in diabetes mellitus (DM), is known to cause cognitive decline in some of these patients, often affecting their overall quality of life. Some medicinal plants used in folk medicine for DM have also been reported effective in treating some of its co-morbidities, including cognitive decline. Plants of the *Helichrysum* genus (Asteraceae family) are well known in South African traditional medicine for their diverse health benefits, which make them potential sources of biologically active compounds. Thus, in the current study, the composition of bioactive compounds, as well as the antidiabetic and neuroprotective effects of selected *Helichrysum* plants viz: *Helichrysum pandurifolium* Schrank, *Helichrysum foetidum, Helichrysum petiolare* and *Helichrysum cymocum*, were investigated.

Liquid chromatography-mass spectrometry (LC-MS) analysis was first used to characterise the aqueous acetone extracts of each plant, followed by bioactivity evaluation. LC-MS analysis and phytochemical screening revealed *H. petiolare* extract has the highest content of phenolics, saturated fatty acids, polyunsaturated fatty acids and total flavonoids. The best nitric oxide scavenging activity and total antioxidant capacity compared with other species were also demonstrated. Furthermore, the flavonoid composition varied in all extracts, with *H. petiolare* and *H. pandurifolium* Schrank extracts having the highest number of flavonoids. Thus, the aqueous acetone extract of *H. petiolare* (AAHPE) was further investigated for antidiabetic and neuroprotective potentials. The AutoDock Vina tool was used for molecular docking simulation.

Treatment with AAHPE (25-75 µg/mL) improved the cell viability and increased the concentration-dependent percentage glucose uptake in the insulin-resistant HepG2 cell line significantly compared with the control. The highest AAHPE concentration (75 µg/mL) showed

higher glucose uptake activity than the standard drug, metformin. Furthermore, AAHPE was found to inhibit both α-amylase and α-glucosidase enzymes *in vitro*, as corroborated by molecular docking results that showed strong binding (ΔG) of AAHPE flavonoids to α-amylase and α-glucosidase compared with the drug, acarbose (ΔG for flavonoids = -7.2 to -9.6 Kcal/mol vs ΔG for acarbose = -6.1 Kcal/mol) for α-amylase; (ΔG for flavonoids = -7.3 to -9.0 Kcal/mol vs ΔG for acarbose = -6.3 Kcal/mol) for α-glucosidase.

Analysis of *in vitro* neuroprotective effects of AHHPE against glucotoxicity induced on SH-SY5Y cells showed improved cell viability at all treatment concentrations (25-100 µg/ml). It also showed reduced reactive oxygen species production (ROS) and increased production of adenosine triphosphate (ATP) compared with cells treated with the standard acetylcholinesterase (AChE) inhibitor drug, donepezil (1 mM) or untreated cells (300 mM glucose). Molecular docking analysis showed that selected AAHPE flavonoids exhibited tight binding forces (better inhibitory profiles) with AChE compared with donepezil (-8.3 Kcal/mol). The flavonoids include 3, 5-dicaffeoylquinic acid (-9.9 Kcal/mol), isorhamnetin 3-galactoside (-8.8 Kcal/mol), 4,5-dicaffeoylquinic acid (-8.6 Kcal/mol), methyl 3, 5-di-O-caffeoyl quinate (-8.6 Kcal/mol), 3-caffeoylquinic acid (-8.4 Kcal/mol), quercetin-3-glucoside (-8.4 Kcal/mol) and sinocrassosideA1 (-8.4 Kcal/mol).

Thus, the AAHPE and its bioactive phytocompounds, especially flavonoids, with their potential antioxidant effects, more effective glycaemic control than metformin, and better neuroprotective effects than donepezil against DM-associated disorders (e.g cognitive decline). Hence, they could be developed as commercially available dietary herbal supplements for managing postprandial hyperglycaemia.

Dedication

This work is dedicated to God Almighty for showing me mercy beyond my imagination.

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List of acronyms and abbreviations

AAHPE	Aqueous acetone *Helichrysum petiolare* extract
AAHCE	Aqueous acetone *Helichrysum cymocum* extract
AAHFE	Aqueous acetone *Helichrysum foetidum* extract
AAHPSE	Aqueous acetone *Helichrysum pandurifolium Schrank* extract
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ADMET	Absorption, distribution, metabolism, excretion, and toxicity
AGEs	Advanced glycation end-products
ATP	Adenosine triphosphate
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CAM	Complementary alternative medicine
CAT	Catalase
CNS	Central nervous system
CVD	Cardiovascular disease
DACD	Diabetes-associated cognitive decline
DCM	Methanol and dichloromethane
DE	Diabetic encephalopathy
DM	Diabetes mellitus
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimetthyl Sulfoxide

DN	Diabetic neuropathy
DPNP	Diabetic peripheral neuropathic pain
DPPH	1,1 diphenyl-2-picrylhydrazyl
EPTT	Endpoint titration technique
FAs	Fatty acids
FN-1	Fibronectin
FRET	Fluorescence resonance energy transfer assay
GC-MS	Gas chromatography mass spectrometry
HSV1	Simplex virus type 1
IC_{50}	Inhibitory concentration
IGF	Insulin growth factor
IL-1	Interleukin-1
IL-6	Interleukin-6
INT	p-Iodo nitro tetrazolium
LC-MS	Liquid chromatography mass spectrometry
MAPK	Mitogen-activated protein kinase
MDA	Malonaldehyde
mg/dL	Milligram per decilitre
mmol/L	Millimole per Litre
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MUFA	Monounsaturated fatty acid
MV-EA	Measles virus Strain Edmonston A
MV-EA	Edmonston virus strain A

NADPH	Nicotinamide adenine dinucleotide phosphate
ND	Neurodegenerative diseases
NFkB	Nuclear factor Kappa B
NO	Nitric oxide
PAI-1	Plasminogen activator inhibitor
PARP	Poly ADP-Ribose polymerase
PBS	Phosphate-buffered saline
PD	Parkinson disease
pH	Hydrogen ion concentration
PI3K/AKT	Phosphatidylinositol-3-Kinase/ protein kinase B
PKC	Protein kinase C
PPAR-γ	Nuclear proliferator-activated receptor gamma
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species
S.F. A7	Semliki forest virus A7
SFA	Saturated fatty acid
SOD	Superoxide dismutase
SRB	Sulforhodamine B
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TBHQ	Tert-butyl hydroquinone
TFC	Total flavonoids content
TPC	Total phenolic content

USA	United States of America
USD	United States Dollars
VEGF	Vascular endothelial growth factor
VSVT2	Vesicular stomatitis virus T2
WHO	World Health Organization

CHAPTER ONE

Background information on diabetes mellitus (DM) and neurodegenerative diseases (ND), mainly the type, causes, and prevalence, is provided in this chapter. Diabetes mellitus is highlighted as a risk factor for cognitive decline or dementia, which affects the neurochemistry, neurophysiology and structural architecture of the central nervous system (CNS). Some common mechanisms for DM and ND are discussed. The importance of medicinal plants as safe and effective sources of potential new drugs for DM-associated co-morbid conditions like cognitive decline is also highlighted. Furthermore, the aim and objectives, significance and rationale of the study is outlined in this chapter, and a layout of the thesis structure is presented.

1.0 Introduction

Diabetes mellitus and ND co-morbidity have become a recent concern because of the many devastating effects, globally. These two disease entities appear to be age-related and can be more specifically referred to as diabetes-associated cognitive decline (DACD). With improvements in living standards worldwide, DACD has frequently been reported in the ageing population as DM co-existing with such ND as Alzheimer's disease (AD), Parkinson's disease (PD), Amyloid lateral sclerosis and Huntington's disease.

Emerging evidence has revealed the propensity or susceptibility of ageing cognitive decline or dementia in the older diabetic population compared with aged populations without DM (Pugazhenthi et al., 2017). The co-morbid condition is associated with an increasing and devastating effect on the health-care delivery system and well-being of the patients and reducing the quality of life. DM is a metabolic disorder of multiple aetiology, characterised by hyperglycaemia caused by defects in insulin secretion, insulin action or both of these. The complications of DM are potentially debilitating, leading to high morbidity and mortality, affecting many people worldwide.

About 366 million people had diabetes as of 2011, and this figure is projected to increase to 642 million in 2040 (Ogurtsova et al., 2017). There are many long-term micro- and macrovascular complications associated with DM, including retinopathy, cardiomyopathy, nephropathy, neuropathy, and encephalopathy, with devastating implications (Parsamanesh et al., 2018). Evidence of complications in patients with DM, shows that the peripheral nervous system is often affected. New and more revealing data now indicate that the complications of DM also affect the CNS negatively, resulting in a dysfunctional CNS, which is characterised by reduced cognitive function and the decline in memory and mental speed or alertness (Biessels et al., 2006; Mijnhout et al., 2006; Wang et al., 2010). The brain's neurons are affected in which there is a slow progressive alteration in cerebral function and structure leading to neurodegeneration (Grieco et al., 2019; Shi et al., 2019).

Neurodegenerative diseases are debilitating conditions resulting from the neurons' progressive loss and or death. ND is an incurable condition in which the neurons in the human brain are tremendously affected. ND consist of a range of conditions that include dementia, AD, PD, Huntington's disease, amyotrophic lateral sclerosis and mild cognitive impairment.

In the United States of America (USA), 5.3 million persons were affected by AD while 1 million persons had PD, the projections for expected dementia-related conditions are 65.7 million cases for 2030 and 115.4 million cases for 2050 (Shi et al., 2019). In the USA, 53.2 million are living with dementia, and the projection of ND in the USA is alarming, which cost additional expenses of about $157–$215 billion in 2010 (Hurd et al., 2013, Alzheimer's Association,2019).

Neurodegenerative disease, which involves the degradation of the brain part or region, is linked to hereditary, toxicity, metabolic or infectious state. A rising prevalence of ND is

presently costing significant medical, social and economic burdens because of the extensive care and support needed for its management.

Although ND and DM are unrelated, several studies have revealed a close association between DM and a decline in brain function and performance, which means that neurodegeneration is a complication and also an evident in DM patients (Cherbuin & Walsh, 2019; Nasrolahi et al., 2019). Therefore, DM is found to precipitate the burden of ND by increasing the risk of all types of dementia, AD, and mild cognitive impairment by about 1.5-fold, while patients with DM, compared to those without, are prone to developing PD by 2.2-fold (Zhao & Townsend, 2009). One of the features of DM is insulin resistance, which has been established as a risk factor of ND, specifically AD (Nasrolahi et al., 2019).

Furthermore, DM and neurodegenerative disease's unrelated disease pathology are seemingly complex in nature, with culminating evidence revealing shared molecular mechanisms in both conditions. Strong evidence revealed DM as an independent risk factor for cognitive decline or dementia of the CNS, affecting the neurochemistry, neurophysiology, and structural architecture of the CNS. The defective or impaired CNS homeostasis from induced chronic monosaccharide toxicity or chronic hyperglycaemia alters memory performance, cognitive flexibility and psychomotor functions (Rahigude et al., 2012). These implicated neurological complications of the CNS occur in about 70% of patients with DM (Yin et al., 2014), which further support the secure link of this disease to co-morbidity. Chronic hyperglycaemia, a dysregulated monosaccharide, which is evident in insulin perturbations or resistance occurring in the peripheral tissue and the brain, plays a crucial role in DM-related cognitive decline (Williamson et al., 2012).

Recently, attention has been devoted to an essential biomolecule, monosaccharide, which is needed for cell physiology. However, evidence has shown that sugar or

monosaccharide toxicity resulting from excess consumption of glucose, fructose and galactose is very harmful, as the damage caused leads to many diseases similar to those caused by alcohol and tobacco (Aragno & Mastrocola, 2017; Kovacic & Somanathan, 2013) to the body system. The CNS, especially the brain, is not an exception to the damage that sugar toxicity induces in chronic hyperglycaemic states, which aggravates the incidence of diabetic neuropathy and encephalopathy, resulting in cognitive decline, dementia and ageing (Liu, Y. Y. et al., 2013; You & Kim, 2019).

Many mechanisms are implicated in DM associated ND conditions. For example, glucose, one of the monosaccharides, produces excess ROS in chronic hyperglycaemia leading to oxidative stress. This ROS generation and other multiple biochemical pathways, such as mitochondrial pro-inflammatory induction, polyol pathway up-regulation, protein kinase C (PKC) enhancement, activation of hexosamine biosynthetic pathway, and stimulation of the formation of advanced glycation end-products (AGEs) (Rahimi et al., 2018; Yang et al., 2017), induce toxicity and increase neuronal damage or death via peroxidation of membrane lipid, DNA damage and protein oxidation.

Efforts are still in top gear to unravel the actual mechanism by which excess consumption of monosaccharides induces neurodegeneration as the incidence of AD, PD, and several other neurologic disorders appear to be higher in persons with T2DM suggesting shared cellular and molecular mechanisms. Some shared mechanisms include oxidative stress, apoptosis, inflammation, mitochondrial dysfunction, AGEs formation, insulin resistance, impaired insulin signalling and amyloid deposition (Ramesh Kandimalla et al., 2017; Moreira, 2012; Zhao & Townsend, 2009).

Historically, medicinal plants are as old as man, and knowledge about medicinal plants is passed on from generation to generation and from one country to another. In ancient times,

the Chinese, Egyptians, Indians, Greeks, Romans, and old Slavs were comprehensive with the importance of many medicinal plants (Abdel-Mageid et al., 2018; Petrovska, 2012). All over the world today, plants, in general, serve beneficial purposes to humankind, such as shelter, food, fibre, clothing, and therapy. The importance of medicinal plants in treating human and animal diseases cannot be overemphasized. About 80% of the world's population in the last three decades solely depended on using medicinal plants or herbs for treating and curing several disease conditions (David et al., 2015).

In the last two decades, US$60 billion and US$83 billion were generated from in year 2000 and 2008 respectively from medicinal plants, with a projection of US$5trillion by the year 2050 from medicinal plant herbs products (Morozov et al., 2019; Zhang et al., 2012) as revealed by the data from the convention of biological diversity. The renewed interest in medicinal plant products is propelled by emerging side effects of modern synthetic drugs, high cost and reduced potency against chronic and long-term disease. However, medicinal plant products are generally regarded as safe, potent, readily available and cheap or affordable (Peltzer et al., 2016).

Medicinal plants are traditionally used to treat ailments, such as cough, catarrh, diarrhoea, infections caused by fungi, viruses, and bacteria, diabetes mellitus, malaria, asthma, menstrual pain, chest pain, skin and kidney disorders, constipation, stomach pain, and yellow fever. Hence, scientific investigation on the traditional use of medicinal plant products, endorsing folklore or traditional claims, is on the increase (Mahomoodally, 2013). Consequently, this knowledge leads to predicting and validating the undeniable advantage of medicinal plants in the treatment of diseases.

The World Health Organization (WHO) attested to the increased integration of traditional medicine into the health delivery system through scientific investigation (Segneanu

et al., 2017). Medicinal plants serve as essential therapeutic agents because of the bioactive compounds that are inherent to them. These bioactive compounds include phenolics, flavonoids, saponins, alkaloids, terpenes, and fatty acids, found in leaves, roots, seeds, whole plant, stem, and bark of medicinal plants.

The use of medicinal plants' bioactive components or compounds as therapeutic agents is critical in the area of biomedical and natural product research. Thus, profiling the bioactive particles is vital in research, entailing sequential procedures from plant selection, collection and identification, extraction and the isolation of bioactive molecules, identification and structural elucidation to biological and pharmacological screening (Segneanu et al., 2017). These active compounds are responsible for pharmacological and biological activities, such as antibacterial, anticataleptic, antidiabetic, antihyperlipidaemic, anti-fungal, antimicrobial, anticancer, and antioxidant anti-inflammatory, diuretic, hepatoprotective, neuroprotective.

The plant antioxidant is the repository for most diseases or ailment's treatment, management, and cure, which will continue to play a significant role considering their safety and efficacy. Both traditional and modern approaches to medicinal plants in disease treatment leads to drug discovery and development. New/modern drug discovery and development approaches are more significant than traditional approaches, which are complicated and time-consuming.

These new approaches use computational techniques, which are termed computer-aided drug design, computational drug design, computer-aided molecular, *in silico* drug design, computer-aided molecular modelling, computer-aided rational drug design. Computational drug techniques give insight into drug effectiveness in pharmacokinetics, pharmacodynamics, and toxicity. Hence, these approaches have recently gained momentum and implementation in the scientific world, specifically the pharmaceutical industry. Both experimental and

computational strategies are valid and complimentary in drug discovery and development. The use of information from chemical and biological entities such as ligands or targets helps identify and optimize new drugs. Furthermore, these new drugs with compounds having undesirable characteristics are eliminated (poor activity or poor absorption, distribution, metabolism, excretion, and toxicity ADMET).

Drug discovery and development is streamlined with computational techniques showing significant benefits. Overall, enriched compounds with drug-likeness, lead-likeness, active qualities, and elimination of molecules with inactive, reactive, toxic, or poor ADMET/PK properties are highly desired in drug discovery and development. Hence, it is necessary to explore new medicinal plants and their constituents for experimental and computational strategies in drug discovery and development for anti-diabetic and neuroprotection studies.

1.1 Aim of the study

To investigate the anti-diabetic and neuroprotective potential or effects of *Helichrysum* species extract using HepG2 and SH-SY5Y lines and *in silico* approach.

1.2 Objectives of the study

Objective 1: To determine the flavonoid composition of four *Helichrysum petiolare, Helichrysum cymocum, Helichrysum foetidum* and *Helichrysum pandurifolium* Schrank species using LC-MS analysis.

Objective 2: To determine the four selected *Helichrysum* extracts' antioxidant activity, total phenolics, total flavonoids, total antioxidant, and fatty acid compositions.

Objective 3: To investigate the antidiabetic potential of the aqueous acetone *Helichrysum petiolare* extract (AAHPE) with molecular docking relevance in DM in drug discovery and development.

Objective 4: To evaluate the neuroprotective potential of the AAHPE against glucotoxicity with molecular docking relevance in cognitive decline in drug discovery and development.

1.3 Significance of the study

Diabetes mellitus is not considered a neurological disease as such. However, DM has an incredibly negative influence on peripheral and CNS functions and cognition, leading to the co-morbid condition referred to as Type 3 diabetes. Diabetes mellitus complication is implicated in the brain, which occurs as cognitive decline, and it is estimated that DM doubles the risk for dementia.

Diabetes-induced cognitive impairment has increased, and more attention is needed to abate this condition. The development of potential targets of diabetes-induced cognitive impairment is essential to ensuring prognostic outcomes in understanding the pathophysiological changes in the disease condition. The knowledge about the DM-linked cognitive decline co-morbidity would prompt the science world about novel molecules discovery, giving hope of recovery and better health status to affected patients or patients prone to this co-morbid condition.

Overall, because DM contributes to ND, evidently as cognitive decline, the development of safe, potent, affordable, and available medicinal plants is critical in ensuring healthy living in co-morbid disease conditions. The great diversity of available medicinal plants would provide the basis for developing novel therapeutic interventions for treating this disease condition.

1.4 Rationale for the study

The increase in the incidence of ND is considered one of the causative events of DM. The menace of DM would likely contribute to dysfunctional cognitive function. Presently, the world is battling because over 366 million people suffer from DM, which is expected to increase to 552 million by 2030 (Ramesh Kandimalla et al., 2017). This increase is a concern because DM affects all age groups, especially those still actively contributing to the country's gross domestic product (GDP). The condition could render them less productive, with a negative impact on their physical well-being and their cognitive well-being.

This co-morbid condition has a significant economic impact on medical resources, a burden that the patients cannot afford because of few available interventions, which are expensive. Therefore, there is a need for the critical development of new and potent therapeutic interventions through medicinal plants, which will provide more options for treating these co-morbid conditions.

This work provided insight into the studies of DM and neurodegenerative conditions with the discovery and development of the drug from *Helichrysum* species of medicinal plants, fostering more scientific knowledge and breakthrough.

1.5 Thesis structure

Chapter One: An overview of the literature on diabetes mellitus and ND, including mechanisms related to co-morbid conditions is provided in this chapter. Drug discovery and development with experimental and *in silico* approaches, using medicinal plants are highlighted as the crux of the research.

Chapter Two: The general overview of the literature on diabetes mellitus and its complications is discussed. Medicinal plants' properties and specific actions for drug discovery and development, especially in diabetes mellitus and its complications are reviewed.

Chapter Three: This is a published manuscript, discussing the literature review of some selected *Helichrysum* species. We aim to provide a comprehensive literature review, using different engine searches of the databases and printed materials on the existing knowledge of traditional and scientific investigations on selected *Helichrysum* species. Overall, the medicinal properties and *in vitro* biological activities of selected *Helichrysum* species from South Africa is the crux of the discussion:

> Akinyede, K.A.; Cupido, C.N.; Hughes, G.D.; Oguntibeju, O.O.; Ekpo, O.E. Medicinal Properties and In Vitro Biological Activities of Selected *Helichrysum* Species from South Africa: A Review. *Plants* **2021**, *10*, 1566. https://doi.org/10.3390/ plants10081566

Chapter Four: An insight into the bioactive flavonoids' analysis and determination of the cytotoxicity or cell viability of the selected *Helichrysum* species extracts on selected cell lines is gives in this chapter. This work has not been published.

Chapter Five: In this chapter, we elaborate on the importance of medicinal plant constituents, especially the antioxidants and fatty acids constituents. In the published manuscript, we investigated the antioxidants and GC-MS analysis, which identify and quantify the fatty acids of the selected *Helichrysum* species that were carried out:

> Akinyede, K.A.; Hughes, G.D.; Ekpo, O.E.; Oguntibeju, O.O. Comparative Study of the Antioxidant Constituents, Activities and the GC-MS Quantification and Identification of Fatty Acids of Four Selected *Helichrysum* Species. Plants 2022, 11, 998. https://doi.org/10.3390/plants11080998

Chapter Six: We investigated the *in vitro* antidiabetic effect of AAHPE, using insulin resistance HepG2 cell line and molecular docking flavonoids constituent as an antidiabetic

therapy. In this published work, we provided a lead way for drug discovery and development from *Helichrysum petiolare*:

> Akinyede, K.A.; Oyewusi, H.A.; Hughes, G.D.; Ekpo, O.E.; Oguntibeju, O.O. In Vitro Evaluation of the Anti-Diabetic Potential of Aqueous Acetone *Helichrysum petiolare* Extract (AAHPE) with Molecular Docking Relevance in Diabetes Mellitus. Molecules 2022, 27, 155. https://doi.org/10.3390/molecules27010155

Chapter Seven: The potential use of the AAHPE was explored. Investigation of an *in vitro* evaluation of the neuroprotective potential of AAHPE against glucotoxicity and molecular docking relevance in cognitive decline was carried out. This work has not been published.

Chapter Eight: The conclusion of the study and recommendations are given in this chapter while highlighting work to be done in the future.

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CHAPTER TWO

Comprehensive information about DM and its complications are provided in this chapter. It highlights the importance of medicinal plants as potent and relatively safe compared with synthetic drugs that have been explored in the treatment of DM-related complications. Neurodegeneration remains a hallmark of different types of DM-related complications, and various mechanisms are highlighted in this chapter as possible causes of complications of DM.

2.0 Literature review

2.1 An overview of diabetes mellitus

Diabetes mellitus is a severe and endocrine complex metabolic condition characterised by high sugar levels in the blood. In DM, the insulin-producing β cells of the pancreas are dysfunctional, and thus, there is an absolute insulin deficiency, or the bodys' cells become insensitive to insulin production (Salehi et al., 2019; Soumya & Srilatha, 2011). A state of catabolism is triggered, which causes the breakdown of the body's fat, protein and glycogen to produce more sugars that culminate in excess production of sugar, a chronic hyperglycaemia state, and also overproduction of ketones by the liver (Folorunso & Oguntibeju, 2013; Oguntibeju, 2013).

Diabetes mellitus can be classified into type 1 DM (T1DM) and type 2 DM (T2DM); however, DM can also be induced in pregnancy, referred to as gestational DM. T1DM, also known as insulin-dependent DM, is caused by an auto-immune reaction of the beta-cell destroying itself. Alternatively, T2DM is referred to as insulin-dependent diabetes, which is the most common type, where the cell's response to insulin becomes reduced, causing insulin resistance. T2DM is caused by multifactorial factors that are probably genetic (family history, ethnic group) and lifestyle in nature (obesity, unhealthy diet, lack of exercise and smoking) (Murea et al., 2012).

The pathogenesis of T1DM is not preventable because of the unusual autoimmunity. Available treatment requires the intake of exogenous insulin to control blood glucose levels (Van den Berghe, 2004). For T2DM, risk factors include excessive caloric food intake, family history, obesity, race, genetic disorder, smoking, sedentary lifestyle, and some drugs or chemicals (Odeyemi & Bradley, 2018). Appropriate diet plans, physical exercise and antidiabetic drugs are thus essential for managing type 2 diabetes.

The global prevalence of DM revealed an almost double increase from 4.7% to 8.5% since 1980 in the adult population, with that in children also on the increase (Salehi et al., 2019). Worldwide, DM affects about 451 million people, while an estimated 693 million is expected to be affected by 2045 (Cho et al., 2018). In Africa, approximately 19.8 million persons were affected by DM, which was responsible for more than 6% of deaths in the African region (Chiwanga et al., 2016). The distribution of the prevalence of DM in the world continents is depicted in Figure 2.1.

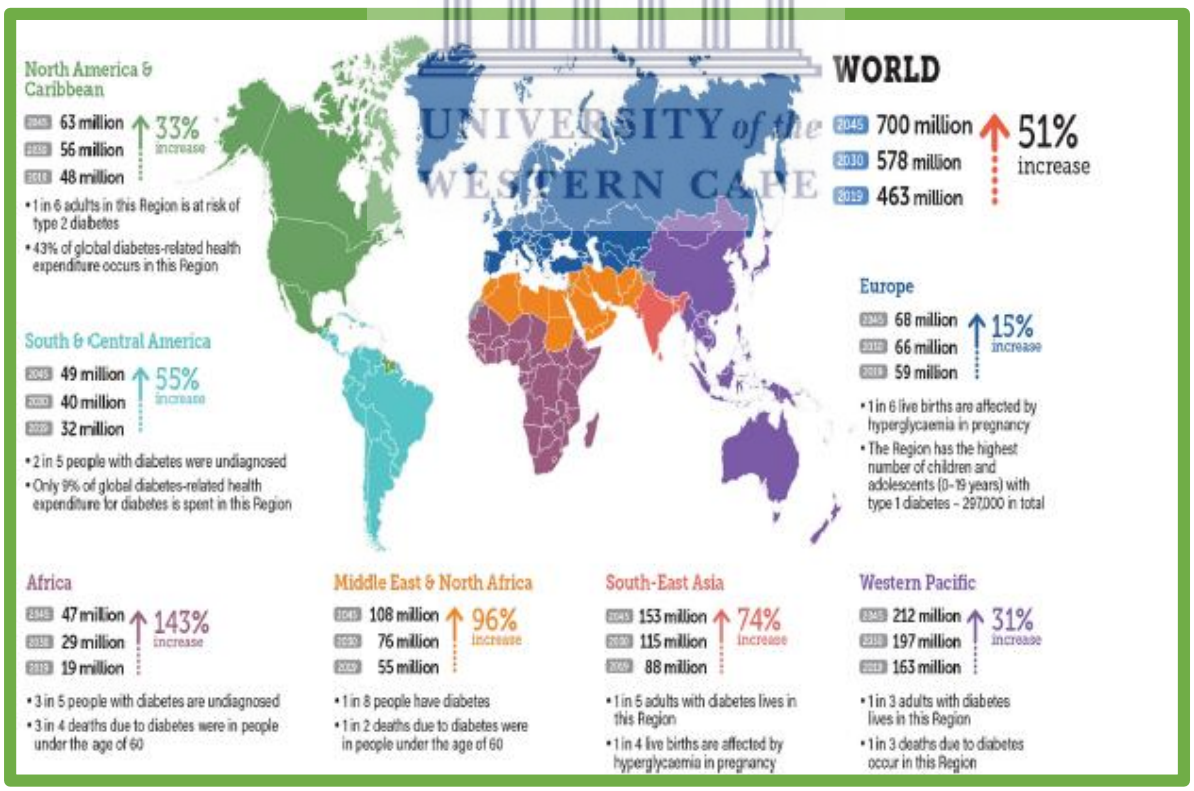

Figure 2.1. World prevalence of diabetes mellitus in adults aged 20-79 years (2019, 2030 and 2045) (Source: Akhter, 2021)

T2DM represents more than 90% of the adult and child population, while the prevalence of T1DM is about 5% (Salehi et al., 2019). Also, there has been an incremental prevalence of DM over the past three decades, and less developed countries (low- and middle-income countries) are more at risk of developing DM in comparison to the developed countries (high-income countries) (Salehi et al., 2019). Recent demographic studies substantiated the incidence and prevalence of DM and projected that about 82 million people 64 years and older in developing countries would be affected by 2030 compared to about 48 million in developed countries (Kakkar, 2016). Furthermore, DM remains a life-threatening condition to people in the developing and developed countries of the world. The WHO's projection revealed that DM would be the seventh leading cause of mortality worldwide by 2030 (Kakkar, 2016).

The impact of DM morbidity and mortality cannot be overemphasised, as the economic implication in terms of expenditure, loss of valuable workforce and time has placed a heavy burden on the economy (Alleman et al., 2015). Globally, approximately $232 billion was expended on the management of diabetes and its complications in 2008 (Van Dieren et al., 2010), while $376 billion was utilised on diabetes in 2010. Projection of about 490 USD billion in 2030 on treating DM and its complications is burdensome (Zhang et al., 2010). The development of diabetes complications increases the overall health-care costs seven times than when complications are absent (Pelletier et al., 2009; Van Dieren et al., 2010). Many factors are currently under review in the expenditure of DM treatment, including region, age group, gender, and the country's income level.

Clinically, the level of blood glucose can be detected using a glucometer. However, an individual is suspected to be suffering from diabetes when the following symptoms are prominent, frequent urination, excessive thirst, intense hunger and tiredness, blurred vision and at later stages, poor wound healing, sexual dysfunction, especially in men, and some infection in the gums (Perkins et al., 2006).

The mentioned DM symptoms can be confirmed when an individual's fasting plasma glucose and glucose tolerance levels are greater or equal to 126 mg/dL (7.0 mmol/L) and 200 mg/dL (11.1 mmol/L), respectively, while a clinical guide for the random plasma glucose level is greater or equal to 200 mg/dL (11.1 mmol/L) on two different consecutive occasions. Also, patients with values between 6.1-7.0 mmol/L and 7.8 mmol/L have impaired fasting glucose and glucose tolerance.

Diabetes mellitus and chronic hyperglycaemia prompt or induce various devastating associated sequelae resulting in a severe, complex and chronic entity, thus, increasing the death rate. The associated sequelae of DM are known as DM complications. Chronic hyperglycaemia in DM is responsible for long-term damage, dysfunction and organ failure commonly experienced by diabetic patients (Tiwari et al., 2013). The damage affects the micro- and macrovascular tissues that constitute the organs/system. The microvascular DM complications include retinopathy, nephropathy and neuropathy, while macrovascular complications are cardiovascular disease (CVD), cerebrovascular accidents and peripheral vascular disease.

Treating diabetes mellitus with synthetic drugs is key to preventing other related complications. However, the associated deleterious side-effects of synthetic treatments have shifted attention to African medicinal herbs and nutraceuticals with potential antidiabetic properties because of their potency and benefits in preventing and treating DM and its related complications. Many African medicinal herbs and nutraceuticals are effective in preclinical studies with limited clinical trials in treating DM and its complications (Venkatakrishnan et al., 2019).

2.2 Neurodegeneration

Neurodegeneration is defined as a persistent, progressive and altered neuronal function and structural stability that eventually leads to the death of neurons and increased neuronal

clearance (Barber et al., 2012). This pathological condition is a tremendously complex entity that involves molecular, anatomical and biochemical events relating to injury, loss and death of neurons. Neurodegeneration is often used in various neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD) and many others because of the loss of neuron activity over a long period. However, a more definitive and succinct definition refers to neurodegeneration as "neurological disorders with heterogeneous clinical and pathological expressions affecting specific subsets of neuron in specific anatomic system arising from unknown reasons and progress in relentless fashion" (Barber et al., 2012; Pathak et al., 2021).

In chronic hyperglycaemia, neurodegeneration affects the brain or peripheral nervous system and other organs such as the eye, kidney, and heart. The features of neurodegeneration in all these organs are reflected by histological, biochemical and functional pathologies—for example, the retinal neurodegeneration in diabetes. Histological studies revealed apoptosis, gross morphological changes in the retina, a reduction in the numbers of surviving amacrine cells, abnormal ganglion cell morphology, centrifugal axon abnormalities, and nerve fibre layer thickness features in the retina undergoing neurodegeneration induced by hyperglycaemia. The biochemical evidence includes nitric oxide determination, the measurement of synapse-specific proteins and synaptic vesicle-associated protein, while the available evidence includes changes in visual function and oscillatory potentials and a reduced scotopic threshold response of the eye nerves (Barber et al., 2012).

The surge in neuronal complications caused by DM has been reported in the literature. With the increasing prevalence of DM and advancement in age across the globe, the chances are that the risk of cognitive impairment in DM will be overwhelming. The complication of memory impairment in diabetes begins with mild cognitive impairment, which probably progresses to AD at a later stage of diabetes (Zilliox et al., 2016). Chronic hyperglycaemia

resulting from impaired insulin levels is suggested to be one of the reasons behind memory impairment in people with diabetes (Jash et al., 2020).

2.3 Mechanisms related to DM and its complications

Cellular mechanisms involved in DM and its complication include oxidative stress, inflammation, the product of Advanced glycation end-products (AGEs) and activation of some signals in pathways such as mitogen-activated protein kinase (MAPK), vascular endothelial growth factor (VEGF), poly ADP-ribose polymerase (PARP), interleukin-6 (IL-6), and interleukin-1 (Garud & Kulkarni, 2017; Laddha & Kulkarni, 2018; Suryavanshi & Kulkarni, 2017). Perturbation in the polyol pathway, hexosamine pathway and activation of PKC are other significant pathways implicated in diabetic-related complications (Laddha & Kulkarni, 2018; Sharma et al., 2017). The dysfunctional and dysregulated pathways in diabetes promote the complications that affect different organs in the body, primarily through hyperglycaemia-sustained oxidative stress. The understanding and targeting mechanisms in diabetes and its complications could be a pointer towards prevention, management and treatment interventions. These mechanisms can be exploited in monitoring clinical prognosis when administering medications.

Chronic hyperglycaemia in DM plays a vital role in diabetes-related complications, making the disease aetiology complex with multiple inter-related pathways through which these complications progress. A different line of scientific evidence has implicated oxidative stress, polyol pathway, hexosamine pathway, PKC pathway, AGEs pathway and inflammation mechanisms in DM-related complications (see Figure 2.2). Oxidative stress that causes free radical generation is central mechanism, leading to activations of many other pathways that cause DM-related complications. DM-mediated complications from chronic hyperglycaemia affect many parts of the body, ranging from small to large vessels, tissue and organs. These

complications include diabetic cardiomyopathy, diabetic neuropathy, diabetic retinopathy, diabetic nephropathy, diabetic encephalopathy and liver complications that further increase DM mortality and morbidity (Pourhanifeh et al., 2020).

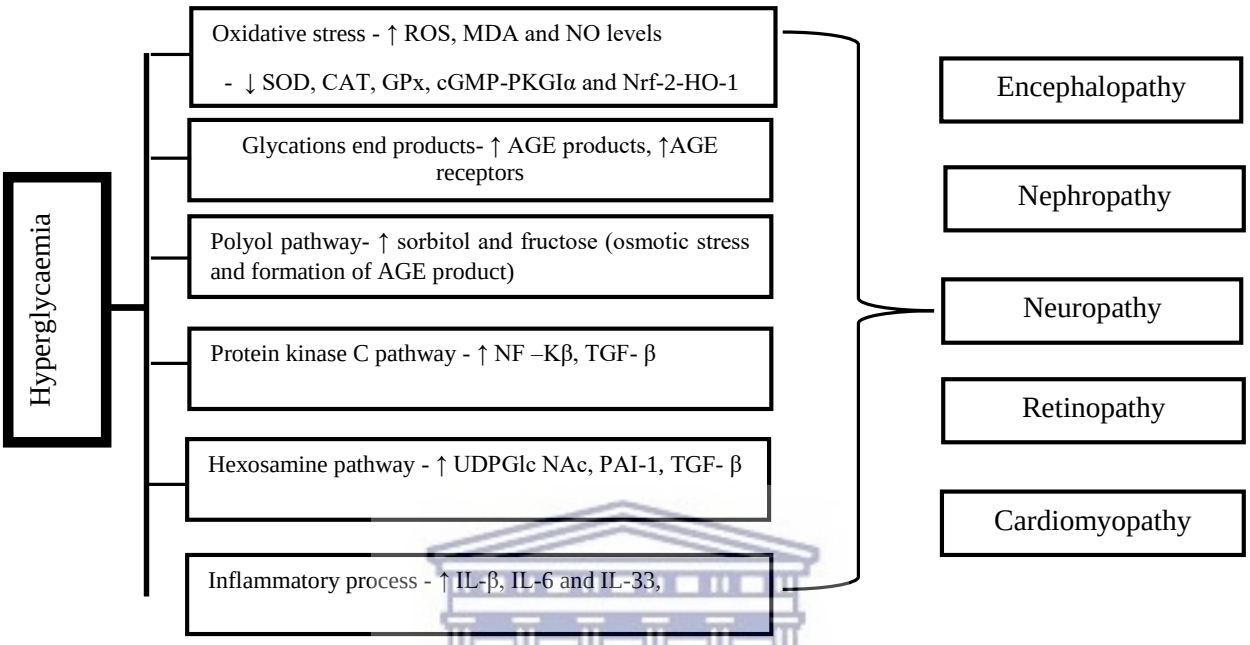

Figure 2.2. The synopsis description of the associated mechanisms in chronic hyperglycaemia involved in the pathophysiology of DM-related complications. [Adapted from (Nedeljkovic & Ali, 2017) with slight modification.]

2.3.1 Diabetic cardiomyopathy

Diabetic cardiomyopathy is one of the complications in DM in which there are structural and functional alterations in the myocardium while other cardiac-associated risk factors such as coronary heart disease or high blood pressure are conspicuously absent (Jia et al., 2018). Diabetic cardiomyopathy, in the long run, leads to heart failure, an early diastolic and late systolic dysfunction because of distinct features of fibrosis and stiffness associated with the myocardium.

The pathogenesis of diabetic cardiomyopathy is multifaceted, propagating the damage caused by this complication. Various pathogenic factors such as chronic hyperglycaemia, insulin resistance and altered cardiac metabolic signalling are implicated, with their effect

stimulating many other pathways ranging from impaired vascular endothelial, adrenergic activity, dysfunctional mitochondria calcium (Ca^{2+}) homeostasis, renin-angiotensin activation, myocardial ischemia/functional hypoxia, oxidative stress, inflammation, mitochondrial dysfunction cell stress to endoplasmic reticulum organelles and ultimately cardiac cell death (Pourhanifeh et al., 2020).

Cardiac cell death is linked to excessive ROS production from chronic hyperglycaemia. The ROS-induced oxidative stress cause damage to the important cell component, DNA, lipid and protein. As a result, apoptosis is activated, autophagy in the fibre muscles is inhibited, and myocardial mitochondrial biogenesis and complex I, III and IV activity of the mitochondrial enzymes are suppressed, as ROS increases in chronic hyperglycaemia (Hu et al., 2009). Chronic hyperglycaemia is a life-threatening risk factor that needs to be controlled to reach an acceptable glycaemic level. An effective therapy that ameliorates other causatives of diabetic cardiomyopathy and blood glucose control would help reverse the damage significantly (Pourhanifeh et al., 2020). Scientific investigations illustrating diabetic cardiomyopathy showed different associated causative factors, as shown in Table 2.1.

Table 2.1. Reported scientific investigations of the mechanisms and treatments of diabetic cardiomyopathy

Treatment and dosage	Animal model	Mechanism	Effect	References
Astragalus L. polysaccharides extract, 1 g/kg given orally	STZ-induced diabetic hammester rat	↓ Ang II , pro-MMP-2 MMP-2 and p-ERK1/2 expression	Protection of myocardial ultrastructure	(Chen et al., 2010)
Panax quinquefolius L. 200 mg/kg oral gavage	Streptozotocin-induced diabetic mice	↓mRNA expressions of atrial natriuretic factor and brain natriuretic factor	Prevent the diabetes-induced cardiac biochemical and functional changes	(Sen et al., 2013)
Ficus religiosa L. extract,200 mg/kg orally administered	STZ-induced diabetic rat	↓TGF-β, TNFα and oxidative stress markers	Improve treatment of cardiomyopathy	(Sharma et al., 2014)
Anthocyanin, 250 mg/kg given orally	STZ-induced diabetic rat.	↓ COX-2,TLR4, p-NF-kB,ANP and IL-6	Improvement of cardiac cell inflammation, hypertrophy and attenuation of cardiac fibrosis	(Y. F. Chen et al., 2016)
Terminalia arjuna Wight &Arn extract, 500 mg/kg given orally	Streptozotocin (STZ)-induced diabetic rats.	↓Endothelin 1 (ET-1), tumor necrosis factor-a (TNF-a), and interleukin 6 (IL-6)	Improvement of the myocardial function	(Khaliq et al., 2013)
Abroma augusta L. extract, 100 and 200 mg/kg orally given	Streptozotocin (STZ)-induced diabetic rats	↓IL-1β, IL-6, TNF-α NF-κB and PKCs, Bad, and Bcl-2 caspase cascade	prophylactic role against cardiomyopathy	(Khanra et al., 2015)
Grape Seed Proanthocyanidins extract, 250 mg/kg orally given	Streptozotocin (STZ)-induced diabetic rats	↓ RAGE, NF-kB and TGF-β_1	Amelioration of glycation-associated cardiac damage and improvement of LV myocardium	(Cheng et al., 2007)
Wogonin, 10 mg/kg given intraperitoneally	STZ-induced diabetic mice	↓ IL-1β, IL-6, TNFα, PAI-1,and NF-kB signalling	Mitigation of hyperglycemia-related cardiomyocyte impairment	(Khan et al., 2016)
Cortex Mori Radicis (MCR) extract	Streptozotocin (STZ)-induced diabetic rats	↓ER stress ERK and p38 MAPK activation	Amelioration of diabetic cardiomyopathy	(Lian et al., 2017)

↑ Means: Activation, up-regulation or high expression

↓ Means: Inhibition, deactivation, blockade, down-regulation or low expression.

2.3.2 Diabetic retinopathy

Diabetic retinopathy (DR) is a prevalent microvascular complication where the retinal vasculature is altered. Over the years, diabetes patients develop biochemical and metabolic abnormalities in the retina, thus damaging the tiny blood capillary vessels in the eye (Al-Kharashiv, 2018; Laddha & Kulkarni, 2018). The risk of developing DR is doubled in DM patients compared to non-DM patients. Diabetes patients' vision is impaired, and the development of cataract and glaucoma evident (Harris & Eastman, 2000; Sharma et al., 2005). Although, most occurrences of DR are asymptomatic, this risk factor remains the leading cause of vision loss in DM patients globally (Momeni et al., 2015; Williams et al., 2004).

In terms of the number of DR patients, the population increases along with the same trend because of the high prevalence of DM worldwide (Lee et al., 2015). The aetiology of DR is not limited to chronic hyperglycaemia, oxidative stress or inflammation (Al-Kharashi, 2018). Other confounding factors that induce the development of DR are AGEs, growth factors, and excessive vitreous and circulating cytokines.

Several investigations have supported inflammation as the main causative of early-stage DR (Santiago et al., 2018) because ROS and cytokines trigger chronic inflammations. The markers of the inflammation such as IL-1, IL-6, COX2, nuclear factor kappa B(NFkB), intercellular adhesion molecule-1 and VEGF increase in the retina and vitreous humours of diabetic patients and animals to induce early-stage DR.

Progression to late-stage DR involves oxidative stress stimulating VEGF during retinal hypoxia (Arjamaa & Nikinmaa, 2006). Oxidative stress, inflammation and autophagy mechanisms have been implicated in DR in several studies. It has been observed that halting or diminishing such pathways is not enough in the treatment of DR. Thus, there is a need for preventive strategies that could prevent this underlying mechanism of DR through the use of herbal medicines and botanicals (see Table 2.2).

Table 2.2. Reported scientific investigations of the mechanisms and treatments of diabetic retinopathy

Treatment and dosage	Animal Model	Mechanism(s)	Effect	References
KIOM-79 is an herbal mixture, 50 mg/kg of KIOM-79 orally administered	Zucker diabetic fatty (ZDF) rats	↓AGE, apoptosis, NF-kB	Amelioration of retinal microvascular dysfunction	(Kim et al., 2012)
Dendrobium chrysotoxum Lindl. extract, 30–300 mg/kg orally administered	STZ-induced diabetic rats	↓VEGF, VEGFR2, MMP 2/9, PDGF A/B, bFGF and IGF-1	Ameliorate retinal inflammation	(Gong et al., 2014)
Alpha Mangostin supplement, 200 mg/kg by oral gavage	Type 2 diabetic rat	↓ MDA, AGEs, RAGE, TNF-α, And VEGF	Restoration of ocular blood flow (OBF) and blood-retinal barrier (BRB) permeability	(Jariyapongskul et al., 2015)
Moringa oleifera Lam. (MO), 100 mg/kg, by oral gavage	Streptozotocin-induced diabetic rats	↓ TNF-α, IL-1b) VEGF and PKC-b)	Prevention of diabetes-induced retinal dysfunction	(Kumar Gupta et al., 2013)
Zingiber zerumbet L. extract, 200 and 300 mg/kg orally administered	STZ-diabetic rats	↓ (IL)-1α, IL-6 (NF-kB), VEGF, p38 (MAPK)	Improvement of retinal structural change and inhibiting retinal inflammation	(Hong et al., 2016)
Niaspan, 40 mg/kg orally administered	STZ-diabetic rats	↓VEGF/VEGFR, VCAM-1/CD45, apoptosis and BRB breakdown. ↑ tight junction proteins and Ang-1/Tie-2 expression	Promote the vascular repair and inhibits inflammation of the retinal	(Wang & Yan, 2016)
Lisosan G (LG), 0.5 g/kg orally administered	Streptozotocin-induced diabetic rats	↓apoptosis, VEGF expression, Oxidative stress, and inflammatory markers ↑ BRB integrity	Protective actions against neural and vascular defects In diabetic retinopathy	(Liu et al., 2019)
Extract of *Magifera indica L.* and *Vietnamese Coriander*, 2, 10, and 50 mg/kg orally given	Streptozotocin-induced diabetic rats	↓ oxidative stress, aldose reductase, p38MAPK, ERK1/2, and VEGF	Protection against diabetic cataract and diabetic retinopathy	(Wattanathorn et al., 2017)
Typhae pollen polysaccharides (TPP), TPP 0.4 g/kg orally administered	STZ-induced diabetic rats	↓ IL-6, TNF-α VEGF and bFGF expression	Amelioration of diabetic retinopathy by inhibiting inflammation and improving blood circulation	(Lei et al., 2018)
Lycopus lucidus Ex Benth. extract	STZ-induced diabetic rats	↓ p38MAPK/NF-kB signaling pathway	Ameliorating oxidative stress, inflammation and angiogenesis DR	(Liu et al., 2019)

↑ Means: Activation, up-regulation or high expression

↓ Means: Inhibition, deactivation, blockade, down-regulation or low expression.

2.3.3 Central nervous system-related complications of diabetes encephalopathy

Although less studied, diabetes encephalopathy (DE) is also a common type of DM compared to the other complications. Chronic hyperglycaemia in DM induces oxidative stress

and inflammation that cause damage to the neurons in different regions of the brain and, over time, results in neuronal death of the brain, an underlying mechanism in DE.

The brain is a vulnerable organ to oxidative stress because of the substantial amount of oxygen needed, high level of polyunsaturated fatty acids (PUFA) content, and a limited and insufficient antioxidant system of the brain to obstruct or tackle ROS that causes or promotes hippocampal and neuronal apoptosis with effect as impaired cognitive function in DM patients (Lee et al., 2020). The function and structure of the brain is the hallmark of cognitive capacity, which is said to be altered in DM patients (Chornenkyy et al., 2019).

The occurrence and development of DE are linked to some pathways, such as the polyol pathway, activated polyol pathway, flux and enhanced hexosamine pathway. Meanwhile, the modification of proteins by N-acetyl glucosamine, activation of PKC isoforms induced by hyperglycaemia and increased aggregation of AGEs remain the associated mechanisms of DE (Brownlee, 2001; Cai et al., 2011). In Table 2.3, studies reporting mechanism(s) that exist alone or in conjunction with others are provided. However, according to Wang and Zhao (2016), a common dependent pathway, hyperglycaemia-induced oxidative stress, is regarded as crucial in DE.

The brain's neurons are subjected to injury, loss or death because cerebrovasculature is altered. The alteration is characterised by acute and chronic vascular and metabolic disturbances, primarily related to the central and peripheral nervous system (Biessels et al., 1994). Such alterations may arise from decreased insulin growth factor (IGF) level, dysfunctional vascular reactivity, and overtly reduced blood flow to the brain, leading to reduced or impaired cognitive function of the hippocampus. DM is parallel to many cognitive-related disorders such as mild dementia, Alzheimer's disease, anxiety, and depression (Panza et al., 2018). Chronic hyperglycaemia in DM-induced neurotoxicity is linked to impaired

neuronal and neurotransmission functions in DE. ROS overstimulation in the brain regions, such as the hippocampus, induces oxidative damage, a vital contributor to most DM complications (Pourhanifeh et al., 2020 Kuhad & Chopra, 2008).

Table 2.3. Reported scientific investigations of the mechanisms and treatments of diabetic encephalopathy

Treatment/dosage	Animal model	Mechanism	Effects	References
Liuwei Dihuang decoction, 1 and 2 g/kg, given orally	STZ-induced diabetic rats	↓Caspase-3, Aβ protein expression, AChE and iNOS activities. ↑IGF-1 and BDNF expression	Neuroprotective action against diabetic encephalopathy	(J.-p. Liu et al., 2013)
Simvastatin, 10 mg/kg or 20 mg/kg administered orally	STZ-induced diabetic mice	↑PPARγ ↓NF-kB p65, Bcl-2/Bax and Caspase-3	Improvement of diabetic related cognitive decline	(Fang et al., 2017)
Genistein, 2.5. 5.0 and 10 mg/kg given intraperitoneally	STZ-induced diabetic mice	↓Oxidative stress markers, TNF-a, IL-1b and nitrites	Protective against diabetic related cognitive dysfunction	(Rajput & Sarkar, 2017)
Formononetin, 25, 50 mg/kg given intraperitoneally	STZ-induced diabetic mice	↓HMGB1/TLR4/NF-κB signaling and NLRP3 inflammasome	Amelioration of STZ-induced cognitive dysfunction	(J. Wang et al., 2018)
Paeonol, 25, 50 and 100 mg/kg administered orally	STZ-induced diabetic rat	↓caspase 3, Aβ protein expression, AChE and iNOS activities ↑IGF-1 and BDNF expression	Amelioration of cognitive deficit	(J. Liu et al., 2013)
Ferulic acid, 15 and 30 mg/kg orally given	STZ-induced diabetic rat	↓PTP 1B and P-IRS expression	Improvement of diabetes induced cognition impairment	(Wang et al., 2017)
Luteolin, 50 and 100 mg/kg administered orally	STZ induced diabetic rat	↓Cholinesterase activity, ↑Antioxidant markers	Improved neuronal injury and cognitive performance	(Y. Liu et al., 2013)
Agmatine, 5-10 mg/kg given intraperitoneally	STZ-induced diabetic rat	↓Cholinesterase activity ↑Antioxidant markers	Improved memory impairment in diabetic rat	(Bhutada et al., 2012)

↑ Means: Activation, up-regulation or high expression

↓ Means: Inhibition, deactivation, blockade, down-regulation or low expression

2.3.4 Diabetic neuropathy

Diabetic neuropathy (DN) is the most common complication of DM, affecting more than 50% of DM patients worldwide and reducing their quality of life (Yang et al., 2019). DN affects the somatic and autonomic nerve of the peripheral nervous system with features ranging from increased motor nerve conduction velocity and sciatic nerve diameter to thermal hyperalgesia (Azmi et al., 2019) that induces tremendous neuropathic pain. Diabetic neuropathy occurs in T1DM and T2DM, with over 50% occurring in T2DM patients and is the

most common complication of DM. Diabetic neuropathy is described as defective peripheral nerve disorder in chronic hyperglycaemia alone without recourse to other causes (Degu et al., 2019). The dysfunctional state of the peripheral nerves could lead to other sequelae such as pains, foot ulcers, infection and non-traumatic amputation (Kasim et al., 2010; Tesfaye et al., 2011). The symptomatic nature of DN could reflect negatively as loss of sensation and strength, while positive symptoms include pricking sensation and pain (Kasim et al., 2010).

The pathogenesis of DN is multifaceted, which differs in T1DM and T2DM patients, as revealed by different lines of studies. (Tesfaye, S., & Selvarajah, D. 2009; Feldman, et al., 2019). Oxidative stress metabolic and vascular disturbances are the frontline cause of DN in T2DM, while hyperglycaemia is the leading cause of DN in T1DM (Tesfaye et al., 2011). The other causes of DN are sodium and potassium channel expression abnormalities, disorders related to metabolic and immunity that activate glial cells, altered blood vessels to prevent blood flow to peripheral nerves, and recently, facilitatory or inhibitory balances, alteration in the CNS (Waldman, 2000). Diabetic neuropathy can occur with or without pain. However, diabetic peripheral neuropathic pain (DPNP) is common in these patients, occurring in more than 10%. DPNP is characterised as a burning, piercing or aching/throbbing pain, while nocturnal pain experienced after a stimulus, such as allodynia, hyperalgesia, or a lack of sensation, often leads to sleep deprivation, anxiety and depression. These sleep symptoms are probably responsible for energy deficiency, affecting the quality of life, and thus dependency on others for carrying out daily activities (Kasim et al., 2010). DPNP poses social, psychological, and physical health challenges, which are severe symptoms of this disease (Tesfaye et al., 2011).

The activation of some biochemical pathways, such as the polyol pathway, glycolysis and oxidation phosphorylation by chronic hyperglycaemia, induces the formation of AGEs, ROS generation and PKC signalling molecules (Ighodaro, 2018). The persistence of

hyperglycaemia in DM contributes to neuropathy and neuropathic pain development because it initiates neuronal derangement linked to ROS generation. The mitochondrial remain a significant site for ROS production, which is stimulated in DM from excess glycolytic activities, hence, increased PKC pathway, altered gene expression and growth factors. (Pourhanifeh et al., 2020). Furhermore, an increase in the formation of AGEs and binding to its receptor initiate inflammation thereby causing oxidative stress. A signalling cascade of receptor LOX1, Toll-like receptor 4 and RAGE increases the oxidant enzyme such as; nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, depicting weakens the antioxidant defence system, hence oxidative stress-induced damage to the neurons are some underlying causative mechanisms (Wang et al., 2015) seen in Table 2.4.

In addition, insulin signalling impairment alters neuronal growth and survival in DN because of its neurotrophic functions (Sima et al., 2004). Neurotrophic signalling maintains sodium-potassium ATPase activity, endothelial nitric oxide synthase and cerebral blood flow to ensure neuronal growth and survival. This neurotrophic signalling is possible by activating phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) signalling. Insulin impairment induces DN, which prevents neuronal repair and decreases nerve growth factors (Sima et al., 2004). Preventive intervention or treatment target involves the induction of brain neurotrophins, sodium-potassium ATPase activity and nerve growth factor (Oh, 2016)

Table 2.4. Reported scientific investigations of the mechanisms and treatments of diabetic neuropathy

Treatment and dosage	Animal model	Mechanism	Effect	References
Olea europaea L. extract, 300 and 500 mg/kg orally administered	STZ-induced diabetic rat	↓oxidative stress markers, caspase 3 and Bax/Bcl2	Attenuation of diabetic neuropathic pain	(Kaeidi et al., 2011)
Quercetin, 10, 20 and 40 mg/kg given orally	STZ-induced diabetic rat	↓oxidative-nitrosative stress markers, TNF-α, IL-1 β and DNA damage	protection against diabetic neuropathy	(Kandhare et al., 2012)
BAY 11-7082, 1 and 3 mg/kg given orally	STZ-induced diabetic rat	↓NF-kB, IkB and p-IkB, IL-6, TNF-α and COX-2. ↑Nrf2/HO-1	Amelioration of diabetic neuropathy	(Kumar et al., 2012)
Hesperetin, 20 and 50 mg/kg given orally	STZ-induced diabetic rat	↓IL-1β, IL-6 and TNF-α	Attenuation of neuropathic pain	(Aswar et al., 2014)
Fragaria X ananassa Duch, 50, 100 and 200 mg/kg given orally	STZ-induced diabetic rat	↓ Kim-1, MDA, TNF-α, IL- 6 and caspase-3	Amelioration of diabetic neuropathy	(Ibrahim & Abd El-Maksoud, 2015)
Pepino polyphenolic extract, 0.5 or 1% given orally	STZ-induced diabetic mice	↓TBARS, ROS, IL-6, TNF-α and AGEs	Amelioration of diabetic peripheral nerves	(Ma et al., 2016)
Rapamycin, 1 µg 3 µg or 10 µg Intrathecal administered	STZ-induced diabetic rat	↓mTOR and DRG Nav1.8	Attenuation of neuropathic pain	(He et al., 2016)
Annona Reticulata L. Bark (or) Ziziphus jujube Mill. Root bark along with insulin	STZ-induced diabetic rat	↓ IL-1b, IL-6, IL-10, TNF-α iNOS, and NFkB	Attenuation of neuropathic pain. Agmatine, an endogenous ligand of imidazoline receptor protects against memory impairment and biochemical alterations in streptozotocin-induced diabetic rats	(Raghuram Kandimalla et al., 2017)
Kaempferol, 5 and 10 mg/k and Eruca sativa, 100, 200 and 400 mg/kg G given orally	STZ-induced diabetic rat	↓oxidative stress markers, nitrosative stress markers and AGEs	Amelioration of the progression of diabetic	(Kishore et al., 2018)
Bogijetong decoction, 400 mg/kg orally given	STZ-induced diabetic rat	↓TNF-α and p38 ↑Erk1/2	Amelioration of the progression of diabetic	(Kim et al., 2017)

↑ Means: Activation, up-regulation or high expression

↓ Means: Inhibition, deactivation, blockade, down-regulation or low expression.

