STRUCTURAL AND FUNCTIONAL PROPERTIES OF BIOACTIVE VEGETABLE LEAF POLYPHENOLS

By

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ABSTRACT

Polyphenols of natural sources especially from plants have increasingly become important due to their ability to bind enzyme proteins, inhibit free radicals and reduce carbohydrate and lipid digestion. Polyphenols have received a lot of interest because they are important alternatives to drugs with potential health benefits. Therefore, the aim of this thesis was to determine the antioxidant, antihypertensive and digestive enzymes inhibitory properties of water-soluble polyphenols of *Amaranthus viridis* (AV), *Solanum macrocarpon* (SM), and *Telfairia occidentalis* (TO) leaves. The leaf polyphenols were extracted using double distilled water for 4 h at 60^{o}C and then freeze dried to yield AV, SM and TO extracts. The three vegetable extracts had strong antioxidant properties, which were measured as ability to scavenge free radicals and chelate metal ions. During a 24-h test, AV, SM and TO extracts reduced systolic blood pressure (SBP) of spontaneously hypertensive rats (SHR) after oral administration of 100 mg/body weight (BW) better than the 500 mg/kg BW dose. All the three vegetable extracts (AV, SM and TO) also inhibited in vitro activities of digestive enzymes (pancreatic lipase, α-amylase, α-glucosidase, chymotrypsin, and trypsin). Results indicate that TO extracts were better inhibitors of the digestive enzymes followed by SM and AV extracts, except for trypsin. The mode of inhibition was identified to be competitive for trypsin, α-amylase, α-glucosidase, chymotrypsin α-amylase, α-glucosidase, chymotrypsin. The TO extract was the most bioactive and was further fractionated into chlorophyll and non-chlorophyll fractions, which were then studied for their antioxidant and enzyme inhibition properties.

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FOREWARD

This thesis was compiled using the manuscript format and it consist of six manuscripts, which followed immediately after the general introduction and literature review chapters. The manuscripts were prepared according to the journal style in which they were published. Manuscript 1 (Journal of Food Biochemistry) determined the influence of nitrogen micro-dosing on phenolic content, antioxidant, and anticholinesterase properties of the aqueous extracts of TO, SM and AV leaf extracts. Manuscript 2 (Journal of Food Bioactive) examined the antihypertensive effect of aqueous polyphenol extracts of AV and TO leaves in spontaneously hypertensive rats. Manuscript 3 (Acta Horticulture) examined the trypsin inhibitory activity of aqueous extracts of AV, SM and TO leaves while manuscript 4 (Current Topics in Nutraceutical Research) studied the effect of SM leaf extract on the blood pressure and heart rate of spontaneously hypertensive rats. Manuscript 5 evaluated the ability of AV, SM and TO extracts to inhibit α-amylase and pancreatic lipase in addition to structural changes to the enzyme protein in the presence of polyphenolic extracts. Manuscript 6 determined the effect of chlorophyll removal on the in vitro antioxidant and antihypertensive properties of TO fractions. Manuscripts 1, 2, 3 and 4 have been published while 5 and 6 are currently under revision for submission to journals. A transition statement is provided after each manuscript that links the next chapter for a consistent flow. The last chapter provides the overall summary of the study, limitations, and possible future directions.

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LIST OF ABBREVIATION

Aβ	Amyloid beta
ABTS	2, 2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ACE	Angiotensin-1 converting enzyme
ACh	Acetylcholine
AD	Alzheimer’s disease
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
ANOVA	Analysis of variance
AT-I	Angiotensin-I
AT-II	Angiotensin-II
ATCI	Acetylcholine iodide
ATP	Adenosine triphophate
AV	*Amaranthus viridis*
BApNA	N-Benzoyl-D-L arginine paranitroanilide
BDE	Bond dissociation enthalpy
BHT	Butylated hydroxyl toluene
BW	Body weight
CAT	Catalase
CD	Circular dichroism
CH	Chlorophyll-enriched fraction
CNS	Central nervous system
CVD	Cardiovascular disease
Da	Dalton
DBP	Diastolic blood pressure
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DNSA	Dinitro-salicylic acid
DPPH	2, 2-Diphenyl-1-picrylhydrazyl
DPP-IV	Dipeptidyl-peptidase-iv
DTNB	5,5-dithio-bis-(2-nitrobenzoic acid

EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
FAPGG	*N*-(3-[2-Furyl]acryloyl)-phenylalanylglycylglycine
FAT	Fertilizer application time
FC	Folin-Ciocalteu
FD	Fertilizer dose
FI	Fluorescence intensity
FRAP	Ferric-reducing antioxidant power
FPLC	Fast Protein Liquid Chromatography
GAE	Gallic acid equivalent
GSH	Reduced glutathione
GP_x	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
HAT	Hydrogen atom transfer
HDL	High density lipoprotein
HOCl	Hypochlorous acid
HPLC	High performance liquid chromatography
HRSA	Hydroxyl radical scavenging activity
IC_{50}	50% Inhibitory concentration
iNOS	Inducible nitric oxide synthase
IP	Ionization potential
kDa	Kilodalton
KNOS	Kinin-nitric oxide system
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
LDL	Low density lipoprotein
MAE	Microwave assisted extraction
MCA	Metal chelation activity
NADPH	Nicotinamide adenine dinucleotide phosphate
NC	Chlorophyll depleted fraction
NMR	Nuclear magnetic resonance
NO	Nitric oxide

NOS	Nitric oxide synthase
$_1O2$	Singlet oxygen
O2·-	Superoxide anion
·OH	Hydroxyl radicals
OONO	Peroxynitrite
ORAC	Oxygen radical absorbance capacity
PEs	Polyphenolic extracts
POD