2.3.5 Diabetic nephropathy

Diabetic nephropathy affects T1DM and T2DM patients, with microvascular complications most often resulting in end-renal failure (Pourhanifeh et al., 2020). Diabetic nephropathy is depicted by nephron expansion, glomerular hyperfiltration and glomeruloscelorisis that develops from the hardening of the mesangial cell of the kidney

(Bherwani et al., 2016). The pathogenesis of DN is associated with different mechanisms (as showns in Table 2.5) from many lines of evidence, including lipid disorders, oxidative stress, pro-fibrotic and fibrotic cytokines stimulations vis a vis fibronectin FN-1 and plasminogen activator inhibitor PAI-1 and connective tissue growth factor (Wang et al., 2015).

Furthermore, the aggregation of AGEs could prompt RAGEs overexpression, thus culminating in oxidative stress. The aggregated AGEs and RAGEs in serum and the kidney increase the levels of lipid peroxidation and decrease the activities of glutathione peroxidases and superoxide dismutase, causing renal damage complications in diabetes (Lee et al., 2011). It is well established that ROS-induced hyperglycaemia plays significant responsibility in DM complications, including diabetic nephropathy. Hence, the persistency of oxidative stress from ROS-induced damage to the cellular component such as the nuclear DNA and other mitochondrial genetic material of the kidney. The proximal tubule epithelial cells and other kidney cells are susceptible to apoptosis in the hyperglycaemic state from that action of the ROS, thus enhancing the continuous loss of kidney function in diabetic nephropathy (Sifuentes-Franco et al., 2018).

Table 2.5. Reported scientific investigations of the mechanisms and treatments of diabetic nephropathy

Treatment and dosage	Animal Model	Mechanism(s)	Effect	References
Extract of cassiae semen (CS) 200 mg/kg CS orally administered	Streptozotocin (STZ) injected diabetic rat	↓COX-2 mRNA and protein. ↓AGEs, TGF-1 and collagen IV	Inhibit the development of diabetic nephropathy	(Kim et al., 2014)
Oligonol (LMW Polyphenol) 10 or 20 mg/kg orally administered	Streptozotocin (STZ) injected db/ db mice	↓AGE pathway, ↑Bcl-2,survivin, Bcl-2-associated-x protein, cytochrome c and caspase -3	Renoprotective against the development of kidney diabetic complication	(Park et al., 2014)
Allicin 15, 30 and 45 mg/kg/day intra gastric gavage	Streptozotocin (STZ) injected diabetic rats	↓Collagen I, TGF-𝛽1 and p-ERK ½ pathway	Protective against diabetic nephropathy	(Huang et al., 2017)
Calycosin 10 mg/kg/day	Diabetic db/db mice	↓IKBα and NF-Kb, p65 protein	Significantly suppresses diabetes-induced renal inflammation in diabetic renal injury	(Zhang et al., 2019)
Oryeongsan (ORS), Oral gavage of ORS (100 mg/kg/day)	Type 2 diabetic animals (db/db mice)	↓TGF-β1/Smads pathway	Amelioration of insulin resistance and diabetes-associated glomerulosclerosis in db/db mice	(J. J. Yoon et al., 2014)
Spatholobus suberectus Dunn. (SS) extract, SS extract (50 mg/kg) orally administered	Type 2 diabetic animals (db/db mice)	↑(Nrf2), (Glo1) and (NQO1) while ↓AGEs (RAGE)	Amelioration of diabetes-induced renal damage	(Do et al., 2018)
Pueraria tuberosa Roxb Ex Wild. (PTY-2r), PTY-2r extract of 50 mg/100 g and 100 mg/100 g given orally	Streptozotocin (STZ) injected diabetic rat	↑Bcl-2 and antioxidant enzymes. ↓Bax, Caspase 3,cleaved PARP-1	Nephroprotective against diabetes-induced renal damage	(Shukla et al., 2018)
Taraxerol, a pentacyclic triterpenoid, Taraxerol 20 mg/kg administered orally	Rat model of type 2 diabetes (T2D)	↓Polyol Pathway, AGEs, NFkB/PKCs and PARP signaling	Taraxerol exhibited protective effect against diabetes-induced renal damage	(Khanra et al., 2017)
Lyoniresinol 3α-O-β-D-Glucopyranoside LGP1 and LGP2). LGP1 and LGP2 orally administered at 20, 40, 80 mg/kg body weight/day doses	Streptozocin (STZ) induced diabetic mice Streptozotocin	↓Nuclear factor – kB, (NF-Kb) caspase -3-8-9 and Bcl associated X protein (Bax).	Inhibition of diabetic nephropathy progression	(Wen et al., 2013)
DW1029M is a botanical extract,100 mg/kg of DW1029M administered orally	Streptozotocin (STZ) induced diabetic rat	↓AGE formation, RLAR activity, and TGF-1 signaling	It has protective effect against diabetic nephropathy	(J. Yoon et al., 2014)

↑ Activation, up-regulation, or high expression

↓ Inhibition, deactivation, blockade, down-regulation, or low expression.

2.4 Medicinal plants

Medicinal plants have demonstrated many pharmacological effects or actions. The potency and safety profile attributes of medicinal plants, easy accessibility or availability, and

low costs render them a desirable source of treatment for various diseases, including DM, particularly in developing countries (Singab et al., 2014).

One of the complementary alternative treatments used in Africa's developing countries is medicinal herbs and nutraceuticals from plants. As complementary alternative medicine (CAM), African medicinal herbs and botanicals have significantly increased worldwide to manage chronic diseases, including DM and its complications. These herbal products are generally safe because of their natural origin (Han et al., 2019). A variety of plant-based CAM intervention include primary health care, prevention, treatment, and maintenance/management (Thomson et al., 2014). Generally, in today's world, plant-based medicine is emerging in treating various diseases as an adjunct or use-alone therapy (Sofowora et al., 2013)

Medicinal plants have directly or indirectly been derived as a source of some modern drugs, pointing in a direction needing exploration and strengthening to ascertain new favourable lead choices for drug development (Salehi et al., 2019). In developing countries, DM treatment entails medicinal plants use. These plants contain array of phytoconstituents such as flavonoids, terpenoids, saponins, carotenoids, and alkaloids glycosides that possess significant antidiabetic properties (Tran et al., 2020). Using phytoconstituents, aimed at treating DM for their ability to lower blood glucose rapidly and also prevent cardiovascular-related problems, boost the antioxidant system, and improve insulin action and secretion (Surveswaran et al., 2007). These phytoconstituents remain protocol guide of research in clinical trials for drug discovery and development that are insightful for a plausible breakthrough in DM management, which has tremendously helped curtail DM-related complications (Vinayagam & Xu, 2015).

Traditional medicine is very prominent, as more than 80% of traditional healers / practitioners are still actively engaged in treating various ailments in many countries of the

African continent (Kasole et al., 2019). Spiritual belief and alternative treatments remain modalities such as the use of medicinal plants in disease management, including DM. Because of the different side effects associated with increasingly available synthetic drugs, using medicinal plants should be considered for safety and potency (Rai & Kishore, 2009).

2.5 Therapeutic effect of medicinal plants in diabetes mellitus and complications

Many medicinal plants have been used to reduce blood glucose and improve diabetes complications. African herbal medicines and botanicals are being recognised as more scientific information is published from developed and developing countries to support their use in treating DM and complications (Bahmani et al., 2014; Shaw et al., 2012). Scientific evidence through surveys revealed that Africa has numerous medicinal plants with anti-hyperglycaemic, hypoglycaemic, and antidiabetic properties effective in preventing, treating, and managing DM and complications. The traditional knowledge and some scientific validations of antidiabetic African plants have been conducted in the following African countries, Morocco, Algeria, Tunisia, Sudan, Libya, Nigeria, South Africa, Togo, Gabon, Ghana, Senegal and Cameroon (Abo et al., 2008; Ameyaw et al., 2012; Dièye et al., 2008; Karou et al., 2011; Miara et al., 2018; Nole et al., 2016; Oyedemi et al., 2009; Skalli et al., 2019; Tjeck et al., 2017; Van Wyk, 2015; Wannes & Marzouk, 2016; Yagi & Yagi, 2018).

Mohammed et al. (2014) documented the potential antidiabetic plants in all the five regions of Africa, West, East, North, South, and Central Africa, and revealed 185 plant species belonging to 75 families. These medicinal plants have been investigated for antidiabetic effects. In Table 2.6, different families and plant species that received attention in the review by Mohammed et al. (2014) to treat DM and its complications are revealed.

Table 2.6. Different families and species of medicinal plants used in the treatment of DM and its complications

Scientific Name	Parts of the plant used	Mechanism of action	References
Aloe vera (L.) Burm (Asphodelaceae)	Whole plant	Ameliorate oxidative stress	(Jain et al., 2010) (Okyar et al., 2001)
Artemisia roxburghiana Wall. ex Besser (Asteraceae)	Whole plant	Stimulate insulin release from β cells of Langerhans	(Eliud & Peter, 2012)
Aspilia pluriseta Schweinf. ex Engl. (compositae)	Roots	Induce hypoglycemic effect,	(Eidi et al., 2006)
Allium Sativum L. (Alliaceae)	Garlic cloves	Possess insulin-mimetic properties, regulates GLUT4 translocation	(Eidi et al., 2006)
Allium cepa L. (Alliaceae)	Bulbs	Possess insulin-mimetic properties, regulates GLUT4 translocation	(Mathew & Augusti, 1975)
Caesalpinia volkensii Harms (Caesalpiniaceae)	Leaves	Induces hypoglycemic effect	(Njagi et al., 2012)
Bidens pilosa L.	Leaves	Induces antihyperglycemic effect	(Piero et al., 2011)
Salvia coccinia (Lamiaceae)	Whole plant	Stimulate insulin release from β cells of Langerhans	(Hussain et al., 2004)
Monstera deliciosa Liebm (Araceae)	Whole plant	Stimulate insulin release from β cells of Langerhans	(Hussain et al., 2004)
Abies pindrow. Roxyle D .Don (pinaceae)	Whole plant	Stimulate insulin release from β cells of Langerhans	(Hussain et al., 2004)
Camellia sinensis L. (Theaceae)	Leaves	Inhibits rate-limiting gluconeogenic enzymes, PEPCK and G6Pase	(Koyama et al., 2004)
Caylusea abyssinica Fresen. Fisch &Mey	Leaves	Induces anti-glycemic effect	(Piero et al., 2011)
Catha edulis Vahl (Calastraceae)	Leaves	Induce hypoglycemic effect	(Van de Venter et al., 2008)
Catharanthus roseus L. (Apocynaceae)	Juice leaf	Stimulate insulin release from β cells of Langerhans	(Nammi et al., 2003)
Cinnamomum zeylanicum (Lauraceae)	Bark	Stimulate insulin release from β cells of Langerhans	(Verspohl et al., 2005)
Olea europaea L. (oleaceae*)*	Leaves	Potentiate glucose-induce insulin release, increase peripheral uptake of glucose	(Gonzalez et al., 1992)
Cogniauxia podoleana Baillon (Cucurbitaceae)	Leaves	Induce hypoglycemic effect	(Diatewa et al., 2004)
Erythinia abbyssinica Lam ex DC. (Fabaceae)	Stem bark	Induce hypoglycemic effect	(Piero et al., 2011)
Ficus sycomorus L. (Moracea)	Stem bark	Induce hypoglycemic effect	(Olaokun et al., 2013)
Ginkgo biloba L. (Ginkgoaceae)	Whole plant	Stimulate insulin release from β cells of Langerhans	(Kudolo, 2000)
Gymnema sylvestre R. Br. (Asclepiadaceae)	Leaves	Inhibit carbohydrate absorption in the gut	(Mutalik et al., 2005)
Gynura procumbens Merr (Asteraceae)	Leaves	Lower the intestinal absorption of glucose, increase hepatic insulin sensitivity	(Akowuah et al., 2001)
Kleinia squarrosa (Asteraceae)	Stem bark	Induces hypoglycemic effect	(Murugi et al., 2012)
Maesa lancelata (Myrsinaceae)	Fresh fruits	Ameliorates oxidative stress	(Haraguchi et al., 1996)
Moringa stenopetala Baker f. Moringaceae	Leaves	Induces Antiglycemic effect	(Nardos et al., 2011)
Olea europaea L. (Oleaceae)	Leaves	Regulates GLUT4 translocation	(Gonzalez et al., 1992)
Opuntia robusta H.L. (cactaceae)	Fruits	Ameliorates oxidative stress	(Budinsky et al., 2001)

Opuntia streptacantha L. (Cactaceae)	Fruits, stem	Stimulates insulin release	(Frati-Munari et al., 1989)
Pappea capensis Eckl & Zeyh (Sapindaceae)	Leaves and stem bark	Induces anti-hyperglycemic effect	(Karau et al., 2012)
Pentas schimpeniana A.Rich. (Rubiaceae)	Leaves	Induces anti-hyperglycemic effect	(Dinku et al., 2010)
Solanum lycocarpum (solanaceae) A.St. Hil.	Fruits	Induces hypoglycemic effect	(Kar et al., 2006)
Strychnos henningsii Gilg. (Longaniaceae)	Leaves and bark	Induces hypoglycemic effect, Ameliorates oxidative stress	(Oyedemi et al., 2010)
Tetrapleura tetraptera. Schmauch & Thonn (Fabaceae)	Fruits	Induces hypoglycemic effect	(Ojewole & Adewunmi, 2004)

Many African medicinal herbs and botanicals show biological activities or mechanisms as antidiabetic, antihyperglycaemic, hypoglycaemic, and inhibitors of both α-amylase and α-glucosidase (Salehi et al., 2019), among other things. Hence, the aforementioned biological properties attributed to these African herbal medicines and nutraceuticals in preventing, treating, and managing diabetes-related complications are valuable.

2.6 Synthetic drugs for diabetes and its complications

Many classes of oral hypoglycaemia such as sulfonylureas, biguanides, alpha-glucosidase inhibitors, thiazolidinediones and non-sulfonylureas secretagogues act through different mechanisms with anti-hyperglycaemic effects. These oral hypoglycaemic agents are specific in actions and type of DM (Salehi et al., 2019). For example, oral glimepiride, a sulfonylurea, enhances insulin elevation when it binds to sulfonylurea receptors of beta-cell of islets of Langerhans. This mechanism ensures closure of the potassium channels dependent on ATP. At the same time, calcium influx in a depolarised cell membrane and secretion of insulin from the secretory granules is accomplished to reduce blood sugar, which is particularly very effective in treating T2DM (Salehi et al., 2019).

Biguanides, such as the popular metformin, boost the muscle's peripheral tissue to be sensitive to insulin and inhibit liver gluconeogenesis, increasing the stimulation of insulin and sugar utilisation. While acarbose, an inhibitor oral hypoglycaemic agent, acts by inhibiting

pancreatic alpha-amylase and alpha-glucosidase enzymes responsible for the breakdown of polysaccharides to monosaccharides for absorption in the small intestine. This action reduces glucose availability and hence reduces the absorption of postprandial blood sugar levels (Lebovitz, 1997).

The hypoglycaemic class of thiazolidinediones, such as pioglitazone, is potent and selective to a particular receptor for its antidiabetic action; this receptor is called nuclear proliferator-activated receptor gamma (PPAR-γ) is located in the liver, skeletal muscle and adipose tissue, which regulate insulin secretions and circulation for glucose utilisation. This regulation is through the activated PPAR-γ through the transcription of insulin genes. The cumulative action of thiazolidinediones includes increasing tissue sensitivity, both muscle and adipose, to insulin, reducing gluconeogenesis by the liver, and preventing beta-cell death caused by the accumulation of free fatty acids (Koski, 2004). The non-sulfonylureas secretagogues, the last class of oral hypoglycaemic agents such as meglitinide, act similar to thiazolidinediones. However, they bind to different beta-cell receptors to increase insulin secretion (Koski, 2004).

Although these oral hypoglycaemic agents can be used alone, they are also used alongside insulin in DM treatment but are limited in their ability to cure DM entirely and cause a total reversal of DM complications. Furthermore, the bane of using hypoglycaemic agents are the prominent deleterious side effect encountered, drug resistance (reduced efficacy after prolonged use) and drug toxicity (Singab et al., 2014). Therefore, the search for a newer antidiabetic agent with better efficacy and safety profile from medicinal plants is imperative to overcome the overwhelming limitation posed by the available synthetic drug.

2.7 References

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CHAPTER THREE

The repository of medicinal plants is extensive, requiring continued exploration of this flora. Medicinal plants that are neuroprotective and have potentially safe and potent antidiabetic agents need to be discovered and developed, especially those from some of the under-explored species. In this light, we carried out the literature review of some selected *Helichrysum* species in this chapter. In this chapter, the aim is to provide a comprehensive literature review using different engine searches on the database and printed materials of the existing knowledge about traditional and scientific investigations on selected *Helichrysum* species.

plants

MDPI

Review

Medicinal Properties and In Vitro Biological Activities of Selected Helichrysum Species from South Africa: A Review

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Abstract: The genus *Helichrysum* Mill comprises hundreds of species that are mostly flowering perennial shrubs. Some of these plants that belong to the *Helichrysum* species are used in traditional medicine to treat cough, back pain, diabetes, asthma, digestive problems, menstrual pain, chest pain, kidney disorders, skin disorders, wounds, open sores, among other conditions, but, only a few scientific studies are reported in the literature with sufficient information that validates the acclaimed folkloric benefits of these plants. This review, therefore, provides a comprehensive update of the available information on the cytotoxicity, genotoxicity, anti-proliferative, anti-bacterial, antifungal, anti-viral, anti-HIV, anti-malarial, anti-ulcerogenic, anti-tyrosinase, anti-inflammatory, and anti-oxidant activities of selected *Helichrysum* species of interest: *H. petiolare*, *H. cymocum*, *H. foetidum*, and *H. pandurifolium* Schrank, using scientific databases as well as electronic and print sources. The ethnobotanical and morphological characteristics as well as the phytochemical composition and biological activities of these plants are elucidated. The scientific rationale for their current use is discussed based on the evidence in the literature. This review highlights the putative use of the *Helichrysum* species as a reliable source of bioactive compounds for the production of standard commercial drugs to treat many ailments, including those reported in folkloric uses. Further research on the many plants in the genus *Helichrysum* is recommended to explore their economic importance both as edible crops and medicinal botanicals.

Keywords: medicinal plants; phytochemicals; pharmacological actions; *Helichrysum* species

1. Introduction

The genus Helichrysum Mill comprises distinctively of aromatic herbs and shrubs of the family Asteraceae. It has a worldwide distribution but is mainly found in Africa, with its highest diversity in South Africa, where approximately 245 of the 500 known species occur. Other areas of *Helichrysum* diversity include Europe, southwestern Asia, South. India, Sri Lanka, Turkey, and Australia. For ease of identification, the southern African species are divided into 30 informal groups [1].

Plants of the *Helichrysum* genus have been in use for more than 2000 years for various folkloric purposes. The flowers of some members of this genus have a unique

bright-yellow color that depicts their Greek language origins: "helios" and "chryos" which mean "sun"and "gold" respectively. In folkloric medicine, some *Helichrysum* plant parts are either consumed as teas or prepared as "burnt offering" smoke to disinfect the abodes of sick patients and to appeal for blessings from the ancestors in indigenous traditional practices [2]. In general, plants of this genus are known to be used in traditional medicine for the treatment of many ailments, including liver disorders, gall bladder complications, cystitis, jaundice, stomach pain, allergies, infections, colds, cough, skin infections, inflammation, menstrual pain, asthma, arthritis, insomnia, diabetes mellitus, and for wound healing [3–7], most commonly, the scented leaves and flowers.

The therapeutic properties of the *Helichrysum* species are often attributed to their different constituent phytochemicals, especially the essential oils [8,9]. In addition to the essential oils, plants in this species also contain such phytochemicals as terpenoids, phenolics and oxygenated compounds as secondary metabolites, including flavonoids, chalcones, phenolic acids, terpenes and essential oils, pyrone, benzofurans, and phloroglucinols [10]. Thus, these plants are potential reservoirs of bioactive compounds for drug discovery and development. Hitherto, only limited biological effects of the *Helichrysum* phytochemicals have been reported including, the antioxidant, antifungal, anti-inflammatory, anti-bacterial, hepatoprotective, anti-proliferative and anti-diabetic activities [7,11–13].

So far, only a few of the many *Helichrysum* species have been studied; the best known *Helichrysum* species used traditionally to treat different ailments are *H. cymosum*, *H. odoratissimum*, *H. petiolare* and *H. nudifolium*. Only limited information is available in the literature on these plant species on their ornamental, industrial and pharmaceutical applications [14]. Thus, in this review, peer-reviewed information on *Helichrysum petiolare* Hilliard & B. L Burtt, *Helichrysum cymosum* (L.) D. Don, *Helichrysum foetidum* (L.). Moench and *Helichrysum pandurifolium* Schrank from South Africa will be elaborated, including their botanical, ethnopharmacological, phytochemical and bio-scientific profiles.

2. Research Methodology

The keywords relevant to this review, including "*Helichrysum species*" "*Helichrysum petiolare* Hilliard & B. L Burtt", "*Helichrysum cymosum* (L.) D. Don", "*Helichrysum foetidum* (L). Moench", "*Helichrysum pandurifolium* Schrank", "cytotoxicity", "anti-genotoxicity", "anti-proliferative", "anti-bacterial", "anti-fungal", "anti-viral", "anti-malarial", "anti-ulcerogenic", "anti-tyrosinase", "anti-inflammatory", "antioxidant", "phytochemicals", "ethnopharmacology" and "essential oil" were searched for, using different databases including Sci Finder, ISI Web of Knowledge, Science Direct, Google Scholar, PubMed, Scopus, Wiley Online Library and Springer; while online theses, dissertations, and other print materials also provided important information on the traditional use, bioactive phytochemical compositions, biological and pharmacological actions, as well as leads on relevant gaps in research and future directions.

3. Helichrysum *petiolare* and Its Biological Activities

Helichrysum petiolare (*H. petiolare*) is commonly referred to as "Silverbush everlasting plant", and called "kooigoed" in the Afrikaans language of South Africa. This plant is a shrub with gray or silver-gray hair covering the aromatic round-shaped leaf, while its flowers are whitish-creamy [15]. *H. petiolare* is used in South African traditional medicine to treat fever, catarrh, cold, cough, menstrual disorders, kidney-related infections, chest problems, high blood pressure, and erectile dysfunction. In addition, the decoction of the leaves of the plant is used in traditional beauty therapy to refine skin texture/looks and for wound healing [16]. The known phytochemicals present in *H. petiolare* include phenols, flavonoids, and anthocyanins, the extract and its essential oil constituents were reported to show activity against Gram-positive and Gram-negative bacteria [17]. Other biological activities of this plant are discussed below.

3.1. Cytotoxicity/Anti-Proliferative Activity of H. petiolare

The in vitro cytotoxic effects of the chloroform and methanol leaf and stem extracts of *H. petiolare* (1:1 solvent ratio) on transformed kidney epithelial (Graham's) cells, breast cancer cells (MCF-7) and brain cancer (SF-268) cells were studied using the sulforhodamine B (SRB) assay and the results showed percentage growth inhibition of 59%, 33% and 76% for the Graham's, MCF-7, and SF-268 cell lines, respectively [18]. In another study, B16F10 mouse melanoma cells and MeWo human skin melanoma cells were treated with the methanol extract of *H. petiolare*, and the cytotoxicity and cell cycle analysis showed a dose-dependent reduction in cell viability or proliferation, as well as S-phase and M-phase cell cycle arrest, reflecting pro-apoptotic effects.

3.2. Anti-Bacterial Activity of H. petiolare

The anti-bacterial activities of nine *Helichrysum* species of South African origin, including *H. petiolare*, have been reported in such bacterial strains as *Escherichia coli*, *Yersinia enterocolytica*, *Klebsiella pneumomiae* (Gram-positive) and *Staphylococcus aureus* and *Bacillus cereus* (Gram-negative), using disc diffusion assay, with the standard drug neomycin as control [19]. When compared with the control, the methanol and acetone extracts of *H. petiolare* showed significant activities against *S. aureus* and *B. cereus* strains while the essential oils showed no activity against these two bacterial strains. The zone of inhibition is a uniformly circular area on an antibiotic dish that has no bacterial growth [20]. In the study under reference, the inhibition zone values ranged between 2.5 mm to 9.0 mm in the methanol and acetone extract-treated dishes compared with the 6 mm zone of inhibition in the standard drug. The Gram-positive bacteria were unaffected following treatment with the essential oil as well as the methanol and acetone extract of *H. petiolare*. Furthermore, the minimum inhibitory concentration (MIC) assay was used as a confirmatory test for the disc diffusion assay, and the results of this assay showed much improved activity for both the methanol and acetone extracts (<0.25 mg/mL) of *H. petiolare* when compared with the standard drug ciprofloxacin (0.31×10^{-3} mg/mL). However, the MIC assay showed a relatively low activity of the essential oil treatment (8 mg/mL) on the *S. aureus* bacterial strain [19].

In another study, Lourens and co-workers reported on the anti-bacterial effects of the methanol and chloroform (1:1 solvent ratio) extracts of *H. petiolare* on five bacterial strains, namely *B. cereus*, *S. aureus*, *S. epidermidis*, (Gram-positive), *K. pneumomiae*, and *P. aeruginosa* (Gram-negative), with ciprofloxacin as the positive control. The results showed minimum anti-bacterial effects against *S. aureus* and *B. cereus* with a MIC value of 4 mg/mL and 2 mg/mL respectively, compared to the control. However, no anti-bacterial activity was recorded against the remaining bacterial strains in the 96-well microplate assay [21].

3.3. Anti-Inflammatory Activity of H. petiolare

Louren and co-workers investigated the anti-inflammatory properties of the essential oil of *H. petiolare* using the 5-lipoxygenase assay, and the half-maximal inhibitory concentration (IC_{50}) value of 23.05 ± 0.57 µg/mL was obtained for the varying concentrations of hydro-distilled essential oils, indicating potent anti-inflammatory effects. In comparison, the positive control had an IC_{50} value of 5.0 ± 0.50 µg/mL while the methanol and acetone extract of *H. petiolare* showed no anti-inflammatory activity, with an IC_{50} value greater than 100 µg/mL [21].

3.4. Anti-Fungal Activity of H. petiolare

Three strains of human fungi namely, *Cryptococcus neoformanas*, *Candida albicans* and *Alternaria alternate*, were evaluated using the disc diffusion assay. The results showed that only the acetone extract of *H. pertiolare* showed moderate anti-fungal activity against *C. albican* compared with the standard drug Nystatin. In contrast, the essential oil and methanol extract had no anti-fungal activity in the three fungal strains tested [21].

3.5. Anti-Oxidant Activity of H. petiolare

A slight modification of the 1,1 diphenyl -2- picrylhydrazyl (DPPH) free radical scavenging assay [20] was used to determine the anti-oxidant activity of the extracts of *H. petiolare*, in reference to the ascorbic acid standard. The results of treatment with the IC_{50} values of 44.28 µg/mL, 28.70 µg/mL and 2.5 µg/mL of the acetone and methanol extracts as well as the ascorbic acid standard, respectively, showed that more than 50% of the free radicals was inhibited or neutralized.

3.6. Antigenotoxicity Activity of H. petiolare

The results from one study showed genotoxic effects of the methanol extracts of *H. petiolare* on normal Vero cells at concentration ranges of 12.5–200 µg/mL, using the micronucleus assay [22]. In another study, the 90% methanol and dichloromethane (DCM) extracts of *H. petiolare* were screened for aflatoxin B_1-induced mutagenicity using the bacteria-based genotoxic Ames and Vitotox assays [23]. The methanol extract showed significant dose-dependent anti-mutagenic activity against *S. typhimurium* TA100 and TA 98 while the DCM extract had moderate anti-mutagenic effects against *S. typhimurium* TA 100 and more severe effects against *S. typhimurium* TA 98. Cancer remains a global disease today with high mortality and morbidity rate. Mutagens are known to promote the pathogenesis of most cancers via gene mutation and chromosomal aberration and anti-mutagens are known to prevent chromosomal translocation, deletion and inversion [22,23]; hence the use of *H. petiolare* extracts as food supplements could serve as potential carcinoprotection agents, especially cancers induced by aflatoxin-producing fungi, in poorly-processed food crops.

3.7. Anti-Tyrosinase Activity of H. petiolare

A number of skin disorders are known to be promoted by anomalies in such implicated enzymes as tyrosinase and elastase, in the face of damaging free radical effects on the skin [24]. Therefore, compounds that inhibit such enzymes (e.g., tyrosinase inhibitors) could potentially be very useful in the cosmetic industry to prevent skin aging and other undesirable skin conditions [25,26]. *H. petiolare* plant extracts have been investigated for their tyrosinase inhibition activities (25), and results obtained showed moderate tyrosinase inhibition values of 44.3% and 59.2% at 50 µg/mL and 200 µg/mL concentrations respectively, using kojic acid as the reference control [25]. The results obtained tend to suggest that phytochemicals from the extracts of *H. petiolare* could inhibit free radical accumulation and modulate tyrosinase activity.

In another study, treatment with the ethanol extract of *H. petiolare* was found to be nontoxic to human dermal fibroblast (MRHF) cells using the Hoechst 3342/propidium iodide stain and resulted in decreased ROS and NO production following lipopolysaccharide-induced damage in the RAW 246.7 cell line. Additionally, weak inhibitory effects of the extract on the enzymes of collagenase, elastase and tyrosinase at various concentrations was reported in another study, compared to the positive control. In addition, this plant extract effectively inhibited protein glycation, indicating its potential use as an anti-aging agent for the skin [27].

4. *Helichrysum cymosum* and Its Biological Activities

Helichrysum cymosum (*H. cymosum*) is a plant endemic to South Africa and known commonly as "gold carpet" or "yellow-tipped strawflower" in English. Its local South African names include "goue tapyt" (in Afrikaans) and "impehho" (in isiXhosa). This plant is widely distributed in the Eastern Cape, KwaZulu-Natal, and Western Cape Provinces, and grows as a short, wooly shrub with grayish silver leaves and bright yellow-colored flowers in flat heads. This species is divided into two subspecies, namely the *H. cymosum* subsp. *cymosum* and *H. cymosum* subsp. *calvum* Hilliard [28,29], with distinct characteristics in the flowers, fimbrils, ovary, pappus, and plant length [29]. Traditionally, the aerial parts, leaves, roots, and leaves of this plant are used to treat different ailments ranging from cough, catarrh, colds, headache, menstrual pain, fever, wounds, flatulence, pulmonary problems, skin

infections, pertussis vomiting as well as to improve immunity, and appetite [15,29]. The reported biological and pharmacological activities of *H. cymosum* include anti-bacterial, anti-fungal, anti-inflammatory, anti-malarial, anti-oxidant, and cytotoxic effects [15,29], some of which are briefly discussed below.

4.1. Cytotity of H. cymosum

Vuuren et al. investigated the toxicity of the essential oil and acetone extract of *H. cymosum* as well as one of its isolated compounds, helihumulone on the transformed kidney epithelial cell line, using the tetrazolium-based cell proliferative or viability assay (MTT) [30]. The results showed that the essential oil was the most toxic extract, with an IC_{50} value of 17.47 ± 33.0 µg/mL compared to 172.2 ± 10.08 µg/mL for the acetone extract and 57.05 ± 3.04 µg/mL for helihumulone, respectively. In another study, the cytotoxic effects of *H. cymocum* on the kidney Vero cell line were evaluated using the XTT assay with varying concentrations of different solvent extracts of the plant: 3.13, 6.25, 12.50, 25.00, 50.00, 100.00, 200.00 and 400.00 µg/mL. These results showed that compared to the positive control Zearlenone, with an IC_{50} value of 1.33 ± 0.32 µg/mL, the chloroform extract (IC_{50} value 36.52 ± 0.27 µg/mL) was more toxic to the Vero cells than the methanol-water (50% water/methanol and 50% chloroform) extract with an IC_{50} value 59.74 ± 0.14 µg/mL [31].

4.2. Anti-Oxidant Activity of H. cymosum

Francois (2016) investigated the anti-oxidant capacity of the essential oils isolated from *H. cymosum* leaves using the 1, 1 diphenyl 1- picrylhydrazyl (DPPH) assay. Results obtained showed that 50% free radical scavenging activity was achieved by the 6.3 g/L concentration of this plant compared to the same effects by the 7.0 mg/L concentration of the positive control, butylated hydroxytoluene (BHT), a known compound used as a food anti-oxidant additive [29]. Thus, the extract of this plant had more anti-oxidant effects than BHT, indicating potential use as food additives. No other anti-oxidant studies involving *H. cymosum* were found in the literature at the time of writing this review, hence more studies are recommended.

4.3. Anti-Malarial Activity of H. cymocum

Vuuren et al. reported on the anti-plasmodium activity of the acetone crude extract, the essential oil, and the isolated compound helihumulone from *H. cymocum* on the falciparum parasite, relative to the effects of two standard anti-malaria drugs (quinine and chloroquine). The results from the G3-H hypoxanthine incorporation assay showed that when compared to the two standard anti-malarial drugs, quinine (IC_{50} value 0.13 ± 0.04 µg/mL) and chloroquine (IC_{50} value 0.09 ± 0.02 µg/mL), the essential oils had the least IC_{50} value (1.25 ± 0.77 µg/mL) followed by helihumulone (14.89 ± 1.88 µg/mL) and then the acetone crude extract (60.76 ± 2.83 µg/mL). Considering the relatively low IC_{50} value of the essential oils of *H. cymocum*, their potential use as natural sources of anti-malarial treatment agents is plausible [30].

4.4. Anti-Fungal Activity of H. cymocum

The anti-fungal activity of the essential oil and acetone extract of *H. cymocum* as well as the isolated compound, helihumulone against *C. neoformans* and *C. albicans* was investigated using the microdilution technique, with a potent anti-fungal agent amphotericin B as the positive control. The MIC values used ranged from 0.03–4.0 mg/mL, and the results showed that the 0.03 mg/mL and 0.063 mg/mL MICs of the helihumulone were the most potent against *C. neoformans* and *C. albicans* [32]. Another study on the anti-fungal activity of the isolated essential oils of *H. cymocum* showed that over 50% zone of inhibition was achieved against *Penicillium oxalicum* while a 6 mm to 9 mm area of inhibition was reported for *C. albicans* [33,34].

4.5. Anti-Bacterial Activity of H. cymocum

Bougatsosa et al. reported on the anti-bacterial activities of the essential oils of *H. cymocum* against the Gram-positive bacteria *S. aureus* and *S. epidermis* as well as four Gram-negative bacteria, *E. coli*, *Enterobacter cloacae*, *K. pneumoniae* and *Pseudomonas aeruginosa*. The MIC results from the dilution technique showed that the essential oils of *H. cymocum* were not sensitive (completely inactive) to all the bacteria strains tested when compared to the essential oils of a closely-related plant species *H. fulgidum* (L.) Wild, which have been reported to be highly sensitive to the same bacterial strains tested [34]. Similar studies by Sindambiwe et al. showed that the 80% ethanol extract of *H. cymocum* was not sensitive to the bacteria *Proteus vulgaris*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *Salmonella paratyphi*, *B. cereus*, *Mycobacterium fortuitum* and *S. aureus*, whereas *Streptococcus pyogenes* showed a MIC value of 5 mg/mL which indicates some sensitivity [35]. The results of another anti-bacterial activity study using the disc diffusion assay showed a zone of inhibition value of 7 mm and 5 mm respectively for *S. aureus*, 8 mm and 5 mm respectively for *B. cereus*, and no inhibition by the essential oils [32]. In yet another study, the isolated compound helihumulone, was found to have the highest anti-bacterial activity against the bacteria *Enterococcus faecalis*, *B. cereus*, *B. subtilis*, *S. aureus*, *P. aeruginosa*, *E. coli*, *Y. enterocolitica*, and *K. pneumonia* at the concentration range of 0.02–0.12 mg/mL, using the p-iodo nitro tetrazolium (INT) microplate method, when compared with the essential oil (1.0–8.0 mg/mL) and acetone extract (0.1–0.3 mg/mL) of *H. cymocum*. The acetone extract activity was reported to be six times more than that of the essential oils [33].

4.6. Anti-Inflammatory Activity of H. cymocum

Standford and co-workers investigated the inhibitory effects of the ethanol and aqueous extracts of *H. cymocum* to prostaglandin, a known marker of inflammation, using the in vitro cyclooxygenase assay. The results showed that, whereas the standard anti-inflammatory drug indomethacin showed 60% inhibition, the aqueous extract showed 52% and the ethanol extract showed 100% inhibitory activity [36]. In addition, other related plant species like *Helichrysum excisum* and *Helichrysum felinum* were reported to exhibit anti-inflammatory properties; the acetone extract and essential oil from *H. excisum* showed anti-inflammatory properties at IC_{50} concentrations of 35.09 ± 1.12 µg/mℓ and 27.62 ± 0.43 µg/mL respectively, using the 5-lipoxygenase assay, while the anti-inflammatory properties reported for the acetone extract and essential oil from *H. felinum*, occurred at IC_{50} concentrations of 38.72 ± 2.94 µg/mL and 22.87 ± 7.59 µg/mL respectively, in comparison with control with IC_{50} of 5.00 ± 0.50 µg/mL [37].

4.7. Anti-Viral Activity of H. cymocum

The virucidal activity of the ethanol extract of *H. cymocum* against selected viruses was studied using the 50% endpoint titration technique (EPTT). This assay involves the determination of virus titer reduction in the presence of two-fold dilutions of test compounds on monolayers of cells grown in plastic or glass Petri dishes [38]. The results obtained showed that the extract was effective against the simplex virus type 1 (HSV1), the measles virus strain Edmonston A (MV-EA) as well as the Semliki forest virus A_7 (S.F. A_7) [35]. In addition, the anti-viral bioactivity of the methanol/water and chloroform (50% water/methanol and 50% chloroform) extracts of the aerial parts of *H. cymocum* were investigated in one study using colorimetric cell-based, a cytopathic effect inhibition assay. The results showed cytopathic effects against Vero cells at a final toxic concentration of 400 µg/mL, comparedwith the positive control acyclovir with anti-viral activity of 0.75 µg/mL [31].

5. *Helichrysum foetidum* and Its Biological Activities

H. foetidum is a robust herb commonly referred to as the stinging starflower with a characteristic pungent smell. Its leaves are elliptic, sparsely hairy, white-woolly, and are arranged in groups at the base of the stem while its many flower heads are borne in broad, leafy spreading umbrella-like

inflorescences [39]. This plant is native to South Africa and has since been introduced to other parts of the world, including Spain, Portugal, and Southern Brazil [40]. *H. foetidum* is used in African tradomedical practices to induce a trance, mainly due to its hallucinatory effects. It is also used for the treatment of wounds, for dressing the circumcised penis in traditional ceremonies, for infected sores, for menstrual pains, herpes, eye infection, influenza, among other ailments [39]. The chemical composition of *H. foetidum* includes diterpenoid, Kaur-16-en-18-oic acid, flavonoid, apigenin 7,4 O-dihydroxy-5-methoxy-flavanone, apigenin 7-O- β-D- glucoside and 6-methoxy-2^0, 4,4-trihydroxy-chalcone helichrysetins, as well as glucosylated helichrysins [41]. Its biological activities include anti-bacterial [42], anti-fungal [43], anti-viral [35], antioxidant, anti-ulcerogenic, and cytotoxic effects [43].

5.1. Cytotoxicity of H. foetidum

The in vitro cytotoxic effects of the isolated compounds of *H. foetidum* on the human prostate cancer cell line (PC-3) were evaluated using the XTT assay. These compounds were found to have no effects on PC-3 viability at the concentrations. It was reported that the compound reduced the viability of cancer cells and induced apoptosis at the concentrations of 50 nM or 50 µM of the isolated compound [42]. In another study, the transformed human kidney epithelial (Graham's) cells, MCF-7 breast adenocarcinoma cells, and SF-268 glioblastoma cells were reported to be sensitive to the leaf and stem extracts of chloroform and methanol (solvent ratio 1:1), with a significant reduction in cell viability (24.9%) in MCF-7 cells, at a concentration of 0.1 mg/mL of the extract [18].

5.2. Anti-Ulcerogenic Properties of H. foetidum

Malolo et al. reported on the potential of the methanol extract and the isolated compounds from *H. foetidum*, to inhibit the enzyme protease, through the in vitro protease pepsin inhibition assay known as the fluorescence resonance energy transfer (FRET) assay [43]. Pepsin is the main acid protease of the stomach and is implicated in peptic ulcer disease, reflux oesophagitis, and excessive stomach acid secretion, although *Helicobacter pylori* bacteria are also involved in the pathogenesis of most gastric diseases. Following treatment with the methanol extract of *H. foetidum*, the FRET assay showed 17.8%, 35.6% and 37.6% inhibitory activity against pepsin at the concentrations of 10 µg/mL, 25 µg/mL and 50 µg/mL, respectively; however, no action was detected against subtilisin, a known alkaline protease [43,44]. Furthermore, out of the six isolated compounds tested in this study, only apigenin-7-β-D-glucoside and 6^0 methoxy-2^0,4 dihydroxychalcone-4^0-*O*-β-D-glucoside showed moderate inhibition range of 37.4% to 46.3% at 50 µg/mL concentration [43].

5.3. Anti-Bacterial Activity of H. foetidum

Steenkamp et al. (2014) investigated the anti-bacterial activity of the methanol, and aqueous extracts of *H. foetidum* against *S. aureus*, *S. pyrogenes*, *E. coli*, and *P. aeruginosa*, and the results showed a MIC value of not more than 4 mg/mL in all the bacterial strains tested [45]. In addition, the anti-bacterial activity of the methanol extract and six isolated compounds from *H. foetidum* against *B. subtilis* bacteria was evaluated in another study, using a fluorescence-based anti-bacterial inhibition assay [43]. The results obtained showed MIC values of 85.4% and 21.8% for the methanol extract, at the concentration range of 1.0 mg/mL and 0.1 mg/mL respectively, while the isolated compounds displayed growth inhibition in the range of 75.0% to 85.0%.

5.4. Anti-Fungal Activity of H. foetidum

Cladosporium cucumerrinum is a known fungus that causes diseases in plants, leading to reduced crop yield. The in vitro anti-fungal activity of the methanol extract and six isolated compounds from *H. foetidum* against this pathogen was reported [45]. The concentrations of 50 µg/cm, 100 µg/cm, 200 µg/cm and 400 µg/cm of the methanol extract of *Helichrysum foetidum* used in a bioautograpy assay on silica gel plates, indicated that zones of growth inhibition against the fungus were well developed

on bioautograpy plate, showing a significant anti-fungal property [45]. All the six isolated compounds showed growth inhibition against *C. cucumerrinum* in the range of 70% to 56% at 1.0 mg/mL concentrations in the assay [45].

5.5. Anti-Oxidant Activity of H. foetidum

Bruno et al. reported the anti-oxidant activity of the methanol extract of *H. foetidum* using an array of in vitro assays [46], including the 2-2' azinobin-3-ethlbenzothiazoline-6sulphonic acid (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging, and the β-carotene/linoleic acid assay. The anti-oxidant activity of the methanol extract of *H. foetidum* determined by the ABTS and DPPH assays, occurred at the IC_{50} concentrations of 0.5 ± 0.1 µg, 0.6 ± 0.1 µg, and 1.5 ± 0.3 µg respectively, compared with the known antioxidant compounds, Trolox or BHT, used as the standards, with IC_{50} values of 1.3 µg and 1.5 µg respectively. On the other hand, the scavenging of hydrogen peroxide (HRPO) test, the superoxide anion scavenging (SAS) test and the hypochlorous acid scavenging (taurine) test showed anti-oxidant activity at IC_{50} concentrations of 15.0 ± 2.0 µg, 34.0 ± 6.0 µg, and 24.0 ± 3.0 µg respectively. The anti-oxidant activity of medicinal plants is commonly attributed to the presence of phenols, flavonoids, flavanols, and oligomeric proanthocyanidins, which in this study, were quantified to be 580 ± 87 mg/g, 460 ± 69 mg/g, 12 ± 2 mg/g, and 5 ± 1 mg/g respectively, in the methanol extract of *H. foetidum* [46].

5.6. Anti-Viral Activities of H. foetidum

The 50% endpoint titration technique (50% EPTT) was adopted by Sindambiwe et al. (1999) to evaluate the anti-viral activities of the aqueous and 80% ethanol extracts of the whole plant parts of *H. foetidum*. Virucidal activities against HSV1 and the Semliki Forest virus A7 (SFA7) were reported but not against the measles virus strain Edmonston A (MV-EA) and the vesicular stomatitis virus T2 (VSVT2) [35].

6. *Helichrysum pandurifolium* Schrank and Its Biological Activities

The common name of *H. pandurifolium* Schrank is *Hottentotskruie* or *Hottentotskooigoed* or fiddle leaf-strawflower, with the stem and leaf parts often prepared as infusions for the traditional treatment of such ailments as respiratory conditions, cough, heart conditions, kidney stones and other kidney-related conditions [10]. The plant is a tall, slender, loosely-branched and soft shrub with orbicular-to-ovate leaves that abruptly narrow at a broad petiole-like base. The flowers are yellow, with the outer and inner parts of the involucre bracts being light-brown and white-pink, respectively. The apex of this herbal plant is sharp, while its fruits have a pappus with an array of feathery bristles [14,40].

Currently, information on the phytochemicals and pharmacological actions of *H. pandurifolium Schrank* is scanty in the literature, a possible indication that this species is not well-studied scientifically.

7. Common Phytochemicals Present in the Selected *Helichrysum* Species

Phytochemicals refer to structurally diverse secondary metabolites or compounds that are produced by plants or non-pathogenic endophytic microorganisms in plants, to serve a protective role against any form of insults by pathogens (bacteria, fungi and viruses), through participation in the body's anti-oxidant defensive and protective mechanisms such as free-radical scavenging [47,48]. Phytochemicals, therefore, confer essential pharmacological or biological functions on medicinal plants. In 1967, phytochemical studies of the *helichrysum* genus were done, and the helichrysum *dendroideum* species was the first to be explored [49], leading to the identification of many chemical secondary metabolites. Scientific reports on 63 *helichrysum* species of South African origin have shown many isolated compounds, including acylphloroglucinol, humulone derivatives, flavonoids, 8-hyroxyflavonols, α-pyrones, chalcone, and pyranochalcones [37,50,51]; essential oils, benzofurans, oxygenated compounds are present in the many species of this plant genus [10]. Representative of some of the compounds

present in *H. petiolare*, *H. cymosum*, *H. foetidum* and *H. pandurifolium Schrank* are depicted in Figures *3*.1–*3*.4. While the biological functions of the associated compounds including that of the essential oils are listed in Tables *3*.1–*3*.5 in this review.

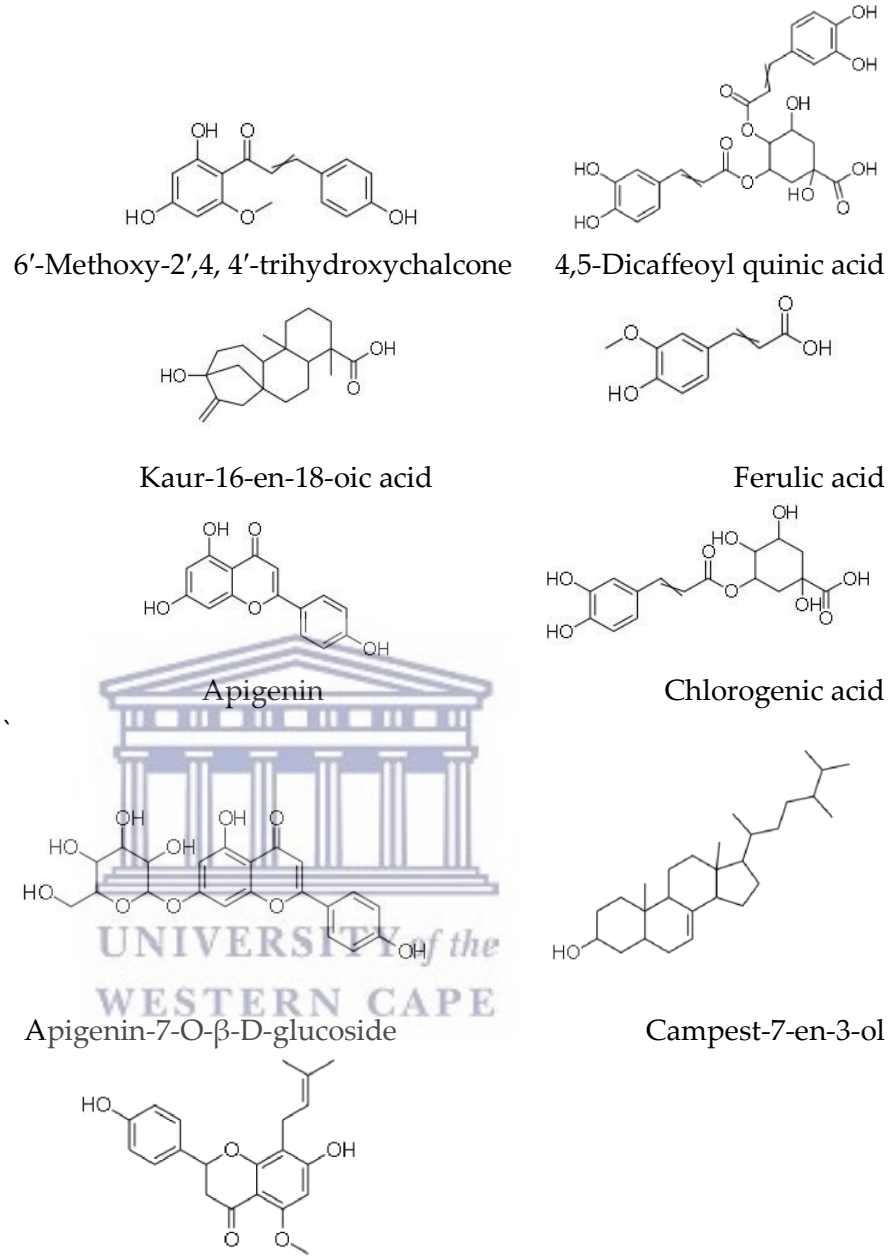

Figure 3.1. Structures of phytochemicals isolated from plants of *Helichrysum foetidum*.

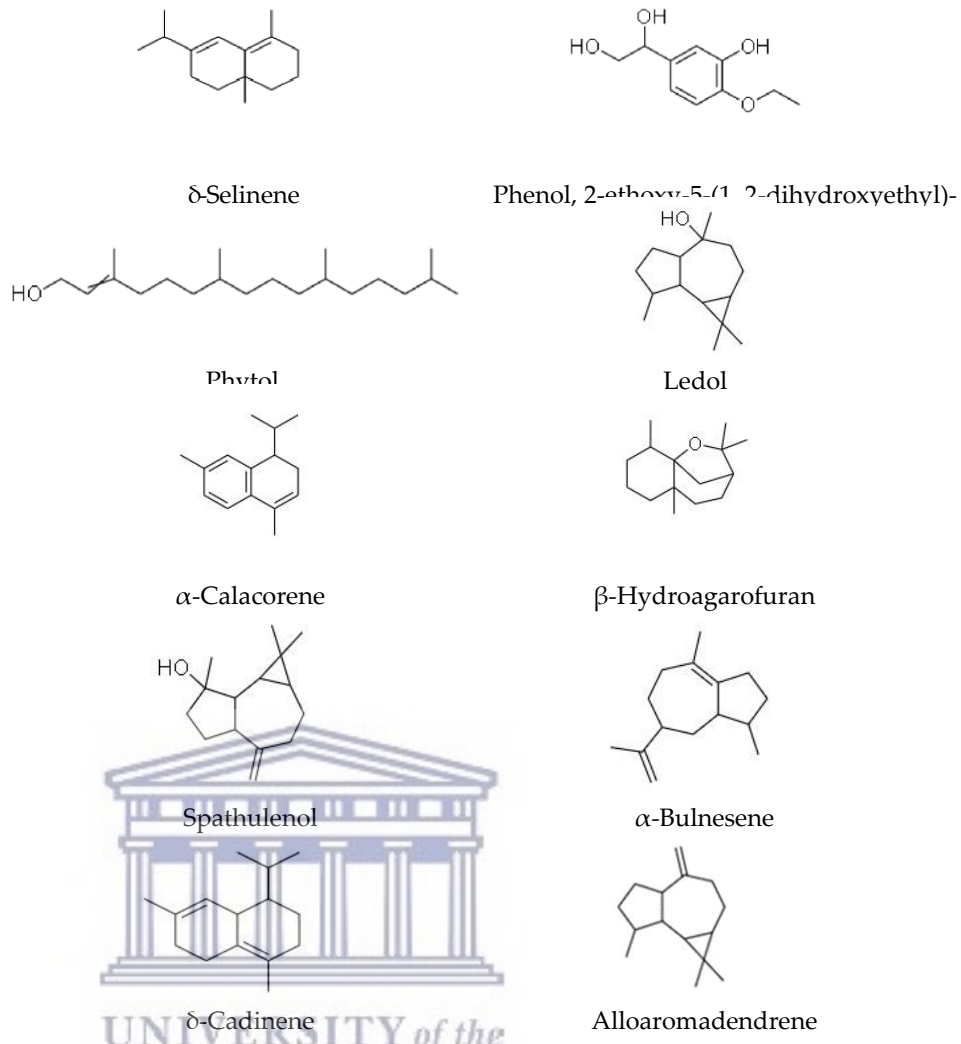

Figure 3.2. Structures of phytochemicals isolated from *Helichrysum petiolare*.

Table 3.1. Description of bioactivity of the compounds of *Helichrysum cymosum*.

Compounds	Biological Functions
Helihumulone	anti-bacterial and anti-mycotic [15,52]
(Z)-β-ocimene	Molluscidal and leshimanicidal agents [53]
Trans-caryophyllene	Anti-malarial [15,36]
1, 8-cineole	Anti-inflammatory, antioxidant, anticancer, analgesic [54]
α-humulene	Anti-Proliferative [55]
(E)-β-ocimene	Molluscidal and leshimanicidal agents [53]
Caryophyllene oxide	Anti-malarial [15,36]
β-caryophyllene	Anti-malarial [15,36]
Δ-3-carene	AChE inhibition, anti-inflammatory Anti-fungal [56,57]
5-hydroxy-8-methoxy-7-prenyloxyflavanone	Anti-viral [30]

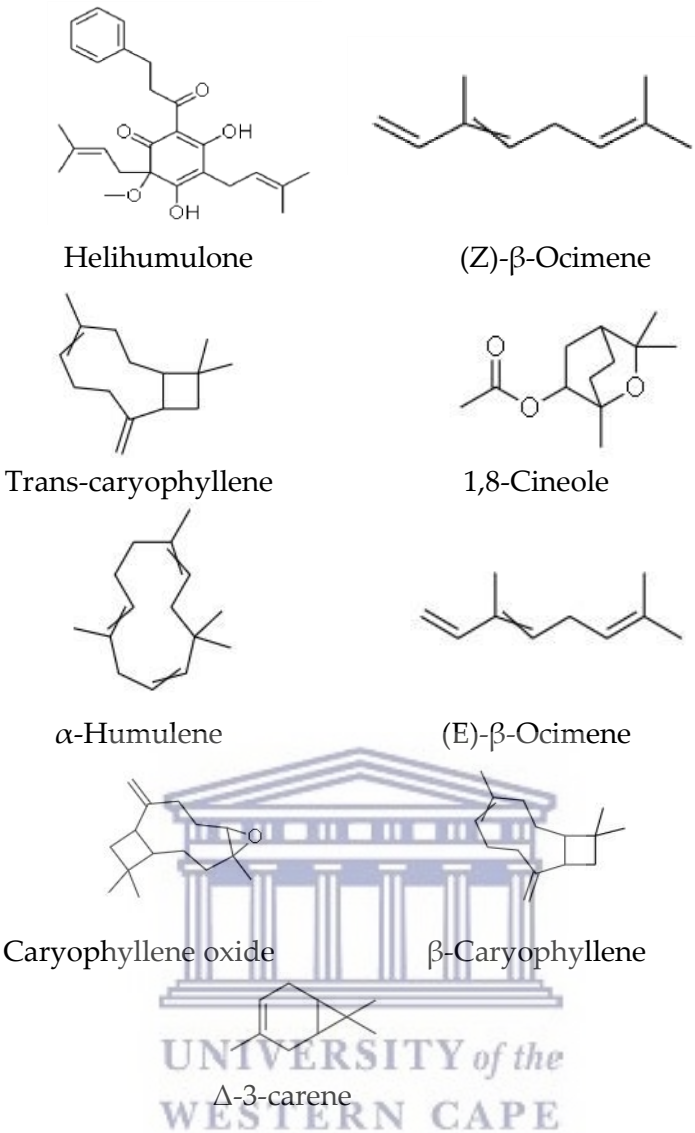

Figure 3.3. Structures of phytochemicals isolated from *Helichrysum cymosum*.

Table 3.2. Description of bioactivity of the compounds *Helichrysum foetidum*.

Compounds	Biological Functions
6^0-methoxy-2^0, 4, 4^0-trihydroxychalcone	Anti-bacterial, anti-fungal, anti-ulcerogenic [43]
6^0-methoxy-2^0,4-dihydroxychalcone- 4^0-O-β-D-glucoside	Anti-bacterial, anti-fungal, anti-ulcerogenic [43]
Kaur-16-en-18-oic acid	Anti-bacterial, anti-fungal, anti-ulcerogenic [43]
Apigenin	Anti-bacterial, anti-fungal, anti-ulcerogenic [43]
Apigenin-7-O-β-D-glucoside	Anti-bacterial, anti-fungal, anti-ulcerogenic [43]
7,4^0-dihydroxy-5-methoxy-flavanone	Anti-bacterial, anti-fungal, anti-ulcerogenic [43]
4,5 –diacaffeoyl quinic acid	Anti-bacterial, anti-fungal, anti-ulcerogenic [43]
Ferulic acid	Anti-inflammatory, anti-oxidant, anti-diabetic, anti-hypertensive [58]
Chlorogenic acid	Anti-oxidant, anti-inflammatory, Anti-bacterial, anti-mutagenic and anti-cancer [59]
Campest-7-en-3-ol	Anti-bacterial, antifungal, anti-ulcerogenic [43]

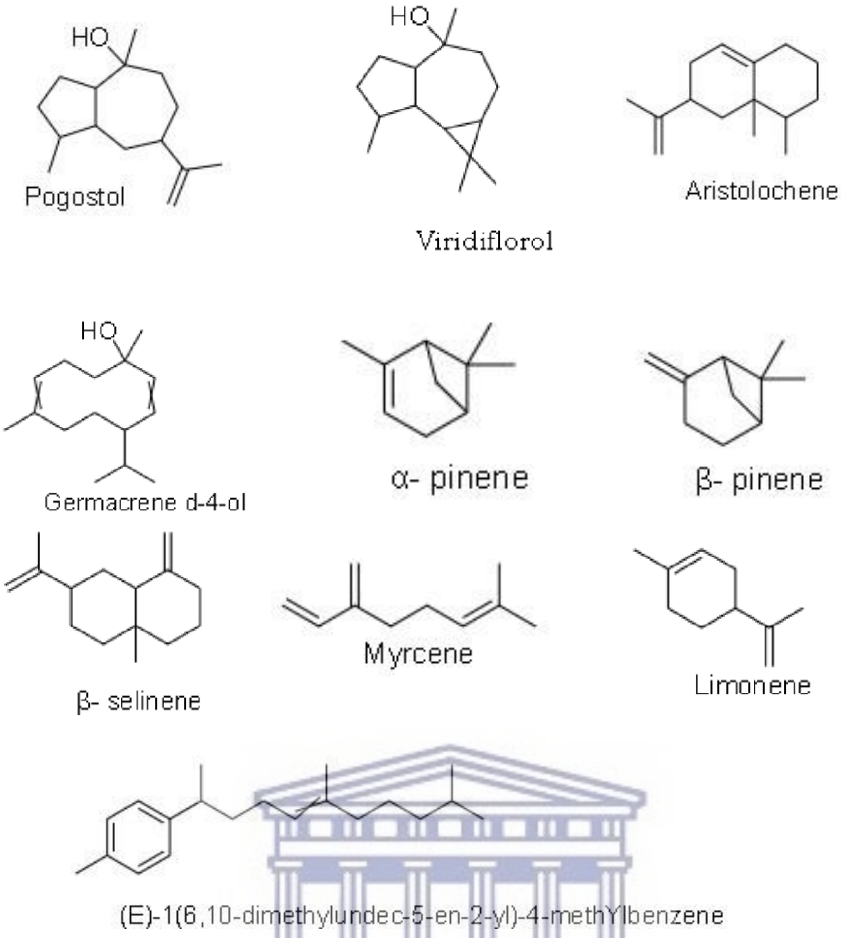

Figure 3.4. Structures of phytochemicals isolated from *H. pandurifolium*.

Table 3.3. Description of bioactivity of the compounds of *Helichrysum petiolare*.

Compounds	Biological Functions
Alloaromadendrene	Anti-microbial, anti-viral, anti-diabetic, anti-inflammatory [60]
δ-Selinene	anti-fungal [61]
Phenol, 2-ethoxy-5- (1, 2-dihydroxyethyl) -	N/A
Phytol	Anti-oxidant, anti-microbial, anti-convulsant, cytotoxic, anti-inflammatory [62]
Ledol	N/A
α-Calacorene	Anti-microbial, anti-oxidant [63]
β-Hydroagarofuran	Anti-bacterial and anti-mycotic [15]
Spathulenol	Anti-inflammatory, anti-nociceptive [64,65]
α-Bulnesene	Anti-platelet aggregation agent [66]
δ-Cadinene	Anti-malarial [67]

Not available: N/A.

Table 3.4. Description of bioactivity of the compounds of *H. pandurifolium*.

Compounds	Biological Functions
Viridiflorol	Anti-fungal, anti-bacterial [14]
Pogostol	N/A [14]
α-pinene	N/A [14]
β-pinene	N/A [14]
Aristolochene	N/A [14]
(E)-1(6,10-dimethylundec-5-en-2-yl)-4-methylbenzene	N/A [14]
Z-β-ocimene	N/A [14]
δ-cadinene	N/A [14]
Germacrene d-4-ol	N/A [14]
1,8-cineole	N/A [14]

Not available: N/A.

8. Essential Oils Present in Selected *Helichrysum* Species

Essential oils (EOs) are the condensed or concentrated hydrophobic liquids that contain volatile chemical compounds derived from plants. The variety of compounds in EOs depends on such factors as the plant species, plant part used (leaves, fruits, roots), harvest period, environmental conditions (land fertility, humidity, temperature) and the extraction technique employed [68].

One distinct characteristic of the *helichrysum* genus is its aromatic nature and most of the species in this genus have been reported to be rich in essential oils (EOs) containing many compounds [10]. Many EOs are prescribed as alternative medicine (in aromatherapy) based on the healing effects of their aromatic compounds [69]. Monoterpenes, sesquiterpenes and diterpenes are some of the broad groups of compounds present in the EOs of most plants, including the *helichrysum* genus; these compounds are largely responsible for the reported anti-fungal, anti-bacterial, anti-diabetic, anti-inflammatory, anti-ulcer, anti-cancer, anti-oxidant, anti-nociceptive, and anti-spasmodic properties associated with these plants.

Table 3.5. The common phytochemical compounds present in the essential oils of selected *Helichrysum species*.

Helichrysum Species	Plant Parts	Compounds	Method of Analysis	Pharmacological Activity	References
H. petiolare  **A growing *Helichrysum petiolare* plant [70] SANBI available online http://pza.sanbi.org/helichrysum-petiolare (accessed on 19 June 2021)**	Leaves	α-pinene (6.8%), 1, 8-cineole (22.4), p-cymene (9.8%) and β-caryophyllene (14%)	G.C.-M.S.	Anti-fungal, anti-inflammatory	[38]
	Whole plant	(*E*)-Longipinane (11.79%), *trans*-Geranylgeraniol (11.68%), Phytol (11.28%) Geranyllinalool (11.13%) and α-Eicosane (12.07%)	G.C.-M.S.	Anti-microbial, anti-inflammatory	[71]
H. cymosum  **A growing *Helichrysum cymosum* plant [72] SANBI available online: http://pza.sanbi.org/helichrysum-cymosum-subsp-cymosum (accessed on 19 June 2021)**	Leaves, Flowers	Δ-3-carene (16.1%), β-caryophyllene (12.0%)	G.C., G.C.-M.S.	Anti-fungal	[73]
	Flowers	Monoterpenes (77.9%)	G.C.-M.S. G.C., G.C.-M.S.	Anti-inflammatory	[15]
	Leaves, Flowers	(Z) -β- ocimene	G.C.-M.S.	-	[74]
	Leaves	α-pinene (12.4%), 1, 8-cineole (20.4%), β-caryophyllene (10.8%)		Anti-bacterial	[38]

Table 3.5. *Cont.*

Helichrysum Species	Plant Parts	Compounds	Method of Analysis	Pharmacological Activity	References
H. foetidum  **A growing *Helichrysum foetidum* plant [39] SANBI available online http://pza.sanbi.org/helichrysum-foetidum (accessed on 19 June 2021)**	Leaves, flower	B-pinene (3.1%), Trans-Sabiene hydrate (1.8%), 4-terpineol (3.1%), β-caryophyllene (2.5%)	G.C.-M.S.	Anti-microbial Anti-inflammatory	[75]
H. pandurifolium  **A growing *Helichrysum pandurifolium* plant [76] iNaturalist. Available online: https://www.inaturalist.org/observations/23571154 (accessed on 19 June 2021)**	N/A	N/A	N/A	N/A	

Gas chromatography: G.C.; Gas chromatography mass spectrometry: G.C.-M.S.; Not available: N/A.

9. Conclusions

Plants belonging to the *Helichrysum* genus are a vital source of traditional medicines in many parts of the world, including South Africa. The many novel chemical compounds present in the extracts of these plants and their essential oils account for most of their pharmacological actions. This review highlighted the reported diversity of the *Helichrysum* genus and the ethnomedicinal and biological activities of some of its species. However, only limited scientific reports are available in the literature on some species, hence further multidisciplinary studies by botanists, chemists, ethnopharmacologists and medical scientists are required on all the plants in this genus, as they appear to be potential sources of useful bioactive medicinal compounds that could be exploited in the drug discovery and development value chain.

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CHAPTER FOUR

The importance of secondary metabolites, specifically flavonoids, with an array of health benefits used in the nutraceutical, pharmaceutical, medicinal, and cosmetic industries is explained in this chapter. We identified and quantified different flavonoids from selected *Helichrysum* species extracts and evaluated the effect of these plant extracts on different cell lines to determine the cytotoxicity or cell viability properties. It was revealed that *Helichrysum* species could be further explored as essential phytochemical-based drugs.

Abstract

Distinctive aromatic herbs and shrubs, the *Helichrysum* genus of the Asteraceae family are widely distributed globally, with origin from South Africa, are well known for their health benefits attributed to the array of bioactive secondary metabolites. Flavonoids are crucial secondary metabolites with indispensable uses in nutraceutical, pharmaceutical, medicinal and cosmetic industries. Hence, this work aims to identify and quantify the flavonoid constituents from 90% aqueous acetone extract of four selected *Helichrysum* species. These are aqueous acetone *Helichrysum petiolare* extract (AAHPE), aqueous acetone *Helichrysum pandurifolium Schrank* extract (AAHPSE), aqueous acetone *Helichrysum foetidum* extract (AAHFE), and aqueous acetone *Helichrysum cymocum* extract (AAHCE). *In vitro* cell survival or viability determined the effect of these extracts on SHSH-5Y and HepG2 cell lines. The identification and quantification using LC-MS analysis were performed, while MTT cytotoxicity determined the cell survival. Thirty-eight (38) flavonoids, which are all present in AAHPE and AAHPSE, were identified, while flavonoids such as arbutin and quercetin-3-O-(feruloyl) sophoroside were absent in AAHFE and only compound, dactylin was absent in AAHCE. It is worth mentioning that the extracts' effect was cell line dependent, offering somewhat survival and

protection. Overall, AAHPE has profound cell survival or protection potential in both cell lines, which could be attributed to more and high flavonoid content compared with other extracts. The result indicated that these *Helichrysum* species could be further explored as essential phytochemical-based drugs.

Keywords: Flavonoids, *Helichrysum*, LC-MS, cytotoxicity, medicinal plants, phytochemicals

4.0 The analysis of bioactive flavonoids and determination of cytotoxicity activity of selected *helichrysum* species extracts

4.1 Introduction

Natural compounds are numerous, and the largest known classes in plants are flavonoids and their glycosides. Flavonoids are ubiquitous secondary metabolites of diphenylpropane (C6-C3-C6), having different numbers of hydroxyl groups attached to the ring structure. Flavonoids generally consist of fifteen carbons aromatic rings A and B linked to a heterocyclic C-ring. Flavonoids are classified into flavonols, flavones, isoflavones, anthocyanidins and flavanols (Ignat et al., 2011; Kumar, 2017). The biological and physiological functions of flavonoids are numerous, with growing interest because of their antioxidant activities mainly linked to their structure (Zhang et al., 2020).

The chemical structure of flavonoids makes them hydrogen-donating (radical scavenging) and metal-chelating agents, which protect the plant against pathogen attack, ultraviolet light and oxidative cell injury (Cuyckens & Claeys, 2004; Ignat et al., 2011; Kumar, 2017). While for specific biological or pharmacological activities, flavonoids functions include anti-inflammatory, antioxidant, anticancer, antidiabetic, antihypertensive neuroprotective, hepatoprotective, antiviral, anti-ulcer, antibacterial, antifungal (Cuyckens & Claeys, 2004; Panche et al., 2016; Raffa et al., 2017; Salih et al., 2017).

Flavonoids are broad-spectrum components with vast application in the nutraceutical, pharmaceutical, medicinal and cosmetics industries, stimulating essential cellular enzyme processes; this ability promotes their different functions in these industries. The health-promoting effects or roles of flavonoids in various disease conditions indicate its immense contribution as outlined in scientific investigations (Albalawi et al., 2015; Beking & Vieira, 2010; LeJeune et al., 2015; Marzocchella et al., 2011). Human and animal health disease

conditions are ameliorated or abrogated with flavonoids with immense therapeutic and chemopreventive properties (Panche et al., 2016.

Flavonoids are innumerable, consisting of over 10, 000 structures dependent on different factors, such as plant species, developmental stage, organ, and growth conditions. The analysis of the flavonoids is cumbersome because of the array or diversity of these different structures. They have three-ring structures (C6-C3-C6), partially originating from hydroxylation, methylation, and glycosylation that determine their functionalities. Flavonoids are semi-polar compounds with non-polar core structures having various polar hydroxyl groups and carbohydrate moieties already modified; hence, their extraction is favoured by polar solvents such as methanol, ethanol, acetonitrile, and acetone. (Stalikas, 2007). Also, mixtures of polar organic solvents with water increase the extraction and efficiency of glycosylated flavonoids and other more polar phenolic acids in medicinal plants.

The analysis of flavonoids constituents with liquid chromatography (LC) is prompted by the characteristic's hydroxylation, methylation, glycosylation of the flavonoids, while Mass Spectrometry (MS) analysis is prompted by the aromatic rings and phenolics hydroxyl group constituents. There are many established methods such as spectrophotometry capillary electrophoresis immunofluorescence that quantify flavonoids; however, the LC-MS method has a better profile in terms of specificity, resolution, accuracy, and lower disturbance (Yang et al., 2009; Zhang et al., 2020). Over the years, flavonoids have been studied, and the focus has been on the antioxidant effect, pharmacological effect, and the effect of the different extractions utilised (Lee et al., 2018; Zhang et al., 2020).

However, limited quantitative studies or investigations of flavonoids warrant or propel research on quantifying flavonoids (Tang et al., 2014). Biological samples like medicinal plant extract are quantified using target analysis (Lu et al., 2008). Thus, LC-MS remain a veritable

analysis method, especially for flavonoids, which could be explored in the selected *Helichrysum* species from South Africa.

Helichrysum petiolare is referred to as a silverbush everlasting plant species, a shrub plant with grey or silver-grey hair covering a round-shaped leaf and the white coloured flower containing various phytochemicals, such as phenolic, flavonoids, essential oils, among other things, with reported traditional and scientific investigations (Akinyede et al., 2021).

Helichrysum pandurifolium Schrank, known as fiddle leaf-strawflower, is a tall, slender, loosely branched, soft shrub with orbicular-to-ovate leaves that are narrow at the broad petiole-like base. The flowers are yellow, and the outer and inner parts of the involucre bracts, respectively, light brown and whitish pink. This species' phytochemical and pharmacological actions remain elusive (Akinyede et al., 2021).

Helichrysum cymocum is a yellow-tipped strawflower or gold carpet that grows very short, having woolly, greyish silver leaves and bright, yellow-coloured flowers in flat heads. Various traditional uses and pharmacological activities are highlighted in recent work (Akinyede et al., 2021). *Helichrysum foetidum* has a characteristic pungent smell and is referred to as a stinging starflower. The leaves are elliptic, sparsely hairy, white-woolly arranged from base of the stem in groups, and its many flowers are broad. The folkloric and pharmacological use shows its potential in treating many ailments attributed to many of its phytochemicals, especially flavonoids (Akinyede et al., 2021).

The extracts of *Helichrysum* species have been reported for their antioxidant activities; and information on the crude extract for potentially treating many disease conditions (Akinyede et al., 2021). However, some of the phytochemical constituents are under-explored. Thus, this work aims at the bioactive flavonoid constituents by using LC-MS analysis to identify and quantify the flavonoids of AAHPE, AAHPSE, AAHFE and AAHCE. In addition, we

investigated the effects of these extract on the cell survival or viability on selected cell line vis a vis HepG2 and SH-SY5Y.

4.2. Material and Methods

4.2.1. Collection of plant material

Four *Helichrysum* species were collected from the Western Cape in the environment of the Cape Peninsula University of Technology (CPUT), Bellville, in October 2020 and samples were identified by Prof. Christopher N. Cupido of the Department of Botany, University of Fort Hare, Alice, South Africa. The accession numbers of the *Helichrysum* species in this study were *Helichrysum petiolare*-UFH-2020-10-01, *Helichrysum cymosum*-UFH 2020-10-02, *Helichrysum foetidum*-UFH, 2020-10-03 and *Helichrysum pandurifolium*-UFH 2020-10-04.

4.2.2. Plant extraction

The leaves of the plants were cleaned and air-dried to a constant weight, and the dried samples were pulverised using an electronic blender, grounded and weighed. The powdered plant materials in conical flasks were soaked and subjected to intermittent stirring in 90% aqueous acetone and warmed in the water bath at 60°C for two hours (Nasr et al., 2019). The mixture was filtered with Whatman cellulose filter paper under pressure using a pump, and the plant material was subjected to a second extraction after soaking overnight and the filtrate pooled before rotary evaporation. The final residue or extract obtained was allowed to dry in the fume cupboard and stored at -20°C until required for use as AAHPE, AAHPSE, AAHFE and AAHCE. In addition, we investigated the effect of these extracts on the cell survival or viability of selected cell lines vis-a-vis HepG2 and SH-SY5Y.

4.2.3 Liquid chromatography-mass spectrometry analysis.

Liquid chromatography-mass spectrometry analysis was performed using the method of Stander et al. (2017), with slight modification. The UPLC-MS analysis was performed with

Waters Synapt G2 quadrupole time-of-flight (QTOF) mass spectrometer (MS) connected to a water Acquity ultra-performance liquid chromatography (UPLC) (Waters, Milford, MA, USA). Electrospray ionisation was used in negative mode with a cone voltage of 15V, desolvation temperature of 275°C, desolvation gas at 650 L/h, and the rest of the MS settings optimised for the best resolution and sensitivity.

Data were acquired by scanning from 150 to 1500 m/z both resolution and MSE mode. Two channels of MS data were obtained in MSE mode, first at low collision energy (4V) and then using a collision energy ramp (40–1000) to obtain the fragmentation data. Leucine enkephalin was used as a locked mass (reference mass) for accurate mass determination, and the instrument was calibrated with sodium formate. Separations were achieved on a 150 mm HSST3 column. The injection volume of 3 µL was used as a mobile phase consisting of 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid as solvent B. The gradient started at 100% solvent A for one minute and changed to 28% B over 22 minutes linearly. It changes to 40% B over the 50s, and a wash step of 1.5 minutes at 100% B was achieved after re-equilibration to the initial condition for four minutes. The flow rate was 0.3 ml/min, and the column temperature of 55°C was maintained.

4.2.4. Cell viability assay

The survival or proliferation of the cells was determined using the colourimetric [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] (MTT) dye reduction assay. SH-SY5Y and HepG2 cells were seeded at a density of 5000 cells per well and allowed to attach for 24 hours. The AAHPE, AAHPSE, AAHFE and AAHCE were treated in increasing concentrations of (25–100 µg/mL) for 48 hours, with blank (only medium) and negative control (seeded cells without treatment).

After 48 hours, all wells, including the treated, blank and control, were incubated with 10 µl of the MTT solution (5 mg/mL) for four hours. The medium was carefully removed from

each well, and 100 µL of DMSO (Dimethyl Sulfoxide) to solubilize the purple formazan crystals was added. Optical density (OD) was read at 570 nm using a microplate reader (BMG Labtech Omega® POLARStar), and the mean cell proliferation was calculated relative to control. The IC_{50} was calculated using GraphPad Prism6 software (GraphPad Software, San Diego, CA, USA) from three experimental repeats.

$$\text{Percentage (\%) cell proliferation} = \frac{\text{Optical density of treated cells}}{\text{Optical density of control cells}} \times 100\%$$

4.3. Results

The LC-MS based flavonoids of the AAHPE, AAHPSE, AAHFE and AAHCE in negative modes revealed the presence of 38 flavonoidss as shown in Table 4.1.

Table 4.1. Liquid chromatography-mass spectrometry analysis of bioactive flavonoid constituents of AAHPE, AAHPE, AAHFE and AAHCE

s/n	Bioactive compounds	Average R_t(min)	AAHPE Conc. (mg/g)	AAHPSE Conc. (mg/g)	AAHFE Conc. (mg/g)	AAHCE Conc. (mg/g)
1	Arbutin	5.475	1279.2	330.3	0.0	2.0
2	Protocatechuic acid 4-O-glucoside	7.913	182.9	151.7	0.5	10.5
3	3-Caffeoylquinic acid (neochlorogenic acid)	9.609	120.2	169.2	9.8	118.7
4	Chlorogenic acid (5-caffeoylquinic acid)	11.975	826.7	413.7	13.3	570.8
5	Caffeic acid derivative	12.758	134.4	132.0	21.6	99.1
6	UNPD8659	13.638	131.8	44.7	0.0	3.1
7	Dactylin	14.234	55.0	41.3	0.4	0.0
8	1,3-Dicaffeoylquinic acid	14.433	388.7	37.5	3.8	17.7
9	UNPD204949	14,769	78.6	23.7	0.4	2.7
10	UNPD114029	14.968	167.4	29.4	0.4	68.6
11	Quercetin-3-O-(feruloyl) sophoroside	15.203	71.6	68.5	0.0	0.3
12	5-Feruloyl quinic acid	15.296	411.6	108.9	2.1	57.8
13	3-O-p-Coumaroylquinic acid	15.54	82.7	79.0	0.7	21.2
14	Sakuranin	15.562	3.5	0.5	609.6	97.0
15	Myricetin 3-galactoside	15.779	402.5	77.2	1.6	49.1
16	4-Feruloyl quinic acid	16.016	363.2	10.6	1.1	3.7
17	Quercetin 3-galactoside	16.285	586.3	298.4	5.0	159.9
18	3-O-Caffeoyl-4-O-methylquinic acid	16.515	407.1	155.8	6.9	178.9
19	Sinapic acid	17.052	14.9	289.4	60.9	48.8
20	Engeletin	17.334	749.7	60.4	2.0	20.0

21	Rutin	17.426	12.8	690.8	1.1	2.0
22	Quercetin 3-glucoside	17.656	118.5	11.5	6.5	418.6
23	Kaempferol galactoside (Trifolin)	18.06	166.2	75.1	3.6	1.5
24	UNPD80025	18.352	104.2	29.3	0.0	0.7
25	1,4-Dicaffeoylquinic acid	18.633	258.9	34.2	7.1	34.5
26	3,4-Dicaffeoylquinic acid	18.797	535.3	482.5	29.8	997.9
27	3,5-Dicaffeoylquinic acid	19.236	1727.3	873.5	159.4	790.4
28	Kaempferol glucoside (Astragalin)	20.078	13.4	77.5	796.3	7.0
29	4,5-Dicaffeoylquinic acid	20.34	1209.1	1173.2	81.0	1779.6
30	Sakuranetin	20927	6.1	7.2	932.4	864.6
31	Sinocrassoside A1	21.036	127.1	2.5	2.5	82.6
32	Cascaroside C	21.652	116.3	4.9	0.4	14.0
33	Feruloyl quinic acid	22.12	120.5	29.2	2.8	7.2
34	3,5-Dicaffeoylquinic methyl ester	23.102	319.0	346.1	20.8	255.3
35	Sakuranin isomer	23.403	4.5	7.9	2620.4	84.8
36	Kaempferol	24.086	27.1	353.4	1965.9	149.7
37	Luteolin	24.32	1.9	0.8	215.1	1.1
38	Sakuranetin isomer	24.447	2.0	12.9	3174.7	1449.8

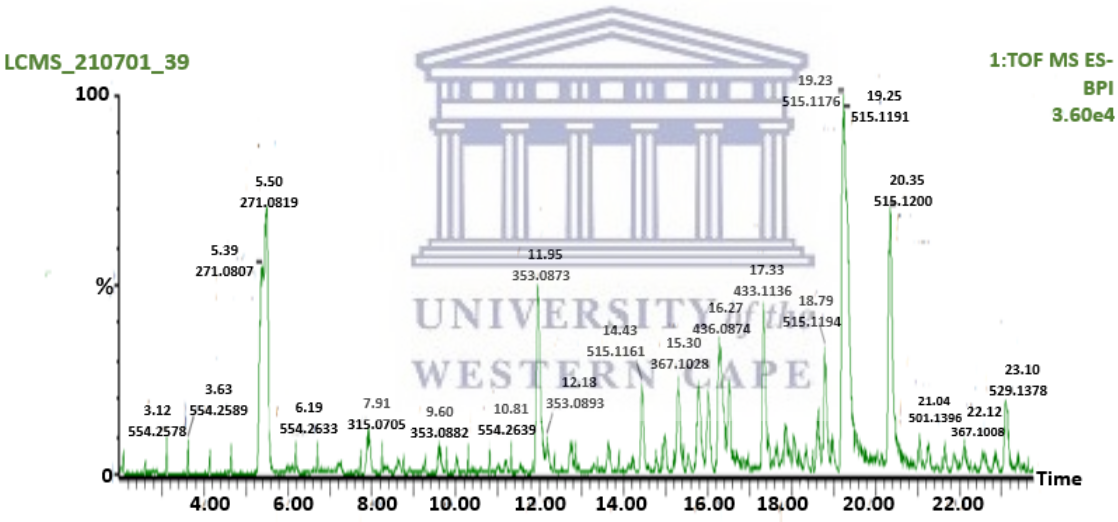


Figure 4.1. Liquid chromatography-mass spectrometry chromatogram of AAHPE

The quantitative assessment of the flavonoid constituents is indicated in their concentrations. The quantitative assessment indicated the following predominant compounds, 3,5-dicaffeoylquinic acid (1727.3 mg/g), arubitin (1279.7 mg/g), 4,5-caffeoylquinic acid (1209.1 mg/g), 5-caffeoylquinic acid (826.7 mg/g), engeletin (747.9 mg/g), quercetin 3-galactoside (586.3 mg/g), 5-feruloyl quinic acid (411.6 mg/g), 3-O-caffeoyl-4-O-methylquinic

acid (407.1 mg/g), myricetin 3-galactoside (402.5 mg/g), dicaffeoylquinic acid (388.7 mg/g) in AAHPE as revealed in the LC-MS chromatogram (Figure 4.1).

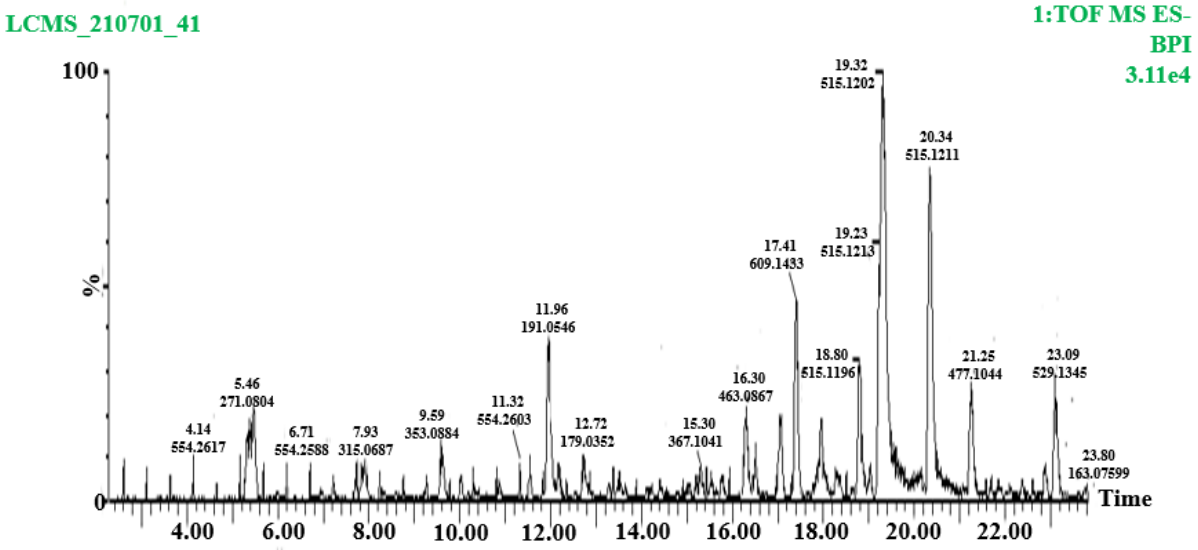


Figure 4.2. Liquid chromatography-mass spectrometry chromatogram of AAHPSE

For AAHPSE, the quantitative assessment indicated the following predominant compounds in different concentrations that include 4,5-dicaffeoylquinic acid (1173.2 mg/g) 3,5-dicaffeoylquinic acid (873.5 mg/g), rutin (690.8mg/g), 3,4-dicaffeoylquinic acid(482.5 mg/g), chlorogenic acid (5-caffeoylquinic acid) (413.7 mg/g), kaempferol (353.4 mg/g) arbutin- (330.3 mg/g), quercetin 3-galactoside-(298.4 mg/g), sinapic acid(289.4 mg/g) 3-caffeoylquinic acid (neochlorogenic acid) (169.2 mg/g) as revealed in the LC-MS chromatogram (Figure 4.2).

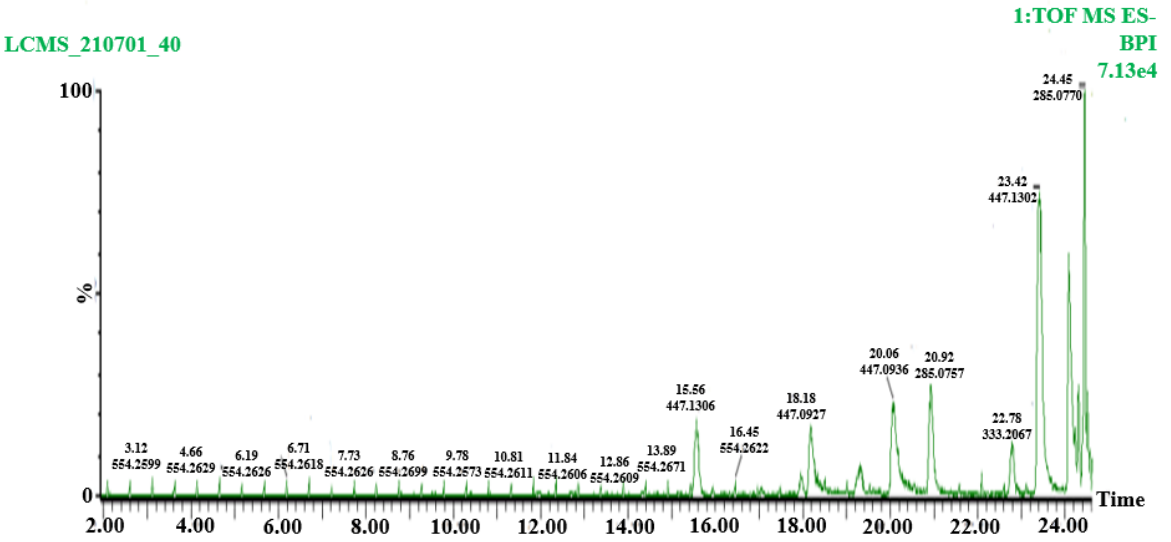


Figure 4.3. Liquid chromatography-mass spectrometry chromatogram of AAHFE

Also, the AAHFE quantitative assessment indicated the following predominant compounds in different concentrations including the sakuranetin isomer (3174.7 mg/g), sakuranin isomer- (2620.4 mg/g), kaempferol (1965.9 mg/g), sakuranetin (932.4 mg/g), kaempferol glucoside (Astragalin) (796.3 mg/g), sakuranin (609.6 mg/g), luteolin (215.1 mg/g), 3,5-dicaffeoylquinic acid (159.4 mg/g), 4,5-dicaffeoylquinic acid (81.0 mg/g), sinapic acid (60.9 mg/g) as revealed in the LC-MS chromatogram (Figure 4.3).

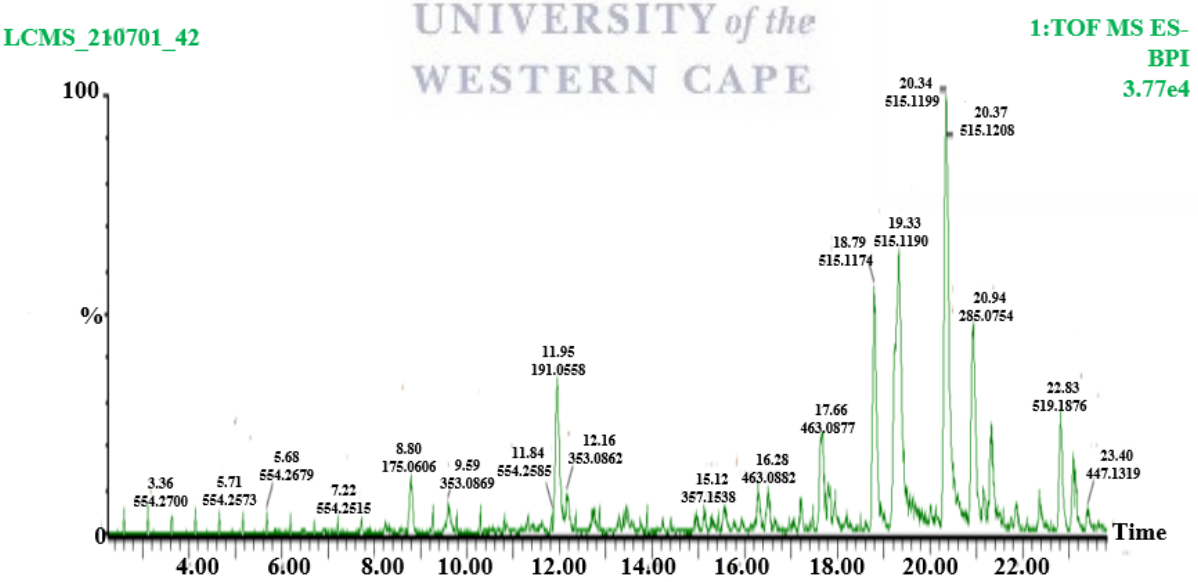


Figure 4.4. Liquid chromatography-mass spectrometry chromatogram of AAHCE

While in AAHCE, the quantitative assessment indicated the following predominant compounds in different concentrations of 4,5-dicaffeoylquinic acid (1779.6 mg/g), sakuranetin isomer (1449.8 mg/g), 3,4-dicaffeoylquinic acid (997.9mg/g), sakuranetin (864.6 mg/g), 3,5-dicaffeoylquinic acid (790.4 mg/g), 3-caffeoylquinic acid (570.8 mg/g), quercetin 3-glucoside 418.6 mg/g), 3,5-dicaffeoylquinic methyl ester 255.3 (mg/g), quercetin 3-galactoside (159.9 mg/g), kaempferol (149.7 mg/g) as revealed in the LC-MS chromatogram (Figure 4.4). Overall, AAHPE has the highest constituents of flavonoids in all the *Helichrysum* species, and with subsequent safety obtained, the AAHPE was then chosen for the study.

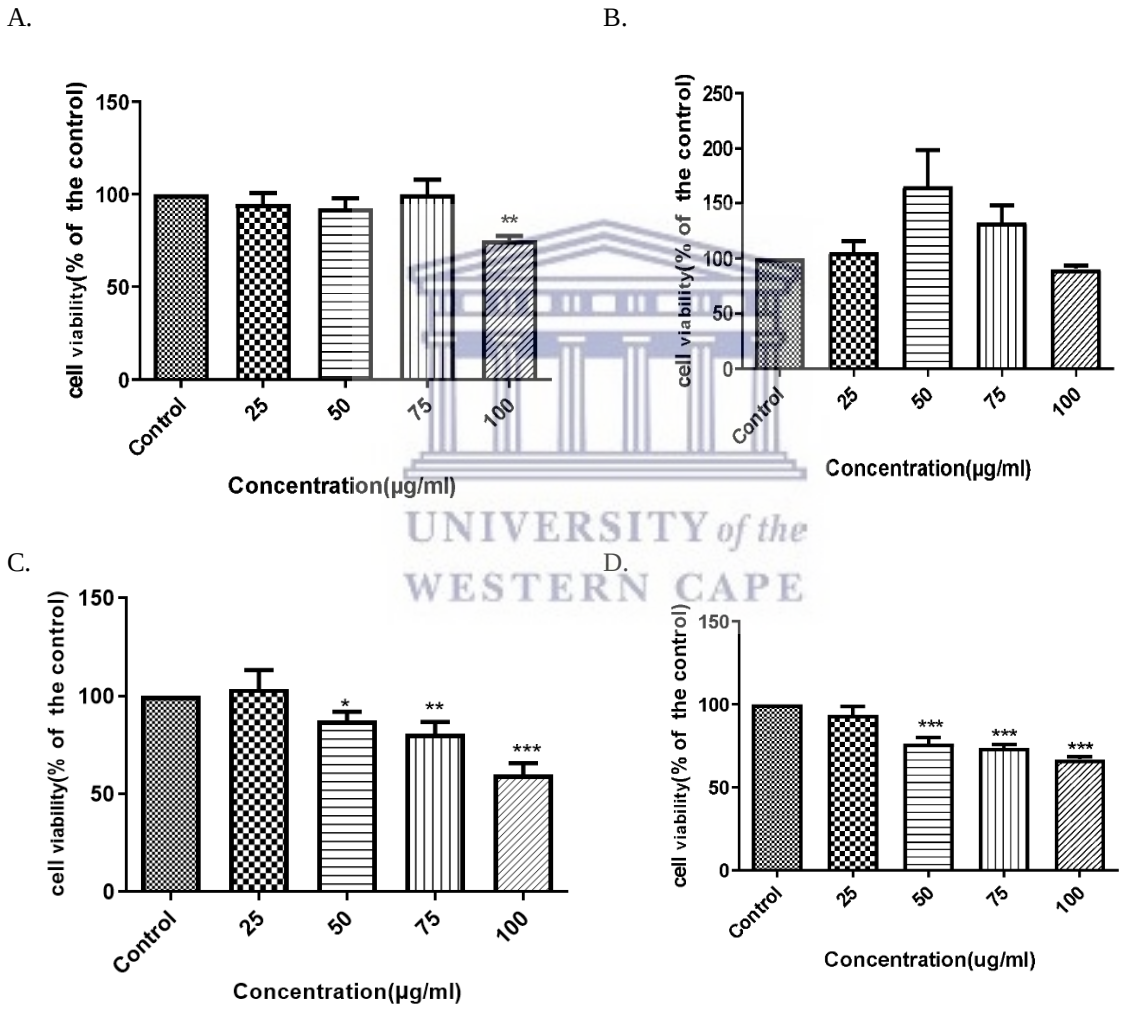
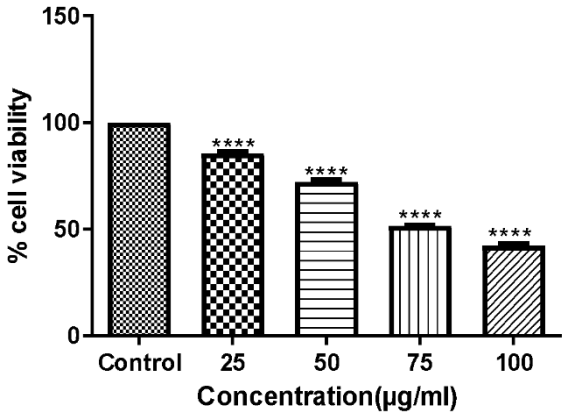


Figure 4.5. Cytotoxicity study of *Helichrysum* species on SH-SY5Y cell. (a*) Helichrysum pandurifolium* extract (b) *Helichrysum petiolare* extract (c) *Helichrysum foetidum* extract (d) *Helichrysum cynmosum* extract. The values are expressed as the mean ± SD (n=3) *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 compared with the control.

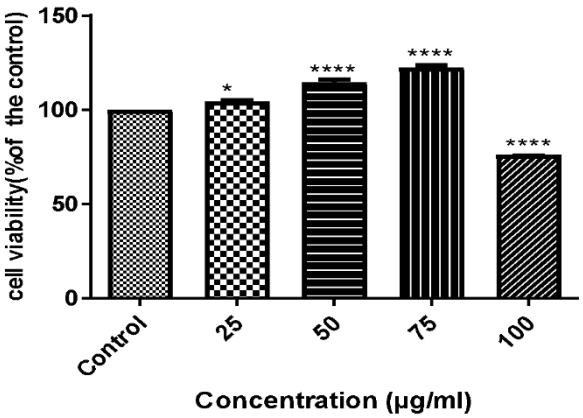
The different extracts of *Helichrysum* species of 25–100 µg/ml concentrations were used for treating SH-SY5Y cells for 48 hours. In the *H. pandurifolium* extract, 25–75 µg/mL concentrations had over 80% to 100% cell viability, while 100 µg/mL had less than 75% cell viability (Figure 4.5a). The *H. petiolare* extract showed that all the concentrations (25–100 µg/mL) tested had over 100% cell viability (Figure 4.5b). The decline in cell viability was noticeable in the 100 µg/mL concentration, which still had over 90% viability. *H. foetidum* extract shows cell viability of over 100% from the lowest concentration of 25 µg/mL and over 85% in 50 µg/mL, which declined to 80% cell viability in 75 µg/mL; however, a reduction of 60% viability was observed in 100 µg/mL concentration (Figure 4.5c). The *H. cynmosum* extract had over 90% cell viability at 25 µg/mL concentration, while 50 µg/mL, 75 µg/mL, and 100 µg/mL concentration had 95%, 73% and 66% cell viability on the SHY-SY cell line (Figure 4.5d). However, we decided to use 25–100 µg/mL for a further study being the concentrations with the highest viability among the *Helichrysum* species tested on the SH-SY5Y cell line.

We have indicated that *H. petiolare* was chosen because it is proven to have a better profile of safety based on the result of cytotoxicity from other *Helichrysum* species. Data were expressed relative to 100% of the control, mean ± standard deviation; the experiment was carried out at three independent experiments, analysed with one-way ANOVA followed by the Dunnet's test with a significant difference.

A.



B.



C. D.

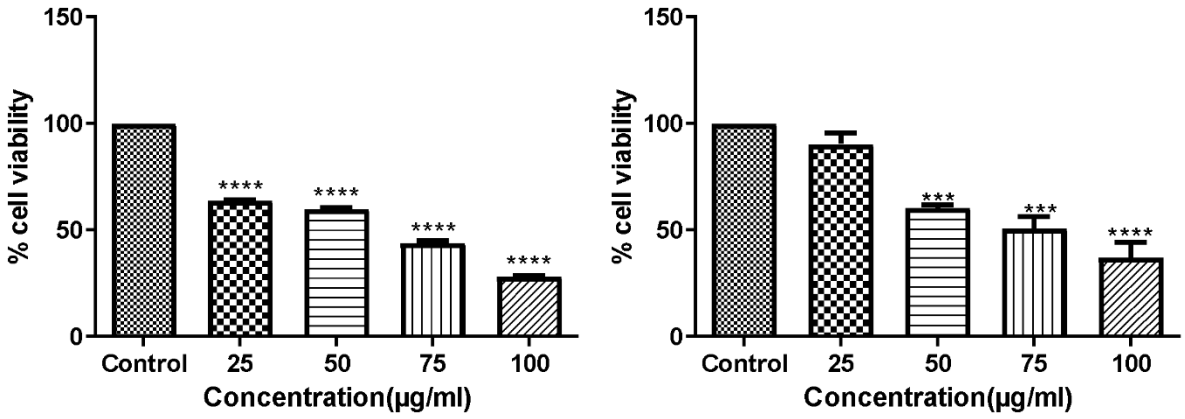


Figure 4. 6. Cytotoxicity study of *Helichrysum* species on HepG2 cell line. (a) *Helichrysum pandurifolium* extract (b) *Helichrysum petiolare* extract (c) *Helichrysum foetidum* extract (d) *Helichrysum cynmosum* extract. The values are expressed as the mean ± SD (n=3) *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 compared with the control.

The different extracts of *Helichrysum* species of 25–100 µg/mL concentrations were used for treating the HepG2 cell line for 48 hours. In *H. pandurifolium* extract, the decrease in cell viability was concentration-dependent, which revealed that concentrations 25 µg/mL, 50 µg/mL, 75 µg/mL and 100 µg/mL had cell viability of 85%, 71%, 52% and 42% respectively (Figure 4.5a). The *H. petiolare* extract showed that the concentrations (25–75 µg/mL) had over 75% cell viability but declined to 50% for the 100 µg/mL concentration (Figure 4.5b). The *H. foetidum* extract showed an appreciable decline in cell viability of the concentrations (25–100 µg/mL) ranging from 90% to 33% cell viability (Figure 4.5c). *H. cymosum* showed more appreciable decline in cell viability of the concentrations, 25–100 µg/mL, ranging from 66–27% cell viability (Figure 4.5d).

We decided to use 25–75 µg/mL for a further study being the concentrations with the highest viability of *H. petiolare* on the HepG2 cell line. *H. petiolare* was chosen because it is proven to have a better profile of safety based on the result of cytotoxicity from other *Helichrysum* species. Data were expressed relative to 100% of the control, mean ± standard deviation; the experiment was carried out at three independent experiments, analysed with one-way ANOVA followed by the Dunnet's test with a significant difference.

4.4. Discussion

The need for new or novel substitute drugs is essential and medicinal plants remain one of the most available, valuable, and suitable natural sources. Thus, exploring different classes of compounds is pivotal to the invention of new novel drugs. A class of compound, flavonoids, from medicinal plants, have not been fully elucidated for different disease conditions. Flavonoids have recently gained much attention in research relating to their use for treating and managing disease conditions because of their associated antioxidant activities. Since most disease conditions have been linked to oxidative stress, flavonoids are the repository or mainstay of compounds to mitigate or halt free radical scavenging events associated with the pathophysiology of such diseases.

We report the flavonoids of aqueous acetone extract of four selected *Helichrysum* species (*H. petiolare, H. pandurifolium, H. foetidum* and *H. cymocum)* using LC-MS. The results revealed that AAHPE and AAHPSE had all 38 flavonoids. The AAHPE showed a higher concentration of almost all the flavonoid constituents than other Helichrysum species (Table 4.1). While results also revealed that AAHFE does not demonstrate the presence of some flavonoids such as arbutin, quercetin-3-O-(feruloyl) sophoroside and two other unidentified compounds (UNPD8659 and UNPD80025) (Table 4.1) that can be studied in the future.

Furthermore, AAHCE does not reveal the presence of dactylin (Table 4.1). It is noteworthy that AAHFE revealed the presence of sakuranin, sakuranin isomer, kaempferol glucoside (Astragalin), luteolin and sakuranetin isomer in higher concentrations (Table 4.1). These results showed that *Helichrysum* species revealed different concentrations of flavonoids that could be advantageous in using these plant species to treat the various disease conditions.

As reported by many researchers, the extracts of some *Helichrysum* species have been used to treat diabetes and other disease conditions (Akinyede et al., 2021). Generally, flavonoid

constituents correlated to antidiabetic and cognitive decline ameliorative properties (Ademosun et al., 2016; Heidarianpour et al., 2021).

Extracts of the *Helichrysum* species did not induce cytotoxicity in SH-SY5Y; but a relative cytotoxic effect on HepG2 cell lines tested following 48 hours of exposure, as shown in this study. Noteworthy is that AAHPE showed more than 80% cell viability of all the concentrations tested on SH-SY5Y for AAHPE. However, different results were obtained for HepG2. The cell viability using MTT implies the ability of the active enzyme of the cell, mitochondrial dehydrogenase, to reduce or convert the blue formazan to purple colour. The degree or the intensity of the purple colour showed the enzyme's activity.

Although in most cytotoxicity assays, the benchmark of 80% indicates relative safety, which can further be used to set the therapeutic dose for the protective experimental study (Iso/TC 194, 2009; Nwakiban et al., 2020). All the concentrations of AAHPE (25–100 µg/ml) had 80% cell viability in the SH-SY5Y cell line. Different results were also obtained for the other extracts of *Helichrysum* species with varying cell viability differing from AAHPE.

Importantly, not all *Helichrysum* species used in this study showed cell viability potential on SH-SY5Y and HepG2 cell lines; however, pronounced cell viability was seen with AAHPE compared with other *Helichrysum* species extracts investigated in this study. The biological actions of the *Helichrysum* species could probably be because of flavonoids, compounds with unique chemical structures, which could be because of high concentrations and more of the identified flavonoids in AAHPE. Hence, the unique nature of the flavonoid contents of these *Helichrysum* species may contribute significantly to the discovery of phytochemical-based drugs.

The present study showed that the extract's flavonoid composition occurs in different amounts in the species investigated. Other phytochemicals were excluded except for flavonoids. Hence, it is plausible to suggest that the flavonoid contents may be related to most

of the biological activities demonstrated by *Helichrysum* species (Akinyede et al., 2021; Ben Haj Yahia et al., 2019; Popoolaet al., 2015; Tabassum et al., 2017).

Flavonoids identified from the *Helichrysum* species in this work, such as arubitin, quercetin, kaempferol, luteolin, engeletin, dicaffeoylquinic acid, chlorogenic acid, sinapic acid and other compounds possess hypoglycaemic or antidiabetics potential (Alaofi, 2020; Alkhalidy et al., 2018; Altındağ et al., 2021; Brahmachari, 2011; Choi et al., 2014; Li et al., 2021; Stolf et al., 2017). While the flavonoids, rutin, kaempferol glucoside (Astragalin), Kaempferol galactoside (Trifolin), 4,5-dicaffeoylquinic acid, quercetin 3-galactoside, kaempferol and other compounds present in these *Helichrysum* species are well known for their neuroprotective potential against cognitive decline conditions (Cheng et al., 2010; Khan et al., 2018; Lee, S. G. et al., 2011; Pan et al., 2020; Singh & Hembrom, 2018).

Thus, the cell viability results of AAHPE on SH-SY5Y and HepG2 cell lines probably revealed a cytoprotective effect in this study that could be attributed to the flavonoids' content of some of the *Helichrysum* species (Ishige et al., 2001; Jing et al., 2015). The cytoprotective effect linked to flavonoids' characteristics as potent free radical scavengers and their ability to interfere with the cell's oxidative/antioxidative potential promotes cell survival and proliferation (Chen et al., 2002; Granado-Serrano et al., 2007; Martín et al., 2014).

In conclusion, this work revealed the flavonoid constituents of AAHPE, AAHPSE, AAHFE and AAHCE using LC-MS analysis further supports the antioxidant activities of these species in our previous study (Akinyede et al 2022). The AAHPE has the highest cumulative amount of flavonoids in all the species of the *Helichrysum* investigated, which may give the prospect for its use. We could explore the variations in the distribution of the noticeable flavonoid components in oxidative stress-related conditions linked to the antioxidant activities. The antioxidant activities of these plant species could be a potential for drug discovery and development in future studies.

The findings on the cell survival or protective activities of the four species of *Helichrysum* investigated was found to support the claim that this plant family could provide new insights into the treatment and management of diabetes and the cognitive decline condition being oxidative stress-related disease if explored further. In future research, we hope to purify the extracts that showed promising activities to isolate the pure compounds responsible for the observed effects of those extracts and test these compounds on the mechanism of action in such disease conditions.

4.5 References

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CHAPTER FIVE

The importance of the medicinal plant constituents, especially the antioxidants and fatty acids constituents, is highlighted in addressing many disease conditions in this chapter. Natural antioxidants are said to be safe and potent compared with synthetic antioxidants. Safe and effective antioxidants, which promote health and are used to treat various ailments, need to be discovered and developed from nature. The relevance of antioxidants in treating oxidative stress-related disease conditions of diabetes and cognitive decline such as DACD cannot be overemphasized. Therefore, investigations of the antioxidants and GC-MS analysis that identify and quantify the selected *Helichrysum* species' fatty acids were investigated.

plants

Article

Comparative Study of the Antioxidant Constituents, Activities and the GC-MS Quantification and Identification of Fatty Acids of Four Selected *Helichrysum* Species

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Abstract: *Helichrysum* Mill. (Asteraceae) is a plant genus comprising distinctively of aromatic plants of about 500–600 species. Since most of these plants have not been previously studied, extensive profiling helps to validate their folkloric uses and determine their potential value as sources of plantderived drug candidates. This study, therefore, aims to investigate the antioxidant activity (DPPH, NO, FRAP); total antioxidant capacity, total phenolic, total flavonoid, and fatty acid compositions of the aqueous acetone extracts from four *Helichrysum* plants namely, *Helichrysum pandurifolium*, *Helichrysum foetidum*, *Helichrysum petiolare*, and *Helichrysum cymocum*. The results obtained showed that the *H. cymocum* extract had the best DPPH radical scavenging activity (IC_{50} = 11.85 ± 3.20 µg/mL) and *H. petiolare* extract had the best nitric oxide scavenging activity (IC_{50} = 20.81 ± 3.73 µg/mL), while *H. pandurifolium* Schrank extract (0.636 ± 0.005 µg/mL) demonstrated the best ferrous reducing power, all of which are comparable with results from ascorbic acid used as the standard. The IC_{50} values of the radical scavenging activity ranged from 11.85–41.13 µg/mL (DPPH), 20.81–36.19 µg/mL (NO), and 0.505–0.636 µg/mL (FRAP), for all the plants studied. The *H. petiolare* has the highest total antioxidant capacity (48.50 ± 1.55 mg/g), highest total phenolic content (54.69 ± 0.23 mg/g), and highest total flavonoid content (56.19 ± 1.01 mg/g) compared with other species. The fatty acid methyl esters were analysed using gas chromatography-mass spectrometry (GC-MS). The results obtained showed variations in the fatty acid composition of the plant extracts, with *H. petiolare* having the highest saturated fatty acid (SFA) content (7184 µg/g) and polyunsaturated fatty acid (PUFA) content (7005.5 µg/g). In addition, *H. foetidum* had the highest monounsaturated fatty acid (MUFA) content (1150.3 µg/g), while *H. cymocum* had the highest PUFA:SFA ratio of 1.202. In conclusion, the findings from this study revealed that *H. pandurifolium* Schrank, *H. foetidum*, *H. petiolare*, *and H. cymocum* are repositories of natural bioactive compounds with potential health-promoting benefits that need to be investigated, for both their antioxidant activity in a number of disease conditions and for further exploration in drug discovery and development projects.

Keywords: helichrysum; antioxidant; fatty acids; drug discovery and development; DPPH radical scavenging; total phenolics

1. Introduction

The discovery of natural, safe, and very effective antioxidants has highlighted the need to address health-related problems in recent years. The effectiveness and safety

of antioxidant use and the integrity of the body's antioxidant system are linked to healthy living and the prevention of both life and non-life-threatening diseases. Exogenous or dietary antioxidants work in tandem with the body's antioxidant system to protect against or combat reactive oxygen species (ROS),also known as free radicals, which cause oxidative stress [1,2]. The overproduction of ROS, such as superoxide anion (O_2^{-}), per hydroxy radical ($HOO^{\cdot}$), hydroxyl radical ($HO^{\cdot}$) singlet oxygen ($^1O^2$), and hydrogen peroxide (H_2O_2), involve consistent or persistent electron reductive pathways to molecular oxygen [3,4]. As a result, chain reactions or processes are created, which cause lipid peroxidation, leading to damage to cell membrane phospholipids, DNA, and protein molecules which are often implicated as oxidative stress inducers in cancer, diabetes mellitus, inflammation, stroke, immunosuppression, anaemia, and neurodegenerative diseases [5].

Antioxidants are chemicals that counteract the imbalances caused by oxidative processes, triggering a defence mechanism against the overproduction of free radicals. Natural, safe, and potent antioxidants that provide defence against the harmful effects or actions of free radicals are gaining immense interest in medical research, as they offer protection against free radicals. Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butyl hydroquinone (TBHQ), are linked to high levels of toxicity in humans and are generally expensive [6–9]. For instance, BHA, a synthetic phenolic antioxidant widely utilised in various sectors, affects endocrine functions, causing significant alterations in oestrogen secretion and steroid hormone homeostasis [10–12]. Hence, antioxidants in plant or other natural sources, with few or no adverse effects, are preferred alternatives to synthetic antioxidants, especially because they are affordable and readily available. Fatty acids (FAs) are essential chemical constituents in the cells, which serve as fuel for many biological and metabolic activities, including muscular contraction, and have both nutritional and medicinal values. Medicinal plants are excellent sources of fatty acids in nature and occur in different forms like saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) [13]. Most of the components of medicinal plants, including fatty acids, terpenes, alkaloids, tannins, terpenoids, saponins, have been shown in various studies to prevent and treat many oxidative stress-related disease conditions [14,15].

The genus *Helichrysum* Mill is a distinctively aromatic medicinal plant of the family Asteraceae, well-distributed in many countries worldwide, including South Africa. *Helichrysum* Mill consists of approximately 600 plant species with at least one-third (245 species) available in South Africa. Many of these species differ in morphology and are therefore classified into 30 different groups. Plants in this genus have been traditionally used for the treatment of such human ailments as cold, cough, skin infections, inflammation, insomnia, cystitis, jaundice, stomach pain, menstrual pain, asthma, arthritis disorders, diabetes mellitus wound healing, etc. The in vitro antioxidant, antifungal, anti-inflammatory, antibacterial, hepatoprotective, anti-proliferative, and anti-diabetic properties of some species in this genus have been previously studied [16,17], however the therapeutic potential of

Helichrysum petiolare & B. L Burtt, *Helichrysum cymocum (L)* D. Don, *Helichrysum foetidum (L).* Moench, *Helichrysum pandurifolium* Schrank as sources of antioxidants, fatty acids, and other constituents has not been fully explored [18]. Only the antioxidant activities of the acetone and methanol extracts of *H. petiolare,* the essential oils from the leaves of *H. cymocum,* and the methanolic extract of *H. foetidum* have been reported in the literature [19–21]. Therefore, in this study, GC-MS analysis of the extracts of four selected *Helichrysum* species was done to

identify and quantify total phenolic content (TPC), total flavonoids content (TFC), antioxidant activity as well as total fatty acids and lipids.

2. Methods

2.1. Collection of Plant Material

Four *Helichrysum* species were collected from the Western Cape in the environment of the Cape Peninsula University of Technology (CPUT), Bellville, in October 2020, and samples were identified by Prof. Christopher N. Cupido of the Department of Botany, University of Fort Hare, Alice, South Africa. The accession numbers of the Helichrysum species in this study were: *Helichrysum petiolare*-UFH-2020-10-01, *Helichrysum cymosum*- UFH 2020-10-02, *Helichrysum foetidum*-UFH 2020-10-03, and *Helichrysum pandurifolium*- UFH 2020-10-04.

2.2. Plant Extraction

The leaves of the plants were cleaned and air-dried to a constant weight and the dried samples were pulverised using an electronic blender, grounded, and weighed. The powdered plant materials in conical flasks were soaked and subjected to intermittent stirring in 90% aqueous acetone and warmed in the water bath at 60 °C for 2 h with slight modification [22]. The mixture was filtered with Whatman cellulose filter paper under pressure using a pump and the plant material was subjected to a second extraction after soaking overnight and the filtrate pooled before rotary evaporation. The final residue or extract obtained was allowed to dry in the fume cupboard and stored at −20 °C until required for use. (1 mg extract dissolved in 1 ml acetone is used in subsequent analysis).

2.3. Chemicals and Reagents

Acetone and other solvents used in this work were purchased from Merck (Darmstadt, Germany), while 1,1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, gallic acid, quercetin, $FeCl_3$, $AlCl_3$, and Folin- Ciocalteu reagent were purchased from Sigma chemical Co. (St. Louis, MO, USA). All chemicals used including solvents were of analytical grade.

2.4. In Vitro Evaluation

DPPH Radical Scavenging Activity Assay

The DPPH radical scavenging activity assay was performed as previously described [23]. Briefly, a 2000 µL stock concentration of DPPH (0.004 g in 100 mL methanol) was added to aliquots of 500 µL plant extracts at different concentrations (10–250 µg/mL) and the reaction mixture was shaken in the dark for 30 min at room temperature. The controls contained the DPPH solution without the plant extract, while methanol was used as the blank. A decrease in absorbance of the test mixture read at 517 nm will result from quenching of DPPH free radicals after the exposure time interval. The following formula is used to determine the scavenging effects of the plant extracts:

$$\% \text{ inhibition} = [A_0 - A_1] \times 100/[A_0] \quad (1)$$

where A_0 is the absorbance of the blank and A_1 is the absorbance of the extract.

2.5. Nitric Oxide Scavenging Activity Assay

Sodium nitroprusside generates Nitric oxide (NO) in aqueous physiological pH, measured in the Greiss reaction that produces nitrite ions as previously described [24]. Briefly, 4000 µL of the plant extract or standard solution at different concentrations (10–250 µg/mL) was added to 1000

µL of Sodium nitroprusside solution and 2000 µL of the mixture was added to 1200 µL of the Griess reagent containing 1% sulphanilamide, 0.1% naphthyl ethylenediamine dihydrochloride and 2% H_3PO_4. The absorbance of the chromophore formed during diazotisation of nitrite with sulphanilamide, and its subsequent coupling with naphthyl ethylenediamine dihydrochloride, was measured at 550 nm. The percentage (%) inhibition activity was calculated from the following equation, with ascorbic acid as the standard:

$$\% \text{ inhibition} = [A_0 - A_1] \times 100/[A_0] \quad (2)$$

where A_0 is the absorbance of the control while A_1 is the absorbance of the extract or standard.

2.6. Reducing Power Assay

Different concentrations of plant extracts (10–250 µg/mL) and corresponding concentrations of standard ascorbic acid were added to 2500 µL and 2500 µL of phosphate buffer (pH 6.6) and 1% potassium ferricyanide, respectively. Incubation of the mixture was done at 50 ◦C for 20 min after which, 2500 µL of 10% trichloroacetic acid was added to the mixture and centrifuged at 3000 rpm for 10 min. Thereafter, 2500 µL of the supernatant was added to 2500 µL distilled water and 500 µL of freshly prepared 0.1% ferric chloride solution [25], and absorbance was read at 700 nm. Ascorbic acid was used as the standard at the various concentrations.

2.7. Estimation of Total Phenolic Compounds

The Folin–Ciocalteu reagent method was used to determine the phenolic content as previously described [26]. Briefly, 500 µL of the plant extracts and 100 µL of Folin–Ciocalteu reagent (0.5 N) were added and incubated for 15 min at room temperature after which 2500 µL of sodium carbonate (7.5% w/v) was added to the mixture (plant extract + Folin– Ciocalteu) and incubated for 30 min at room temperature, and absorbance read at 760 nm. Phenolic concentration was expressed as gallic acid equivalent (GAE) (mg/g of dry mass) as the reference value.

2.8. Total Flavonoid Content Estimation

Aluminium chloride solution was used to determine the flavonoid content as previously described, with quercetin as the standard [27]. Briefly, 1000 µL of a 100 µg/mL extract stock solution was added to 3000 µL of methanol and mixed with 200 µL of 10% aluminium chloride, 200 µL of 1 M potassium acetate, and 5600 µL of distilled water. The mixture was incubated at room temperature for 30 min, and absorbance read at 415 nm. The calibration curve was prepared from quercetin solutions in methanol, at the various concentrations.

2.9. Determination of Total Antioxidant Capacity

The plant extract (3000 µL) was added to 3000 µL of the reagent solution containing 0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate as previously described [28]. The tubes containing the mixture were capped and incubated in the water bath at 95 ◦C for 90 min and allowed to cool at room temperature, followed by an absorbance reading at 695 nm against the blank.

2.10. GC-MS/MS Quantification

2.10.1. Analysis of Phenolic Acids and Phenolic Aldehydes in *H. pandurifolium, H. foetidum, H. petiolare,* and *H. cymocum* Aqueous Acetone Extracts

Sample Preparation

The extraction of 100 mg plant extracts was done for 3 h at 60 ◦C using 1 ml of 70% methanol, and 130 µL of the extract was freeze-dried and derivatised using 30 µL N, O-Bis (trimethylsilyl)trifluoroacetamide (BSTFA), and 100 µL acetonitrile at 60 ◦C for 30 min. The sample was then transferred into a 2 mL GC vial, and 1 µL was injected onto the GC-MS/MS in splitless mode.

Chromatographic Separation

Helium gas at a flow rate of 1 mL/min, injector temperature maintained at 250 ◦C, and separation of the analytes was performed on a non-polar Rxi-5Sil MS (30 m, 0.25 mm ID, 0.25 µm film thickness) (instrument type, Trace 1300, Thermo Scientific, Waltham, MA, USA) coupled to triple quadrupole mass spectrometer (TSQ 8000, Thermo Scientific). The oven temperature was programmed as follows: 100 ◦C for 4 min, then ramped to 180 ◦C at 10 ◦C/min rate and held for 2 min before finally ramped at 20 ◦C/min until 320 ◦C and held for 5 min. The mass spectrometer detector (MSD) operated in tandem mass spectrometry (MS/MS) mode, the source, and quad temperature were maintained at 250 ◦C and 150 ◦C, respectively. The transfer temperature was maintained at 250 ◦C.

2.10.2. Analysis of Fatty Acid Methyl Esters (FAMEs) in *H. pandurifolium, H. foetidum, H. petiolare,* and *H. cymocum* Aqueous Acetone Extracts

Sample Preparation

Briefly, 100 mg of the plant extract was vortexed and sonicated at room temperature for 30 min in a mixture of 1 m of chloroform and 1 mL of methanol. This was centrifuged at 3000 rpm for 1 min after which 500 µL of the chloroform fraction (bottom layer) was completely dried with a gentle stream of nitrogen, reconstituted and vortexed with 500 µL of methyl tert-butyl ether (MTBE), and 100 µL was derivatised with 30 µL of trimethyl sulfonium hydroxide (TMSH). Thereafter, 1 µL of the derivatised sample was injected into the GC-MS, in a 5:1 split ratio.

Chromatographic Separation

Helium gas at a flow rate of 1.2 mL/min, injector temperature maintained at 240˚C and separation of the FAMEs was performed on a polar RT- 2560 (100 m, 0.25 mm ID, 0.20 µm film thickness) capillary column (instrument type, 6890 N, Agilent technologies network) coupled to Agilent technologies inert XL EI/CI Mass Selective Detector (MSD) (5975, Agilent Technologies Inc., Palo Alto, CA, USA). The oven temperature was programmed as follows: 100 ˚C for 4 min, then ramped to 240 ˚C at 3 ˚C/min rate and held for 10 min. The mass spectrometer detector (MSD) operated in scan mode, and the source and quad temperature were maintained at 250 ˚C and 150 ˚C, respectively. The transfer temperature was maintained at 250 ˚C. The mass spectrometer was operated under electron impact (EI) mode at ionisation energy of 70 Ev, scanning from 40 to 650 *m*/*z*.

2.11. Statistical Analysis

The mean ± SD from three experimental observations in triplicates of data were used for statistical analysis. The in vitro antioxidant assays were analysed using the ANOVA test, followed by Tukey's test, with statistical significance at ($p < 0.05$).

3. Results

3.1. In Vitro Antioxidant Capacities and Profiles of the Four Helichrysum Species

The antioxidant capacity of the aqueous acetone extracts of four *Helichrysum* species, *namely H. pandurifolium, H. foetidum H. petiolare, and H. cymocum*, were investigated.

3.2. DPPH Scavenging Activity

The radical inhibitory or scavenging activity of the selected aqueous acetone extracts of the Helichrysum species (10–250 µg/mL) was concentration-dependent. Results showed that the extracts caused increased activity with increasing concentrations as shown in Table 5.1, which is in tandem with results from previous studies [6,29]. Figure 5.1 shows IC_{50} values of 14.17 ± 1.77 µg/mL *(H. pandurifolium)*, 41.13 ± 3.62 µg/mL *(H. foetidum)*, 23.57 ± 2.59 µg/mL (*H. petiolare)*, and 11.85 ± 3.20 µg/mL *(H. cymocum)* respectively. Hence, the ranking order for the scavenging free radical activity could be represented as *H. cymocum* >*H. pandurifolium* > *H. petiolare* >*H. foetidum* for these extracts, with ascorbic acid used as the standard, showing the best radical scavenging activity and an IC_{50} value of 2.66 µg/mL compared with all four selected *Helichrysum* species.

3.3. Nitric Oxide Scavenging Activity

The scavenging effects of the four Helichrysum species on nitric oxide were *concentrationdependent* (Table 5.2) and Figure 5.2 shows IC_{50} values for the nitric oxide scavenging activity for *H. pandurifolium* (36.19 ± 2.08 µg/mL), *H. foetidum* (24.31 ± 3.67 µg/mL), *H. petiolare* (20.81 ± 3.73 µg/mL), and *H. cymocum* (24.68 ± 4.78 µg/mL), respectively. Thus, the ranking order for the scavenging free radical activity could be represented as *H. petiolare* > *H. foetidum* > *H. cymocum* > *H. pandurifolium* in scavenging nitric oxide, whereas the ascorbic showed excellent potency of inhibiting nitric oxide with IC_{50} of 0.86 µg/mL.

Table 5.1. DPPH scavenging activity of aqueous acetone extracts of *H. pandurifolium, H. foetidum H. petiolare, and H. cymocum*.

Extract Concentration (μg/mL)	*H. pandurifolium*	*H. foetidum*	*H. petiolare*	*H. cymocum*	Ascorbic Acid
10	33.04 ± 1.78 ^{bde}	19.06 ± 3.22 ^{acde}	27.08 ± 5.33 ^{bde}	47.31 ± 1.41 ^{bce}	73.77 ± 5.10 ^{abcd}
25	76.53 ± 2.65 ^{bcd}	35.39 ± 2.34 ^{ade}	41.72 ± 8.80 ^{ade}	58.86 ± 6.3 ^{abce}	83.38 ± 0.79 ^{bcd}
50	91.10 ± 0.67 ^{b}	53.50 ± 3.52 ^{acde}	86.31 ± 1.81 ^{be}	88.67 ± 0.46 ^{b}	94.11 ± 0.55 ^{bc}
125	91.61 ± 1.11 ^{b}	81.83 ± 0.67 ^{ace}	89.62 ± 0.45 ^{b}	85. 58 ± 2.67 ^{e}	94.63 ± 0.67 ^{bd}
250	90.36 ± 1.00	85.28 ± 1.34 ^{e}	89.18 ± 0.59	89.55 ± 1.22	94.99 ± 0.55 ^{b}

Data is presented as a mean ± SD value (n = 3); ($^{a–e}$) represents significance ($p < 0.05$) when compared among the groups.

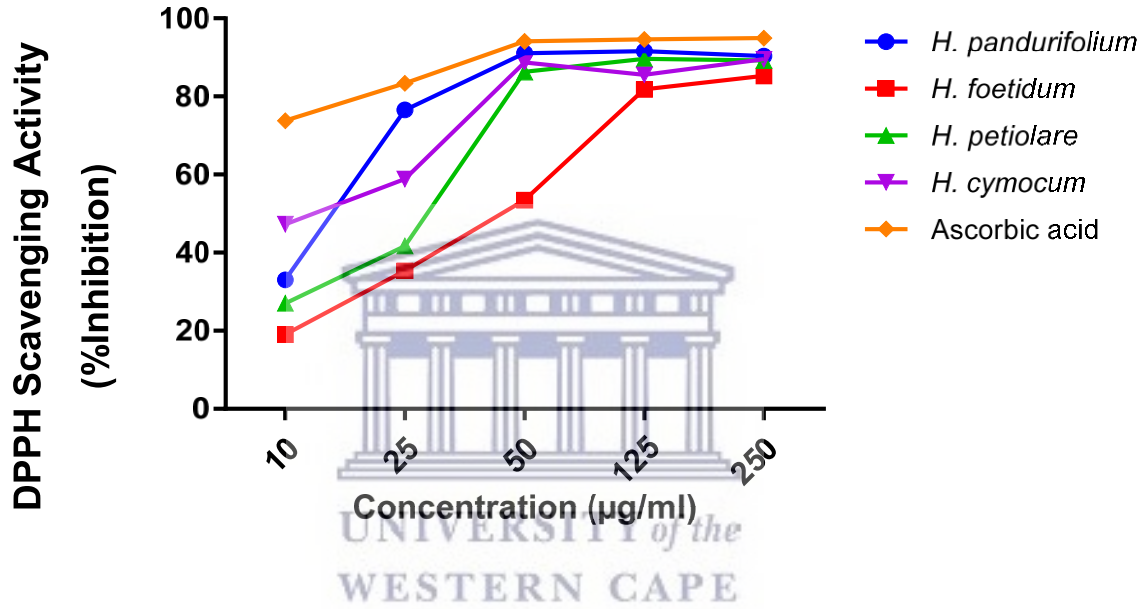

Figure 5.1. Percentage (%) inhibition of aqueous acetone extract of *H. pandurifolium, H. foetidum H. petiolare, and H. cymocum*.

Table 5.2. Nitric oxide scavenging activity of *H. pandurifolium, H. foetidum H. petiolare, and H. cymocum* aqueous acetone extracts.

Extract Concentration (μg/mL)	*H. pandurifolium*	*H. foetidum*	*H. petiolare*	*H. cymocum*	Ascorbic Acid
10	33.27 ± 0.50 ^{bcde}	42.85 ± 0.29 ^{ade}	44.24 ± 0.50 ^{ade}	40.52 ± 0.58 ^{abce}	79.58 ± 0.87 ^{abcd}
25	41.78 ± 2.05 ^{bcde}	47.95 ± 0.66 ^{acde}	50.79 ± 1.47 ^{abe}	50.09 ±0.38 ^{abe}	86.14 ± 0.48 ^{abcd}
50	54.51 ± 0.85 ^{cde}	54.44 ± 0.38 ^{cde}	56.27 ± 0.66 ^{bde}	52.17 ± 1.14 ^{abce}	91.37 ± 0.61 ^{abcd}
125	68.24 ± 0.87 ^{bcde}	70.63 ± 0.58 ^{ade}	71.52 ± 0.61 ^{ade}	76.05 ± 0.29 ^{abce}	93.63 ± 0.40 ^{abcd}
250	78.64 ± 0.38 ^{cde}	80.34 ± 0.38 ^{ce}	82.67 ± 0.58 ^{abe}	81.98 ± 0.48 ^{ae}	95.71 ± 0.40 ^{abcd}

Data are presented as a mean ± SD value (n = 3); ($^{a–e}$) represents significance ($p < 0.05$) when compared among the groups.

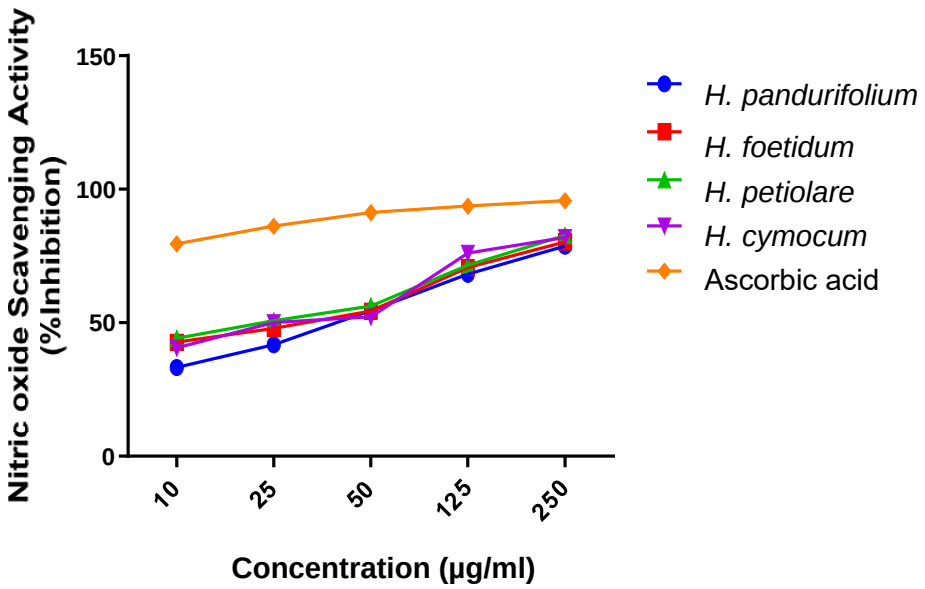

Figure 5.2. Percentage (%) inhibition of aqueous acetone extract of *H. pandurifolium, H. foetidum H. petiolare, and H. cymocum*

3.4. Reducing Power Activity

The reducing power dose-response curves of all extracts (10–250 µg/mL) of the selected *Helichrysum* species are concentration-dependent, as shown in Table 5.3. The ranking order for the reducing power at the highest concentration of 250 µg/mL indicates that *H. cymocum* > *H. foetidum* > *H. pandurifolium* > *H. petiolare* of 0.636 µg/mL, 0.619 µg/mL, 0.602 µg/mL and 0.505 µg/mL, respectively (Figure 3). The ascorbic acid has the highest value of 0.853 µg/mL at the highest concentration of 250 µg/mL.

Table 5.3. Reducing power activity of aqueous acetone extracts of *H. pandurifolium, H. foetidum H. petiolare, and H. cymocum*.

Extract Concentration (µg/mL)	*H. pandurifolium*	*H. foetidum*	*H. petiolare*	*H. cymocum*	Ascorbic Acid
10	0.140 ± 0.002 ^{bcde}	0.181 ± 0.003 ^{acde}	0.158 ± 0.003 ^{abe}	0.152 ± 0.003 ^{abe}	0.262 ± 0.005 ^{abcd}
25	0.224 ± 0.003 ^{bd}	0.296 ± 0.006 ^{acde}	0.234 ± 0.007 ^{bde}	0.174 ± 0.003 ^{bce}	0.398 ± 0.005 ^{abcd}
50	0.307 ± 0.004 ^{bcde}	0.480 ± 0.004 ^{acde}	0.282 ± 0.003 ^{abde}	0.363 ± 0.004 a^{bce}	0.572 ± 0.005 ^{abcd}
125	0.522 ± 0.003 ^{bcde}	0.549 ± 0.002 ^{acde}	0.429 ± 0.005 ^{abde}	0.537 ± 0.004 ^{abce}	0.763 ± 0.008 ^{abcd}
250	0.602 ± 0.007 ^{bcde}	0.619 ± 0.005 ^{acde}	0.505 ± 0.010 ^{abde}	0.636 ± 0.005 ^{bce}	0.853 ± 0.008 ^{abcd}

Each value represents mean ± SD value (n = 3); ($^{a–e}$) indicates significance at ($p < 0.05$) among groups.

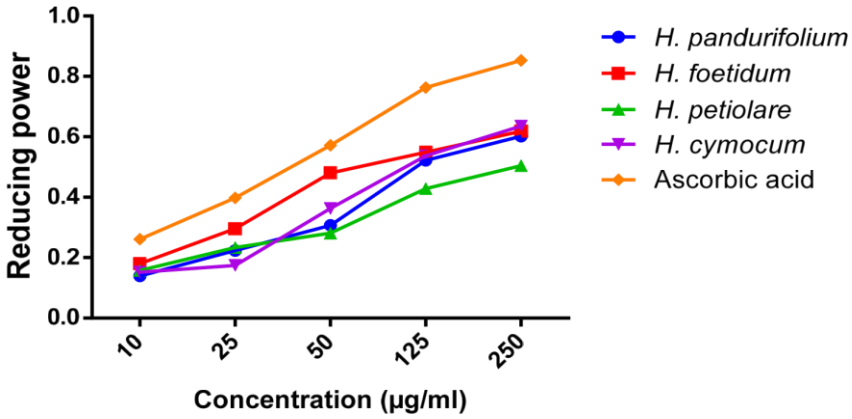

Figure 3. Reducing power activity of aqueous acetone extract of *H. pandurifolium, H. foetidum H. petiolare, and H. cymocum*.

Figure 5.3. reducing power activity of aqueous acetone extract of *H. panddurifolium, H. petiolare, and H. cymocum.*

3.5. Total Antioxidant Capacity (TAC), Total Flavonoid (TF), and Total Phenolic (TP) Content

Figure 5.4 shows total antioxidant capacity (TAC) of *H. pandurifolium* (26.11 ± 3.38), *H. foetidum* (47.44 ± 0.41 mg/g), *H. petiolare* (48.50 ± 1.55 mg/g), and *H. cymocum* (30.82 ± 4.44 mg/g) extracts, respectively, while the flavonoid content was 51.65 ± 0.40 mg/g for *H. pandurifolium*, 46.59 ± 0.75 mg/g for *H. foetidum*, 56.19 ± 1.01 mg/g for *H. petiolare*, and 49.65 ± 0.74 mg/g for *H. cymocum*. On the other hand, the phenolic content was 53.11 ± 0.47 mg/g for *H. pandurifolium*, 42.14 ± 0. 50 mg/g for *H. foetidum*, 54.69 ± 0.23 mg/g for *H. petiolare*, and 47.93 ± 0.57 mg/g for *H. cymocum* acetone extracts, respectively. Overall, *H. petiolare* has the best antioxidant capacity,total flavonoids, and total phenolics compared with other species (Figure 5.4). Previous studies have shown that many factors, including genetic diversity, biological, environmental, seasonal variations as well as the harvesting period, may account for any differences or similarities seen in the results of TF, TP, and TAC of plant extracts for the same plant species [30–32].

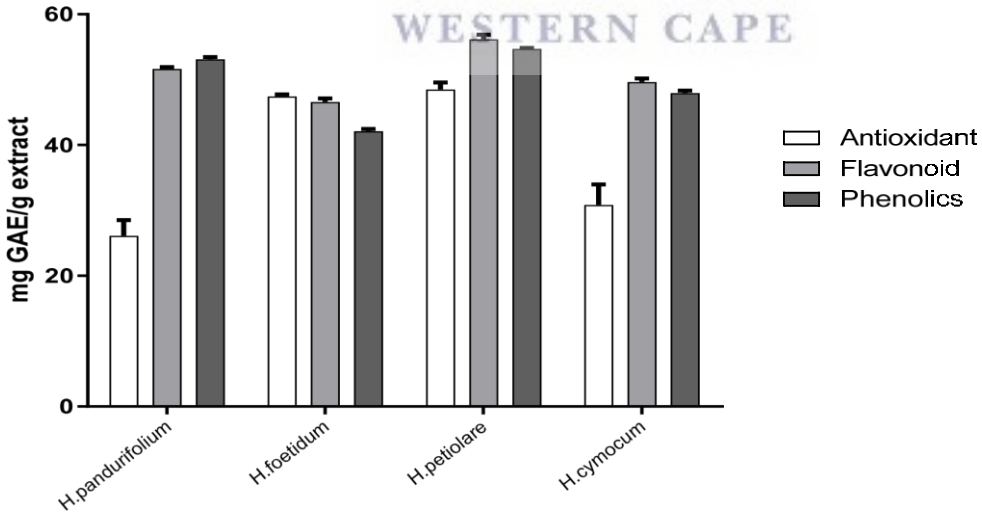

Figure 5.4. Antioxidant profiles of the aqueous acetone extracts of *H. pandurfolium, H. foetidum, H. petiolare, and H. cymocum.* Data is illustrated as mean ± SD (*n* = 3).

3.6. Total Phenolic Acid and Phenolic Aldehyde Composition

In Table 5.4, the composition of the phenolics and their aldehyde content in the four different species vary. The *vanillin* (78.5 µg/g), protocatechuic acid (297.5 µg/g), coniferaldhyde (13.5 µg/g), and caffeic acid (1424.3 µg/g) were more in the aqueous acetone extract of *H. petiolare* in comparison with that of *H. pandurifolium, H. foetidum, and H. cymocum*. It was also observed that syringaldehyde (23.1 µg/g), m-coumaric acid (0.392 µg/g), and ferulic acid (144.3 µg/g) were higher in *H. pandurifolium* compared with that of *H. petiolare, H. foetidum, and H. cymocum*. Additionally, vanillic acid (43.5 µg/g) and p-coumaric acid (0.122 µg/g) showed a higher concentration in *H. foetidum* compared with the other species, meanwhile, syringic acid (13.7 µg/g) and gallic acid (685.7 µg/g) were higher in the aqueous acetone extract of *H. cymocum* in comparison with *H. pandurifolium, H. petiolare,* and *H. foetidum* as showed in Table 5.4. Hence, it is important to know that each component was determined using an external standard calibration by GC-MS/MS.

Table 5.4. Phenolic acid and phenolic aldehyde composition (µg/g) of the acetone extracts of *H. pandurifolium, H. foetidum, H. petiolare, and H. cymocum*.

TMS Derivative	Mass Spectrum		Plant Extracts (Concentration, µg/g)			
	Pseudomolecular Ions	Fragment Ions	*H. pandurifolium*	*H. foetidum*	*H. petiolare*	*H. cymocum*
Vanillin	224	209	70.1	41.9	78.5	27.5
trans-cinnamic acid	220	205	2.22	2.12	29.6	46.4
Syringaldehyde	254	224	23.1	8.23	14.1	8.11
Vanillic acid	312	282	10.6	43.5	14.8	13.6
Protocatechuic acid	355	311	94.6	84.5	297.5	115.9
Coniferaldehyde	250	235	3.7	1.19	13.5	2.19
m-coumaric acid	293	249	0.392	0.259	0.192	0.237
p-coumaric acid	308	203	0.064	0.122	0.046	0.022
Syringic_Acid	342	312	2.02	4.51	2.95	13.7
Gallic_acid	458	281	31.5	13.7	180.6	685.7
Sinapinaldehyde	280	265	11.2	74.3	18.2	16.9
Ferulic_Acid	338	308	144.3	61.2	98.4	53.7
Caffeic_Acid	396	219	1328.2	928.6	1424.3	1363.2

Legend: the acetone extracts of *H. pandurifolium, H. foetidum, H. petiolare, and H. cymocum* were identified by authentic certified reference materials (CRMs) and compared with calibration standards.

3.7. Composition of Saturated Fatty Acids

Table 5.5 shows the amount (µg/g) of the individual fatty acid and classes of fatty acids of the aqueous acetone extract of *H. pandurifolium, H. foetidum, H. petiolare, and H. cymocum.* Eleven saturated fatty acids (C_{12}–C_{24}), two monounsaturated fatty acids ($C_{16:1}$, $C_{18:1\ n\ 9\ (cis)}$), and two polyunsaturated fatty acids ($C_{18:2\ n\ 6\ (cis)}$, C18:3n3) were identified in the extracts of the four plants. The amount of the fatty acids varied widely, viz, 3.1 to 728.3 µg/g (for saturated fatty acids), 76.7 to 1057.9 µg/g (for monounsaturated fatty acids), and 624.9 to 4688.6 µg/g (for polyunsaturated fatty acids), respectively. The PUFA:SFA ratios are

0.604 (*H. pandurifolium*), 0.726 (*H. foetidum*), 0.975 (*H. petiolare*), and 1.202 (*H. cymocum*) with attributable health benefits. Table 5.6 depicts the GC-MS chromatograms of the fatty acids of aqueous acetone extract of *H.pandurifolium, H.foetidum H.petiolare,* and *H.cymocum* while Figure 5.5 shows the area of the peaks, ratio area, and the retention time (R, time) of aqueous acetone extract of *H. pandurifolium, H. foetidum H. petiolare, and H. cymocum.*

Table 5.5. Composition (µg/g) of saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, and total fatty acids of the acetone extracts of *H. pandurifolium, H. foetidum H. petiolare*, and *H. cymocum*.

	SFA												*MUFA*			*PUFA*								
Concentration (µg/g)																					**PUFA:SFA**	**n − 6**	**n − 3**	**(n − 6)/(n − 3)**	**TFA**
	C12:0	**C13:0**		**C14:0**	**C15:0**	**C16:0**	**C17:0**	**C18:0**	**C20:0**		**C21:0**	**C22:0**	**C24:0**	**C16:1**	**C18:1n9 (cis)**	**C18:2n6 (cis)**	**C18:3n3**	**SFA**	**MUFA**	**PUFA**					
H. pandurifolium	18.6	12.8		451.8	126.3	2446.8	223.1	572.1	240.2		39.0	286.9	166.4	76.7	595.9	2144.1	624.9	4584.0	672.7	2768.9	0.604	2144.1	624.9	3.43	8025.6
H. foetidum						2128.7							467.8	92.4	1057.9	2759.6	1147.9	5379.8	1150.3	3907.5	0.726	2759.6	1147.9	2.40	10437.5
H. petiolare		25.3	493.2	349.4		3064.0			466.9	83.5	497.6		656.2	286.4	740.3	4688.6	2316.8	7182.0	1026.7	7005.5	0.975	4688.6	2316.8	2.02	15214.2
		7.9	621.6	318.8					695.8	100.0	705.7														
H. cymocum	17.3	3.1		372.0	135.4	2487.8	218.4	563.7	329.3		54.1	439.4	238.0	90.1	516.7	3796.9	2041.3	4858.5	606.8	5838.2	1.202	3796.9	2041.3	1.86	11303.5

SFA—Saturated fatty acids; MUFA—Monounsaturated fatty acids; PUFA—Polyunsaturated fatty acids; TFA—Total fatty acids.

Table 5.6. Shows fatty acids, area of the peaks, ratio area, and the retention time (R, time) obtained from gas chromatography-mass spectrometry (GC-MS) analysis of the aqueous acetone extract of *H. pandurifolium, H. foetidum H. petiolare, and H. cymocum*.

		H. pandurifolium			***H. foetidum***			***H. petiolare***			***H. cymocum***		
S/N	**Name**	**R. Time**	**Area**	**Area Ratio**	**R. Time**	**Area**	**Area Ratio**	**R. Time**	**Area**	**Area Ratio**	**R. Time**	**Area**	**Area Ratio**
1	C12:0	26.83	129390	0.329	26.83	669469	1.043	26.84	120139	0.336	26.84	196681	0.379
2	C13:0	29.48	84201	0.214	29.47	297564	0.464	29.47	55448	0.155	29.49	67203	0.129
3	C14:0	32.02	1908002	4.854	32.02	5051443	7.869	32.02	2077992	5.817	32.02	2843872	5.478
4	C15:0	34.47	492091	1.252	34.47	3564716	5.553	34.47	1036856	2.903	34.47	985986	1.899
5	C16:0	36.84	11057456	28.128	36.86	23592767	36.752	36.84	11014044	30.834	36.86	20445571	39.383
6	C16:1	38.35	206058	0.524	38.34	462768	0.891	38.42	670890	1.878	38.34	462768	0.891
7	C17	39.06	1269355	3.229	39.07	2190159	3.412	39.06	1175330	3.29	39.07	2097968	4.041
8	C18:0	41.22	3120778	7.939	41.28	8058156	12.553	41.22	3107496	8.699	41.24	5352632	10.311
9	C18:1n9c	42.46	2106853	5.359	42.48	8959105	13.956	42.45	2075141	5.809	42.46	3297951	6.353
10	C19:1STD	43.28	393119	N/A	43.33	641949	N/A	43.28	357208	N/A	42.28	519143	N/A
11	C182n6c	44.35	5314280	13.518	44.37	16679594	25.983	44.35	9199072	25.753	44.37	16995730	32.738
12	C20:0	45.26	1337418	3.402	45.31	5943454	9.258	45.26	2891326	8.094	45.27	3170962	6.108
13	C18:3n3	46.48	1700259	4.325	46.52	7547519	11.757	46.48	4938911	13.826	46.5	9919610	19.108
14	C21:0	47.16	187898	0.478	47.19	1035681	1.613	47.15	398686	1.116	47.16	490302	0.944
15	C22:0	49	1673819	4.258	49.04	6573385	10.24	49	3058160	8.561	49.01	4354287	8.387
16	C24	52.48	1797182	4.572	52.53	10192435	15.877	52.48	4709445	13.184	52.49	4136416	7.968

The peaks, ratio area, and the retention time obtained from gas chromatography-mass spectrometry (GC- MS) are described by the National Institute of Standards and Technology (NIST) library to that of a known compound. This is depicted in the different compositions of fatty acids in the above table.

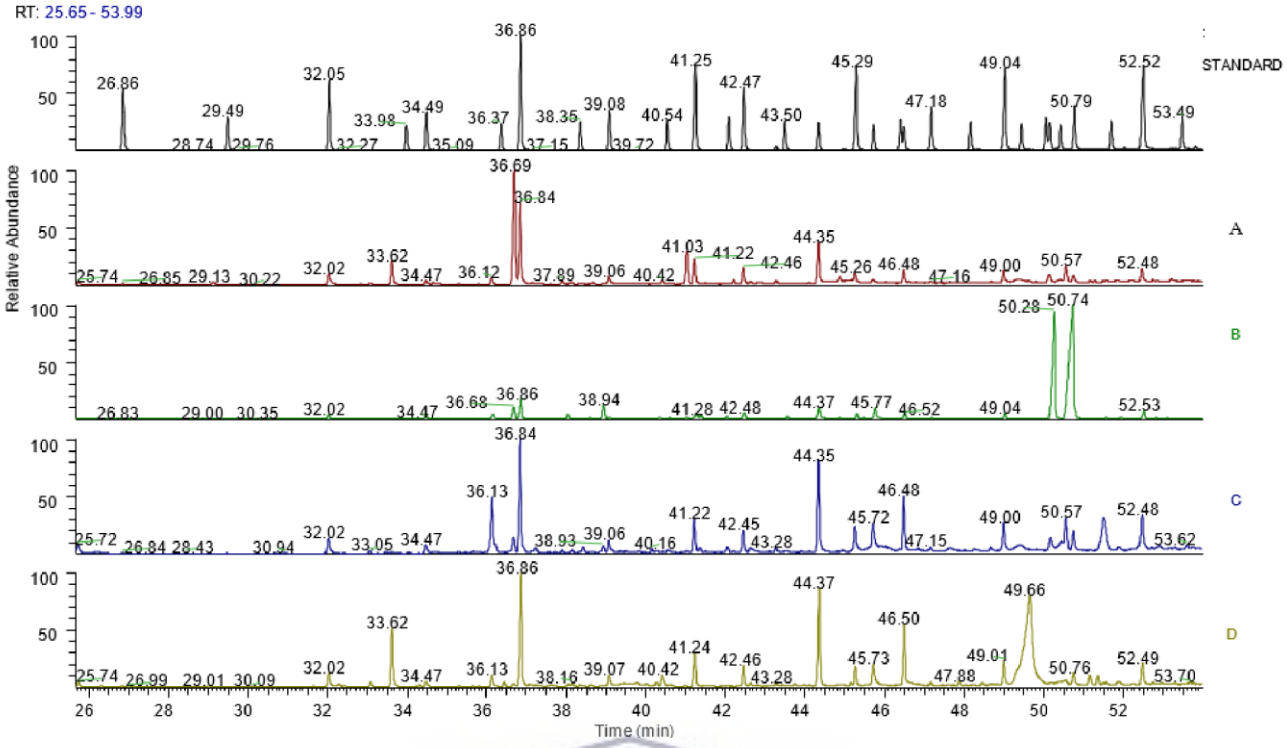

Figure 5.5. Shows the GC-MS chromatogram of the fatty acids of aqueous acetone extract of *H.pandurifolium, H.foetidum H.petiolare, and H.cymocum* with regularly labelled signals detected by the GC-MS detector. LEGEND: (**A**)—FAMEs chromatogram of *H. pandurifolium;* (**B**)—FAMEs chromatogram of *H. foetidum;* (**C**)—FAMEs chromatogram of *H. petiolare;* (**D**)—FAMEs chromatogram of *H. cymocum*.

4. Discussion

Antioxidants, fatty acids, and other constituents of medicinal plants have been reported to be beneficial for preventing, alleviating, or treating, oxidative stress-induced diseases [33,34]. Antioxidants are known to be involved in halting redox imbalances by activating the antioxidant defence system to scavenge free radicals through a number of mechanisms, including increased chain-breaking antioxidant activity (synergistic effect), conversion of unstable hydroperoxides in a non–radical pathway to stable components (reducing effect), singlet oxygen (quencher), conversion of pro-oxidant metal derivatives to stable products (metallic chelation), inactivation of pro-oxidant enzymes, decreased activity of free radical oxidation reactions, and inactivation of autoxidation of chain reactions [35,36].

Antioxidants have great therapeutic value as anti-viral, anti-fungal, anti-bacterial, antitumoural, anti-cancer, anti-angiogenic, anti-inflammatory, anti-allergic, anti-diabetic, and neuroprotective actions [37–40]. Flavonoids and phenolics are the main phytocompounds present in most medicinal plants, with more than 4500 flavonoid compounds having been identified [41] and over 8000 phenolic compounds reported [42,43]. Flavonoids and phenolics with potent antioxidant activities have been shown to effectively modulate oxidative stress-related diseases through clearly-defined mechanisms of action [6]. Apart from their medicinal use, antioxidants are also used in the food industry to preserve and improve the shelf-lives of most foods [44].

The determination of the antioxidant potential of plant extracts is dependent on the different methods used and their underlying mechanisms, which explains the multiplicity of techniques in most related studies [45,46]. Therefore, DPPH scavenging activity, nitric oxide scavenging activity, and reducing power activity were used in this study to investigate the antioxidant activities of the aqueous acetone extracts of *H. pandurifolium, H. foetidum H. petiolare, and H. cymocum*. The phenolic antioxidants have been shown to disrupt the formation of ROS and other free radicals by the transfer of hydrogen atoms from its hydroxyl group [47] while the antioxidant flavonoids are known to stabilise ROS via their scavenging actions through the oxidation of free radicals into more stable but less active or reactive radicals [47]. In this study, the aqueous acetone extracts of *H. pandurifolium, H. foetidum, H. petiolare, and H. cymocum* produced radical decolourisation of the DPPH solution because of the high free radical scavenging activity of the plant extracts [5,48].

The results reveal that the extracts tested have a dose-dependent activity. In fact, at the concentration of 250 µg/mL, the aqueous acetone extracts tested reduce the DPPH radical with an excellent percentage of 90.36 ± 1.00%, 85.28 ± 1.34%, 89.18 ± 0.59%, 89.55 ± 1.22%, 89.55 ± 1.22% for aqueous acetone extracts of *H. pandurifolium, H. foetidum H. petiolare, and H. cymocum*, respectively. Additionally, the IC_{50} is inversely proportional to the antioxidant capacity of a compound. However, the lowest value of IC_{50} indicates a strong antioxidant capacity of a compound. *H. Cymocum* showed the lowest IC_{50} values of 11.85 ± 3.20 µg/mL which had better antioxidant activity compared with *H. pandurifolium, H. foetidum*, and *H. petiolare*, (Figure 5.1). The antioxidant power of the aqueous acetone extracts could be explained by the presence of phenolic compounds including flavonoids present in the species of *Helichrysum* studied and which are known as antioxidant substances with the ability to trap radical species and reactive forms of oxygen. (Figure 5.1).

The results of the IC_{50} DPPH assay of the methanolic extracts of similar species namely *H. dasyanthum, H. excisum*, and *H. felinum* were 12.33, 13.67, and 20.71 µg/mL, respectively, which were within the range of the IC_{50} obtained in our study, as reported by Lourens et al. [21]. However, only the *H. pandurufolium* of IC_{50} (41.13 ± 3.62 µg/mL) is similar and in agreement with those reported [49] with the species name, *H. chionophilum, H. plicatum subsp. plicatum, and H. arenarium subsp. Aucheri* having IC_{50} of 40.5, 48.0, and 47.6 µg/mL, respectively. From literature, the flavonoids are the main compounds in the helichrysum genus with remarkable antioxidant activity, as reported [49,50].

The reaction of sodium nitroprusside with oxygen produces nitric oxide and nitrite that scavenge free radicals via diazotisation with a sulphanilamide acid coupled reaction, producing a pink colour [51]. The antioxidant activities in NO assay involve the donation of protons to the nitrite radicals that show decreased absorbance. In line with the antioxidant activity, the nitric oxide scavenging revealed dose-dependent activity. It is worth mentioning here that all the doses are highly significant among the groups. Although *H. petiolare* (20.81 ± 3.73 µg/mL) with the lowest IC_{50} indicates the best nitric oxide scavenging effect and good antioxidant compared with the IC_{50} of *H. pandurifolium* (36.19 ± 2.08 µg/mL), *H. foetidum* (24.31 ± 3.67 µg/mL), and *H. cymocum* (24.68 ± 4.78 µg/mL). At the concentration of 250 µg/mL, the aqueous acetone extracts tested have NO scavenging activity with an excellent percentage of 78.64 ± 0.38%, 80.34 ± 0.38%, 82.67 ± 0.58%, 81.98 ± 0.48%, and 89.55 ± 1.22% for aqueous acetone extracts of *H. pandurifolium, H. foetidum H. petiolare, and H. cymocum*, respectively. The difference in the antioxidant of aqueous acetone extracts of *H. pandurifolium, H. foetidum H. petiolare, and H. cymocum* could be attributed to the variation

in the chemical composition. Indeed, several types of bioactive compounds known for their antioxidant activity [52,53] are identified in *H. petiolare* with high levels of some compounds, including phenolic (caffeic acid, coniferaldehyde, protocatechuic acid, vanillin) compared to the other species (Table 5.4). The reducing power of natural products or plant extracts indicates their potential to transfer electrons from Fe^{3+} to Fe^{2+}, which is synonymous with the antioxidant activity and is linked to reductones that donate a hydrogen atom to break the free radical chain, thus preventing peroxide formation [54]. The colour change from yellow to various shades of green and blue following treatment is dependent on the reducing power of the plant extract, with the blue colour indicating the highest reducing power. Thus, with increasing concentration of the aqueous acetone extract of *H. pandurifolium, H. foetidum, H. petiolare, and H. cymocum,* the observed blue colour indicates greater reducing power, which is similar to results in previous studies [55,56].

Consequently, the decrease in absorbance observed is an indication of the extent of nitrite radical scavenging potentials [57] and this could be attributed to components such as flavonoids, as reported in previous studies [58,59]. Similarly, the aqueous acetone extracts of *H. pandurifolium, H. foetidum H. petiolare,* and *H. cymocum* can act as natural antioxidants with relative activities scavenging free radical species. The reducing ability or potential is synonymous with the free radical scavenging activity of the plant extracts which is attributable to different amounts of the plant's phytochemicals constituents [6] Overall, the antioxidant activities of these plant extracts are attributed to the constituents of total phenolic, total flavonoid, and total antioxidant capacity.

The fatty acid and lipid composition of the aqueous acetone extracts of *H. pandurifolium, H. foetidum, H. petiolare, and H. cymocum* were determined by fatty acid methyl esters (FAMEs) analysis involving the derivatisation, which was analysed by gas chromatography [60]. Previous studies have shown that geographical location, plant species, and seasonal changes could influence the fatty acid content of plants [61,62]. Unsaturated (monounsaturated and polyunsaturated) fatty acids have been reported to ameliorate cardiovascular diseases, modulate inflammation and support the immune system against cancer, diabetes mellitus, neurodegenerative diseases, etc. [63,64]. This study has shown that the aqueous acetone extracts of *H. pandurifolium, H. foetidum H. petiolare, and H. cymocum* contain various amounts of fatty acids with different compositions, as previously reported [61,65]. Our results showed two monounsaturated (MUFA) and two polyunsaturated fatty acids (PUFA), most of which cannot be synthesised by the human body and are only available in dietary sources, making them of great nutritional health benefit [65,66]. Stearic acid (C18:0), oleic acid (C18:1n9 (cis)), and linoleic acid (C18:2n9 (cis)), with known health benefits, were high in the aqueous acetone extracts of *H. pandurifolium, H. foetidum H. petiolare*, and *H. cymocum* as revealed in Table 5.5, which is similar to findings from previous studies that involved different extractants, different parts, and different *Helichrysum* species, e.g., *H. chionophilum* and *H. plicatum subsp.* [65]. The high dietary fatty acid ratio of PUFA:SFA are implicated in oxidative stress and are prone to lipid peroxidation because

PUFA is highly susceptible, however, raising the PUFA/SFA ratio in the body helps to prevent cardiovascular disease (CVD) and conditions [67]. The PUFA/SFA varied considerably in the aqueous acetone extracts of *H. pandurifolium, H. foetidum H. petiolare, and H. cymocum* having 0.604, 0.726, 0.975, and 1.202 (for PUFA/SFA), respectively in our study, and these were seen to be comparable with the values in some seaweed plants considered to be of great health benefits in literature [13]. To the best of our knowledge, no study has

reported the comparative study of antioxidant activities, constituents, and fatty acid compositions of four selected aqueous acetone extracts of the *Helichrysum* species. However, few studies have investigated the antioxidant activity of one species of this plant [18]. The many folkloric benefits of the plants in the *Helichrysum* species are under-explored in scientific investigations [18]. Natural, plant-based fatty acids are considered to be the best sources of dietary fatty acids because it has been recommended to prevent cardiovascular (CVD) and other disease conditions [67]. Thus, they could serve as potential sources of effective nutraceutical compounds for the prevention of various disease conditions.

In conclusion, our work provides relevant information on the phenolic, flavonoid, antioxidant capacity, and fatty acid profiles of the aqueous acetone extracts of *H. pandurifolium*, *H. foetidum H. petiolare*, *and H. cymocum* which demonstrate significant antioxidant activities. Since these constituents have been reported in previous studies to be effective in the prevention and treatment of various diseases, further research leading to possible drug discovery and development from these four *Helichrysum* species, especially for diabetes and its related cognitive decline conditions, is encouraged.

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CHAPTER SIX

An investigation into the discovery and development of phytochemical-based drugs from the *Helichrysum petiolare* specie is described in this chapter. The α-amylase and α-glucosidase as inhibitors from the extract and flavonoids constituents of *Helichrysum petiolare in silico* study of antidiabetic potential of the flavonoid's constituents were investigated. The research for this chapter entails the experimental and computational strategies that are validatory and complimentary in drug discovery and development. Hence, the *in vitro* antidiabetic study of aqueous acetone *Helichrysum petiolare* extract (AAHPE) using insulin resistance HepG2 cell line and molecular docking of flavonoids' constituent as an antidiabetic therapy was studied. We revealed the potential use of the *H. petiolare* plant extract and its phytochemicals that could be explored for developing potent and safe α-amylase and α-glucosidase inhibitors to treat postprandial glycaemic levels in diabetic patients.

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In Vitro Evaluation of the Anti-Diabetic Potential of Aqueous Acetone *Helichrysum petiolare* Extract (AAHPE) with Molecular Docking Relevance in Diabetes Mellitus

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Abstract: Diabetes mellitus (DM) is a chronic metabolic condition that can lead to significant complications and a high fatality rate worldwide. Efforts are ramping up to find and develop novel α-glucosidase and α-amylase inhibitors that are both effective and potentially safe. Traditional methodologies are being replaced with new techniques that are less complicated and less time demanding; yet, both the experimental and computational strategies are viable and complementary in drug discovery and development. As a result, this study was conducted to investigate the in vitro anti-diabetic potential of aqueous acetone *Helichrysum petiolare* and B.L Burtt extract (AAHPE) using a 2-NBDG, 2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-deoxy-D-glucose uptake assay. In addition, we performed molecular docking of the flavonoid constituents identified and quantified by liquid chromatography-mass spectrometry (LC-MS) from AAHPE with the potential to serve as effective and safe α-amylase and α-glucosidase inhibitors, which are important in drug discovery and development. The results showed that AAHPE is a potential inhibitor of both α-amylase and α-glucosidase, with IC_{50} values of 46.50 ± 6.17 (µg/mL) and 37.81 ± 5.15 (µg/mL), respectively. This is demonstrated by a significant increase in the glucose uptake activity percentage in a concentrationdependent manner compared to the control, with the highest AAHPE concentration of 75 µg/mL of glucose uptake activity being higher than metformin, a standard anti-diabetic drug, in the insulinresistant HepG2 cell line. The molecular docking results displayed that the constituents strongly bind α-amylase and α-glucosidase while achieving better binding affinities that ranged from ΔG = −7.2 to −9.6 kcal/mol (compared with acarbose ΔG = −6.1 kcal/mol) for α-amylase, and ΔG = −7.3 to −9.0 kcal/mol (compared with acarbose ΔG = −6.3 kcal/mol) for α-glucosidase. This study revealed the potential use of the *H. petiolare* plant extract and its phytochemicals, which could be explored to develop potent and safe α-amylase and α-glucosidase inhibitors to treat postprandial glycemic levels in diabetic patients.

Keywords: glucose uptake; drug discovery and development; α-amylase and α-glucosidase inhibitors; diabetes mellitus

1. Introduction

Diabetes mellitus (DM) is a complex global public health condition and, after cancer and cardiovascular disease, is the third most common chronic and non-contagious disease [1]. Insulin dysfunction results from a lack of pancreatic cells to release insulin (Type 1 DM) or an inadequate insulin response (Type 2 DM), both of which are known as insulin resistance (IR). IR reduces insulin-sensitive activity and is the leading cause of Type 2 diabetes worldwide, accounting for 90–95 percent of cases, and is thought to be produced by a prolonged hyperglycemic state. The contribution of reactive oxygen species (ROS) generated by the oxidative stress (OS) induced by chronic hyperglycemia is linked to the onset and progression of diabetes and its associated complications [2,3]. Chronic hyperglycemia produces antioxidant imbalances in the body; therefore, treating DM requires exogenous antioxidants, notably flavonoids, which are bioactive components of many medicinal plants. Medicinal plants are the repository of many bioactive compounds; hence, their excellent nutritional values and biological or pharmacological activities are mainly due to flavonoids [4]. Flavonoids are important bioactive plant compounds with a wide range of nutritional and health benefits as well as biological activities, such as anti-atherosclerosis, anti-inflammatory, anti-diabetic, neuroprotective, antioxidant, anti-proliferative, antimicrobial, and hepatoprotective [5–10] activities. The biological effects of flavonoids are due to their interactions with various proteins and enzymes, including cytochrome P450, laminin receptor, phospholipase A2, α-amylase, α-glucosidase [4], and many others. Interactions between flavonoids and α-amylase and/or α-glucosidase (protein or enzyme) are specific inhibitory mechanisms of prospective anti-diabetic drugs [4,11]. Flavonoids also influence insulin production, insulin signaling, carbohydrate metabolism/digestion, and glucose absorption in insulin-sensitive tissues through a variety of intracellular signaling pathways [12,13].

Digestive enzymes α-amylase and α-glucosidase that hydrolyze starch are implicated in postprandial hyperglycemia; thus, inhibition of these enzymes reduces glucose release and absorption in the small intestine. This significant anti-diabetic effect attributed to the inhibition of α-amylase and α-glucosidase could be achieved through the biological activities of flavonoids [14,15]. Acarbose, miglitol, and voglibose are examples of synthetic medicines that block α-amylase, α-glucosidase, while biguanides, such as metformin, are used to control postprandial hyperglycemia. However, their usage is restricted or discouraged because of diarrhea, stomach pain, flatulence, and other adverse effects. As a result, novel inhibitors with improved safety and efficacy profiles to reduce postprandial glycemic levels effectively and efficiently should be discovered and developed from natural products, particularly medicinal plants. Natural medicinal plant products exhibit a greater chemical variety and engage a larger chemical space than synthetic medications. Natural product pharmacophores serve as the foundation for newly synthesized pharmaceuticals with lower hydrophobicity and higher stereochemical richness than drugs derived entirely from synthetic materials [16]. The bioactive components of natural products or medicinal plants are essential therapeutic agents in biomedical and natural product research. As a result, research into their bioactive profiling is critical. It comprises a series of steps, including plant selection, collection and identification, bioactive molecule extraction and isolation, structural elucidation, and biological and pharmacological screening [17].

The *Helichrysum* genus is an aromatic plant in the *Asteraceae* family, with roughly 500–600 species found globally, including in South Africa. Diabetes mellitus, cough, cold, wound, renal disease, skin infections, chest pain, menstruation pain, fever, and hypertension have all been treated with the *Helichrysum* genera locally in the past. The antioxidant, antibacterial, antifungal, anti-ulcerogenic, anti-tyrosinase, and anti-proliferative characteristics of the *Helichrysum* genus have been investigated in scientific studies [18,19]. Many complex bioactive chemicals, such as phloroglucinols and their derivatives, chalcones, flavonoids, α-pyrones, essential oils, and terpenoids, may be found in this massive plant genus [20,21], whereas for *Helichrysum petiolare*, phenolics, flavonoids, and anthocyanins are the dominant phytochemicals [18,22,23]. A recent review indicated that some species of *Helichrysum* are under-explored for drug discovery and development [18] in many human diseases

or disorders, such as diabetes mellitus. Previous reports have affirmed that a few species, such as *H. nudifolium* L. Less, *H. odoratissimum* L. Sweet, and *H. petiolare* H and B.L have traditionally been used to treat DM [24]. *Helichrysum plicatum ssp. plicatum* and *Helichrysum graveolens* have also been scientifically investigated for the treatment of DM [25,26] in drug discovery and development as an effective anti-diabetic agent. *Helichrysum petiolare* H and B.L is commonly called the silverbush everlasting plant and is called kooigoed in the Afrikaans dialect of South Africa. *Helichrysum petiolare* is a shrub with silver-grey hair covering its aromatic round-shaped leaves with whitish-cream flowers. Our previous study has profiled the antioxidant activities, total phenol, total flavonoids, and fatty acid composition of the aqueous acetone of *Helichrysum petiolare* extract (AAHPE) [in the press]. Therefore, we aimed to provide evidence for the cytotoxic screening of this extract, inhibitory activity of α-amylase and α-glucosidase, glucose uptake activity, and LC-MS analysis of flavonoids of this extract. The molecule docking of the bioactive flavonoid's interactions for possible drug discovery and development of safe and effective anti-diabetic agents from *Helichrysum petiolare* H and B.L. was investigated. This research ultimately aims at providing and ascertaining the possible use of *Helichrysum petiolare* H and B.L extract and its phytochemicals as an alternative source of anti-diabetic agents in the treatment of postprandial hyperglycemia in type 2 diabetes mellitus.

2. Material and Methods

2.1. Chemicals

All chemicals used in this study, including metformin, 2NBDG, 2-(*N*-(7-Nitrobenz-2oxa-1,3-diazol-4-yl) amino)-2-deoxy-D-glucose, acetone, DMSO, α-amylase and α-glucosidase enzymes, 3,5-dinitro salicylic acid, sodium hydroxide, sodium potassium tartrate, and sodium chloride, were high-grade quality of minimum of 95% and were purchased from Sigma Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany). The cell culture media and reagents have been indicated in the manuscript in the cell line and culture condition section.

2.2. Collection of Plant Material

Helichrysum petiolare with accession number UFH-2020-10-01 was collected from Cape Peninsula University (CPUT), Bellville, in October 2020, Western Cape, South Africa and identified by Prof. Christopher N. Cupido of the Department of Botany, University of Fort Hare, Alice, South Africa.

2.3. Plant Extraction

As previously reported by Nas et al. (2019), the leaves of the plants were cleaned and air-dried to a constant weight. The dried plant sample was pulverized using an electronic blender, and the ground plant was weighed. The powdered plant materials were soaked in 90% aqueous acetone in conical flasks, subjected to intermittent stirring, and warmed in the water bath at 60 °C for 2 h [27]. The mixture was filtered through Whatman cellulose filter paper under pressure using a pump. The plant material was subjected to a second extraction by soaking overnight, and the filtrates were pooled together before being subjected to a rotary evaporator. The residue or extract obtained was allowed to dry in the fume cupboard. The residual extracts were stored at −20 °C until required for use. A percentage yield of 6.3% was obtained after 97.6 g of plant material underwent the extraction process to give 6.155 g of the extract.

2.4. Determination of the Enzymatic Inhibitory Activity of AAHPE

2.4.1. α-Amylase Inhibitory Assay

The inhibition of α- glucosidase by plant extract as described by Ali et al. (2006) using a spectrometric method [28]. In their study, the same concentration range (10–250 µg/mL) was used for the plant extract and acarbose. Here, different concentrations of plant extracts in DMSO were mixed

with 4.8 mL of distilled water and 1.2 mL of 0.5% *w*/*v* soluble potato starch in 20 mM phosphate buffer (pH 6.9) containing 6.7 mM sodium chloride in test tubes. At 0 min, 600 µL of enzyme solution was added (4 units/ml in distilled water); after 3 min, 600 µL of the mixture was transferred into another test tube containing 300 µL of DNSA (1 g of 3,5-dinitrosalicylic acid (96 mM), 30 g of sodium potassium tartrate, and 20 mL of 2N sodium hydroxide to a final volume of 100 mL in distilled water, and transferred to a hot water bath maintained at 85–90 °C for 15 min. The reaction mixture in each test tube was diluted with 2.7 mL distilled water and an absorbance measurement (Thermo spectronic spectrophotometer model-biomate 3, Madison, WI, USA) was performed at 540 nm. For concentration, the blank incubation was prepared by replacing the enzyme solution with 600 µL of distilled water at the start of the reaction. The control incubations, representing 100% enzyme activity, were conducted in the same way by replacing the plant extract with 120 µL DMSO. All the tests were in triplicate. Net absorbance (A) due to maltose generated was calculated as Equation (1):

$$\text{A450 nm plant extract} = \text{A450 nm Test} - \text{A450 nm blank} \quad (1)$$

Thus, from the value obtained, the percentage (*w*/*v*) of maltose generated was calculated from the equation obtained from the maltose standard calibration curve (0–0.1% *w*/*v* maltose). The level of the inhibition (%) was calculated as Equation (2):

$$\%\text{inhibition} = 100 - \%\text{reaction (at t = 3 min)} \quad (2)$$

where, %reaction = mean maltose in sample × 100/Mean maltose in control

2.4.2. α-Glucosidase Inhibitory Assay

The inhibition of α-glucosidase by plant extract is described with slight modification from [29]. Different concentrations of the plant extract (10–250 µg/mL) were incubated with 0.5 mg of the protein equivalent of crude α-glucosidase enzyme before reaction initiation with 45 mM sucrose as substrate, in a final reaction mixture of 1 mL of 0.1 M phosphate buffer (pH 7.2). The reaction mixture was incubated for 30 min at 37 °C. A total of 1000 µL of Tris base was used to stop the reaction, and α-glucosidase was monitored using the released glucose with glucose oxidase method by absorbance at the wavelength of 450 nm. α-glucosidase inhibitory activity was expressed as percentage inhibition. The enzyme inhibition data were expressed as IC_{50} values, depicting the plant extract concentration that inhibits 50% of α-glucosidase activity.

2.5. Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis of AAHPE

The LCMS analysis was performed using the method of Standers et al., with slight modification [30]. The UPLC-MS analysis was performed with a Waters Synapt G2 quadrupole time-of-flight (QTOF) mass spectrometer (MS) connected to a water Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA). Electrospray ionization was used in negative mode with a cone voltage of 15 V, desolvation temperature of 275 °C, desolvation gas at 650 L/h, and the rest of the MS settings optimized for the best resolution and sensitivity. Data were acquired by scanning from 150 to 1500 *m*/*z* in both resolution and MSE mode. Two channels of MS data were obtained in MSE mode, first at low collision energy (4 V) and the second using a collision energy ramp (40–1000V) to obtain the fragmentation data. Leucine enkephalin was used as a locked mass (reference mass) for accurate mass determination, and the instrument was calibrated with sodium formate. Separations were achieved on a 150 mm HSST3 column. An injection volume of 3 µL was used as a mobile phase consisting of 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid as solvent B. The gradient started at 100% solvent A for 1 min and changed to 28% B over 22 min linearly. It changed to 40% B over 50 s and a wash step of 1.5 min at 100% B was achieved after re-equilibration to the initial condition for 4 min. The flow rate was 0.3 mL/min and a column temperature of 55 °C was maintained. Compounds were quantified relatively against a calibration curve established by injecting a range of catechin standards from 0.5 to 100 mg/L catechin.

2.6. In Vitro Studies

2.6.1. Cell Line and Culture Condition

The human hepatocarcinoma cell line HepG2, ATCC® HB-8065 ™, was obtained from the American Type Culture Collection (ATTC, Manassa, VA, USA). The cell line was cultured in Dulbecco's Modified Eagles Medium (DMEM) 4.5 g/L glucose supplemented with 10% foetal bovine serum (Gibco, Life Technologies Corporation, Paisley, UK), 1% 100 U/mL penicillin, and 100 µg/mL of streptomycin (Lonza Group Ltd., Verviers, Belgium) in sterile 60 mm Petri dishes placed in a 95% O_2 and 5% CO_2 sterile incubator condition for proper growth. At 70–80% cell confluence, the cells were passaged using 0.1% trypsin EDTA (Lonza Group Ltd., Verviers, Belgium).

2.6.2. Cell Cytotoxicity by MTT Assay of AAHPE

In a cytotoxic assay, MTT(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) mechanisms involve the ability of viable cells to reduce the MTT reagent to formazan using the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidoreductase enzyme. The colour change from the yellow MTT salt solution to purple formazan occurs in the mitochondria of viable or living cells. In this assay, powdered MTT at a concentration of 5 mg/mL in PBS was used. HepG2 cells with optimum established cells of 5000 cell/well of 96-well plates were seeded in 100 µL of complete DMEM culture medium for 24 h to attach. After attachment, the cells were treated by replacing the culture medium with 25 µg/mL, 50 µg/mL, 75 µg/mL, and 100 µg/mL of AAHPE concentrations while cells without plant extract served as the control for the 48 h duration. After treatment, 10 µL of MTT solution was added to every well and incubated for 4 h. The supernatant was carefully aspirated and 100 µL of DMSO was added, after which it was read at 570 nm with a BMG Labtech multi-cell plate reader. The cell viability was expressed as a fraction of viable cells relative to the control culture.

2.6.3. NBDG Glucose Uptake Assay

The insulin-resistant HepG2 cell model and glucose uptake were established according to Liu et al.'s [29] method with slight modifications. Briefly, HepG2 cells were cultured in a black 96-well culture plate; after reaching confluence, the cells were treated with 10^{-6} mol/l insulin for 24 h to induce insulin resistance. The IR HepG2 cells were treated with 25 µg/mL, 50 µg/mL, and 75 µg/mL concentrations of plant extracts and 4 mM metformin (induces proliferation, safe dose) for 24 h, after which they were incubated with 100 nM insulin for 30 min. The glucose uptake was measured after incubation with 40 µM 2-NBDG for 30 min. The cells were washed with ice-cold PBS to stop the response, and 2-NBDG fluorescence intensity was measured on a microplate reader (BMG Labtech Omega® POLARStar, Ortenberg, Germany) at 485 nm (excitation) and 528 nm (emission) wavelengths. The experiment was repeated three times.

2.7. In Silico Drug-Likeness Analysis and ADMET Profiling

The physicochemical, pharmacokinetic, and drug-likeness properties of the flavonoid's compounds identified by LC-MS were determined. In silico ADME (adsorption, distribution, metabolism, and excretion) analysis was performed using the European Bioinformatics Institute (EBI) SwissADME online analyzer (http://swissadme.ch/ (accessed on 25 October 2021)) and ADMETlab web server (http://admet.scbdd.com (accessed on 25 October 2021)) [31,32]. The numerological values of the flavonoid compounds and metformin, an anti-diabetic drug, were interpreted by the qualitative units based on the ADMETlab server explanation. The canonical SMILES for structures of all the compounds and metformin were retrieved from the National Center for Biotechnology Information (NCBI) PubChem database (https://pubchem.ncbi.nlm.nih.gov/ (accessed on 25 October 2021)) prior to the analysis.

2.8. Molecular Docking

2.8.1. Protein Preparation

The crystal structures of α-amylase (PDB ID: 2QV4) and α-glucosidase (PDB ID: 3WEL) proteins were retrieved in .pdb format from the Protein Data Bank (PDB) (https: //www.rcsb.org/ (accessed on 25 October 2021)). The PDB is a worldwide archive used to access the 3D structures of biological macromolecules (Burley et al., 2021). In this study, we adopted a single chain of α-amylase and α-glucosidase for docking analysis. MGLTools software was used for protein preparation. The water molecules and co-crystallized ligands were deleted from the macromolecule and polar hydrogens were added.

2.8.2. Ligand Preparation

The initial 3D structures of the selected ligands were retrieved in .sdf format from PubChem (https://pubchem.ncbi.nlm.nih.gov/ (accessed on 25 October 2021)) and Chem-Spider (http://www.chemspider.com/ (accessed on 25 October 2021)).Pub Chem and ChemSpider are publicly accessible repositories for chemical substances and their related biological activities [33,34]. The optimized structures were then converted into .pdb format using open BABEL.

2.8.3. Docking Protocol

The PDB files of both ligands and proteins were converted in an extended PDB format, termed PDBQT, to perform molecular docking analysis using AutoDock 1.5.6 and AutoDock Vina. The docking protocol was used as previously reported by many studies [35,36]. The "Grid" of AutoDock 1.5.6 was used for calculating the grid parameters, and all the data regarding target proteins, ligand, grid size, and geometry were saved in the "TXT" file. Docking was performed with the grid box size set to 60 × 68 × 56 and 84 × 76 × 50 xyz points for α-amylase and α-glucosidase, respectively, with a grid spacing of 1 Å and the grid center designated at dimensions (x, y, and z): 17,453, 61,696, and 14,571 and 13,999, 16,094, and 28,018 for α-amylase and α-glucosidase, respectively. The output PDBQT files were written into a config. (configuration) file. The conformation with the lowest binding energy was considered the most stable conformation of the ligand regarding the bioactive compounds. The results were analyzed using the free version of Biova Discovery Studio 2020 client (Dassault Systèmes BIOVIA, Discovery Studio Modeling Environment, Release 2017, San Diego: Dassault Systèmes, 2016).

2.8.4. Docking Method

The reference ligands were docked in the binding site of the target proteins and compared with those of the co-crystallized ligands of the target proteins (the PDB ligand of 2QV4 and 3WEL was the sulphate ion) to determine the accuracy of the docking protocol. The prepared ligand molecules were docked in the binding site of the refined α-amylase and α-glucosidase models utilizing AutoDock Vina and scored using the scoring function. The protein–ligand interactions were analyzed further for the docked poses of the ligands in the binding sites of the target proteins. The best pose was selected for further analysis of the binding interactions (including H-bond and hydrophobic interactions) of the ligands using PyMOL (The PyMOL Molecular Graphics System, version 2.2.0, Schrodinger, New York, NY, USA, 2018) and Biova Discovery Studio 2020 client (Dassault Systèmes BIOVIA, Discovery Studio Modeling Environment, Release 2017, San Diego: Dassault Systèmes, 2016).

2.9. Statistical Analysis

GraphPad Prism7 software was used for statistical analysis and data were expressed as mean ± SD. The significant difference was set at ($p < 0.05$) and Duncan's test was performed.

3. Results

The results of the α-amylase inhibition assay of AAHPE indicated concentrationdependent inhibitory action, with the highest concentration having the highest inhibition revealed in Figure *6*.1. The IC_{50} of the extract (46.50 ± 6.17 µg/mL) was lower than that for standard acarbose (IC_{50} = 0.32 ± 0.16 µg/mL) as shown in Table *6*.1. In the same vein, the α-glucosidase inhibitory assay of the plant extract also showed concentration-dependent inhibitory potential revealed in Figure *6*.1. The IC_{50} of the plant extract (37.81 ± 5.15 µg/mL) was lower than that of the standard acarbose (IC_{50} = 5.38 ± 2.76 µg/mL) as shown in Table *6*.1. At the highest concentration of 250 µg/mL, we obtained inhibitory activity of 85.25% and 82.77%, respectively, for α-amylase and α-glucosidase. Overall, the plant extract exhibits inhibition action against α-amylase and α-glucosidase and is potentially an anti-diabetic agent, especially in postprandial hyperglycemic conditions.

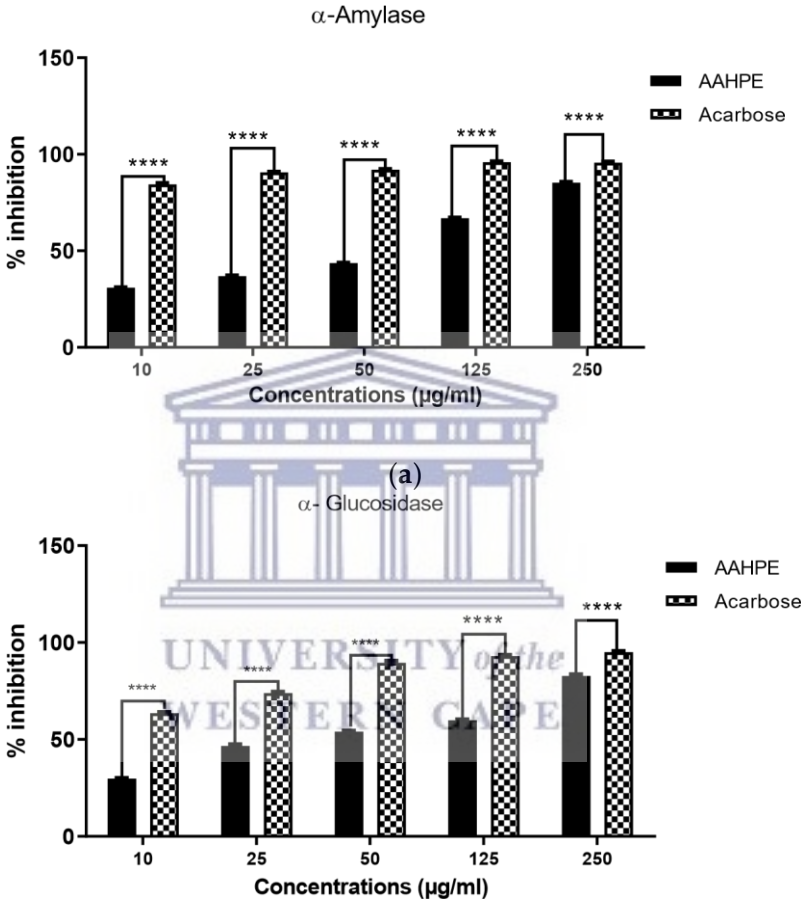

Figure 6.1. (**a**) α-amylase and (**b**) α-glucosidase inhibitory activity of AAHPE, using acarbose as a positive control (10–250 µg/mL). Values are expressed as the mean ± SD (*n* = 3), **** $p < 0.0001$ compared with the control.

Table 6.1. The IC_{50} inhibitory effect of AAHPE and acarbose on the α-amylase and α-glucosidase enzymes.

IC_{50}	AAHPE (µg/mL)	Acarbose (µg/mL)
α-amylase	46.50 ± 6.17	0.32 ± 0.16
α-glucosidase	37.81 ± 5.15	5.38 ± 2.76

3.1. Screening of the Flavonoid's Compound of AAHPE Using LC-MS Analysis

We identified 38 compounds with average R_t (min) and average *m/z* values and concentrations through LC-MS/MS analysis (Supplementary Materials Table S1). Previous studies have identified a positive relationship, or the ability of an increasing concentration of flavonoids, to inhibit α-amylase and α-glucosidase [37]. Hence, 19 different secondary metabolites (flavonoids) identified in Figure *6*.2, LC-MS/MS analysis having concentrations above 100 mg/g and acarbose, an anti-diabetic drug, were docked in this study. The quantitative assessment was indicated in their concentrations (this can be seen in Supplementary Materials Table S1), and the 2D chemical structure of the selected 19 bioactive compounds of AAHPE were illustrated (Supplementary Materials Table S2). The quantitative assessment indicated the following predominant compounds: 3,5-dicaffeoylquinic acid (1727.3 mg/g), arubitin (1279.7 mg/g), 4,5-caffeoylquinic acid (1209.1 mg/g), 5-caffeoylquinic acid (826.7 mg/g), engeletin (747.9 mg/g), quercetin-3-galactoside (586.3 mg/g), 5-feruloyl quinic acid (411.6 mg/g), 3-*O*-caffeyl-4-*O*-methylquinic acid (407.1 mg/g), myricetin 3-galactoside (402.5 mg/g), and dicaffeoylquinic acid (388.7 mg/g), among others.

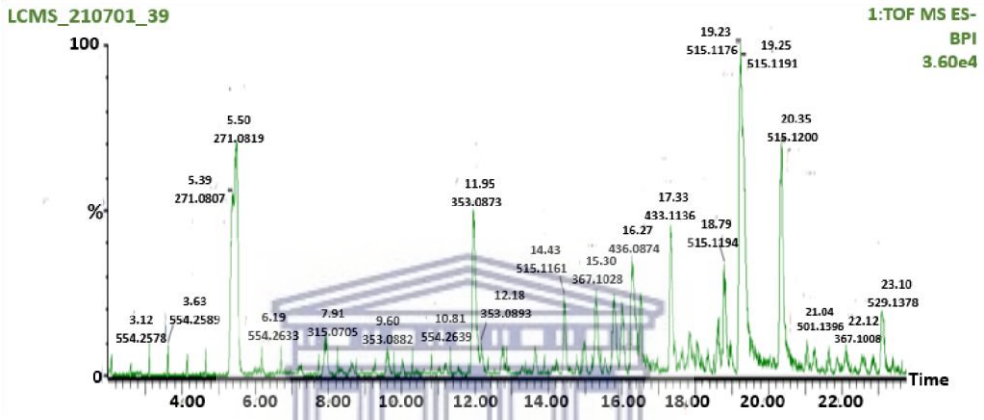

Figure 6.2. LC-MS chromatogram screening of AAHPE.

3.2. The Effect of ACHPE on HepG2 Cell Viability

HepG2 cells were treated with increasing concentrations of AAHPE (25–100 µg/mL) for 48 h. The adherence to the cell viability threshold of 80% was sufficient for our work [38]. The MTT assay showed a slight increase in cell viability with the addition of the AAHPE in a dose-dependent manner between concentrations of 25 and 75 µg/mL, indicating non-toxicity compared to the control. However, at 100 µg/mL, the cell viability reduced by a quarter compared with the control. Thus, cell viability at a concentration of 100 µg/mL is around 75%, indicating slight toxicity. Hence, we adhered to concentrations of 25–75 µg/mL because the cell viability was above the threshold of 80% at these concentrations as shown in Figure *6*.3; thus, they were regarded as very safe doses and were used in the subsequent assay in this study.

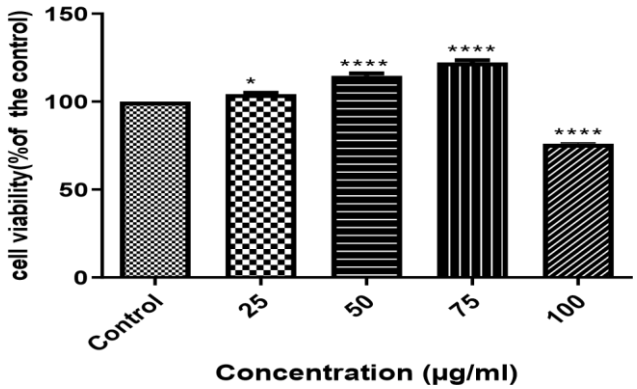

Figure 6.3. Cytotoxic screening of AAHPE at different concentrations. Values are expressed as the mean ± SD (n = 3), * $p < 0.05$ and **** $p < 0.0001$ compared with the control.

3.3. The Effect of AAHPE on Glucose Uptake

The glucose uptake effect in the insulin-resistant HepG2 cells indicated that the standard drug metformin increased the glucose uptake tremendously. There was increase in glucose uptake in concentration-dependent manner treated in insulin-resistant HepG2 cells with AAHPE (25–75 µg/mL). The highest concentration of the plant extract 75 µg/mL is slightly higher in glucose uptake activity than the standard metformin as revealed in Figure *6*.4.

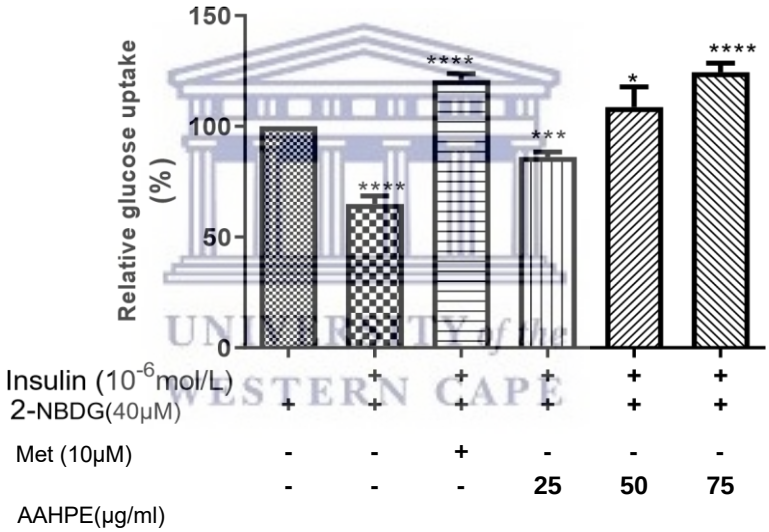

Figure 6.4. Effect of AAHPE on insulin-stimulated glucose uptake in insulin-resistant HepG2 cells. The insulin-resistant cells induced with 10^{-6} mol/L insulin were treated with AAHPE (25–75 µg/mL) concentrations or metformin for 48 h and glucose uptake was measured using fluorescent D-glucose 2-NBDG. Values are mean ± SD, * $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$ significant compared with the control.

In this study, not all the potential compounds satisfied Lipinski's rule of five regarding the octanol-water partition coefficient (LogP ≤ 5), molecular weight (≤500 KDa), number of H-bond donors (≤5), number of H-bond acceptors (≤10), and molecular refractivity (40–130) as tabulated in Table *6*.2. Lipinski's rule of five, referred to as Pfizer's rule, is one of the techniques used to evaluate the drug-likeness of a chemical compound as it delineates the relationship between pharmacokinetics and physicochemical parameters. It determines the potential of the biological activities or pharmacological properties of a chemical compound and its effectiveness as an oral drug in humans [39,40]. We adhered

to compounds with no violations or a minimum of a single violation, as violations of two or more indicate that drug candidates may not be orally active [40,41]. Hence, compounds with predicted poor oral absorption but high potency, as revealed in the Supplementary Materials, could be improved upon using an optimized absorption enhancement approach in future research.

The different ADME properties help with predictions and are essential in drug discovery and development, as shown in Table *6*.3. A significant property is metabolism, which involves the interactions of possible drug candidates with cytochrome P450 (CYP) that ultimately lead to drug elimination through metabolic biotransformation. We observed that all the compounds did not inhibit the different CYP isoforms (CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4) except sinocrassosideA1, which is an inhibitor of CYP1A2 and CYP2C19. Essentially, CYP and P-glycoprotein (P-gp) offer protection to tissues and organisms by processing molecules or drugs synergistically. The inhibition of CYP connotes a significant cause of pharmacokinetics-related drug–drug interactions, with increased toxicity and unwanted side effects predominating because of the reduced clearance and bioaccumulation effect of the drug or metabolite [40,42]. In drug discovery and development, potential drug candidates with unfavourable absorption, distribution, metabolism, and elimination (ADME) parameters are disqualified from clinical trials [31].

Table 6.2. Predicted pharmacokinetic parameters (ADME properties) of the compounds selected from plant LC-MS analysis.

Compounds	$LogP_{o/w}$ (MLOGP)	LogSw (ESOL)	MW (g/mol)	HBA	HBD	Reactivity (40–130)	tPSA	Solubility (mg/mL)	Rotatable Bonds (RoB)	*n* Violation
3-caffeoylquinic acid	0.96	−1.62	354.31	9	6	83.50	164.75	8.50	5	1
3-*O*-Caffeoyl-4-*O*-methylquinic acid	1.47	−1.84	368.34	9	5	87.97	153.75	5.38	6	0
4-Feruloyl quinic acid/5-Feruloylquinic acid	1.47	−1.84	368.34	9	5	87.97	153.75	5.38	6	0
Arbutin	1.07	−0.71	272.25	7	5	62.61	119.61	5.27	3	0
Engeletin	1.77	−3.09	434.49	10	6	103.95	166.14	3.55	3	1
Metformin	0.34	0.29	129.16	2	3	36.93	91.49	2.53	2	0
Protocatechuic acid	1.09	−1.89	154.12	4	3	37.45	777.76	1.99	1	0
SinocrassosideA1	3.36	−4.09	316.43	3	0	92.90	38.83	2.58	0	0
Acarbose	8.56	−2.13	645.60	19	14	136.69	321.17	2.32	9	3

MW: molecular weight, logP: partition coefficient, tPSA: topological polar surface area, logSw: water solubility, HBA: hydrogen bond acceptors, HBD: hydrogen bond donors.

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Table 6.3. ADMET properties of the AAHPE compounds predicted using the SwissADME online analyzer and ADMETlab web server.

Class	Properties	3-Caffeoylquinic Acid	3-*O*-Caffeoyl-4-*O*-Methylquinic Acid	4-/5-Feruloyl Quinic Acid	SinocrassosideA1	Engeletin	Metformin	Protocatechuic Acid	Acarbose	Arbutin
Absorption	Caco-2 permeability (>−5.15 cm/s)	−6.58	−6.331	−6.331	−4.56	−6.581	−5.502	−5.107	−0.8955	−5.954
	Pgp-inhibitor	No	No	No	No	No	No	No	Yes	No
	Pgp-substrate	No	Yes	No	Yes	Yes	No	No	No	No
	HIA (≥30%: high, <30%: low)	Low	High	High	High	High	High	Low	Low	High
	Bioavailability score	0.11	0.11	0.11	0.55	0.55	0.55	0.56	0.11	0.55
	GI absorption	Low	Low	Low	High	Low	High	High	Low	High
	Skin permeation (Log Kp) (cm/s)	−8.76	−8.62	−8.62	−5.65	−8.42	−7.99	−6.39	−5.16	−8.92
Distribution	PPB (90%)	41.961%	41.1349%	41.1349%	97.16.5%	42.5742%	3.9577%	42.6641%	21.138%	36.0499%
	BBB	No	No	No	Yes	No	No	No	No	No
Metabolism	CYP1A2 inhibitor	No	No	No	No	No	No	No	No	No
	CYP1A2 substrate	No	No	No	No	No	No	No	Yes	No
	CYP3A4 inhibitor	No	No	No	No	No	No	No	No	No
	CYP3A4 substrate	Weakly	Weakly	Weakly	Yes	Weakly	No	Yes	Yes	Weakly
	CYP2C9 inhibitor	No	No	No	Yes	No	No	No	No	No
	CYP2C9 substrate	No	No	No	No	No	No	No	No	No
	CYP2C19 inhibitor	No	No	No	Yes	No	No	No	No	No
	CYP2C19 substrate	No	No	No	No	No	No	No	No	No
	CYP2D6 inhibitor	No	No	No	No	No	No	No	Yes	No
	CYP2D6 substrate	No	No	No	No	No	Yes	No	Weakly	No
Excretion	$T_{1/2}$ (>8 h: high; 3 h < Cl < 8 h: moderate; <3 h: low)	0.442	0.565	0.565	1.587	1.213	1.838	0.318	1.32	0.713
	Clearance rate (>15 mL/min/kg: high; 5mL/min/kg < Cl < >15 mL/min/kg: moderate; <5 mL/ min/kg: low)	1.196	1.174	1.174	1.569	1.033	0.911	1.601	0.503	1.526
Toxicity	hERG I/II	No/No	No/No	No/No	No/No	No/No	Yes/Yes	No/No	Ambiguous	No/No
	AMES toxicity	No	No	No	No	No	No	No	No	No
	H-HT (Human hepatotoxicity)	No	No	No	No	No	No	No	No	No
	Skin sensitization	No	No	No	No	No	Yes	No	No	No
	Max. tolerated dose (human) (log mg/kg/day)	−0.134	1.285	1.285	−0.078	0.306	0.902	0.787	0.484	0.485

3.4. Result of Molecular Docking

The present work revealed the binding interaction of 19 selected bioactive compounds in AAHPE and acarbose with α-amylase and α-glucosidase enzymes or protein molecules. Further, an investigation into the interaction was performed in silico using the Auto Dock Vina. Each of the 19 bioactive compounds identified with LC-MS and acarbose (standard drug, positive control) were docked, giving a clear understanding of their interaction with the active sites of both human pancreatic α-amylase (HPA) and human intestinal α-glucosidase (HIG) enzymes. As revealed in Table *6*.4, 19 bioactive compounds present in AAHPE had a higher binding affinity for both HPA and HIG enzymes than the standard drug acarbose. The binding affinity for HPA ranged from ΔG = −7.2 to −9.6 kcal/mol compared to ΔG = −6.1 kcal/mol for acarbose, while the binding affinity for HIP enzymes ranged from ΔG = −7.3 to −9.0 kcal/mol compared to ΔG = −6.3 kcal/mol for acarbose. Arbutin (−7.0 kcal/mol) and protocatechuic acid (−6.6 kcal/mol) showed the least binding affinity for HPA and HIG enzymes, respectively, from the identified bioactive compounds but had higher binding affinity when compared with acarbose. Overall, the binding interactions of all the bioactive compounds were better than the acarbose standard anti-diabetic drug. It is important to note that the more negative the binding free energy value is, the greater the likelihood of the ligand binding to the receptor, as depicted in the interactions of the compounds and acarbose with HPA and HIG represented in Table *6*.4. Among all compounds, sinocrassosideA1 showed the lowest binding energy to both α-amylase and α-glucosidase, whereas arbutin exhibited the highest energy in the case of both enzymes (Table *6*.4). These results show that all selected ligands exhibit good binding affinity with our target proteins. Moreover, we selected acarbose as a standard drug, docked against both targeted proteins. The results were compared with the selected anti-diabetic bioactive compounds (Table *6*.4), which further suggest their affinity for the targeted proteins. This depicts the high possibility of molecular interaction between these bioactive compounds and the enzymes α-amylase and α-glucosidase.

This likely denotes that those bioactive compounds can act individually or synergistically, which results in the good inhibitory activity against HPA and HIG obtained in the in vitro AAHPE inhibition assay.

The detailed interactions of the bioactive compounds of AAHPE with HPA and HIG enzymes was depicted by the visualization and analysis of the docking results, completed using the free version of Biova Discovery studio Visualiser 2020 software. The software revealed the best docking poses, amino acid residues in such interactions, and contributing bond types (conventional hydrogen bond, carbon–hydrogen bond, pi–sigma, pi–pi, pi–alkyl, van der Waals, and others) of the bioactive compounds with HPA and HIG enzymes. From Figures *6*.5 and *6*.6, it was revealed that the bond interactions of the bioactive compounds of AAHPE are numerous and vary. However, van der Waals, carbon–hydrogen, and conventional hydrogen bonds are available in the acarbose interaction with HPA and HIP enzymes. Generally, weak intermolecular interactions, such as hydrophobic and van der Waals bonds, are adjudged to promote greater affinity of the ligand for the target protein with other bonds, thus stabilizing energetically favoured ligands [43]. The best ligand-binding poses in the catalytic domain of HPA and HIG after docking, with the amino acid residues involved in the interaction, are shown in Figures *6*.5 and *6*.6, with a propensity that a set of similar important amino acid residues could be the target to facilitate improved drug efficacy. We observed a similar set of amino acid residues for HPA (Gln63, Thr163, Glu233, and Asp300) and for HIG (Glu109, Lys560, Tyr561, Asn668 and Met801) in the standard drug acarbose with inhibitory potential. However, variation was observed in the interaction of bioactive compounds of AAHPE with the main catalytic residues of Ser774, Arg733, Gln770, Arg392, Gln63, Trp59, and Thr163 for the HPA and HIG enzymes. These additional amino acid residues participating in the

interaction might contribute to the inhibitory activity of these bioactive compounds. Our docking results for the bioactive compounds showed that many were actively involved in hydrogen bonding with eight polar residues, including aspartic acid, serine, histidine, threonine, glutamine, asparagine, glutamic acids, and tyrosine. Other crucial interactions, such as weak van der Waals forces, pi–sigma, and pi–pi interactions, were also found to increase the binding of bioactive compounds with protein binding pockets.

Table 6.4. Predicted binding affinity and detailed docking interactions of α-amylase and α-glucosidase with compounds of AAHPE and acarbose.

Compounds	Binding Affinity (Kcal/mol) αAmylase	No of H-Bonds	H-Bonds Residues with H-Bonds Length (Å)	Binding Affinity (Kcal/mol) αglucosidase	No of H-Bonds	H-Bonds Residues with H-Bonds Length (Å)
3-caffeoylquinic acid	−7.2	4	Ala106 (3.20 Å), Asn105 (3.16 Å), Thr163 (2.65 Å), Gln63 (3.16 Å)	−7.8	6	Gln839 (1.96 Å), Ser774 (2.55 Å), Asn797 (2.68 Å), Thr769 (1.83 Å), Arg773 (2.39 Å) Arg392 (2.78 Å)
3-*O*-Caffeoyl-4-*O*methylquinic acid	−7.4	5	Asp300 (2.27 Å), Thr163 (3.27 Å), Glu233 (2.13 Å), Asp197 (2.94 Å), His305 (2.08 Å)	−7.6	3	Asn797 (2.48 Å), Thr769 (3.15 Å), Arg392 (2.89 Å)
4-Feruloylquinic acid	−7.7	4	Arg195 (3.20 Å), Gln63 (2.70 Å), Thr163 (2.79 Å), Asp300 (2.24 Å)	−7.3	4	Gln839 (2.70 Å), Trp841 (3.25 Å), Thr769 (2.62 Å), Arg392 (2.98 Å)
5-Feruloylquinic acid	−7.7	2	Glu233 (2.59 Å), Asp197 (2.31 Å)	−7.3	4	Gly390 (2.50 Å), Ser774 (2.98 Å), Trp841 (2.88 Å), Arg392 (2.98 Å)
Arbutin	−7.0	3	Glu233 (2.53 Å), Asp197 (2.23 Å), Gln63 (2.96 Å)	−6.8	5	Thr769 (2.71 Å), Trp320 (3.34 Å), Asn797 (2.79 Å), Arg392 (3.28 Å), His387 (2.77 Å)
Engeletin	−8.5	4	Glu233 (2.63 Å), Arg195 (3.34 Å), Gln63 (2.79 Å), His305 (1.97 Å)	−8.4	5	Arg392 (2.83 Å), Tyr319 (3.13 Å), Trp320 (2.91 Å), Arg773 (3.32 Å), Gln770(2.84 Å)
Acarbose	−6.1	4	Glu233 (3.08 Å) Asp300 (2.49 Å), Thr163 (2.93 Å), Gln63 (2.04 Å)	−6.3	4	Glu109 (2.75 Å), Lys560 (2.19 Å), Thr561 (2.29 Å), Met801 (2.29 Å)
Protocatechuic acid	−7.2	3	Glu233 (2.97 Å), Gln63 (3.06 Å), Thr163 (2.57 Å)	−6.6	3	Thr775 (2.65 Å), Ser774 (2.55 Å), Arg773 (2.79 Å)
SinocrassosideA1	−9.6	3	Gln63 (2.50 Å), Asp300 (2.49 Å), Glu233 (2.27 Å)	−9.0	4	Gln839 (2.22 Å), Ser774 (3.08 Å), Glu352 (2.55 Å), Gly390 (1.99 Å)

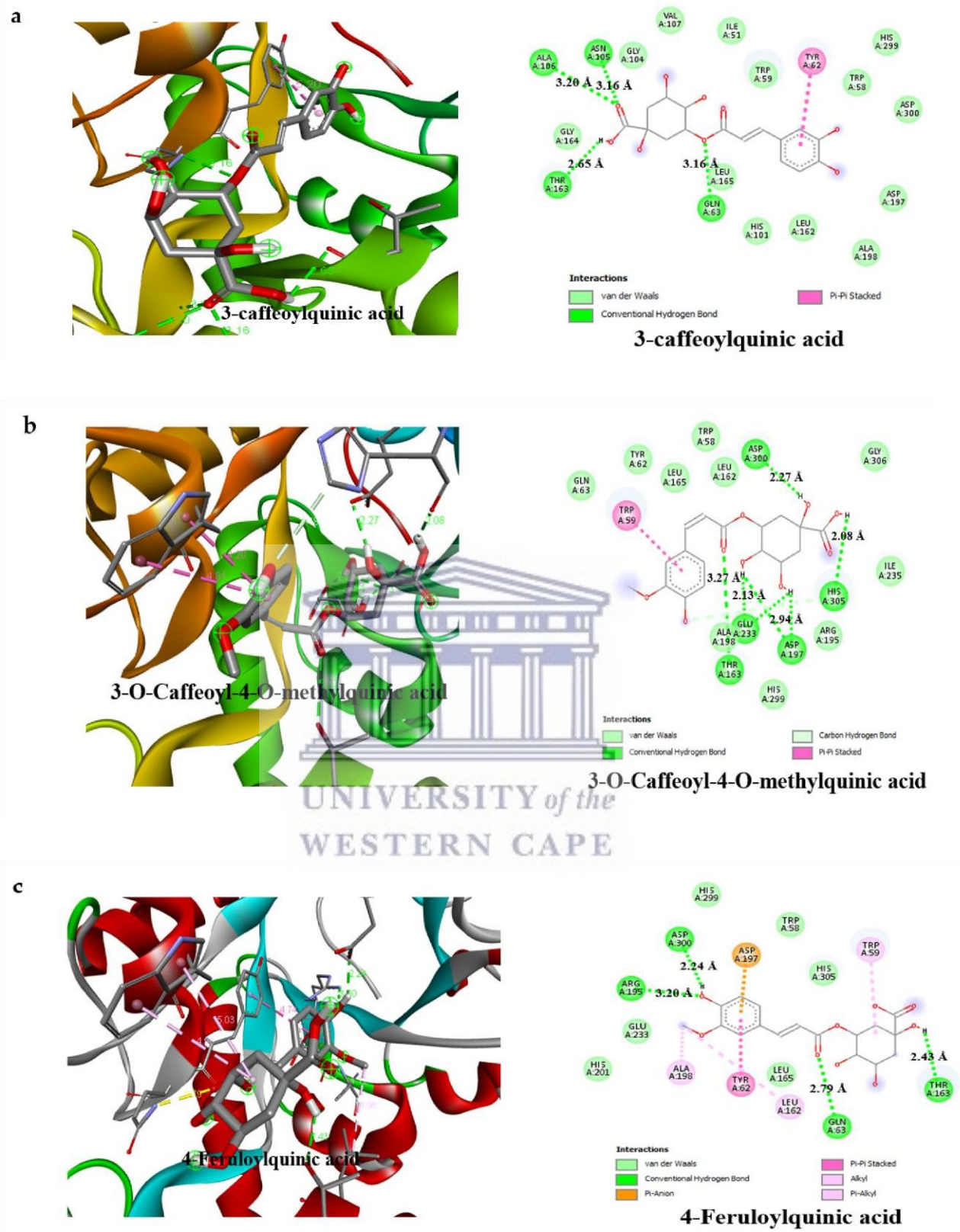

Figure 6.5. *Cont.*

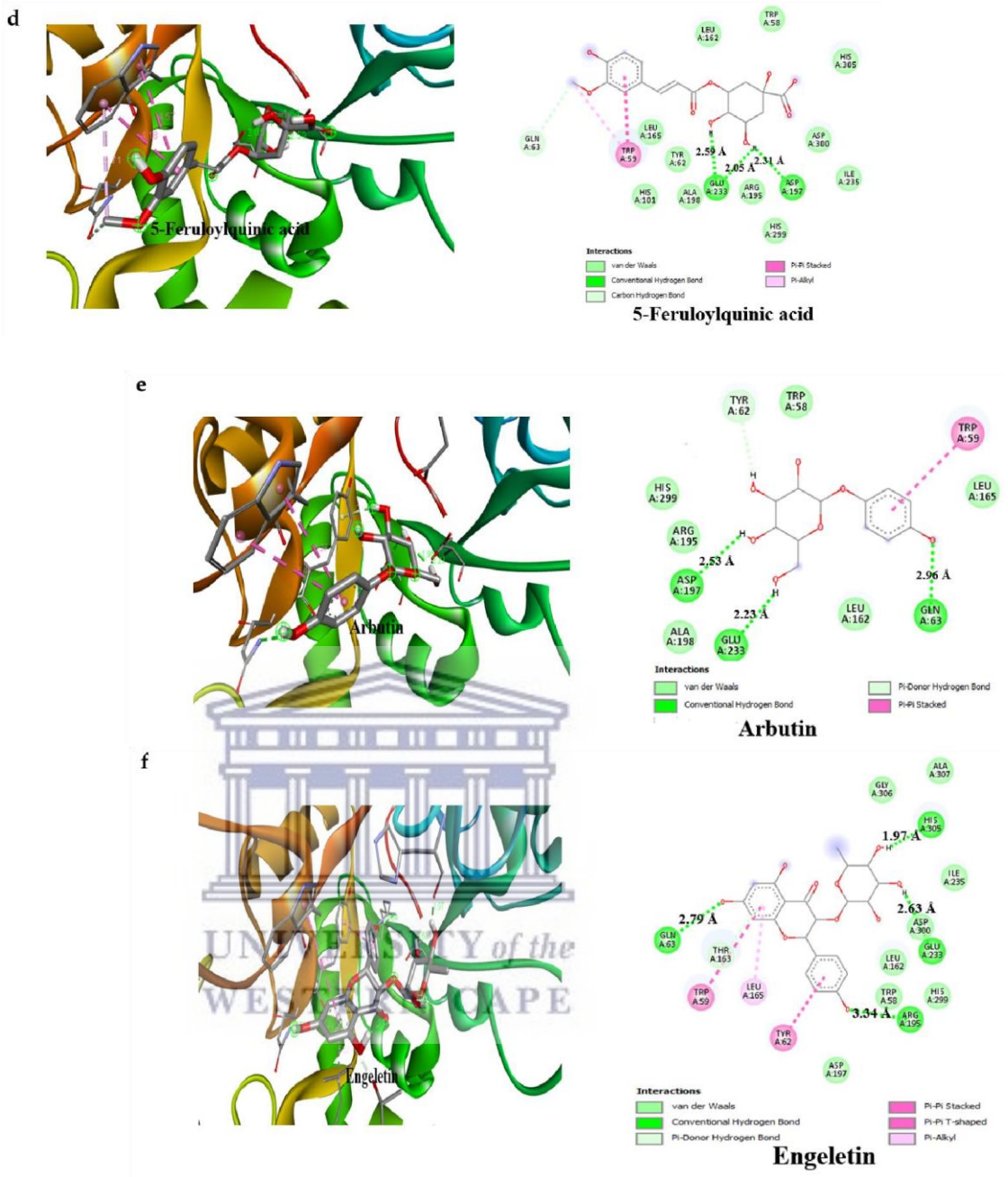

Figure 6.5. *Cont.*

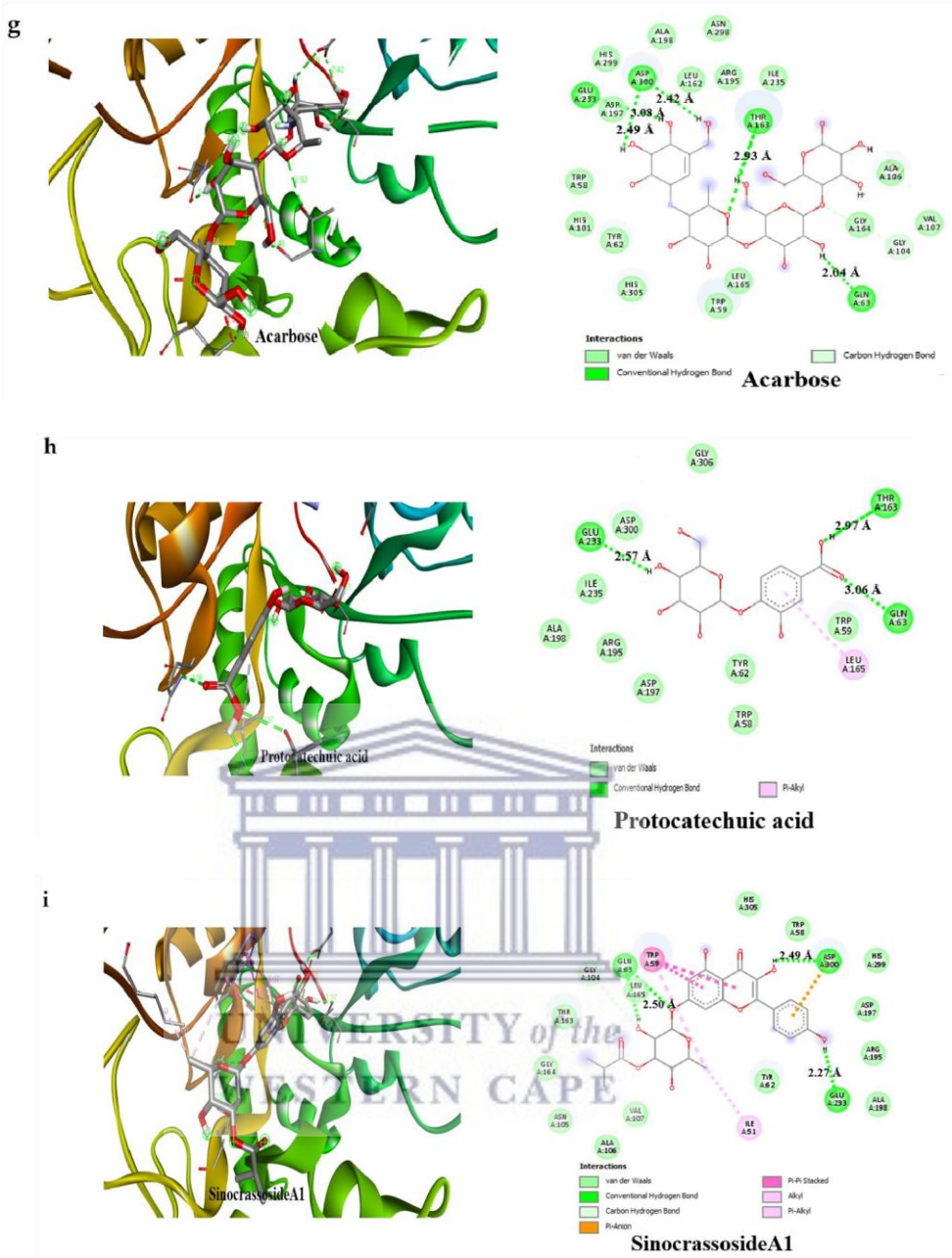

Figure 6.5. Model of the interaction and the 2D Structure of α-amylase protein with (**a**) 3-caffeoylquinic acid, (**b**) 3-*O*-Caffeoyl-4-*O*-methylquinic acid, (**c**) 4-Feruloylquinic acid, (**d**) 5-Feruloylquinic acid, (**e**) Arbutin, (**f**) Engeletin, (**g**) Acarbose, (**h**) Protocatechuic acid, and (**i**) SinocrassosideA1.

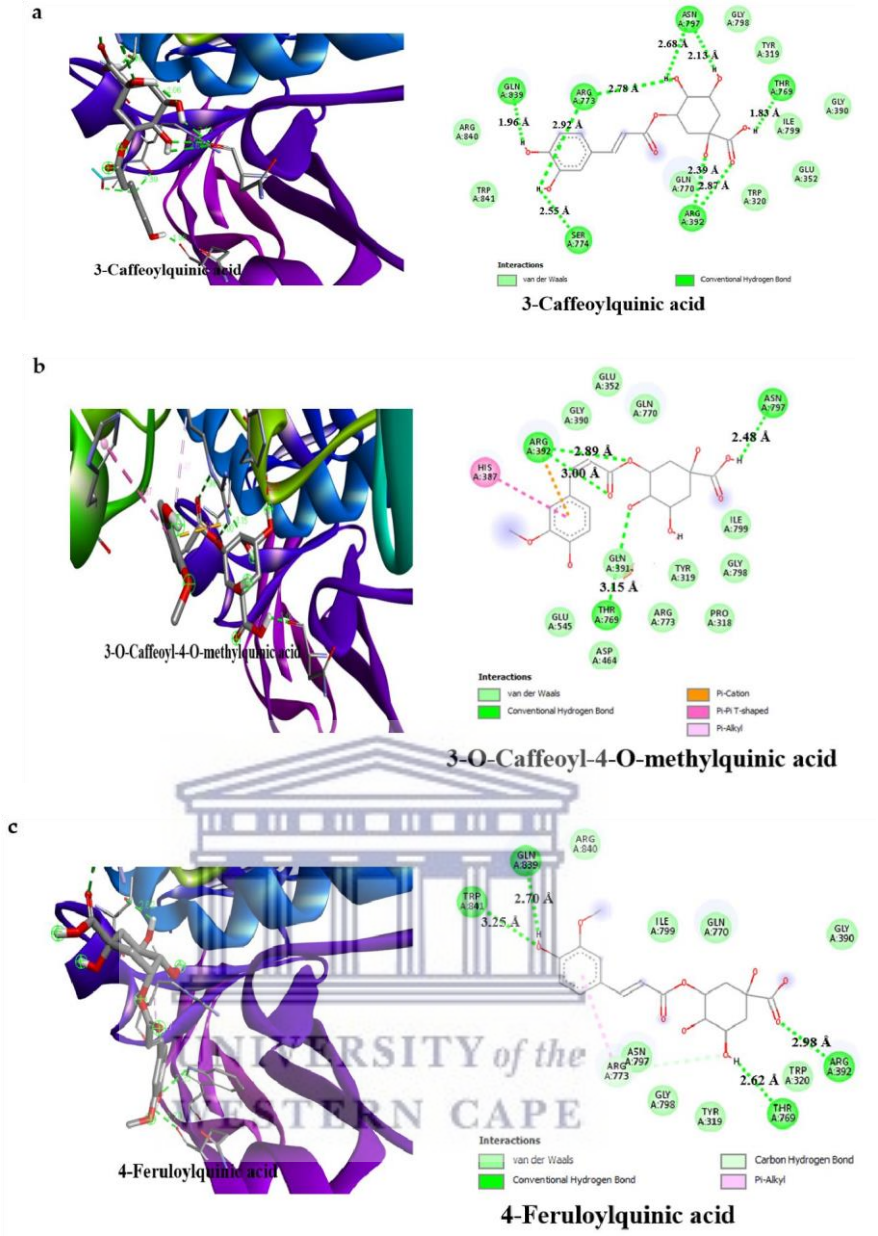

Figure 6.6. *Cont.*

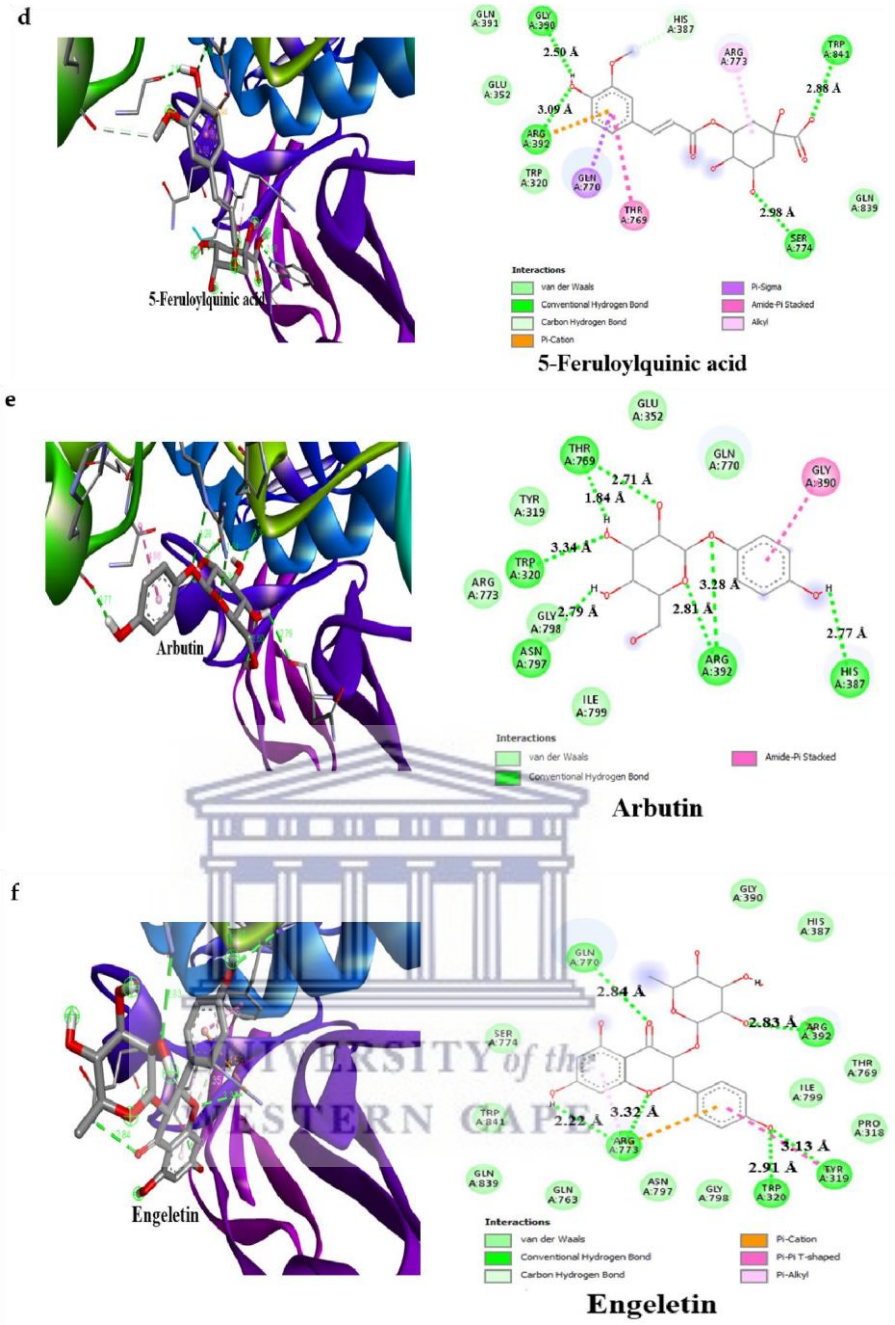

Figure 6.6. *Cont*.

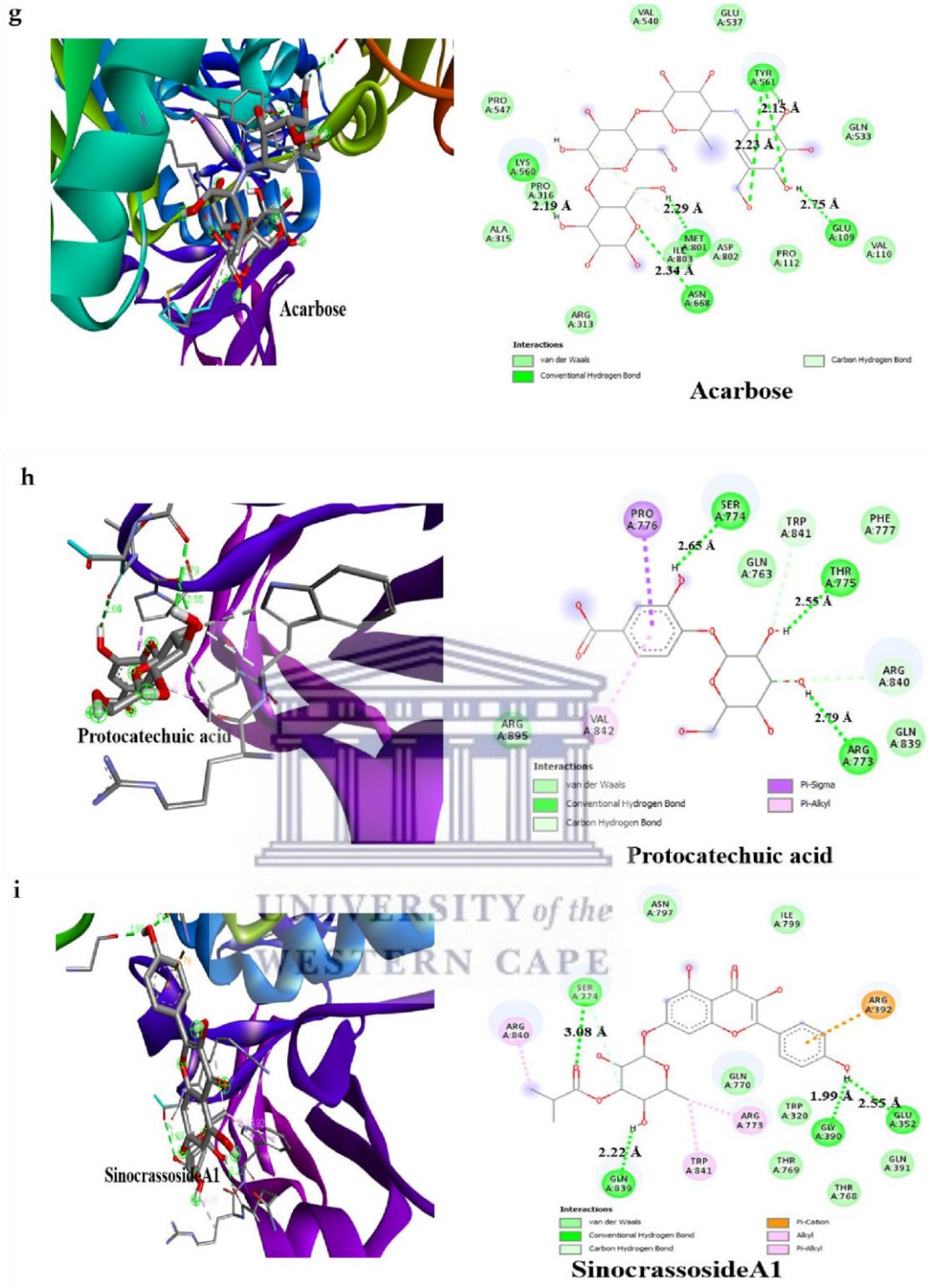

Figure 6.6. Model of the interaction and the 2D Structure of α-glucosidase protein with (**a**) 3-caffeoylquinic acid, (**b**) 3-*O*-Caffeoyl-4-*O*-methylquinic acid, (**c**) 4-Feruloylquinic acid, (**d**) 5Feruloylquinic acid, (**e**) Arbutin, (**f**) Engeletin, (**g**) Acarbose (**h**), Protocatechuic acid, and (**i**) SinocrassosideA1.

4. Discussion

Diabetes mellitus (DM) is a global health concern with metabolic disorders of multiple aetiologies. DM is often characterized by the perturbations of biomolecules, including carbohydrates, fats, and proteins, with defects of insulin secretions or actions, or both [44]. Reducing hepatic glucose generation, increasing insulin production and sensitivity, inhibiting gluconeogenesis, and decreasing glucose absorption are important targets employed in developing synthetic anti-diabetic agents. However, limitations and side effects, such as weight gain, gastrointestinal disorders, headache, peripheral oedema, and hypotension, associated with using these synthetic agents, the cost, and accessibility remain major hindrances [45,46]. Hydrolysis of complex starch, oligosaccharides, and disaccharides by the pancreatic α-amylase, together with glucose uptake by the intestinal α-glucosidase, have been implicated in the postprandial glucose level in type 2 diabetes [47] with its attendant complications. Essentially, the regulation of α-amylase and α-glucosidase biological functions (inhibition) is critical to the treatment regimen. This implies that new drug candidates with potent inhibition of the intestinal α-glucosidase and mild inhibition of pancreatic α-amylase and with a safety profile would be valuable to drug discovery and development for diabetes mellitus.

In the field of drug discovery and development, both experimental and computational strategies are valid and complimentary. The use of information from chemical and biological entities, such as ligands or targets, in the identification and optimization of new drugs, the elimination of compounds with undesirable characteristics (poor activity and/or poor absorption, distribution, metabolism, excretion, and toxicity (ADMET)), and ensuring that the process of drug discovery and development is streamlined are benefits of computational techniques [31,40,48]. Overall, enriched compounds with drug-likeness, leadlikeness, active qualities, and elimination of molecules with inactive, reactive, toxic, or poor ADMET/PK properties are highly desired in drug discovery and development [32,40,48]. In vitro investigation of plant extracts for anti-diabetic potential or activities is essential. The exploration of bioactive compounds present in plant extracts for anti-diabetic activities provides the basis for the discovery of bioactive compounds that are potent inhibitors of intestinal α- glucosidase and mild inhibitors of pancreatic α-amylase with better potency and safety profiles which is urgently necessary.

Helichrysum petiolare H and B.L, commonly called the silverbush everlasting plant, is traditionally used to treat different ailments and has been investigated scientifically to manage disease conditions [18]. Our research on *Helichrysum petiolare* involves drug discovery and development of the plant extract and its polyphenolic bioactive compounds as safe and effective potential anti-diabetic agents, considering their inhibitory activity against α-amylase and α-glucosidase enzymes. Reducing blood sugar, especially the postprandial blood sugar level, is an effective and attractive strategy in treating diabetes mellitus. Many natural products from plant sources are potential therapeutic agents; hence, fast screening of these natural products through molecular docking is important and can quickly meet this demand. Docking represents ligand and protein interactions using a computational approach for molecular recognition. It combines and screens databases for the accuracy of prognostication of protein structures and protein–ligand complexes for principal structure-based drug design [49–51]. Therefore, we evaluated the potential of the anti-diabetic effect of *Helichrysum petiolare* extract and its phytochemicals as α-amylase and α-glucosidase inhibitors. We observed relative safety of the AAHPE via cytotoxic screening, which is similar to other research work [52], and strong inhibitory activity when compared with acarbose. The non-cytotoxicity, good inhibition of α-amylase and α-glucosidase, and excellent glucose uptake activity of AAHPE at

the highest concentration of the extract over metformin indicated anti-diabetic or hypoglycemic properties. The anti-diabetic property of *Helichrysum petiolare* is linked to repositories of the phytochemicals or bioactive compounds [12,52].

It is worth pointing out that from our study, bioactive compounds such as 3-caffeoylquinic acid, 3-*O*-caffeoyl-4-*O*-methylquinic acid, arbutin, engeletin, protocatechuic acid, and sinocrassosideA1 support Lipinski's rule of five, while deviations from the criteria of Lipinski's rule of five for oral bioavailability is indicated by violating more than two of Lipinski's five rules (Supplementary Materials Table S3). In view of this, the drugs formulated using these bioactive compounds that fulfilled Lipinski's rule of five may have some significant advantages over the synthetic compound (acarbose). Interestingly, acarbose violated three out of the five rules and has been reported to be metabolically unstable [53,54].

Natural products are more complex than synthetic compounds, and drugs based on natural product structures exhibit better chemical diversity and occupy larger regions of chemical space than drugs of completely synthetic origins [16,55]. Components or compounds obtained through LC-MS analysis from *Helichrysum petiolare* extract, including protocatechuic acid, arbutin, engeletin, 3-caffeoylquinic acid, are reported to elicit antidiabetic properties through the inhibition of α-amylase and α-glucosidase enzymes [56–58].

In this study, the detailed interaction of the best conformation of the docking results revealed the best docking poses of the bioactive compounds and their binding sites on αamylase and α-glucosidase in Figures *6*.5 and *6*.6, respectively. This involved various bonding interactions, namely conventional hydrogen, carbon–hydrogen, pi–sigma, pi–pi, pi–alkyl, etc. Based on the literature, the strength of the π–π interaction for stabilization of a structural complex is comparable to the strength of hydrogen bond at an excited state [59]. While in the ground state, the loss of the π–π interaction does not affect the active-site conformation, but results in a reduction in the rate of chemical activity approximately 20–30-fold [60]. However, hydrophobic or van der Waals interactions could promote ligand affinity for the target protein [43]. Therefore, this study evaluated the binding affinity between ligand and protein complexes by assessing binding energy, H-bonds, pi–pi interactions, and van der Waals interactions. As shown in Figure *6*.5, the bioactive compounds in AAHPE bind with one or two reported essential binding residues, such as TRP59, ASP197, and GLU233 of α-amylase [61,62]. Aside from these essential amino acid residues, other amino acids, such as Arg195, Thr163, His305, Gln63, His299, Ala106, Asn105, and Asp300, formed hydrogen bonds, as shown in Table *6*.4. In contrast, α-glucosidase interacts with the binding pocket of the following listed amino acid residues: Asp323, Arg392, Ser774, Thr769, His387, Arg773, Asn797, Gly390, Gly839, and Tyr841, which play an important role in phytochemical binding (Supplementary Materials Table S3).

It is noteworthy that sinocrassosideA1 had the lowest binding energy but interacted with the reported important catalytic site amino acid residues TRP59, ASP197, and GLU233 via a conventional hydrogen bond [GLU233 (2.63 Å)], pi–pi interaction (TRP59), and van der Waals interaction (ASP197) (Figure *6*.5i). Meanwhile, sinocrassosideA1 interacted with αglucosidase through four hydrogen bonds (Ser774, Gln839, Gly390, and Glu352) and other bonds, as shown in Figure *6*.6i. Similar interactions were observed for all other bioactive compounds in AAHPE. Similarly, acarbose showed interactions with α-amylase ASP197 via a van der Waals interaction, TRP59 through a carbon hydrogen bond, and GLU233 via a conventional hydrogen bond (Figure *6*.5g), while having the least binding affinity (Table *6*.4). Interestingly, acarbose interacted with the same key amino acid residues involved in catalysis at the active site of α-amylase and α-glucosidase (Figures *6*.5 and *6*.6). This likely implies the

inhibition of α-amylase and α-glucosidase by these bioactive compounds through a similar mode of action as acarbose. The bioactive compounds in the target proteins (α-amylase and α-glucosidase) were less than 3.5 Å, suggesting resilient hydrogen bonding between protein and ligands. Our docking results for the bioactive compounds sinocrassosideA1, arbutin, engeletin, protocatechuic acid (Figures *6*.4 and *6*.5), isorhamnetin-3galactoside, methyl 3,5-di-*O*-caffeoylquinate, 1,4-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, and 5-caffeoylquinic acid, among others (Supplementary Materials Figures S1 and S2), were actively involved in hydrogen bonding with eight polar residues, including aspartic acid, serine, histidine, threonine, glutamine, asparagine, glutamic acids, and tyrosine. Other crucial interactions such as weak van der Waals forces, pi–sigma, and pi–pi interactions were also found to increase the binding of phytochemicals with the protein binding pockets (Figures *6*.5 and *6*.6).

Our docking analysis suggests that most of the bioactive compounds in AAHPE could compete with the substrate for the enzyme's active site in a similar manner as acarbose does [61–63]. Acarbose has been reported in the literature to be a competitive inhibitor of α-amylase and α-glucosidase [62,64,65]. This phenomenon is further confirmed by the present study's findings, as revealed by the similar binding site occupied and binding pose assumed by acarbose (the standard inhibitor) and the bioactive compounds, as shown in Figures *6*.5 and *6*.6. Aside from the binding pose/binding site occupied, all bioactive compounds were bound to the enzymes near the catalytic site domain where acarbose binds (Figures *6*.5 and *6*.6). Moreover, this suggests that these bioactive compounds contribute to the overall α-amylase and α-glucosidase inhibitory effect of the AAHPE, which could be by the same competitive mode of inhibition as acarbose [65].

On the contrary, some bioactive compounds, such 3-caffeoylquinic acid, 4-feruloylquinic acid, and isorhamnetin 3-galactoside, had a higher binding affinity than acarbose towards α-amylase (Supplementary Materials Table S3). A similar result was observed for 3,5dicaffeoylquinic acid with α-glucosidase (Supplementary Materials Table S3). The bioactive compounds did not compete with acarbose for the active site, since they were bound to the proteins at different binding sites, as shown in Figures *6*.5a,c and S2d,h (3-caffeoylquinic acid, 4-feruloylquinic acid and 3,5-dicaffeoylquinic acid, Isorhamnetin 3-galactoside). This indicates their propensity to contribute to the overall α-amylase and α-glucosidase inhibitory effect of the AAHPE via the non-competitive mode of inhibition. Interestingly, all the bioactive compounds in AAHPE investigated in this study inhibit both α-amylase and α-glucosidase, either via the competitive or non-competitive mode of inhibition. This was corroborated with the inhibition assay of AAHPE showing high inhibitory effect, which may be due to the synergetic effect of the bioactive compounds present in the extract. This study is novel and significant to the best of our knowledge as it is the first in silico study on the inhibitory activity of bioactive phytochemicals identified from AAHPE against α-amylase and α-glucosidase as a therapeutic target for the treatment of diabetes mellitus. Findings from this study indicated that the extract of AAHPE and its phytochemicals examined could be a promising therapeutic agent with better therapeutic efficacy than acarbose and could be a potential anti-diabetic agent with strong inhibitory activity against α-amylase and α-glucosidase.

5. Conclusions

This research reports the AAHPE chemical constituents identified using LC-MS/MS analysis as well as the remarkable in vitro anti-diabetic potential of AAHPE. Additionally, a combined physicochemical, pharmacokinetics, drug-like properties, and molecular docking study was performed for α-amylase and α-glucosidase with anti-diabetic constituents of

AAHPE to assess new potential therapeutic drug candidates. LC-MS/MS analysis revealed phytochemicals or bioactive compounds, of which those with high concentrations were chosen for the molecular docking analysis. We affirmed that the phytochemicals sinocrassosideA1, engeletin, 4-feruloylquinic acid, 3-*O*-caffeoyl-4-*O*-methylquinic acid, protocatechuic acid, 3-caffeoylquinic acid, and arbutin are novel in fulfilling Lipski's rules and are potential safe and potent α-amylase and α-glucosidase inhibitors among the array of phytochemicals in this study. The molecular docking showed hydrogen bonds and other interactions as they relate to the importance of binding energy and the stability of complexes of these phytochemicals and various amino acid residues in the active site of the two enzymes that confer them as α-amylase and α-glucosidase inhibitors. Further investigations of in vivo and clinical trials are warranted. Conclusively, we postulate that these bioactive compounds should be considered as safe and potent inhibitors of diabetes mellitus, controlling postprandial hyperglycemia.

Supplementary Materials: The following are available online, Table S1. LC-MS analysis of bioactive constituents of aqueous-acetone extract of *Helichrysum petiolare* (AAHPE), Table S2. Chemical structure (2D) of bioactive compounds in the aqueous-acetone extract of *Helichrysum petiolare* (AAHPE), Table S3. Predicted binding affinity and detailed docking interactions of α-amylase and α-glucosidase with compounds of AAHPE and Acarbose, Figure S1. Model of the Interaction and the 2D Structure of α-amylase protein and compounds of AAHPE, Figure S2. Model of the Interaction and the 2D Structure of α- glucosidase protein and compounds of AAHPE.

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CHAPTER SEVEN

Neurodegenerative disease is attributed to be one of the causative events of the complications of DM, which is described and further highlighted in this chapter. Potential drug discovery and development from medicinal plants with dual actions for treating the co-morbid condition, DM-linked cognitive decline, is imperative in research. In the previous chapter, inhibitory α-amylase and α-glucosidase of the extract, potent glucose uptake, and the potential of flavonoid constituents of the AAHPE *in silico* study displayed its safe and potent antidiabetic activity.

The research in this chapter entails the experimental and computational strategies, validation and complimentary drug discovery and development. Because of the potential antidiabetic action of AAHPE, the extract was also explored as a neuroprotective agent in glucotoxicity, which could be a possible treatment for cognitive decline conditions in diabetes. Hence, *in vitro* evaluation of the neuroprotective potential of AAHPE, with molecular docking of flavonoids and its relevance in cognitive decline, was investigated.

Abstract

The glucotoxicity or hyperglycaemia condition has been linked to complication-related cognitive decline. The predisposition of cognition decline because of DM is debilitating and denoted as DACD. Drug discovery and development with dual actions could be a treatment and preventive regimens for this co-morbid condition (DM and ND). Hence, the aim and objective of this study is to investigate the neuroprotective ability of AAHPE against the glucotoxicity effect on the SH-SY5Y cell line and to determine the bioactivity of the flavonoid constituents of AAHPE as anti-cholinesterase or cholinesterase inhibitor using molecular docking. The *in vitro* assays vis-a-vis neuroprotection, ROS, ATP assays were carried out, and reported bioactive components were docked against acetylcholinesterase (AChE) enzyme. The result revealed that AAHPE improves

cell viability, reverses ROS production, and increases ATP production, indicating its neuroprotective potential against glucotoxicity in the SH-SY5Y cell line. The inhibitory profiles of the compounds were compared with the standard AChE inhibitor, donepezil. In the docking studies, conformational site analysis and docking parameters like binding energy and interaction were determined using AutoDock Vina. Docking studies conducted on 3,5-dicaffeoylquinic acid (-9.9 Kcal/mol), isorhamnetin 3-galactoside (-8.8 Kcal/mol), 4,5-dicaffeoylquinic acid (-8.6 Kcal/mol), methyl 3,5-di-O-caffeoyl quinate (-8.6 Kcal/mol), 3-caffeoylquinic acid (-8.4 Kcal/mol), quercetin-3-glucoside (-8.4 Kcal/mol), sinocrassosidea1 (-8.4 Kcal/mol) compounds, exhibited tight binding forces with the AChE enzyme compared with donepezil (-8.3 Kcal/mol), the standard drug. Based on the *in silico* studies, there is significant binding interaction of all the bioactive compounds with conventional hydrogen bonds, Pi-Pi bond interactions, and van der Waals forces with the active site of amino acid residues of AChE. However, donepezil showed binding interaction with only Pi-Pi bond interactions and van der Waals forces for their inhibitory ability. The obtained results predicted that AAHPE flavonoid constituents could be utilised as sources of valuable phytocompounds with antioxidant and neuroprotective properties over donepezil to ameliorate cognitive decline induced by glucotoxicity.

Keywords: acetylcholinesterase, molecular docking, glucotoxicity, flavonoids, neuroprotection, cognitive decline

7.0 *In vitro* evaluation of the neuroprotective potential of aqueous acetone *Helichrysum petiolare* extract against glucotoxicity and molecular docking relevance in cognitive decline

7.1 Introduction

In recent times, attention to the occurrence of DM and Neurodegenerative disease (ND) co-morbidity has become a significant priority because of the many devastating effects globally. These two disease entities are age-related and denoted as Diabetes associated cognitive decline (DACD) in a more precise term. Diabetes associated cognitive decline is one of the CNS complications that affect the brain in diabetes. DACD was reported in 2006 for the first time by Mijnhout et al., (2006), and characteristics such as impairment to memory, executive function, language and processing speed are somewhat prominent in this condition (Zhao et al., 2018).

Although DM and ND are unrelated, several studies have revealed a close association between DM and a decline in brain function and performance, which means that neurodegeneration is a complication and is evident in DM patients (Cherbuin & Walsh, 2019; Nasrolahi et al., 2019). Therefore, DM is found to precipitate the burden of ND by increasing the risk of all types of dementia, AD and mild cognitive impairment by about 1.5-fold, while patients with DM are prone to developing PD compared with those without DM by 2.2-fold (Zhao & Townsend, 2009).

Evidence revealed the negative impact of DM on the CNS, with T1DM and T2DM patients prone to developing mild to severe cognitive decline compared to non-diabetic individuals (Biessels et al., 1994; Biessels et al., 2008; Yaffe et al., 2012). In addition, glucotoxicity induced learning and memory dysfunction in diabetic conditions or hyperglycaemia-induced neuronal abnormalities involving some mechanisms. Oxidative stress is implicated in the abnormal release of cytokines, activation of PKC, the influx of polyol pathways, and AGEs in hyperglycaemic-

induced cognitive decline, as reported in a few studies (Sima, 2010; Yaffe et al., 2011; Zhao et al., 2018).

However, hyperglycaemia or glucotoxicity induces or serves as an anchor to other factors that lead to cognitive decline in diabetic patients (Wang et al., 2014). Reactive oxygen species induced oxidative stress is involved in the pathogenesis of diabetes. Consequently, oxidative stress causes damage to the brain in hyperglycaemic conditions, which could be linked to mitochondrial dysfunction or integrity. The targeted therapy that ameliorates ROS and maintains the mitochondrial integrity and ATP generation could be beneficial to the prevention of cognitive decline, thus, offering neuroprotection.

Medicinal plants remain a vital source of drug discovery and development with pharmacological properties to treat, cure and manage acute and chronic diseases. The WHO reported that about 80% of people worldwide are dependent on traditional medicine for their essential health requirements (Woo et al., 2012). South Africa manifests a profound diversity of plants of over 20 000 varieties, and around 10.8% of the South African flora are well-recognised in traditional use (Thakur et al., 2019; Van Wyk, 2011). Natural products derived from plant origin are established or known for their potent pharmaceuticals that have been in use in ND (Vazhayil et al., 2014).

Consequently, medicinal plants with antidiabetic potential could offer neuroprotection in glucotoxicity and serve as dual therapeutics, particularly benefiting DACD conditions. Thus, for the first time, *Helichrysum petiolare*, though not utilised in neuroprotection, has been used in previous studies to show its potential antidiabetic properties and is explored in this study. *Helichrysum petiolare*, a shrub with silver-grey hair covering the aromatic round-shaped leaf with

whitish-cream flowers (commonly called Silver Bush Everlasting plant), is well-distributed worldwide with beneficial antioxidant activities and total phenolic and flavonoid constituents.

In general, flavonoid constituents have been adjudged to offer neuroprotection (Ayaz et al., 2019). Several lines of evidence reported flavonoids as essential secondary metabolites that provide neuroprotective effects (Ayaz et al., 2019; Gutierrez-Merino et al., 2011; Pan et al., 2020; Ren et al., 2016; Vauzour et al., 2008). Flavonoids, thus, mediate neuroprotection effects by maintaining neuronal quality and number in the critical brain area and prevent the onset or progression of diseases responsible for the decrease in cognitive function.

The physicochemical, pharmacokinetic, and drug-likeness properties of the flavonoid compounds of the AAHPE identified by LC-MS have been analysed in our previous study (Akinyede et al., 2022). Therefore, in this study, we investigated the neuroprotective ability of the AAHPE against the glucotoxicity effect on SH-SY5Y cell lines and determined the bioactivity of AAHPE's flavonoid constituents as neuroprotective anti-cholinesterase/cholinesterase inhibitor using molecular docking for drug discovery and development.

7.2 Material and methods

7.2.1 Collection of plant material

The *Helichrysum petiolare* species were collected from surrounding areas of the Cape Peninsula University (CPUT), Bellville, in October 2020, Western Cape, South Africa, and identified by Prof. Christopher N. Cupido of the Department of Botany, University of Fort Hare, Alice, South Africa. The accession number, *Helichrysum petiolare*-UFH-2020-10-01, was used for the study.

7.2.2. Plant extraction

The leaves of the plants were cleaned and air-dried to a constant weight. The dried plant sample was pulverised using an electronic blender, and the ground plant weighed. The powdered plant materials in conical flasks were soaked and subjected to intermittent stirring in 90% aqueous acetone and warmed in the water bath at 60°C for two hours with slight modification (Nasr et al., 2019). The mixture was filtered through Whatman cellulose filter paper under pressure using the pump. The plant material was subjected to a second extraction by soaking it overnight and the filtrates pooled together before being subjected to a rotary evaporator while the residue or extract obtained was allowed to dry in the fume cupboard. The residues or extracts were stored at -20°C until required for use as AAHPE.

7.3. Cell lines and culture conditions

The SH-SY5Y cell line was grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% foetal bovine serum (FBS, Gibco, Life Technologies Corporation, Paisley, UK) and 1% 100 U/ml penicillin and 100 µg/ml of streptomycin (Lonza Group Ltd., Verviers, Belgium) in 60 mm dishes. Cells were monitored in a sterile environment and kept in the incubator. The culture medium was changed every three days with or without confluency. Dissociation of cells for seeding or sub-culturing was done at 70-80% confluency using phosphate-buffered saline (PBS) to wash containing 0.1% trypsin EDTA .

The human neuroblastoma SH-SY5Y cell line remains a veritable model established for neurotoxic or neuroprotective studies, particularly when not differentiated (Lee et al., 2015). Several studies relating to neuroprotection have been done on the SH-SY5Y cell line (Barai et al., 2019; Dokumacı & Aycan, 2019; Liu et al., 2009). Factors such as cell condition, toxicants, human

expertise and experimental design impact many neurotoxic and neuroprotective experimental studies (Joshi et al., 2019; Russo et al., 2012; Tieu, 2011).

7.3.1 Treatments

The plant extract was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO, USA) to achieve a 40 mg/ml concentration from which further concentrations were prepared by dilution in cell growth medium to non-toxic concentration of DMSO to the cell. While the different concentration of glucose was prepared with the medium (100–500 mM). Following this, cytotoxicity screening of the plant extract and glucose concentrations was performed to identify non-toxic concentrations for neuroprotection studies.

Briefly, 5 000 cells were seeded per well in 96-well plates and exposed to increasing concentrations (25, 50, 75 and 100 µg/mL) of the plant extract and glucose (100–500 mM). The control cells were treated with a similar amount of DMSO to ensure that the amount of DMSO at the highest treatment concentration was not toxic to the cells, while untreated cells served as a control.

The treatment duration for all experiments was 48 hours, and the concentrations of plant extract (25, 50, 75 and 100 µg/mL) and the 300 mM glucose concentrations were chosen for further studies. For neuroprotection studies, SH-SY5Y cells were grown in 96-well plates and pre-treated with (25, 50, 75 and 100 µg/mL) for two hours before adding 300 mM glucose concentrations and treatment lasted for 48 hours. Cells treated with growth medium were used as controls, and 1 mM donepezil, a known neuroprotective agent, was used as a positive control.

7.3.2 Cell viability assays

In the cytotoxic assay, MTT(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) mechanisms involve the ability of viable cells to reduce MTT reagent to formazan. In

this assay, the viable cell utilised NADPH-dependent oxidoreductase enzyme and MTT powdered of 5 mg/ml in PBS. The colour change from the yellow MTT salt solution to purple formazan occurs in the mitochondria of viable or living cells. SH-SY5Y cell line cells with optimum established cells of 5000 cell/well of 96-well plates were seeded in 100 µl of culture complete DMEM medium for 24 hours to attach.

For the neuroprotection assay, all the groups except the control was pre-treated with various plant concentrations and 1 µM donepezil before the treatment with 300 mM glucose concentration. After treatment, 10 µL of MTT solution was added to every well and incubated for four hours. The supernatant was carefully aspirated, and 100 µL of DMSO added, after which it was read at 570 nm with a BMG Labtech multi-cell plate reader. The cell viability was expressed as a fraction of viable cells relative to the control group.

7.3.3 ATP generation.

SH-SY5Y cell lines (5 000 cells/well) were seeded in 96-well plates and treated for 48 hours with donepezil and different concentrations of the plant after the establishing glucotoxicity of (300 mM). The 96-well plates were incubated, at room temperature, on a plate shaker for five minutes. Intracellular ATP levels were determined using the Mitochondrial ToxGlo™ kit (Promega (G8000) by mixing 10 mL of ATP buffer with an ATP detection according to the supplier's protocol. After the 48-hour treatment, 100 µL of ATP detection solution mixtures were added to each well. ATP content was measured using a luminescent plate reader using Polarstar Omega BMG Labtech.

7.3.4. Assessment of intracellular ROS

Levels of intracellular ROS were determined using the fluorescent probe DCFH-DA dye. Briefly, cell lines (5 000 cells/well) were seeded in 96-well plates and treated after 24 hours with donepezil and different concentrations of the plant. Control cells were incubated with 100 µM H_20_2 as a positive control (values not shown) for 15 minutes. ROS activity in cells was then determined by staining the cells with 20 µM DCFH-DA in 20 mL un-supplemented DMEM and 100 µL added to each well. Cells were incubated for 30 minutes, washed once with 1X PBS, and 100 µL of the PBS added to each well. The fluorescence intensity of DCFH-DA was measured using a (Polarstar Omega BMG Labtech) microplate-reader at excitation wavelength 485 nm and emission wavelength 538 nm.

7.4. Molecular docking

7.4.1. Protein's preparation

Crystal structures of AChE protein (PDB ID: 6O4X) were retrieved in .pdb format from the Protein Data Bank (https://www.rcsb.org/). PDB is a worldwide archive to access the 3D structure of biological macromolecules (Burley et al., 2021). This study adopted a single chain of AChE for docking analysis. The MGL Tools were used for protein preparation. The water molecules and co-crystallised ligands were deleted from the macromolecule and polar hydrogens added.

7.4.2. Ligand's preparation

The initial 3D structures of selected ligands were retrieved in .sdf format from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and (http://www.chemspider.com/). PubChem and ChemSpider are the publicly accessible repository for chemical substances and related biological

activities (Bolton et al., 2008; Williams, 2008). The optimised structures were then converted into .pdb format using open BABEL.

7.4.3. Docking protocol

The PDB files of both ligands and proteins were converted in an extended PDB format, termed PDBQT, to perform molecular docking analysis using AutoDock 1.5.6 and AutoDock Vina. As previously reported, the docking protocol was used (Oyewusi et al., 2021; Oyewusi et al., 2022). We used the "Grid" from AutoDock 1.5.6 for calculating the grid parameters, and all data regarding target proteins, ligand, grid size, and geometry were saved in the "TXT" file.

Docking was performed with the grid box size set to 66 × 70 × 44 AChE protein with a grid spacing of 1 Å, and the grid centre was designated at dimensions (x, y, and z): 99.228, 46.816, and 18.287. The output pdbqt files were written into a config. (Configuration) file. The conformation with the lowest binding energy was considered the most stable conformation of the ligand regarding the bioactive compounds. The results were analysed using free version of the Biova Discovery Studio 2020 client (Dassault Syst`emes BIOVIA, Discovery Studio Modeling Environment, Release 2017, San Diego: Dassault Syst`emes, 2016).

7.5. Docking method

The reference ligands were docked in the binding site of target proteins and compared with those of the co-crystallised ligands of target proteins (PDB ID: 6O4X is sulphate ion) to determine the accuracy of the docking protocol. The prepared ligand molecules were docked in the binding site of the refined AChE model utilising AutoDock Vina and scored by using the scoring function. The protein-ligand interactions were analysed further for the docked poses of the ligands in the binding site of target proteins.

The best flavonoids were selected for further analysis of the binding interactions (including H-bond and hydrophobic interactions) of the ligands using PyMOL (The PyMOL Molecular Graphics System, version 2.2.0, Schrodinger, New York, USA, 2018) and Biova Discovery Studio 2020 client (Dassault Syst`emes BIOVIA, Discovery Studio Modeling Environment, Release 2017, San Diego: Dassault Syst`emes, 2016).

7.6 Statistical analysis

Data were expressed as mean ± SD values with GraphPad Prism 7 used for statistical analysis. The statistical significance ($p < 0.05$) and Duncan's test analysis were used.

7.7 Results

In our experiment, we subjected the SH-SY5Y cell lines to a glucotoxicity assay ranging from 100–500 mM glucose concentrations. The concentrations tested, revealed reduced cell viability in the MTT assay in a dose-dependent manner, as shown in Figure 7.1. There was an appreciable decline in cell viability up to 500 mM; the highest concentration tested has the least viability of about 4%. The concentration of 300 mM, which falls around 50–60% cell viability, was then used for further study.

Moreover, several studies have shown that a concentration between 50–60% cell viability is chosen for further experimental work (Cao et al., 2013; Hsu et al., 2013; Li et al., 2015). Data are expressed relative to 100% of the control, mean ± standard deviation; the test was carried out at three independent experiments, analysed with one-way ANOVA followed by the Dunnet's test with a significant difference.

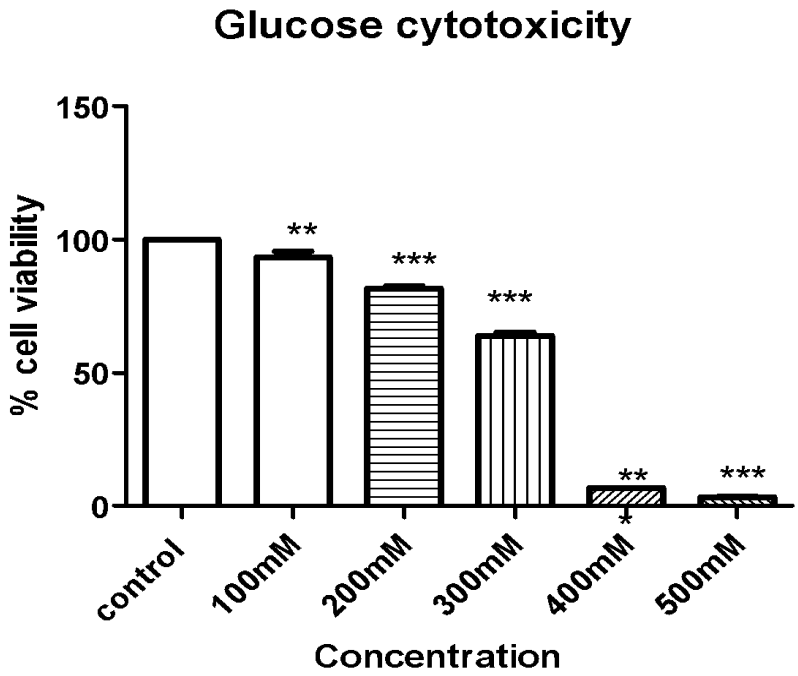


Figure 7.1. The cytotoxic screening of glucose with different concentrations. Values are expressed as the mean ± SD (n=3) *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 compared with the control.

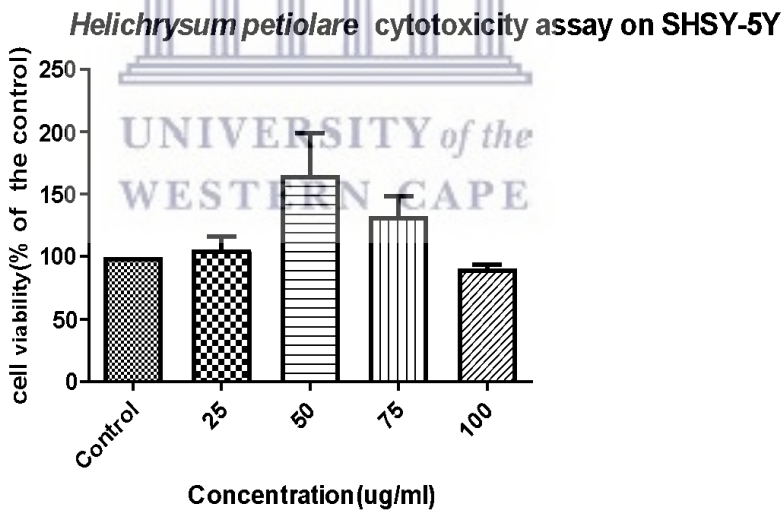


Figure 7.2. The cytotoxic screening of AAHPE with different concentrations on SH-SY5Y. Values are expressed as the mean ± SD (n=3) *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 compared with the control.

The cell viability using MTT implies the ability of the active enzyme of the cell, mitochondrial dehydrogenase, to reduce or convert the blue formazan to purple colour. The degree or the intensity of the purple colour shows the enzyme's activity. In most cytotoxicity assays, the benchmark of 80% indicates relative safety, which can further be used to set the therapeutic dose for the experimental study (Iso/TC 194, 2009; Nwakiban et al., 2020). From Figure 7.2, different extracts of AAHPE of 25–100 µg/ml concentrations were used in treating SH-SY5Y cell for 48 hours.

The AAHPE showed that all the concentrations (25–100 µg/mL) tested had over 80% cell viability. The decline in cell viability was noticeable in the 100 ug/ml concentration, which still had over 80% viability. However, 25–100 µg/mL concentrations were used for further study, indicating that AAHPE has a good safety profile based on the cytotoxicity results. The SH-SY5Y cells treated with donepezil at a concentration of 1 µM showed maximum neuronal viability compared to control cells from previous work and were chosen in this study (Thammasart et al., 2019).

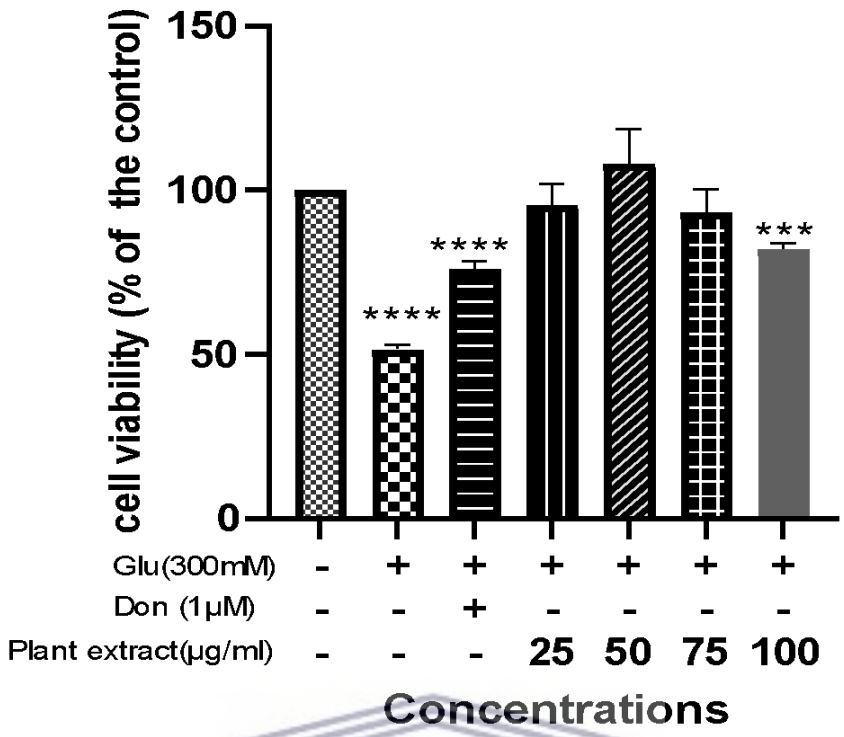


Figure 7.3. The neuroprotection assay of AAHPE of 1 µM donepezil and different concentrations of plant extract (25–100 µg/ml) pre-treated in SH-SY5Y cell line with induced glucotoxicity (300 mM glucose). Values are expressed as the mean ± SD (n=3) *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 compared with the control.

In Figure 7.3, the neuroprotective potential of pre-treated 1 µM donepezil and different concentrations of the AAHPE on the SH-SY5Y cell line with induced glucotoxicity (300 mM glucose) were revealed. Observations indicated that 1 mM donepezil, 25, 50, 75, and 100 µg/mL AAHPE gave cell viability of 76%, 97%, 108% and 93%, respectively, when compared with 51% cell viability obtained in glucotoxicity of the control group. The result shows that all plant extract concentrations tested have a neuroprotective effect more than the donepezil with the best neuroprotection at 50 µg/mL concentration of the AAHPE.

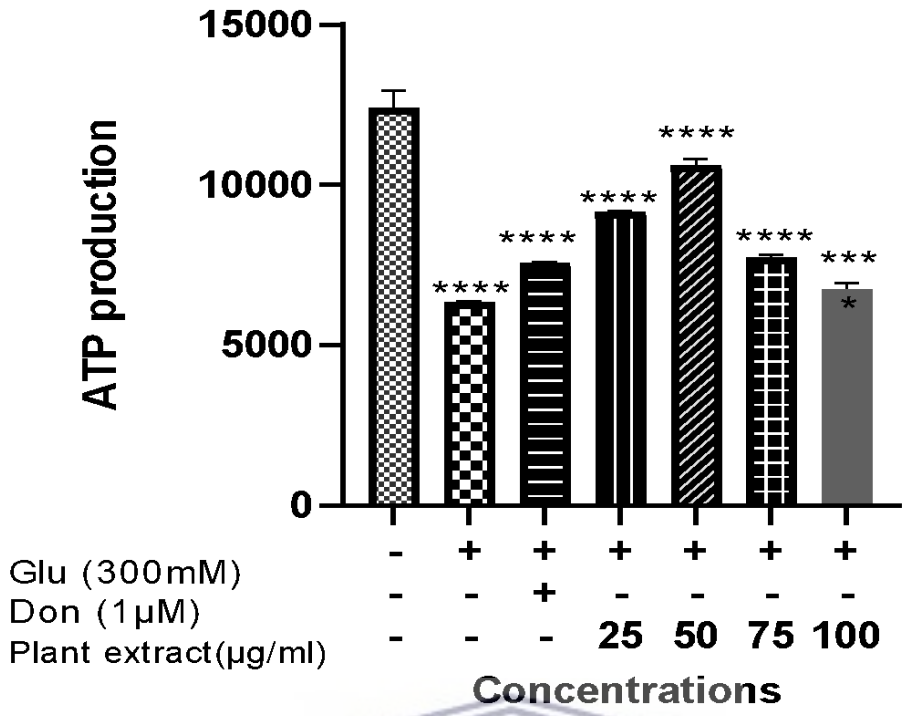


Figure 7.4. The ATP production assay of 1 µM donepezil and different concentrations of AAHPE (25–100 µg/ml) pre-treated SH-SY5Y cell line with induced glucotoxicity (300 mM glucose). Values are expressed as the mean ± SD (n=3) *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 compared with the control.

The level of ATP production in this neuroprotective study is shown in Figure 7.4. Glucose toxicity at 300 mM reduced ATP production of the cell that was about 44%. There was an improvement in ATP production of 68% for donepezil. The plant extract at 25 µg/ml shows a better ATP production of 76% compared with donepezil. The ATP production of 72%, 62% and 47%, which shows improvement, was obtained for the 50, 75, and 100 µg/mL, respectively, for the AAPHE plant extract on the SH-SY5Y cell line compared with 44% ATP production of the toxic glucose group.

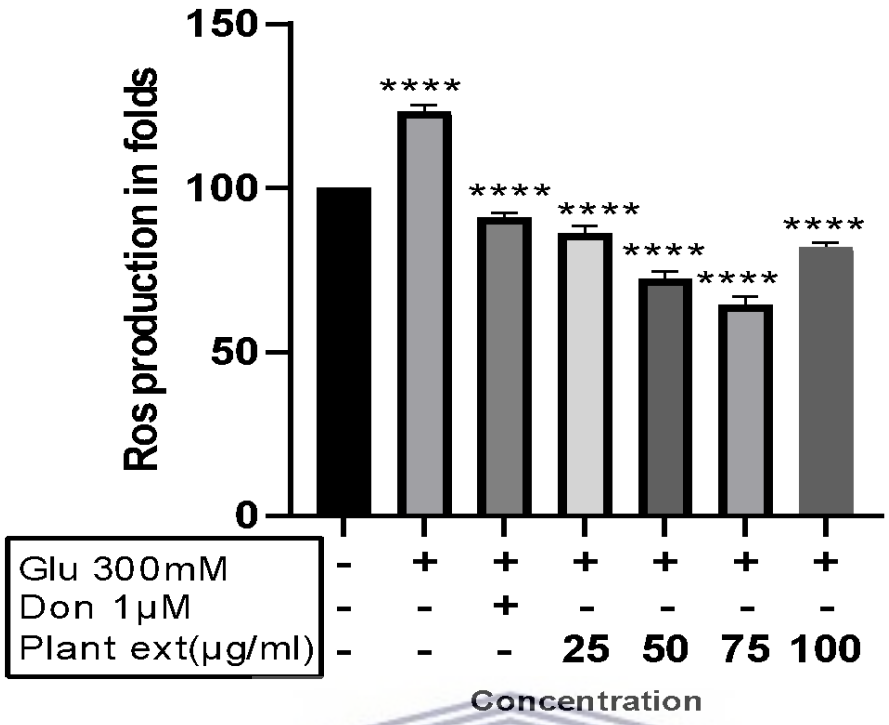


Figure 7.5. The ROS production of 1 µM donepezil and different concentrations of AAHPE (25–100 µg/ml) pre-treated SH-SY5Y cell line in induced glucotoxicity (300 mM glucose). Values are expressed as the mean ± SD (n=3) *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 compared with the control.

As indicated in Figure 7.5, the 300 mM glucotoxicity group (control) revealed the highest reactive oxygen species production. The donepezil and different plant concentrations reduced ROS production significantly ($p < 0.0001$) compared with the control. The plant extraction concentrations showed a dose-dependent reduction in ROS, while donepezil showed a reduced ROS production, although less than the reduced ROS production in any of the plant extract concentrations. The concentration of the plant extract, 75 µg/mL, had the least ROS of all the treated groups. The reduction of ROS production is adjudged to be linked to the antioxidant activity that inhibits free radical species inducing oxidative stress that cause cognitive decline.

The predicted pharmacokinetic parameter and ADMET properties of AAHPE compound, using the SwissADME online analyser and ADMETlab webserver, were done. Lipinski's rule of five is sacrosanct and used to evaluate the drug-likeness of the compounds in AAHPE. The degree of violation of Lipinski's rule indicates the level of oral tolerability (Lipinski et al., 1997) of the compounds of AAHPE, which can further be regarded as a potential candidate for drug discovery and development. The ADME properties act as the backbone that determines the pharmacokinetics, pharmacodynamics, and safety profile of the compounds in AAHPE, which enable us to know those compounds that are potentially qualified for clinical trials in drug discovery and development. The ADMET properties have previously been reported (Akinyede et al., 2022).

Table 7.1. Summary of molecular docking studies of selected LC-MS identified bioactive compounds of AAHPE and donepezil (standard drug) against acetylcholinesterase (AChE)

Compounds	Binding affinity (Kcal/mol)	No of H-bonds	H-bonds residues with H-bonds length (Å)
1,3-Dicaffeoylquinic acid	– 7.6	7	Thr83 (2.04 Å), Tyr124 (3.11 Å), Tyr337 (3.32 Å), Phe295 (3.12 Å), Arg296 (3.10 Å), Trp286 (2.04 Å), Tyr341 (1.85 Å),
1,4-Dicaffeoylquinic acid	– 7.0	3	Thr238 (2.17 Å), Pro235 (2.01 Å), Trp532 (2.11 Å)
3,4-Dicaffeoylquinic acid	– 8.3	4	Gln413 (2.04 Å), Thr238 (2.12 Å), Arg247 (3.23 Å), Trp532 (2.05 Å),
3,5-Dicaffeoylquinic acid	– 9.9	4	Tyr337 (2.47 Å), Phe295 (3.31 Å), Tyr72 (3.12 Å), Ser293 (3.19 Å),
3-Caffeoylquinic acid	– 8.4	5	Ser293 (3.25 Å), Arg296 (2.06 Å), Tyr341 (2.16 Å), Tyr337 (2.57 Å), Tyr124 (1.92 Å),
3-O-Caffeoyl-4-O-methylquinic acid	– 6.9	2	Ser293 (2.98 Å), Tyr124 (2.13 Å),
4,5-Dicaffeoylquinic acid	– 8.6	4	Ser293 (2.88 Å), Phe295 (3.08 Å), Arg296 (3.22 Å), Asn283 (2.63 Å)

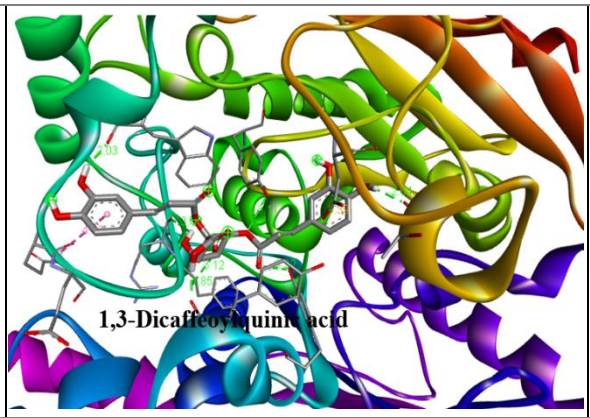
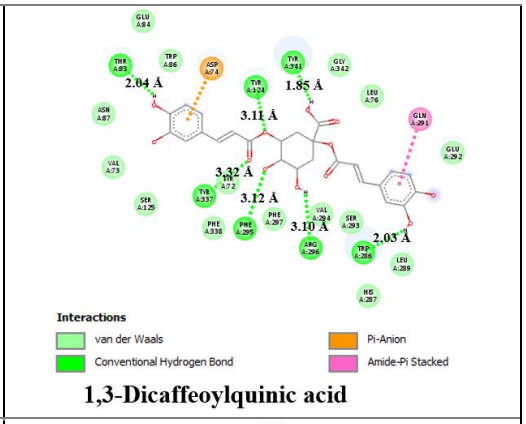
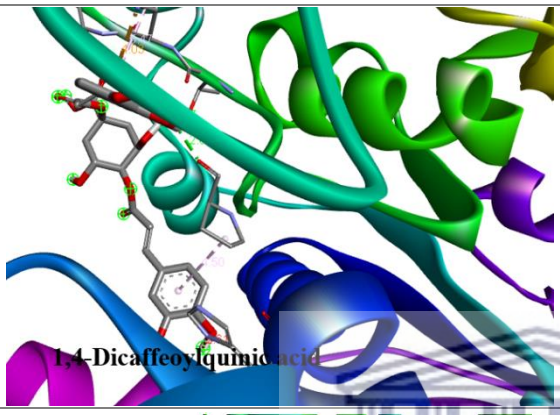
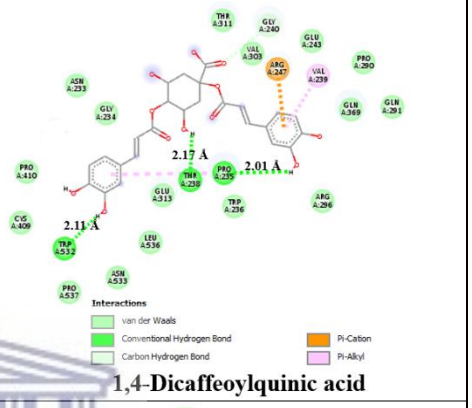
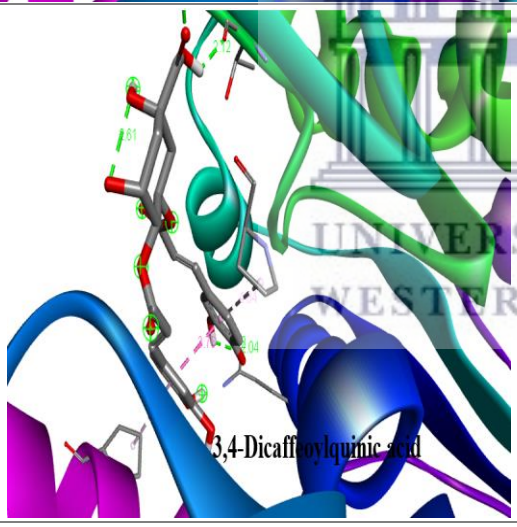
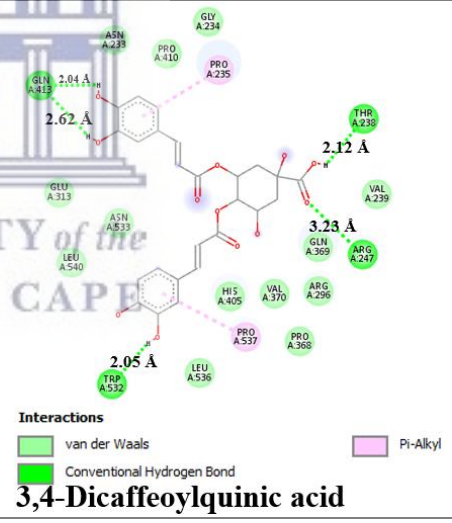
4-Feruloylquinic acid	– 7.7	5	Tyr337 (2.57 Å), Tyr124 (2.74 Å), Tyr341 (2.93 Å), Trp268 (2.62 Å), Gln291 (2.58 Å),
5-Feruloylquinic acid	– 7.9	5	Tyr337 (1.97 Å), Tyr72 (3.14 Å), Tyr341 (2.35 Å), Phe295 (2.92 Å), Arg296 (2.92 Å)
5-Caffeoylquinic acid	– 8.1	4	Ser293 (2.28 Å), Tyr286 (2.64 Å), Tyr124 (2.89 Å), Tyr337 (2.28 Å),
Arbutin	– 8.1	5	Tyr337 (3.04 Å), Tyr124 (2.05 Å), Tyr341 (2.45 Å), Tyr133 (1.79 Å), Trp86 (2.84 Å),
Cascaroside C	– 7.1	5	Asn233 (2.16 Å), Asn533 (2.23 Å), His405 (2.36 Å), Glu313 (2.10 Å), Ser541 (2.88 Å)
Engeletin	– 7.7	4	Pro368 (2.49 Å), His405 (2.13 Å), Ser293 (2.66 Å), Trp286 (2.85 Å)
Isorhamnetin 3-galactoside	– 8.8	4	Ser293 (2.66 Å), Tyr337 (2.66 Å), Phe295 (2.97 Å), Trp286 (2.85 Å),
Kaempferol galactoside (Trifolin)	– 7.3	3	Ser293 (2.78 Å), Tyr124 (3.07 Å), Asp74 (2.51 Å)
Donepezil	– 8.3	–	–
Methyl 3,5-di-O-caffeoyl quinate	– 8.6	6	Ser293 (2.82 Å), Phe295 (3.22 Å), Arg296 (3.24 Å), Trp286 (2.05 Å), His287 (2.73 Å), Tyr337 (2.98 Å)
Myricetin-3-galactoside	– 8.1	5	Arg296 (2.23 Å), Phe295 (2.83 Å), Tyr124 (2.81 Å), Tyr337 (2.26 Å), Tyr72 (3.03 Å)
Protocatechuic acid	– 7.1	6	Tyr124 (2.73 Å), Tyr337 (3.18 Å), Tyr286 (2.53 Å), Arg296 (2.23 Å), Ser293 (2.67 Å), Gln291 (3.03 Å)
Quercetin-3-galactoside	– 8.2	5	Tyr124 (2.26 Å), Tyr337 (3.81 Å), Tyr72 (2.23 Å), Arg296 (2.83 Å), Phe296 (3.03 Å),
Quercetin-3-glucoside	– 8.4	5	Tyr124 (2.71 Å), Tyr337 (2.47 Å), Tyr72 (3.09 Å), Ser293 (2.33 Å), Trp286 (2.19 Å),
SinocrassosideA1	– 8.4	2	Arg296 (2.92 Å), His405 (3.18 Å)

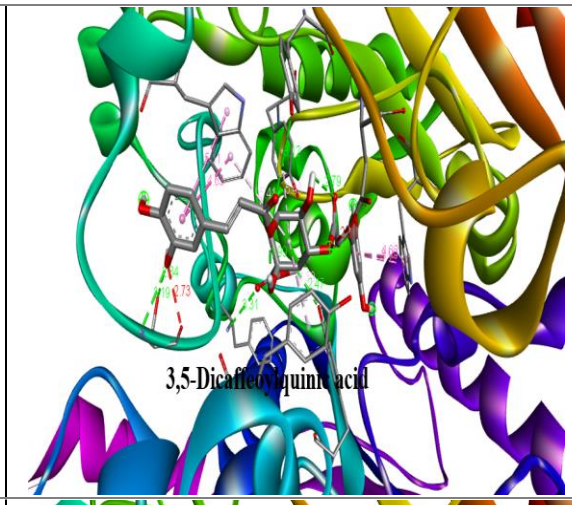
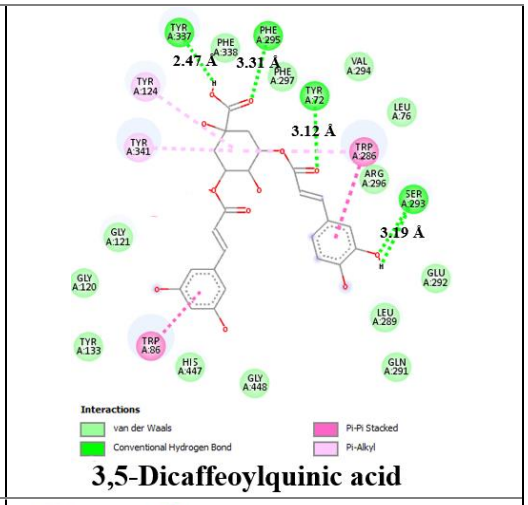
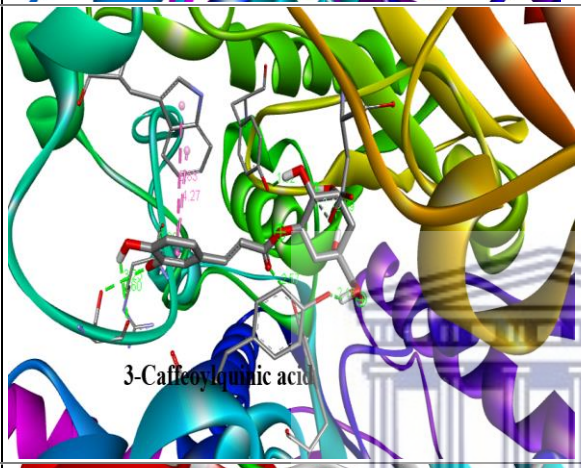
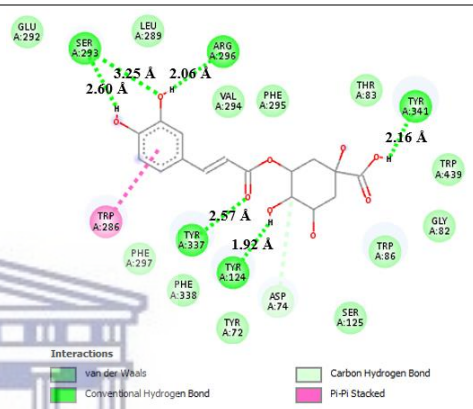
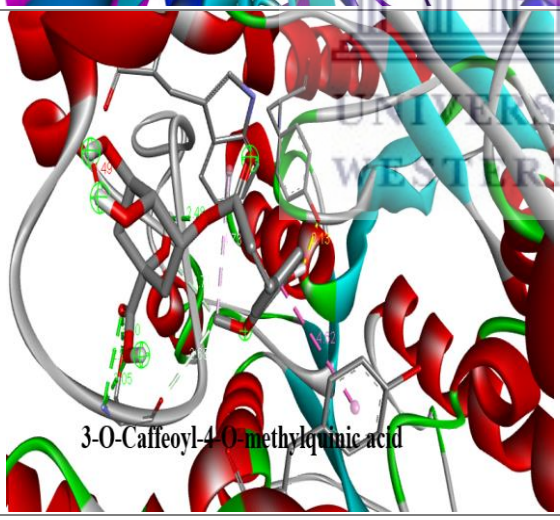
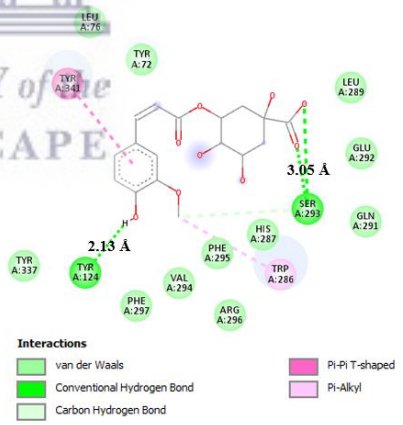
The bioactive compounds in concentrations above 100 mg/g of the AAHPE reported in our previous study (Akinyede et al., 2022) were selected for this present study, showing binding

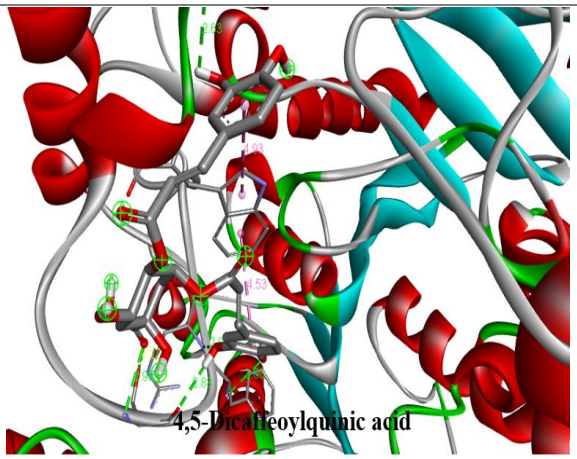
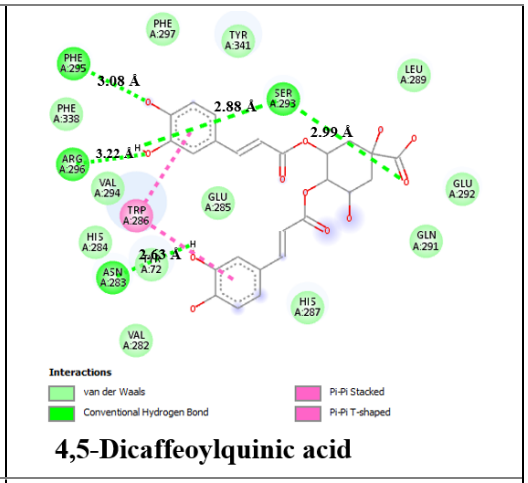
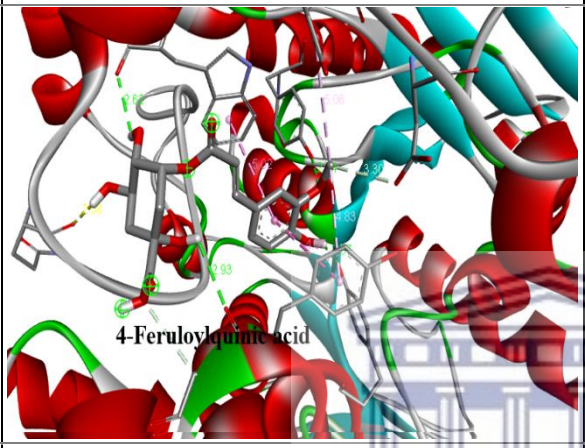
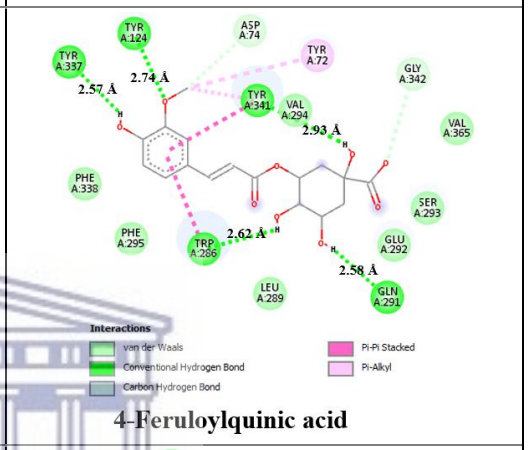
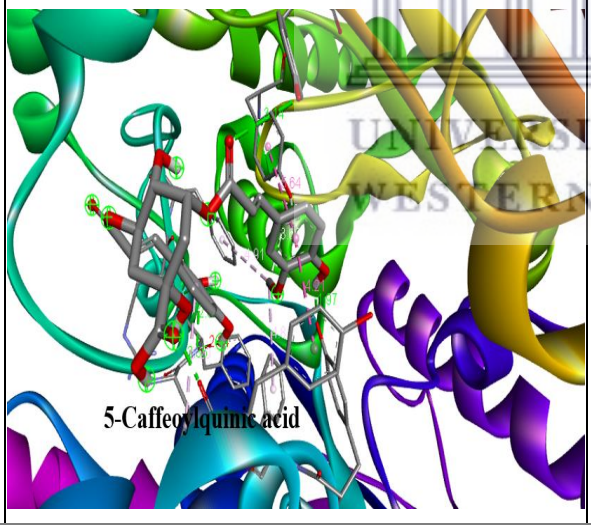
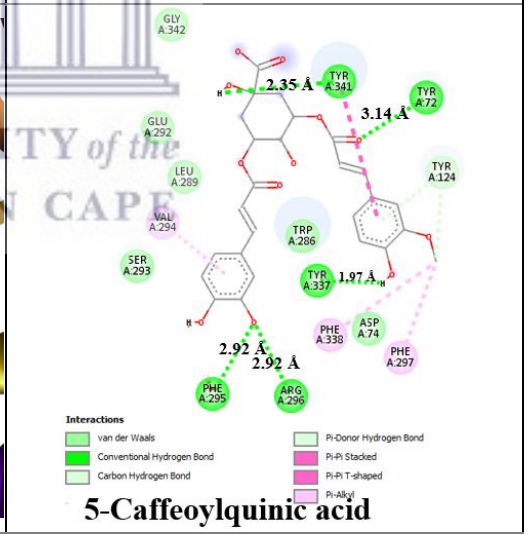
affinity and interactions with the AChE enzyme. While docking results have demonstrated several possible ligand conformations, only the lowest binding energy was chosen. Results of the docking analysis are shown in Table 7.1.

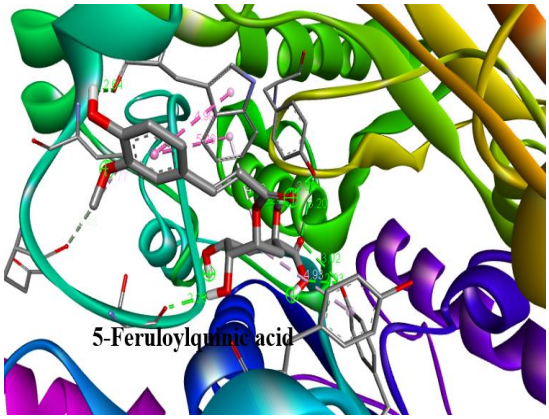
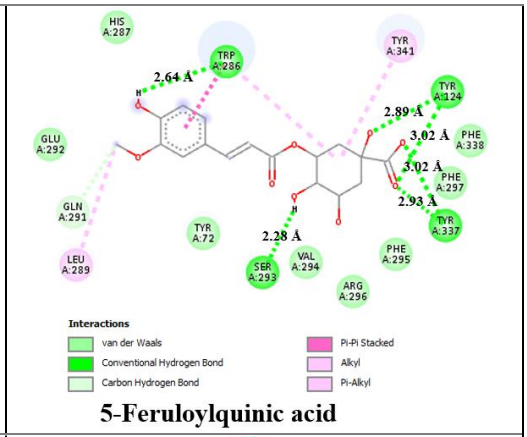
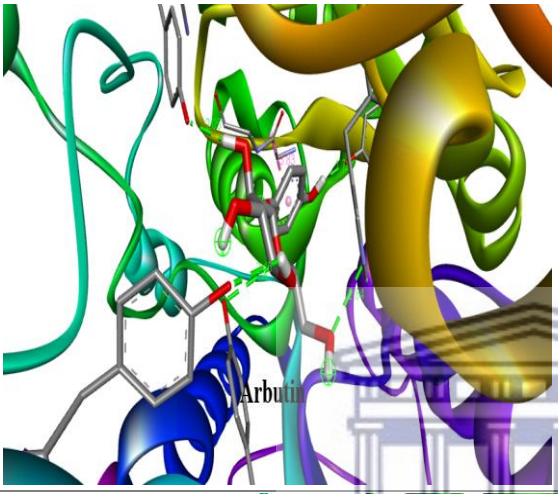
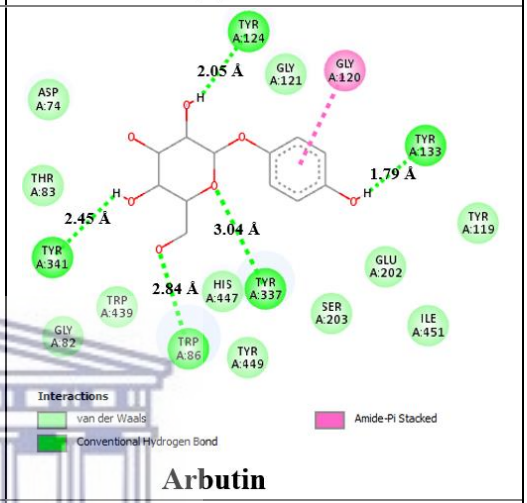
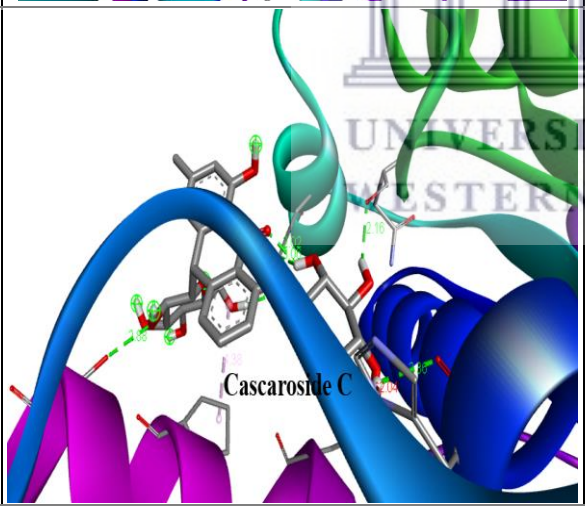
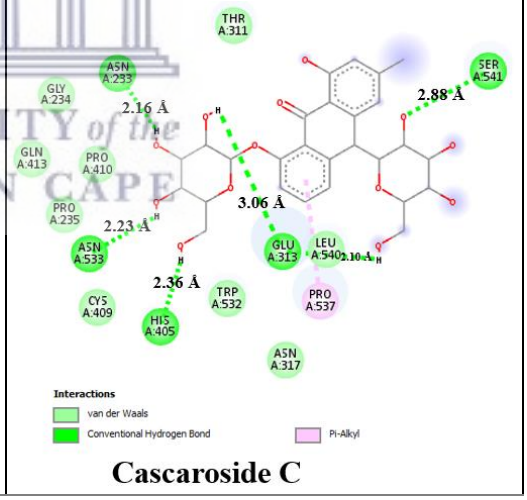
The more negative the binding energy value, the better affinity the ligand has for the protein. The docking study indicated that 3,5-dicaffeoylquinic acid (-9.9 Kcal/mol), isorhamnetin 3-galactoside (-8.8 Kcal/mol), 4,5-dicaffeoylquinic acid (-8.6 Kcal/mol), methyl 3,5-di-O-caffeoyl quinate -8.6 Kcal/mol), 3-caffeoylquinic acid -8.4 Kcal/mol), quercetin-3-glucoside (-8.4 Kcal/mol), sinocrassosideA1 (-8.4 Kcal/mol) showed the strongest binding affinity toward AChE compared with the standard drug, donepezil (-8.3 Kcal/mol). The 3,4-dicaffeoylquinic acid (-8.3 Kcal/mol) has the same binding energy as the standard drug, donepezil (-8.3 Kcal/mol).

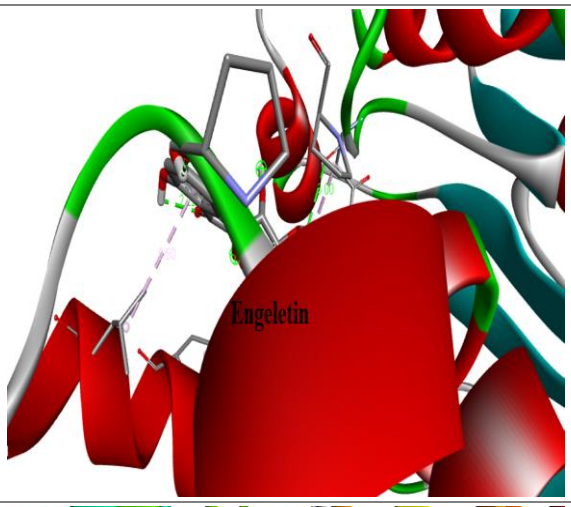
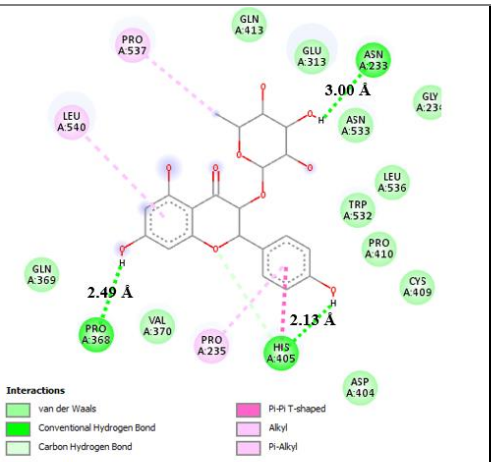
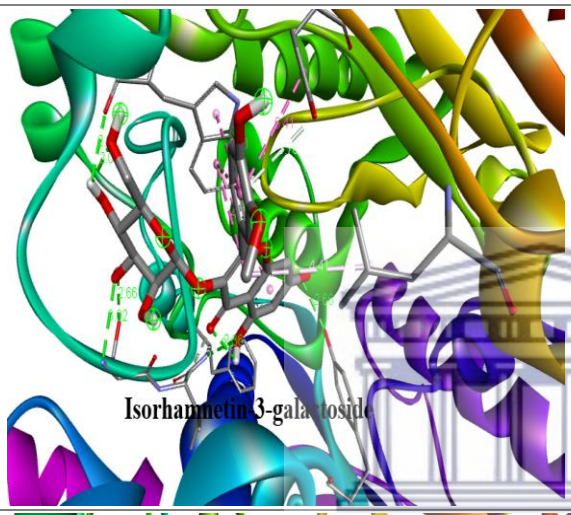
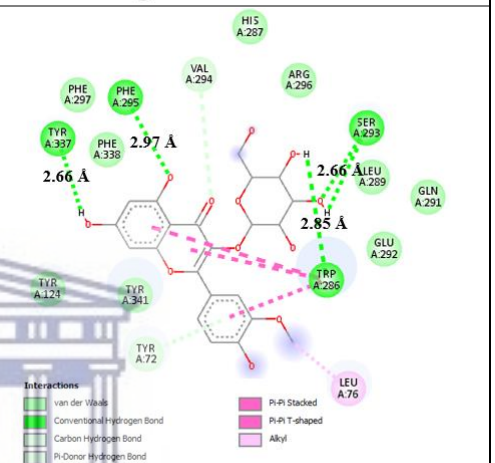
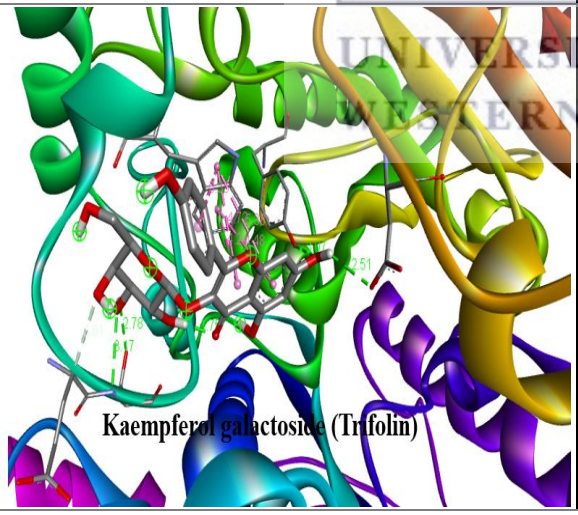
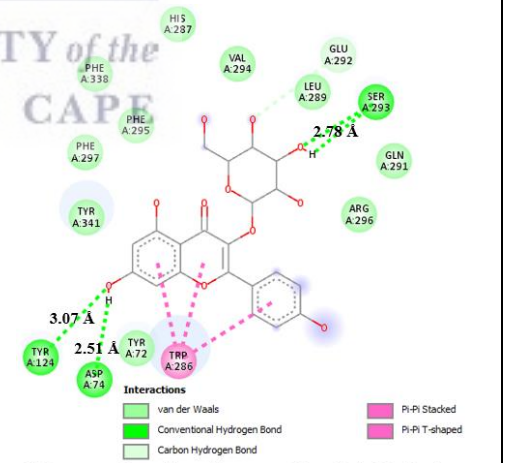
The other bioactive compounds showed a lower binding affinity with the AChE enzyme compared with the standard drug, donepezil, as follows, quercetin-3-galactoside (-8.2 Kcal/mol), 5-caffeoylquinic acid (-8.1 Kcal/mol), myricetin-3-galactoside (-8.1 Kcal/mol), arbutin (-8.1 Kcal/mol), 5-feruloylquinic acid (-7.9 Kcal/mol), 4-feruloylquinic acid (-7.7 Kcal/mol), engeletin (-7.7 Kcal/mol), 1,3-dicaffeoylquinic acid (-7.6 Kcal/mol), kaempferol galactoside (Trifolin) (-7.3 Kcal/mol), cascaroside C (-7.1 Kcal/mol), protocatechuic acid (-7.1 Kcal/mol), 1,4-dicaffeoylquinic acid (-7.0 Kcal/mol) and 3-O-caffeoyl-4-O-methylquinic acid (-6.9 Kcal/mol) (Table 7.1). Worth mentioning is that 3-O-caffeoyl-4-O-methylquinic acid demonstrated the weakest binding affinity toward AChE among the studied ligands. Previous reports also indicated that the standard drug, donepezil, showed the strongest binding affinity than in some flavonoids, which is consistent with our data (Gligorić et al., 2019).

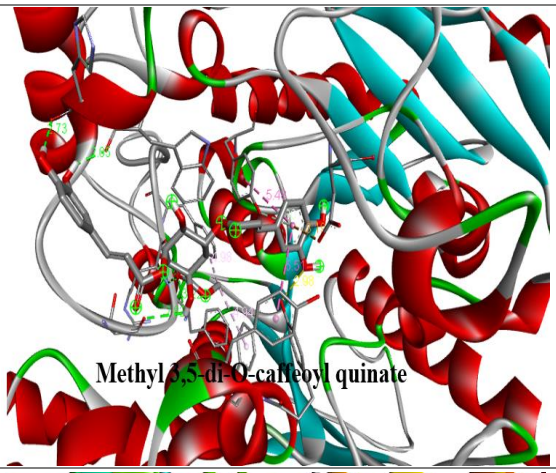
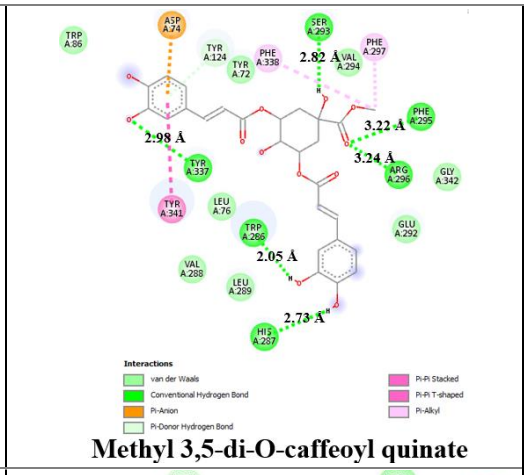
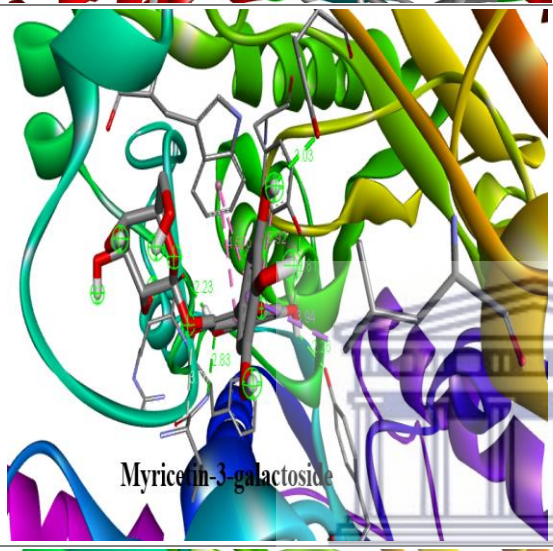
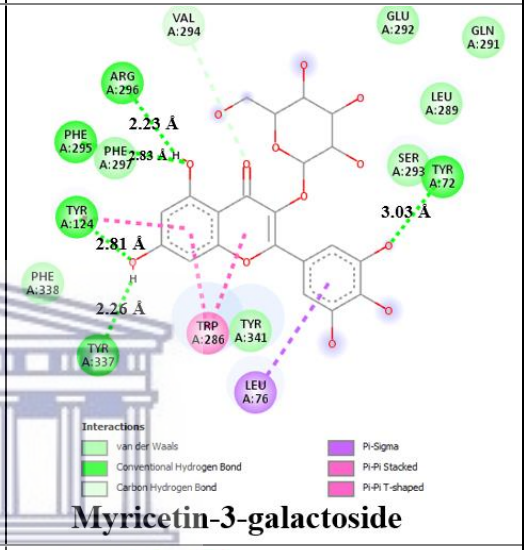
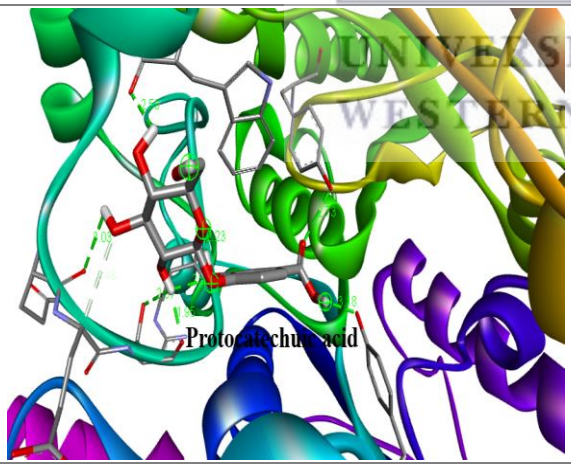
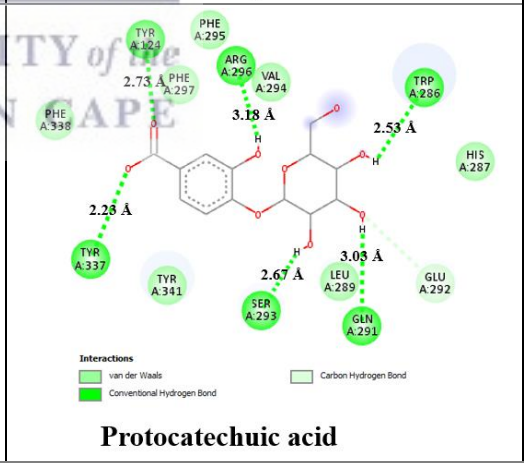
a		
b		
c		

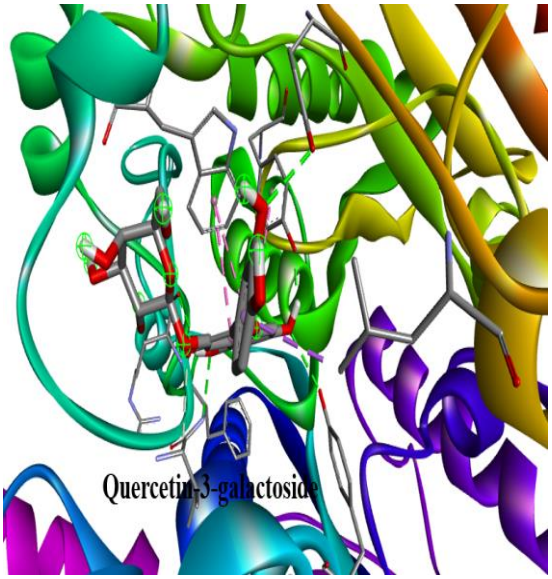
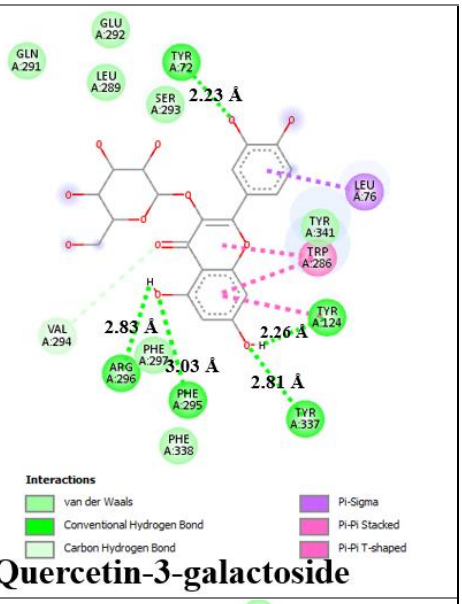
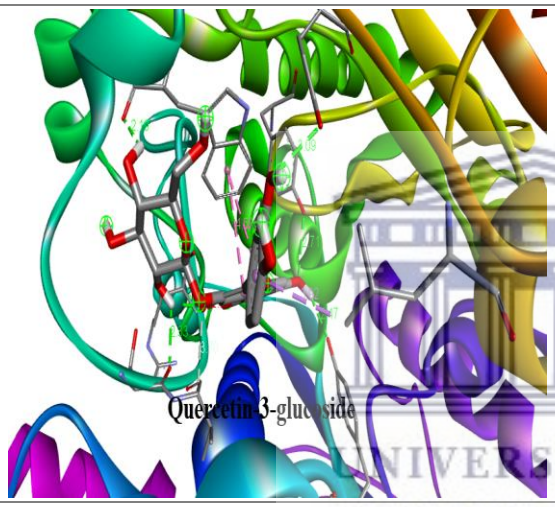
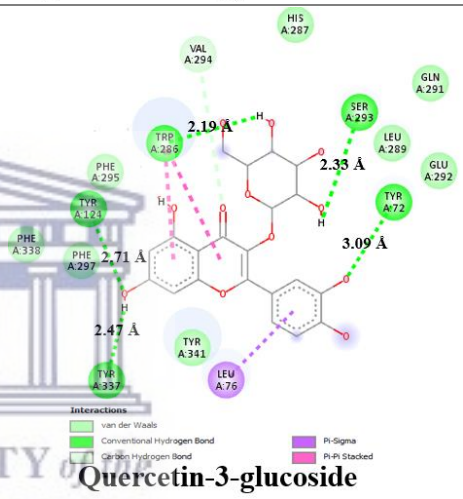
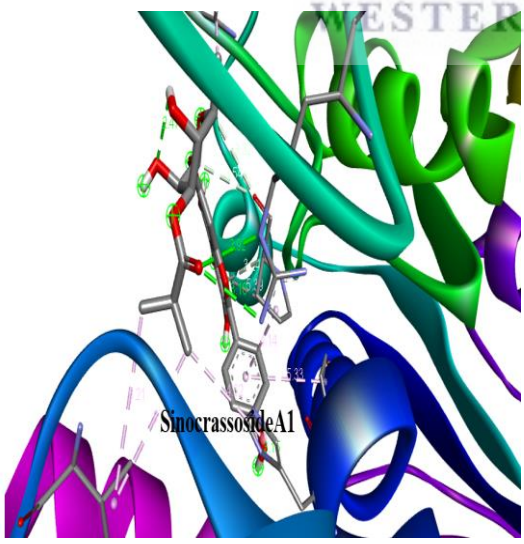
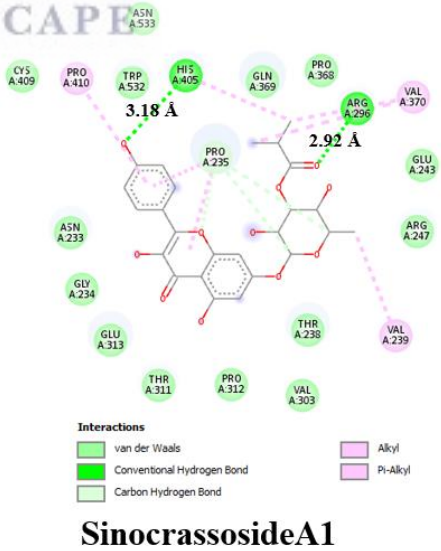
d		 **3,5-Dicaffeoylquinic acid**
e		 **3-Caffeoylquinic acid**
f		 **3-O-Caffeoyl-4-O-methylquinic acid**

g		
h		
i		

j		
k		
l		

m		 **Engeletin**
n		 **Isorhamnetin-3-galactoside**
o		 **Kaempferol galactoside (Trifolin)**

p		
q		
r		

s		
t		
u		

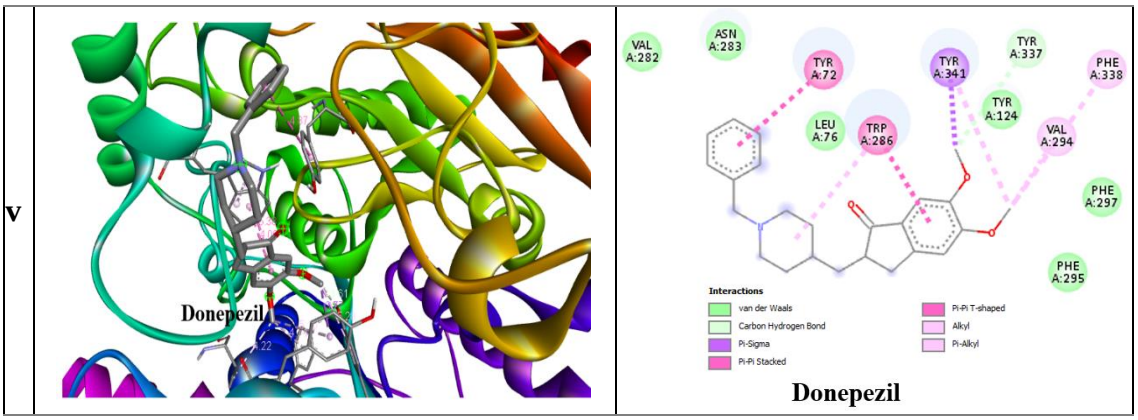


Figure 7.6. Model of the interaction and the 2D structure of acetylcholinesterase protein with (a) 1,3-Dicaffeoylquinic acid (b) 1,4-Dicaffeoylquinic acid (c) 3,4-Dicaffeoylquinic acid (d) 3,5-Dicaffeoylquinic acid (e) 3-Caffeoylquinic acid (f) 3-O-Caffeoyl-4-O-methylquinic acid (g) 4,5-Dicaffeoylquinic acid (h) 4-Feruloylquinic acid (i) 5-Feruloylquinic acid (j) Cascaroside C (k) Arbutin (l) Engeletin (m) Isorhamnetin 3-galactoside (n) Kaempferol galactoside (Trifolin) (o) Methyl 3,5-di-O-caffeoyl quinate (p) Myricetin-3-galactoside (q) Protocatechuic acid (r) Protocatechuic acid (s) Quercetin-3-galactoside (t) Quercetin-3-glucoside (u) SinocrassosideA1 (v) Donepezil.

To get an insight into the interactions of these bioactive compounds with the active site of AChE, 3D and 2D structures of AChE-ligand complexes are shown in Figure 7.6. The nature of the interactions of bioactive compounds AAHPE and the standard drug, donepezil, are presented in this figure (7.6). Generally, all the bioactive compounds interact with the peripheral anionic site and other essential catalytic residues with conventional hydrogen bonds, van der Waals forces and Pi-Pi interaction (Figure 7.6a-u). The standard drug, donepezil, interacts with these amino acids but only with van der Waals forces and Pi-Pi interactions (Figure 7.6v). In addition, all the bioactive compounds showed several hydrogen bonds with prominent amino acid residues of the peripheral anionic site anionic sub-site of AChE and other essential catalytic residues.

7.8. Discussion

The current research probed into the neuroprotective ability of AAHPE against the glucotoxicity effect on SH-SY5Y cell lines and AAHPE's flavonoid constituents as anti-cholinesterase or cholinesterase inhibitors using molecular docking. Over the past few years, a

close association of DM with potential neuronal impairment and cognitive deficits has resulted in learning and memory impairment, psychomotor retardation and altered mental flexibility (Kodumuri et al., 2019). DACD has gained much attention, and the pathophysiology is multifactorial. Oxidative stress from the ROS generation, disruption of calcium homeostasis, higher permeability of the blood-brain barrier and cerebral microvascular damage, impaired glucose metabolism, inflammatory responses of glucotoxicity are some of the proposed risk factors responsible for cognitive decline in DM (Morabito et al., 2014; Wu et al., 2019).

The pathogenesis of DACD involves an excessive production of ROS, which induces oxidative stress causing neuronal damage with defective cognitive performance. The reversal of the ROS production offers neuroprotective ability or potential, which activates antioxidant defence mechanisms. Glucotoxicity induces ROS that causes mitochondrial dysfunction or affects its morphology, indicating cell death which affects cognitive performance (Enogieru et al., 2018; Enogieru et al., 2020).

The mitochondria are the powerhouse of the cell that produces ATP. The ATP powers most of the cellular activities and ensures the proper functioning of the cell. Dysfunctional mitochondria depict that the ATP production of the cell is affected and could initiate cognitive decline from glucotoxicity. Low ATP synthesis is a key factor of neurodegeneration and results from compromised mitochondrial structure seen in the oxidation of biomolecules such as protein, lipids, and mitochondrial DNA. The restoration of mitochondrial integrity with therapeutics indicates reduced cell death, thus, depicting a healthy cellular state and improved ATP production (Cardoso et al., 2017; Carvalho et al., 2015).

The neuroprotection of the AAHPE and donepezil inhibit oxidative stress associated with glucotoxicity, which could be attributed to antioxidant potential or capacity. The expected increase

in ROS production induced by glucotoxicity led to reduced cell viability and invariably cell death, which was ameliorated with antioxidants, and thus increased ATP production and improved cell viability, indicating neuroprotection, which is corroborated in related neuroprotective studies (Nakano et al., 2017; Thammasart et al., 2019).

The impairment of glucose metabolism or glucotoxicity is implicated in early abnormal cognitive abilities or functions, evident in experimental studies (Mittal & Katare, 2016). Brain plasticity, synaptic dysfunction, neurodegeneration, and decreased acetylcholine resulting from increased AChE activity established biochemical links in DM with cognitive decline (Rivera et al., 2005).

The AChE enzyme controls the cholinergic system that is involved in memory formation. AChE is a serine hydrolase that functions to terminate the signal transmission in the cholinergic system, in which the substrate acetylcholine is a neurotransmitter of the cholinergic system. The enzyme's activity breaks the substrate into acetyl and choline, wherein the up-regulation beyond normal limits, ultimately affects cognition. The decrease in the activity of this enzyme with inhibitor use is pivotal to the treatment of cognitive dysfunctions or related neuronal disorders.

Mechanistically, the regular nerve transmission in the synaptic cleft is affected and results in cognitive decline conditions because of over degradation of acetylcholine by the AChE's activity. Hence, inhibition of AChE prevents the breakdown of acetylcholine and, in turn, regulates the level and duration of neurotransmitter actions. Clinically, AChE inhibitors are used to treat cognitive disorders, which increase cholinergic functions by elevating AChE quantity in cholinergic synapses (Tata et al., 2014).

Currently, AChE inhibitors' use includes efficacy, therapeutic range, and safety. These inhibitors, such as donepezil, rivastigmine and galantamine, are clinically used in treating

cognitive decline but display several side effects, including diarrhoea, anorexia, abdominal pain, dizziness, vomiting, headache, syncope, increased cardiac tone causing bradycardia (Birks, 2006; Inglis, 2002; Tayeb et al., 2012). In addition, the clinical effect of these marketed drugs indicates that they are not superior to the other in efficacy (Tayeb et al., 2012). Hence, drug discovery and development in the search for potential inhibitors with higher efficacy and safety than marketed drugs in treating cognitive decline through molecular docking and other predictive tools are imperative.

Docking involves an algorithm of molecular interactions (intermolecular interaction) between the ligand and the specific target. The ligand molecule interacts with the target at a specific binding site by searching for favourable conformations of the protein. The ligand-target binding is determined by the mode of bonding between the amino acid residues of the target protein and the ligand molecule.

Molecular docking remains a quantitative prediction of binding energetics and provides the ranking (score) of docked compounds based on the binding affinity of the ligand-target complex (Das et al., 2017; Huang & Zou, 2010). The binding energetics are mainly based on the hydrogen-bonding pattern of amino acid residues and ligand molecules. As one of the important tools for investigating the active site and elucidating the interactions between ligands and the biological molecule, molecular docking was conducted.

AChE has a narrow 20 Å gorge with two binding sites, the active catalytic site at the bottom of the structure and the peripheral anionic site near the gorge's entrance. The peripheral anionic site of AChE lies at the entrance to the active site gorge, comprising five residues (Tyr 72, Asp74, Tyr124, Trp286 and Tyr341) (Johnson & Moore, 2006). Several additional sub-sites, also important in the catalytic process, have been differentiated within the active site, including the

"anionic" sub-sites (Trp86, Tyr133, Tyr337 and Phe338). The acyl pocket constitutes Phe295 and Phe297, and the oxyanion hole consists of Gly121, Gly122 and Ala 204 (Chen, Y. F. et al., 2016).

Generally, all the bioactive compounds mainly interact with the peripheral anionic site and other important catalytic residues with conventional hydrogen bonds, Van der Waals forces and Pi-Pi interaction (Figure7.1a-u), whereas standard drug donepezil interact with these amino acids only with Van der Waals forces and Pi-Pi interactions (Figure 7.1v). It is worth mentioning that the bioactive compounds forming hydrogen bonds with the target proteins (AChE) were less than 3.5 Å, suggesting the resilience of hydrogen bonding between protein and ligands. The docking results showed that many potential compounds were actively involved in hydrogen bonding with relevant catalytic residues (Tyr72, Asp74, Tyr124, Trp286 and Tyr341) as against the standard drug, donepezil, which does not show hydrogen bonding interactions with any of these amino acids (Islam et al., 2013). This interaction indicates the likelihood of inhibition of AChE by these bioactive compounds via a different mode of action of donepezil. There is propensity to contribute to the overall AChE inhibitory effect of the AAHPE via the non-competitive mode of inhibition. Moreover, this study again suggests that these bioactive compounds' contribution to the overall AChE inhibitory effect of the AAHPE could be via the competitive mode of inhibition as donepezil since these compounds could also interact with AChE through Pi-Pi interactions or van der Waals forces. Notably, these bioactive compounds also bound to these enzymes at their non-catalytic site, a binding pose/binding site different from that of the standard drug (donepezil) (Figure 7.6).

Interestingly, all these bioactive compounds in AAHPE investigated in this study interacted with AChE, predominantly via a combination of conventional hydrogen bonds (Table 7.1), van der Waals forces and Pi-Pi interactions (Figure 7.6). The nature of the interactions of bioactive compounds of AAHPE was different from the standard drug, donepezil, presented in Figure 7.6.

Moreover, several essential catalytic amino acids make Pi-Pi stacking interaction with the phenyl ring of all the bioactive compounds. Some compounds interacted through van der Waals forces except 1,4-dicaffeoylquinic acid 3,4-dicaffeoylquinic acid, cascaroside C and sinocrassosideA1.

Furthermore, these bioactive compounds (1,4-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, cascaroside C and sinocrassoside A1 do not interact with the predicted active site residues and all relevant amino acid residues of the anionic sub-site. However, they formed hydrogen bonds with other amino acids, such as Thr238, Pro235, Trp532, Gln413, Arg247, Asn233, Asn533, His405, Glu313, Ser541, and Arg296 (Table 7.1 and Figures 7.6a, 7.6b, and 7.6u). Comparatively, interactions of the standard drug, donepezil, with relevant catalytic residues (Tyr72, Tyr124, Trp286 and Tyr341) and all relevant amino acid residues (Phe295 and Phe297) are through van der Waals forces and Pi-Pi interactions (Figure 7.6v) (Cardoso et al., 2017).

In nature, the interaction of these amino acids with the standard drug, donepezil, is through hydrophobic interaction. In addition, donepezil showed hydrophobic interactions with some of the significant amino acid residues of the acyl-binding pocket of AChE (Phe295 and Phe297) (Figure 7.6v), which was corroborated in the previous study (Gligorić et al., 2019). Furthermore, all the flavonoid constituents of AAHPE bind AChE at the same active site pocket (Cardoso et al., 2017).

7.9 Conclusions

Epidemiological evidence implies that chronic exposure to hyperglycaemic conditions is responsible for the deterioration of cognition and causes of other mental-related disorders. The causative effect that exists between hyperglycaemia and dementia could be appropriated to different mechanisms.

We affirmed the neuroprotection potential of the AAHPE through an *in vitro* study. The currently available AChE inhibitors have several limitations, and the challenges in terms of

efficacy, therapeutic range, and safety are encompassing. The search for new, effective, safe inhibitors with multiple receptor sites is sacrosanct. In this regard, the evaluation of AAHPE's flavonoids is a rational approach for this study.

In conclusion, we advocate that selected bioactive flavonoid constituents of the AAHPE have great potential in binding affinity in the molecular docking of AChE that can be utilised in drug discovery and development in the pharmaceutical industry. The strong hydrogen bonds and hydrophobic interactions with the significant amino acid residues of the active sites of AChE further support the propensity inhibitory potential of these flavonoids against AChE enzymes, thus conferring improved cognitive function. The *in silico* study has successfully predicted that AAHPE flavonoids have superiority over conventional drugs. This work provides the foundation for further *in vitro* research to develop new therapeutics and study mechanisms of these flavonoid constituents with neuroprotective effects against glucotoxicity-related cognitive decline conditions.

7.10 References

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CHAPTER EIGHT

8.0. Conclusion and recommendations

In this study, AAHPE was explored in drug discovery and development for oxidative-stress-related disease conditions, including diabetic-associated cognitive decline attributable to its repository of antioxidants. The antioxidant activity, the total phenolic, flavonoids, and antioxidant, and the fatty acid compositions of four extracts, *H. petiolare, H. cymocum, H. foetidum* and *H. pandurifolium* were investigated.

The 1, 1 diphenyl-2-picrylhydrazyl radical scavenging activity assay, nitric oxide scavenging activity assay, and reducing power assay revealed the antioxidant activity, while GC-MS analysis of the fatty acid methyl esters indicated the identification and composition of the fatty acids. The antioxidant activities or profile showed a good IC_{50} for all the plant extracts. The total antioxidant, phenolic and flavonoid contents were observed, thus indicating a considerable amount and good antioxidant activity. Although, the fatty acid compositions in the plant extracts varied, *H. petiolare* had the most saturated fatty acid contents and PUFA content, while *H. foetidum* had the highest monounsaturated fatty acid (MUFA) content, and *H. cymocum* had the highest PUFA to MUFA ratio.

In this study, it was revealed that the *Helichrysum* species are prospective natural bioactive chemical repositories. Also, the concentration of flavonoids in all four extracts of *Helichrysum* spp. was identified and quantified. Thirty-eight (38) flavonoids were identified, and only 19 different secondary metabolites (flavonoids), with LC-MS/MS having concentrations above 100 mg/mg, used in subsequent analysis. Study researchers successfully assessed the interaction and mechanism of bioactive compounds (19 flavonoids) of AAHPE against α-amylase, α-

glucosidase and AChE action via *in silico* investigations involving *in silico* pharmacokinetics and substrate docking.

In this study, we successfully evaluated the AAHPE as a potential inhibitor of both α-amylase and α-glucosidase with IC_{50} of 46.50 ± 6.17 and 37.81 ± 5.15 (µg/ml), respectively. This evidence is significant in the percentage increase of glucose uptake activity in a concentration-dependent manner than the control. The highest AAHPE concentration, 75 µg/ml of glucose uptake activity, was elevated compared with metformin, a standard antidiabetic drug in the insulin resistance $HepG_2$ cell line. Furthermore, it was observed that AAHPE extract improves cell viability, reverses ROS production, and increases ATP production in comparison to donepezil, all of which indicated neuroprotection potential from the effect of glucotoxicity in the SH-SY5Y cell line.

The AAHPE bioactive compounds, sinocrassosideA1, engeletin, 4-feruloylquinic acid, 3-O-caffeoyl-4-O-methylquinic acid, protocatechuic acid, 3-caffeoylquinic acid and arbutin, are novel, potentially safe and potent α-amylase and α-glucosidase inhibitors or drugs compared with acarbose. In comparison, the AAHPE bioactive compounds, 3, 5-dicaffeoylquinic acid, isorhamnetin 3-galactoside, 4, 5-dicaffeoylquinic acid, methyl 3, 5-di-O-caffeoyl quinate, 3-caffeoylquinic acid, quercetin-3-glucoside, and sinocrassosideA1 are novel AChE inhibitors to donepezil. The *in silico* pharmacokinetics and molecular docking analysis revealed that only sinocrassoside A1 had a dual inhibitory effect on α-amylase/α-glucosidase and the AChE enzyme. Consequently, this corroborated with the activity of AAHPE with increased glucose uptake for the antidiabetic study and increased cell viability, reduced ROS and increased ATP production in the neuroprotective study.

Generally, it can be construed that we successfully demonstrated the antidiabetic potential in terms of potency and safety of AAHPE on α-amylase and α-glucosidase and AChE of the flavonoid constituents' evaluation, affirming the propensity of discovered and developed drugs. Moreover, the cognitive-related decline was abrogated with the AAHPE, offering neuroprotection and molecular docking of the flavonoid constituents attesting to the inhibition of the AChE enzymes. Hence, it is essential for the treatment and improved cognitive function. It is of significance that the AAHPE and flavonoid bioactive constituents probably possess therapeutic dual-action properties in treating diabetic-associated cognitive decline. In other words, discovering and developing drugs from AAHPE and its compounds can treat and ameliorate DM or ND or when they co-exist, as seen in diabetes-associated cognitive decline.

8.1. Future work and recommendations

- Isolation and characterisation of bioactive compounds of AAHPE are critical to understanding specific or different mechanisms involved in the antidiabetic and neuroprotection effects observed.

- Constituents of flavonoids less than 100 mg/g identified from AAHPE can be subjected to molecular docking, *in silico* analysis, *in vitro* and *in vivo* studies in the near future to determine whether their activity might be directly related to their constituents.

- Molecular dynamic stimulation can be done for the already recognised potent and safe flavonoid constituents of AAHPE in the future, thus providing further validation of flavonoid constituents of AAHPE.

- The results of the flavonoid bioactive compounds of AAHPE based on Lipski's rule of five and the docking experiment could be tested *in vitro* and *in vivo* against α-amylase and α-glucosidase.

- The inhibitory effects of the flavonoid bioactive compounds of AAHPE against the AChE enzyme could be further tested *in vitro* for other neuroprotective mechanisms of action.

Appendices

Appendix 1: Table S1

Supplementary S1: LC-MS analysis of bioactive constituents of aqueous-acetone extract of *Helichrysum petiolare* (AAHPE)

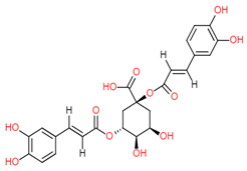
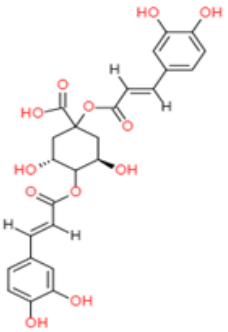
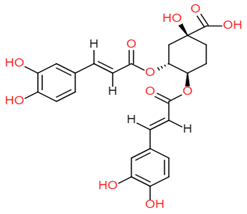
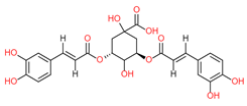
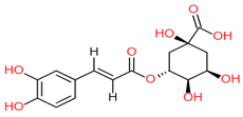
Compound	Name	Average R_t (min)	Average *m/z*	Concentration (mg/g)
1	Arbutin	5,475	271,08157	1279,2
2	Protocatechuic acid 4-O-glucoside	7,913	315,06607	182,9
3	3-caffeoylquinic acid (neochlorogenic acid)	9,609	353,09525	120,2
4	Chlorogenic acid (5-caffeoylquinic acid)	11,975	353,0864	826,7
5	Caffeic acid derivative	12,758	459,1528	134,4
6	UNPD8659	13,638	349,05746	131,8
7	Dactylin	14,234	639,1543	55,0
8	1,3-Dicaffeoylquinic acid	14,433	515,11609	388,7
9	UNPD204949	14,769	377,1803	78,6
10	UNPD114029	14,968	423,09171	167,4
11	quercetin-3-O-(feruloyl) sophoroside	15,203	801,18866	71,6
12	5-Feruloyl quinic acid	15,296	367,10284	411,6
13	3-O-p-Coumaroylquinic acid	15,54	337,09311	82,7

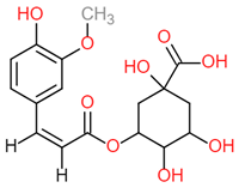
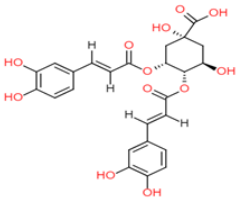
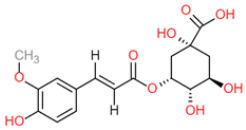
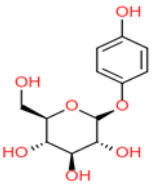
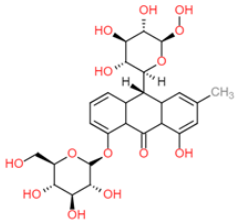
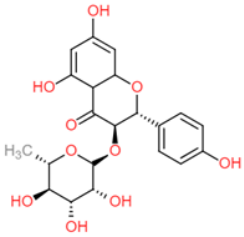
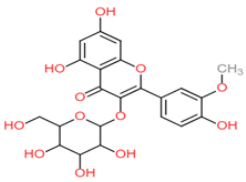
14	Sakuranin	15,562	447,12833	3,5
15	Myricetin 3-galactoside	15,779	479,08148	402,5
16	4-Feruloyl quinic acid	16,016	367,10156	363,2
17	Quercetin 3-galactoside	16,285	463,0874	586,3
18	3-O-Caffeoyl-4-O-methylquinic acid	16,515	367,10147	407,1
19	Sinapic acid	17,052	223,05902	14,9
20	Engeletin	17,334	433,11368	749,7
21	Rutin	17,426	609,14587	12,8
22	Quercetin 3-glucoside	17,656	463,08673	118,5
23	Kaempferol galactoside (Trifolin)	18,06	447,09433	166,2
24	UNPD80025	18,352	493,09372	104,2
25	1,4-Dicaffeoylquinic acid	18,633	515,11823	258,9
26	3,4-Dicaffeoylquinic acid	18,797	515,11768	535,3
27	3,5-Dicaffeoylquinic acid	19,236	515,12036	1727,3
28	Kaempferol glucoside (Astragalin)	20,078	447,09393	13,4
29	4,5-Dicaffeoylquinic acid	20,34	515,11981	1209,1
30	Sakuranetin	20,927	285,07538	6,1

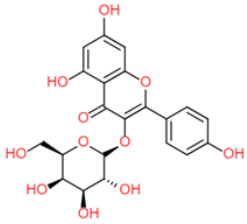
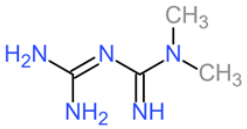
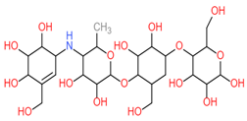
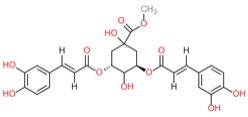
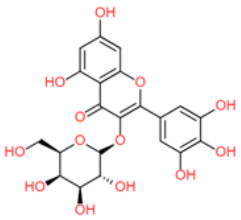
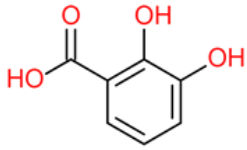
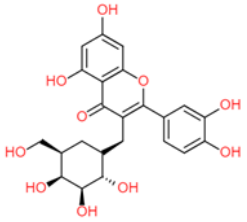
31	Sinocrassoside A1	21,036	501,13861	127,1
32	Cascaroside C	21,652	563,17737	116,3
33	Feruloyl quinic acid	22,12	367,10077	120,5
34	3,5-Dicaffeoylquinic methyl ester	23,102	529,13446	319,0
35	Sakuranin isomer	23,403	447,1297	4,5
36	Kaempferol	24,086	285,04065	27,1
37	Luteolin	24,32	531,15051	1,9
38	Sakuranetin isomer	24,447	285,07715	2,0

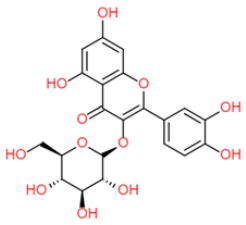
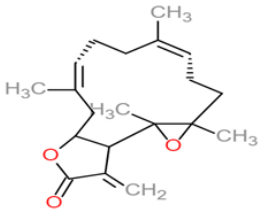
Appendix 2: Table S2

Supplementary S2: Chemical structure (2D) of bioactive compounds in the aqueous-acetone extract of *Helichrysum petiolare* (AAHPE)

Bioactive compounds	PubChem CID/Chem Spider ID	Molecular formula	Calonical SMILES	Chemical structure
1,3-Dicaffeoylquinic acid	6474640	$C_{25}H_{24}O_{12}$	C1C(C(C(CC1(C(=O)O)OC(=O)C=CC2=CC(=C(C=C2)O)O)OC(=O)C=CC3=CC(=C(C=C3)O)O)O)O	
1,4-Dicaffeoylquinic acid	12358846	$C_{25}H_{24}O_{12}$	C1C(C(C(CC1(C(=O)O)OC(=O)C=CC2=CC(=C(C=C2)O)O)O)OC(=O)C=CC3=CC(=C(C=C3)O)O)O	
3,4-Dicaffeoylquinic acid	5281780	$C_{25}H_{24}O_{12}$	C1C(C(C(CC1(C(=O)O)O)OC(=O)C=CC2=CC(=C(C=C2)O)O)OC(=O)C=CC3=CC(=C(C=C3)O)O)O	
3,5-Dicaffeoylquinic acid	6474310	$C_{25}H_{24}O_{12}$	C1C(C[CH](C([CH]1OC(=O)/C=C/C2=CC(=C(C=C2)O)O)O)OC(=O)/C=C/C3=CC(=C(C=C3)O)O)(O)C(=O)O	
3-caffeoylquinic acid	1794427	$C_{16}H_{18}O_9$	C1C(C(C(CC1(C(=O)O)O)OC(=O)C=CC2=CC(=C(C=C2)O)O)O)O	

3-O-Caffeoyl-4-O-methylquinic acid	131752769	$C_{17}H_{20}O_9$	COC1=C(C=CC(=C1)C=CC(=O)OC2CC(CC(C2O)O)(C(=O)O)O)O	
4,5-Dicaffeoylquinic acid	6474309	$C_{25}H_{24}O_{12}$	C1C(C(C(CC1(C(=O)O)O)OC(=O)C=CC2=CC(=C(C=C2)O)O)OC(=O)C=CC3=CC(=C(C=C3)O)O)O	
3-Feruloyl quinic acid/5-Feruloylquinic acid	73210496	$C_{17}H_{20}O_9$	COC1=C(C=CC(=C1)C=CC(=O)OC2CC(CC(C2O)O)(C(=O)O)O)O	
Arbutin	440936	$C_{12}H_{16}O_7$	C1=CC(=CC=C1O)OC2C(C(C(C(O2)CO)O)O)O	
Cascaroside C	46173832	$C_{27}H_{32}O_{13}$	CC1=CC2=C(C(=C1)O)C(=O)C3=C(C2C4C(C(C(C(O4)CO)O)O)O)C=CC=C3OC5C(C(C(C(O5)CO)O)O)O	
Engeletin	6453452	$C_{21}H_{22}O_{10}$	CC1C(C(C(C(O1)OC2C(OC3=CC(=CC(=C3C2=O)O)O)C4=CC=C(C=C4)O)O)O)O	
Isorhamnetin 3-galactoside	13245586	$C_{22}H_{22}O_{12}$	COC1=C(C=CC(=C1)C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)OC4C(C(C(C(O4)CO)O)O)O)O	

Kaempferol galactoside (Trifolin)	5282149	$C_{21}H_{20}O_{11}$	C1=CC(=CC=C1C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)OC4C(C(C(C(O4)CO)O)O)O)O	
Metformin	4091	$C_4H_{11}N_5$	CN(C)C(=N)N=C(N)N	
Acarbose	41774	$C_{25}H_{43}NO_{18}$	CC1C(C(C(C(O1)OC2C(OC(C(C2O)O)OC3C(OC(C(C3O)O)O)CO)CO)O)O)NC4C=C(C(C(C4O)O)O)CO	
Methyl 3,5-di-O-caffeoyl quinate	10075681	$C_{26}H_{26}O_{12}$	COC(=O)C1(CC(C(C(C1)OC(=O)C=CC2=CC(=C(C=C2)O)O)O)OC(=O)C=CC3=CC(=C(C=C3)O)O)O	
Myricetin 3-galactoside	5491408	$C_{21}H_{20}O_{13}$	C1=C(C=C(C(=C1O)O)O)C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)OC4C(C(C(C(O4)CO)O)O)O)O	
Protocatechuic acid 4-0-glucoside	19	$C_7H_6O_4$	C1=CC(=C(C(=C1)O)O)C(=O)O	
Quercetin 3-galactoside	5281643	$C_{21}H_{20}O_{12}$	C1=CC(=C(C=C1C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)OC4C(C(C(C(O4)CO)O)O)O)O)O	

Quercetin 3-glucoside	5280804	$C_{21}H_{20}O_{12}$	C1=CC(=C(C=C1C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)OC4C(C(C(C(O4)CO)O)O)O)O)[O-]	
SinocrassosideA1	643719	$C_{20}H_{28}O_3$	CC1=CCCC2(C(O2)CC3C(CC(=CCC1)C)OC(=O)C3=C)C	

Appendix 3: Table S3

Supplementary S3: Predicted binding affinity and detailed docking interactions of α-amylase and α-glucosidase with compounds of AAHPE *and* Acarbose

Compounds	Binding affinity (Kcal/mol) α-amylase	No of H-bonds	H-bonds residues with H-bonds length (Å)	Binding affinity (Kcal/mol) α-glucosidase	No of H-bonds	H-bonds residues with H-bonds length (A)
1,3-Dicaffeoylquinic acid	– 7.2	2	Asp197 (2.67 Å), Thr163 (2.93 Å)	– 7.6	6	Arg392 (2.99 Å), Thr769 (2.80 Å), Ser774 (2.70 Å), Arg773 (2.56 Å), Gln770 (3.04 Å), Gln839 (2.99 Å)
1,4-Dicaffeoylquinic acid	– 8.9	7	Thr163 (2.75 Å), His305 (2.25 Å), Gln63 (3.26 Å), Asp197 (3.30 Å), Glu233 (2.15 Å), His299 (3.02 Å), Asp300 (2.41 Å)	– 9.2	6	Gln839 (2.53 Å), Arg840 (3.02 Å), Val842 (3.53 Å), Try841 (2.21 Å), Arg773 (2.90 Å), Gly390 (2.98 Å)
3,4-Dicaffeoylquinic acid	– 8.8	4	Thr163 (3.08 Å), Gln63 (3.08 Å), Asp197 (2.75 Å), Glu233 (2.37 Å)	– 7.9	5	Gln770 (2.22 Å), Ser774 (2.81 Å), Trp841 (3.36 Å), Thr769 (3.04 Å), Arg773 (2.69 Å)
3,5-Dicaffeoylquinic acid	– 8.6	4	Thr163 (2.89 Å), Gln63 (1.94 Å), Asp197 (2.61 Å), Glu233 (2.33 Å)	– 8.4	4	Arg552 (3.14 Å), Asp469 (2.47 Å), Asp232 (2.07 Å), Asn496 (2.19 Å)
4,5-Dicaffeoylquinic acid	– 8.7	3	Gln63 (2.70 Å), Thr163 (2.91 Å), Glu233 (3.00 Å)	– 8.4	5	Gln839 (3.02 Å), Ser774 (2.21 Å), Gly390 (2.57 Å), Thr769 (3.08 Å), Arg840 (2.98 Å)
5-caffeoylquinic acid	– 8.4	6	Asp300 (2.49 Å), Try151 (2.95 Å), Glu233 (2.57 Å), His305 (2.08 Å), His299 (2.46 Å), Gln63 (2.93 Å)	– 7.7	4	Glu545 (3.00 Å), Thr769 (2.83 Å), Glu352 (2.97 Å), Arg392 (2.95 Å)
Cascaroside C	– 8.2	3	Glu233 (1.84 Å), Asp197 (2.76 Å), His299 (2.83 Å)	– 7.1	3	Gly770 (3.33 Å), Ser774 (2.83 Å), Arg392 (3.18 Å)

Isorhamnetin 3-galactoside	– 8.5	3	Asp300 (2.25 Å), Gln63 (2.91 Å), His305 (2.92 Å)	– 7.8	5	Thr769 (2.14 Å), Ser774 (2.70 Å), Gln770 (2.18 Å), Arg773 (3.40 Å), Asn797 (3.08Å)
Kaempferol galactoside (Trifolin)	– 8.2	4	Asp197 (2.76 Å), Asp300 (2.05 Å), Gln63 (3.25 Å), Trp59 (2.35 Å)	– 7.3	4	His387 (2.29 Å), Thr769 (2.25 Å), Trp320 (3.08 Å), Arg392 (2.97 Å)
Methyl 3,5-di-O-caffeoyl quinate	– 8.7	6	Glu233 (2.03 Å), Thr163 (2.34 Å), Asp300 (2.93 Å), Gln63 (3.20 Å), His305 (3.17 Å), Trp59 (2.95 Å)	– 8.2	6	Arg388 (2.95 Å), Ser774 (2.87 Å), Thr768 (2.87 Å), Arg392 (3.11 Å), Arg773 (3.20 Å), Arg388 (2.95 Å)
Myricetin-3-galactoside	– 8.3	5	His299 (2.48 Å), His305 (3.22 Å), Trp59 (2.36 Å), Gln63 (3.14 Å), Thr163 (2.72 Å)	– 7.9	5	Trp841 (3.02 Å), Arg392 (3.00 Å), Arg773 (3.29 Å), Gln770 (3.29 Å), Gln839 (2.03 Å)
Quercetin-3-galactoside	– 8.1	3	His305 (2.13 Å), Asp197 (3.00 Å), His299 (3.09 Å)	– 7.7	5	Trp320 (3.11 Å), Gly798 (2.48 Å), Arg773 (3.24 Å), Gln770 (3.35 Å), Ser774 (2.25 Å)
Quercetin-3-glucoside	– 7.8	4	His299 (2.85 Å), Asp197 (2.54 Å), Glu233 (2.56 Å), Arg195 (3.32 Å)	– 7.9	6	Trp320 (3.11 Å), Gln770 (3.35 Å), Ser774 (2.91 Å), Arg773 (3.23 Å), Trp841 (2.93 Å), Gly798 (2.48 Å)

Appendix 4: Figure S1

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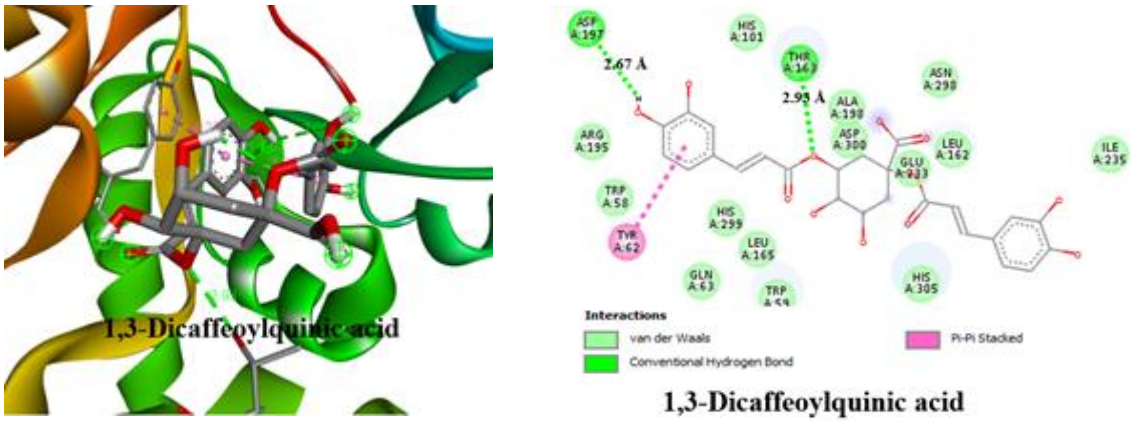

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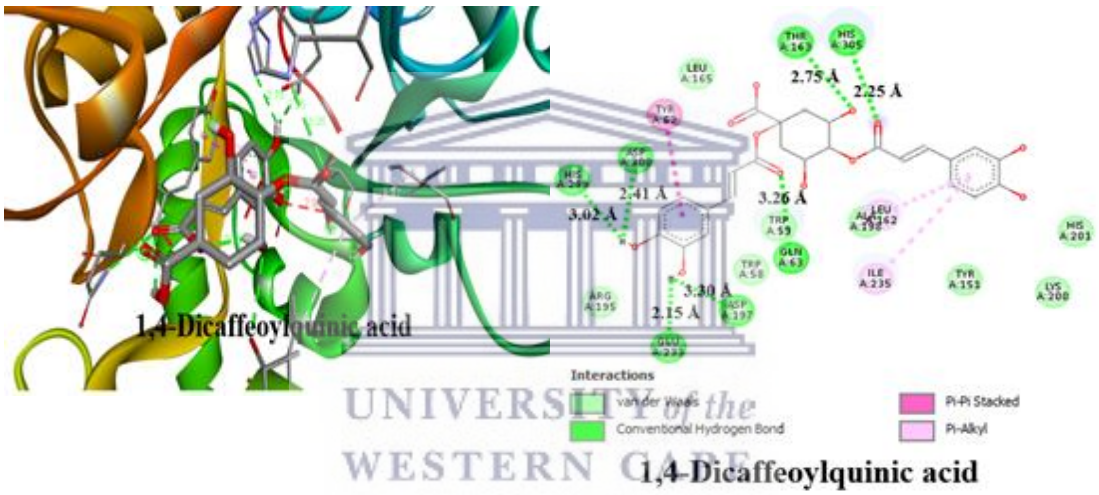

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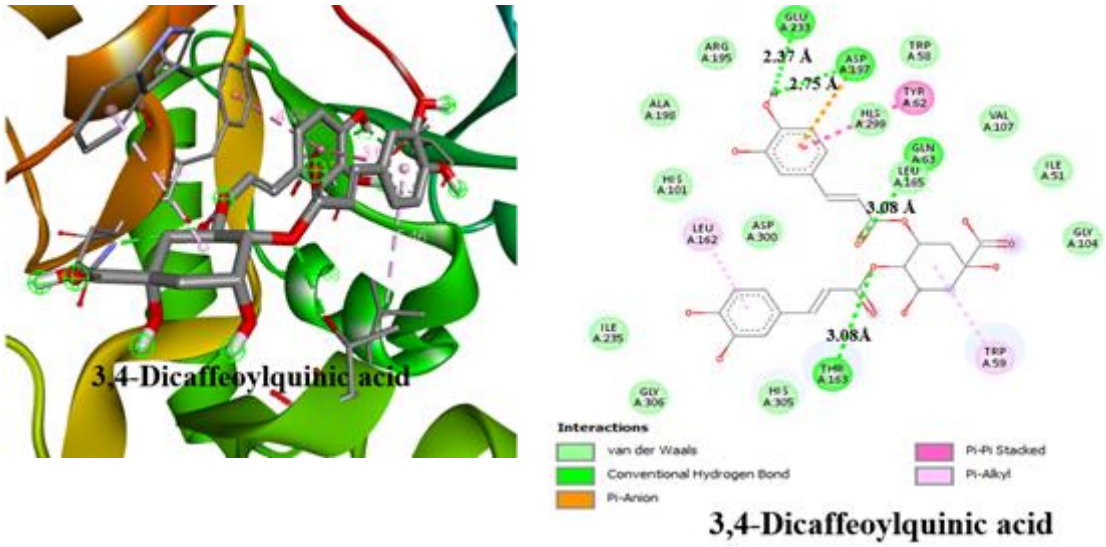

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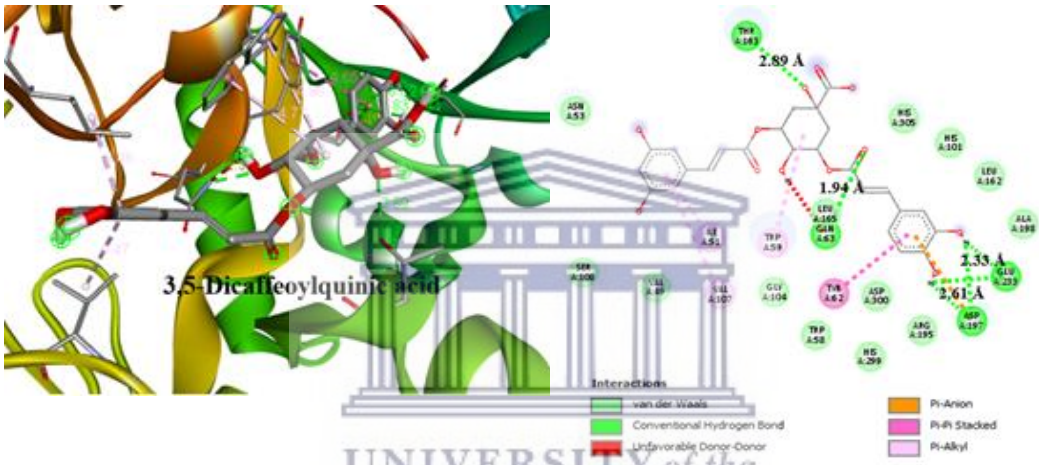

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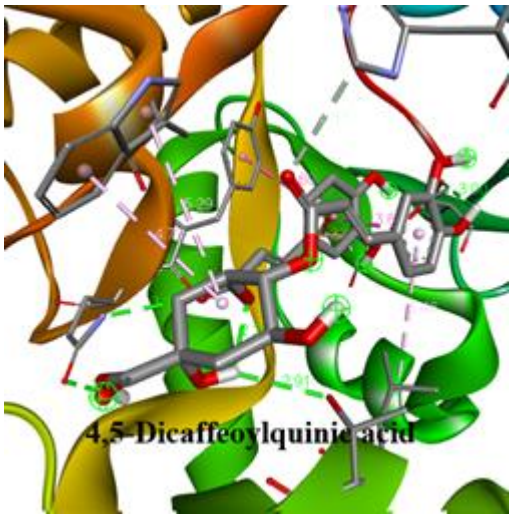

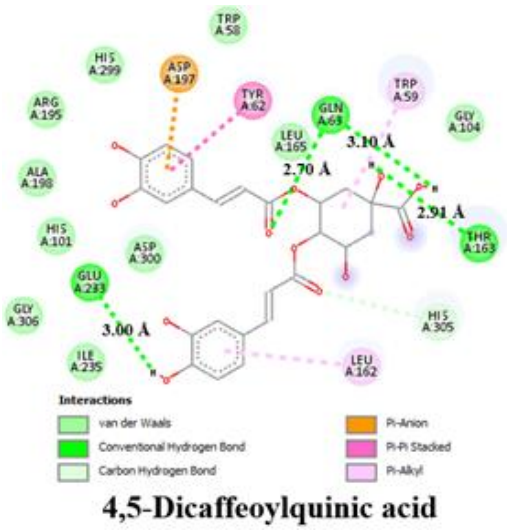

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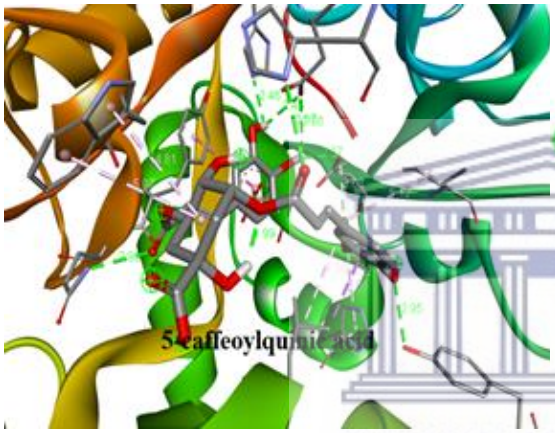

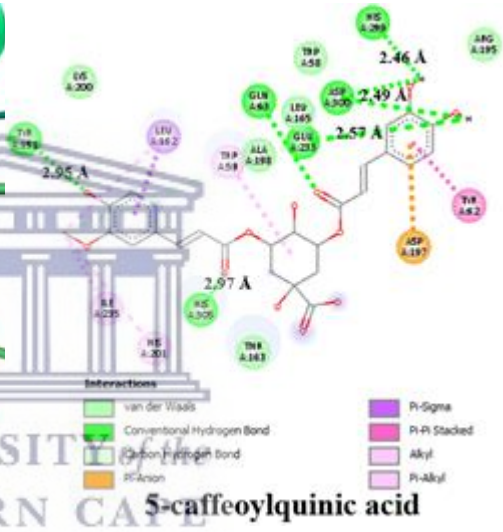

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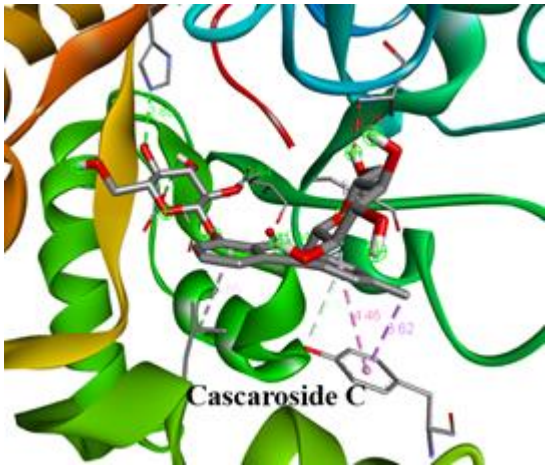

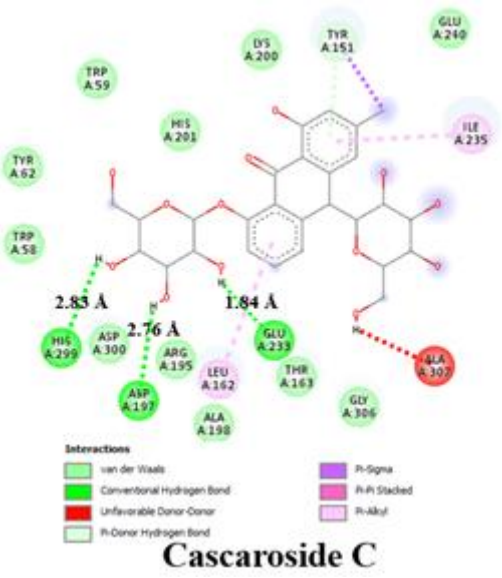

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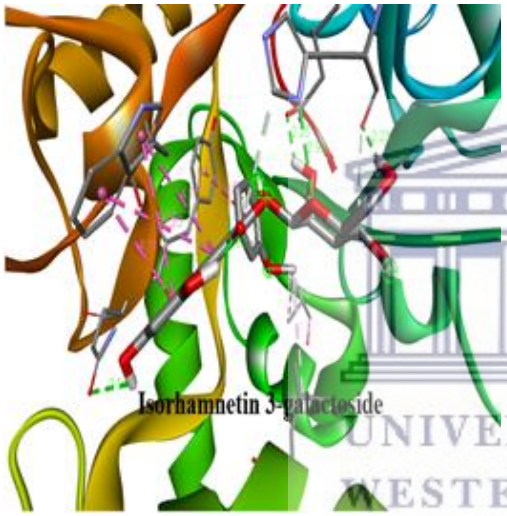

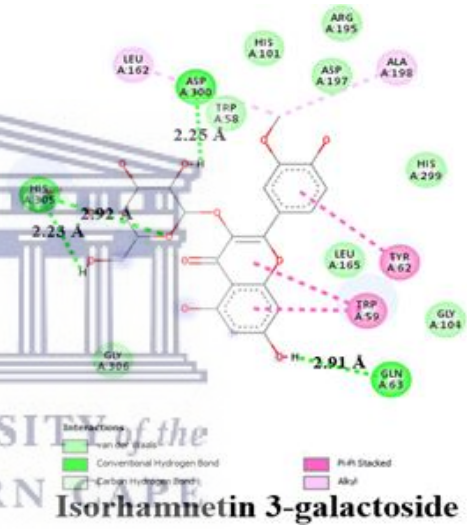

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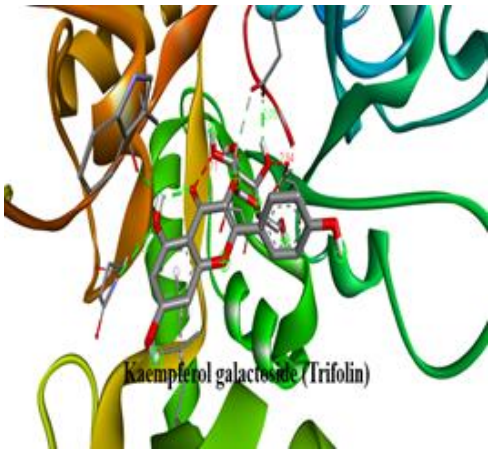

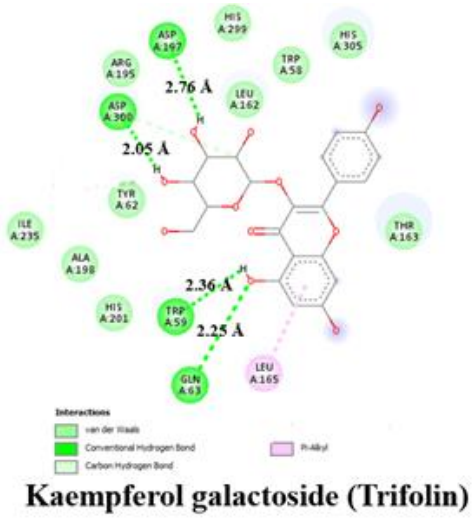

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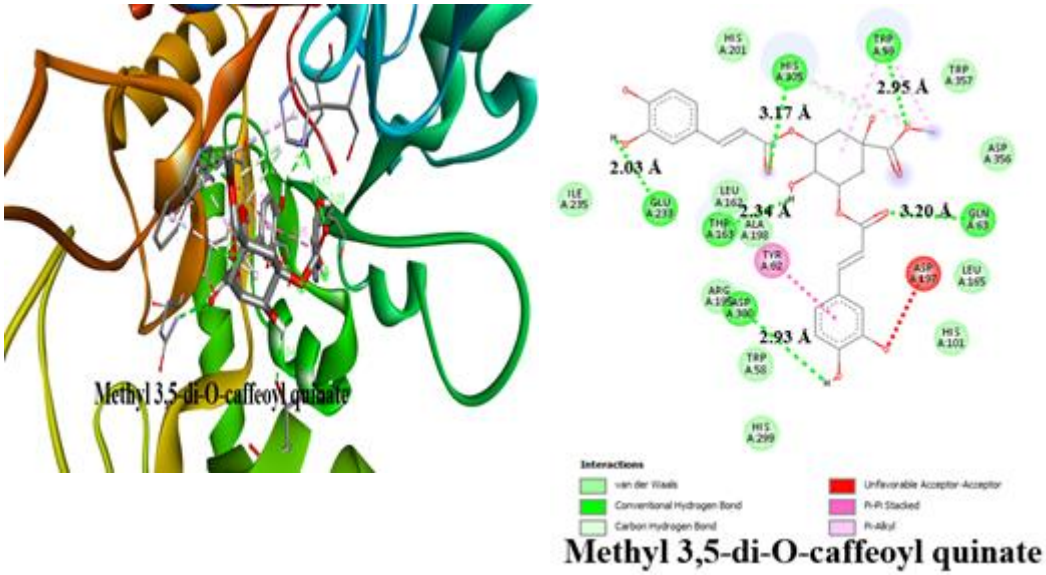

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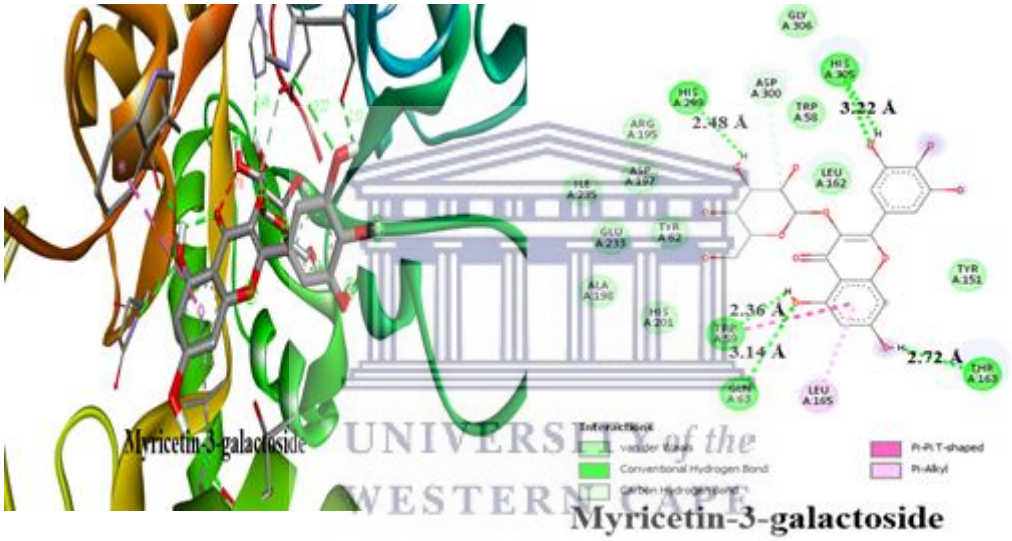

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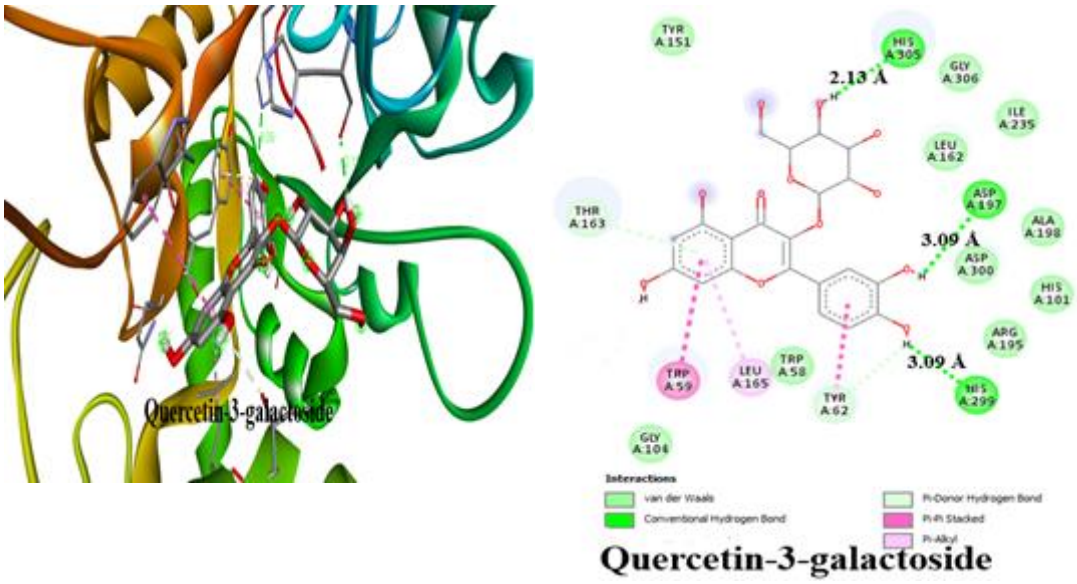

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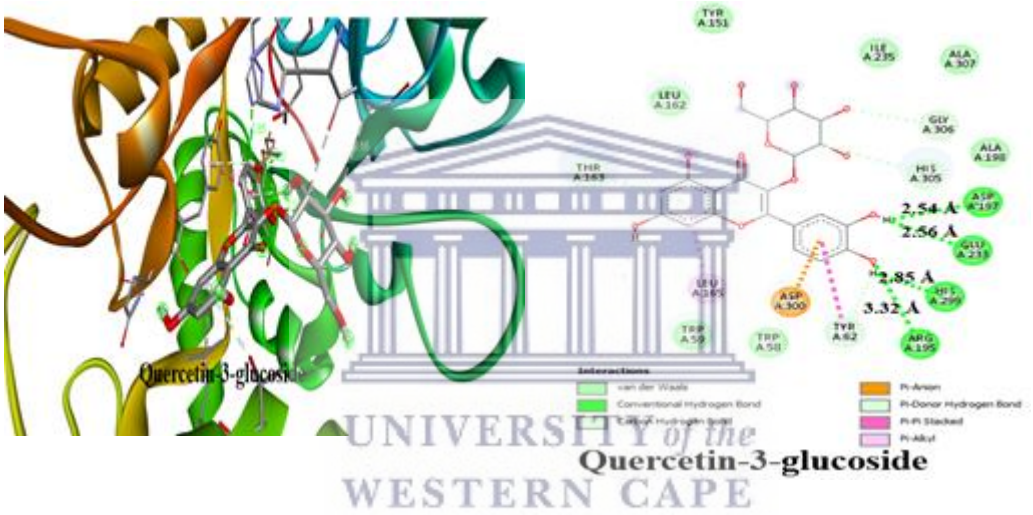

Figure S4. Model of the Interaction and the 2D Structure of α-amylase protein with (a) 1,3-Dicaffeoylquinic acid (b) 1,4-Dicaffeoylquinic acid (c) 3,4-Dicaffeoylquinic acid (d) 3,5-Dicaffeoylquinic acid (e) 4,5-Dicaffeoylquinic acid (f) 5-caffeoylquinic acid (g) Cascaroside C (h) Isorhamnetin 3-galactoside (i) Kaempferol galactoside (Trifolin) (j) Methyl 3,5-di-O-caffeoyl quinate (k) Myricetin-3-galactoside (l) Quercetin-3-galactoside (m) Quercetin-3-glucoside.

Appendix 5: Figure S2

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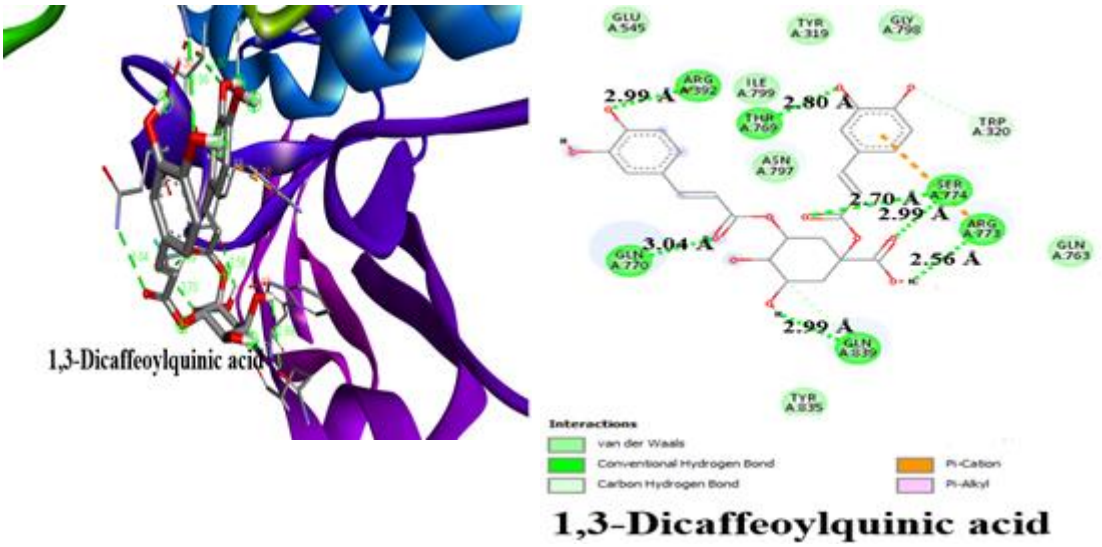

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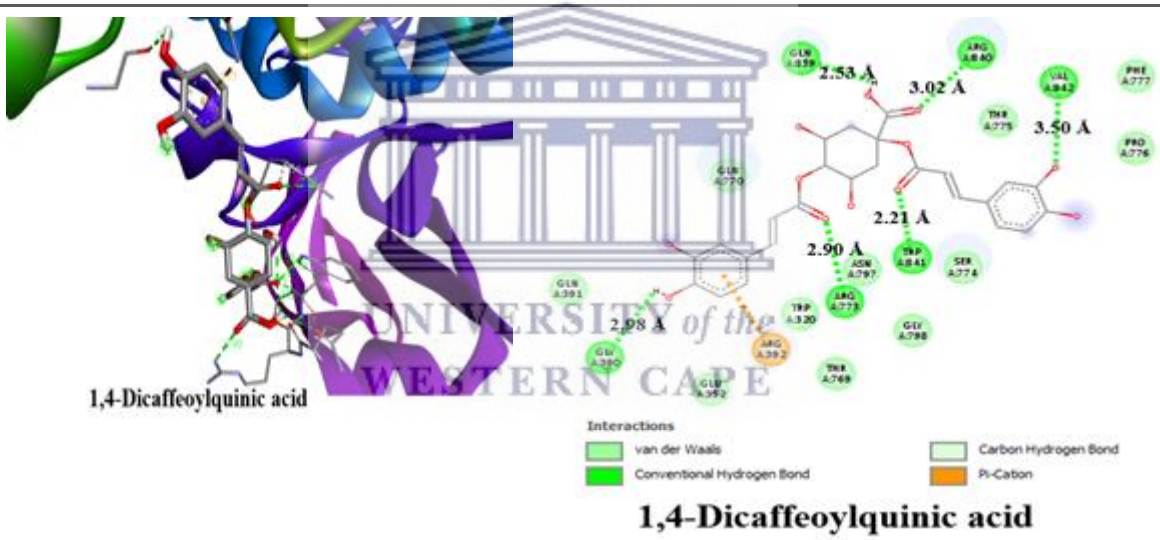

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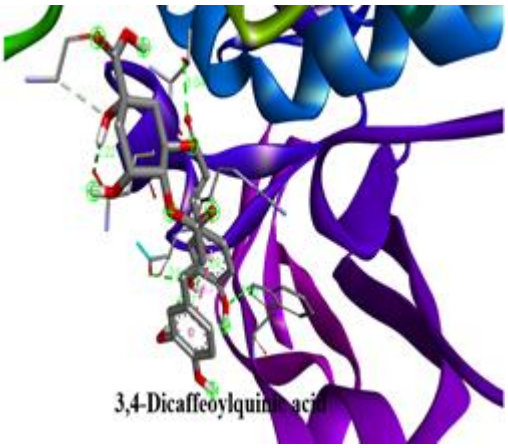

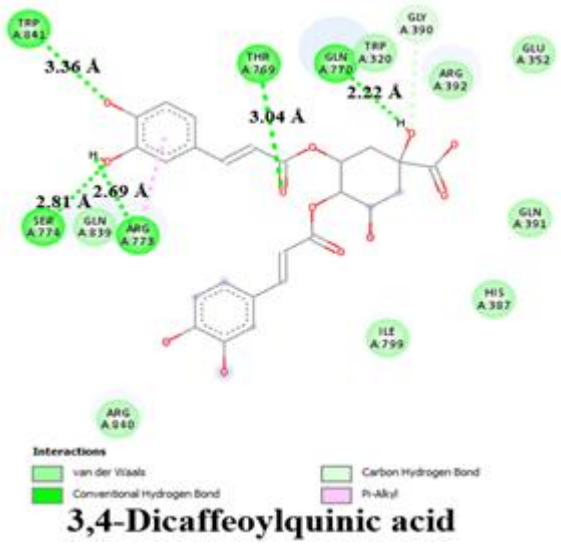

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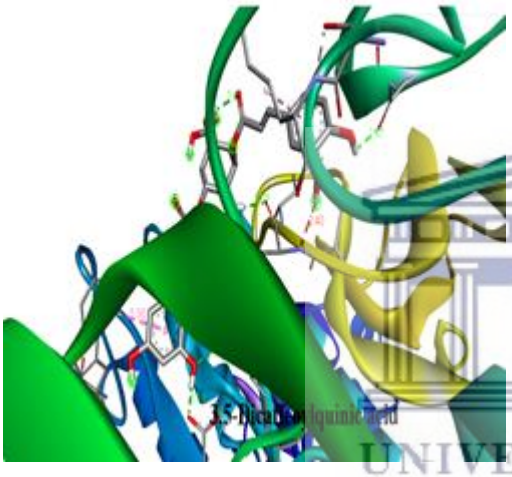

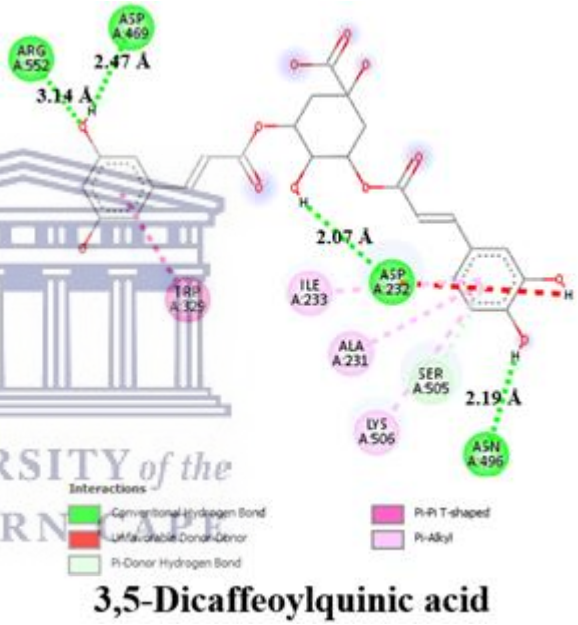

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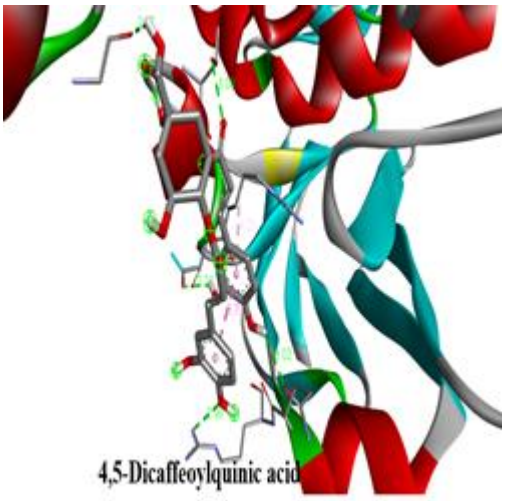

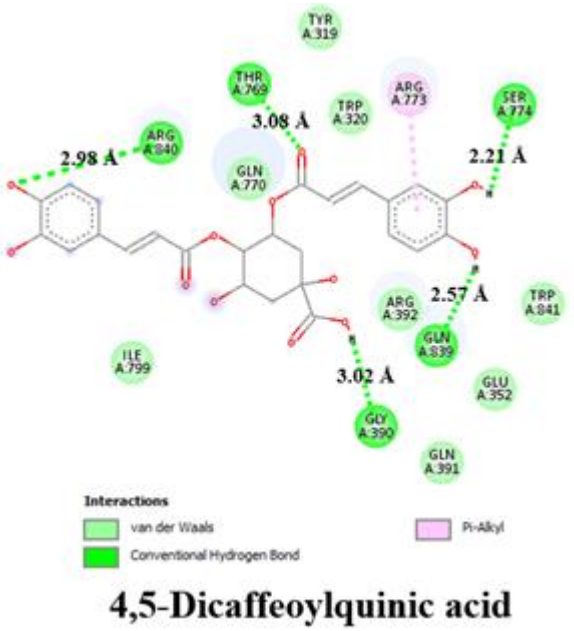

4,5-Dicaffeoylquinic acid

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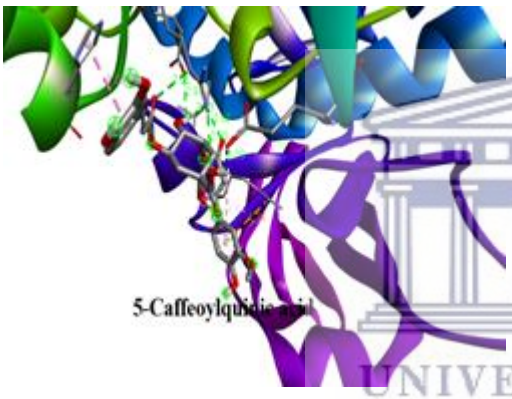

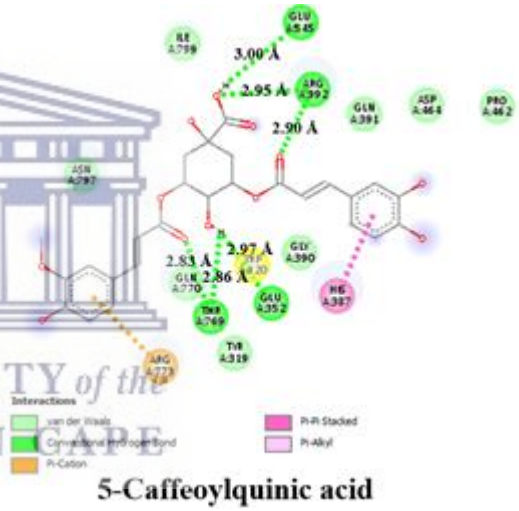

5-Caffeoylquinic acid

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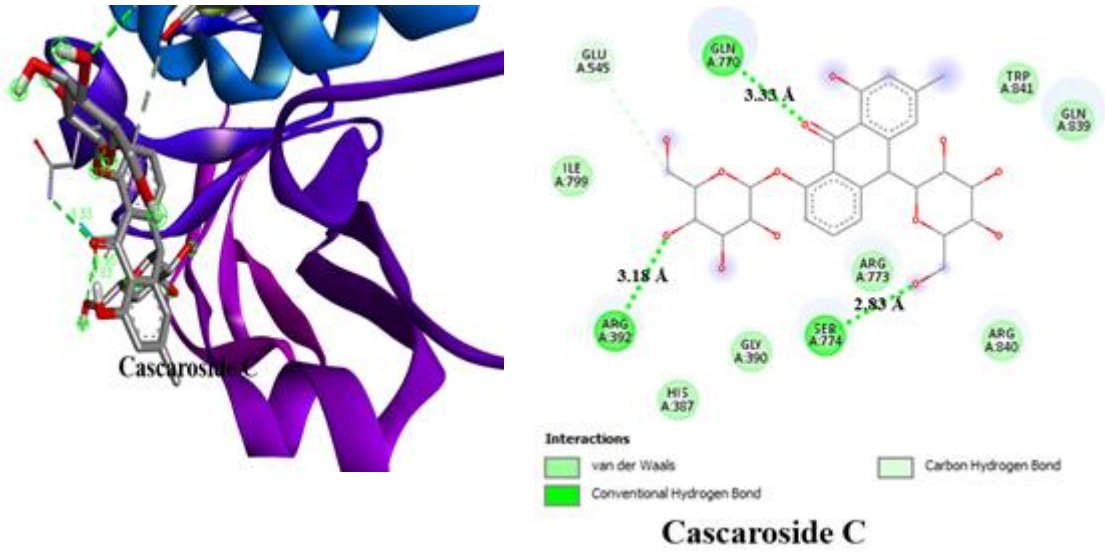
g



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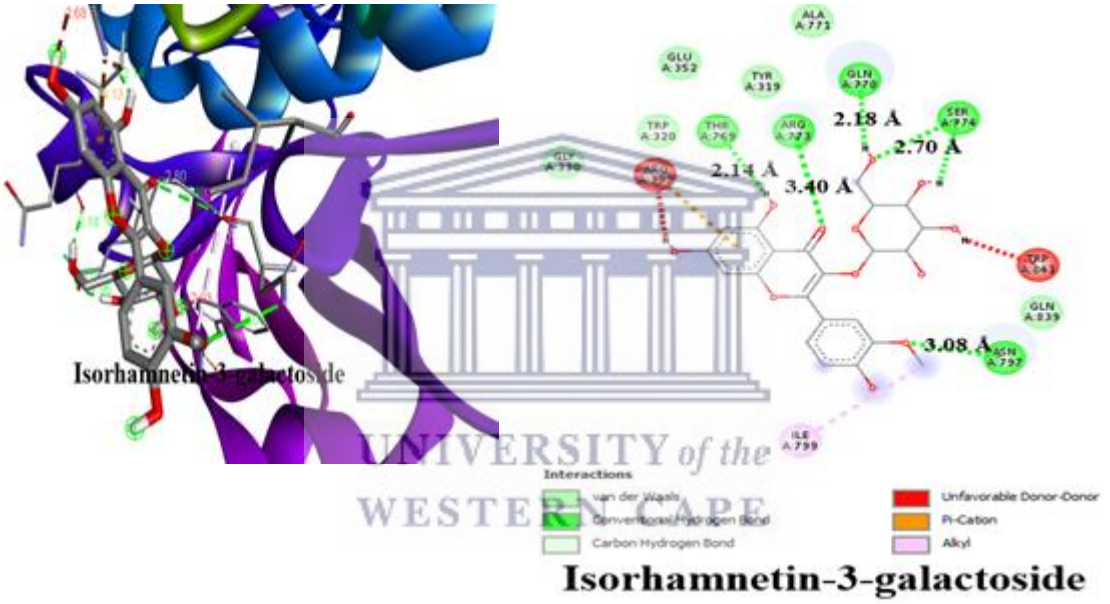

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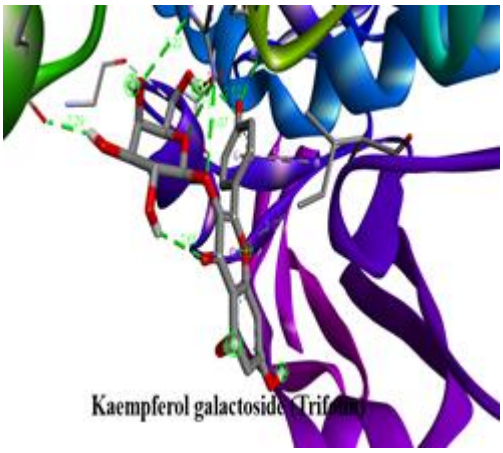

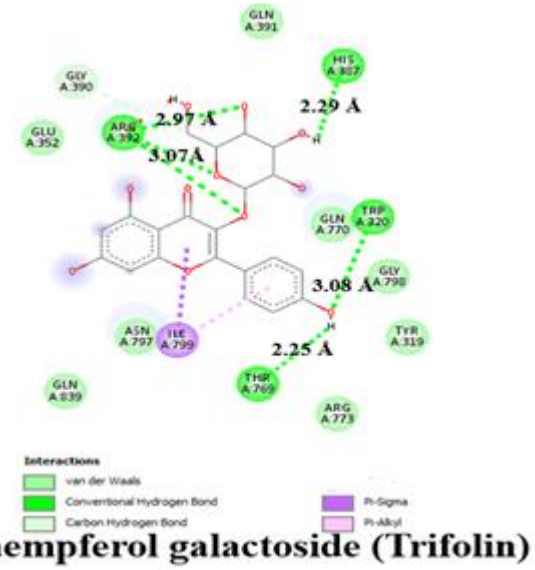

Kaempferol galactoside (Trifolin)

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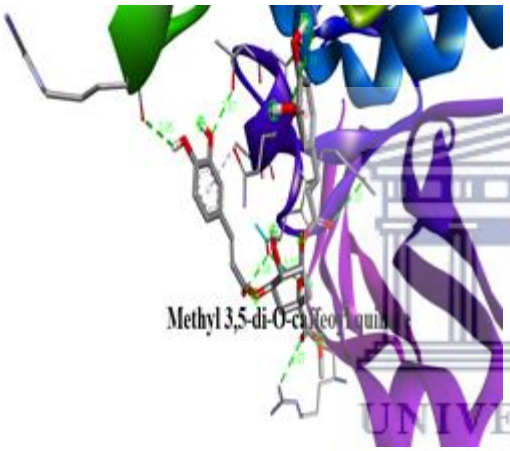

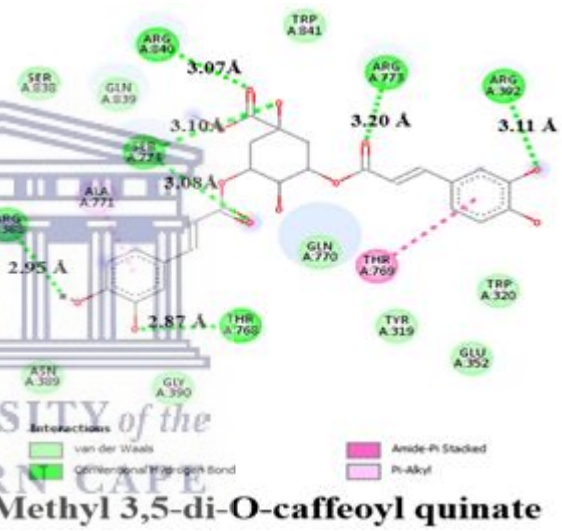

Methyl 3,5-di-O-caffeoyl quinate

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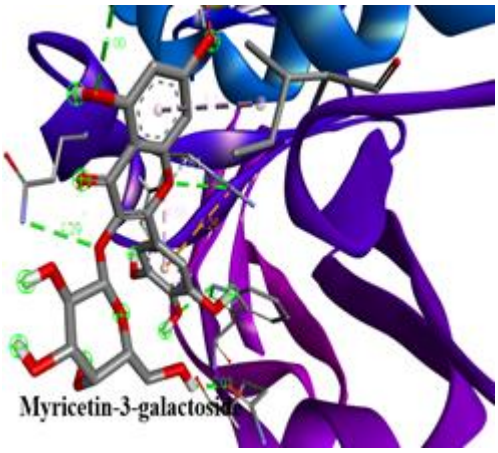

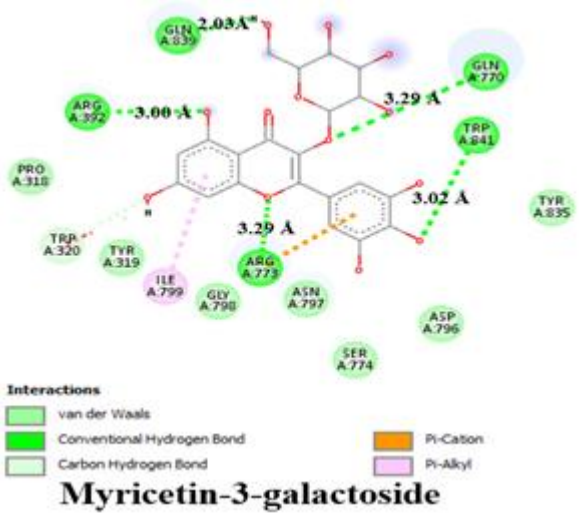

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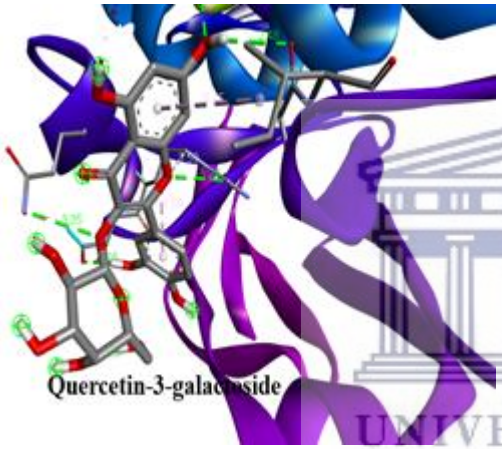

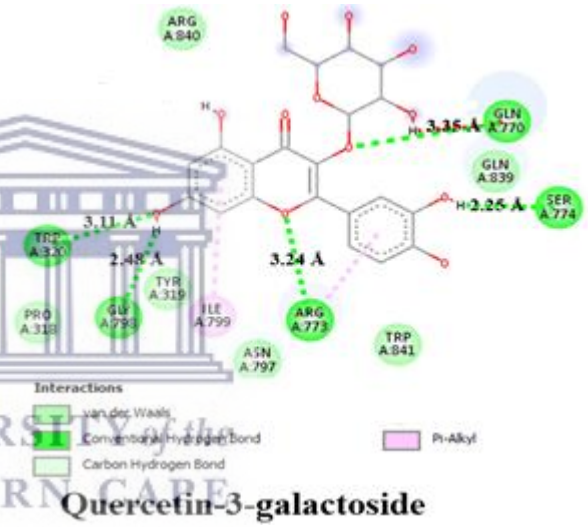

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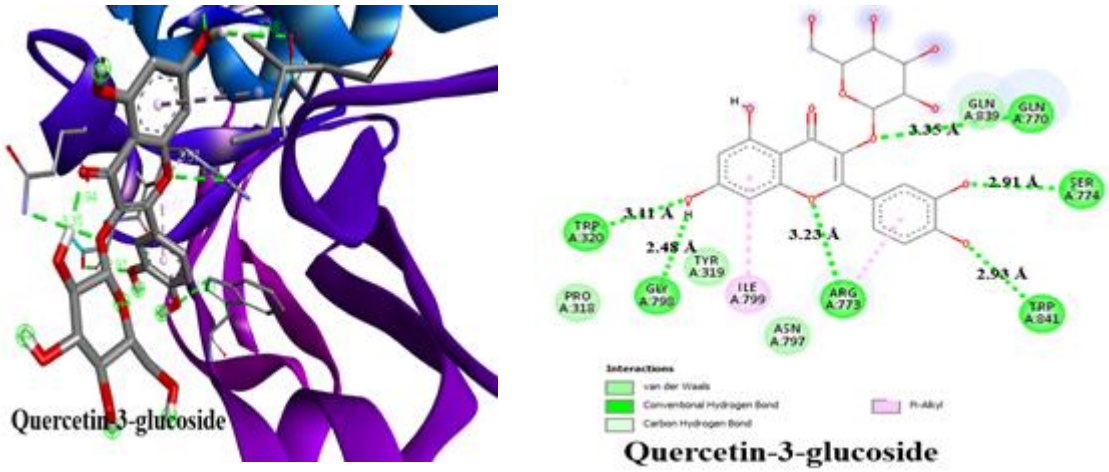

Figure S5. Model of the Interaction and the 2D Structure of α-glucosidase protein with (a) 1,3-Dicaffeoylquinic acid (b) 1,4-Dicaffeoylquinic acid (c) 3,4-Dicaffeoylquinic acid (d) 3,5-Dicaffeoylquinic acid (e) 4,5-Dicaffeoylquinic acid (f) 5-caffeoylquinic acid (g) Cascaroside C (h) Isorhamnetin 3-galactoside (i) Kaempferol galactoside (Trifolin) (j) Methyl 3,5-di-O-caffeoyl quinate (k) Myricetin-3-galactoside (l) Quercetin-3-galactoside (m) Quercetin-3-glucoside.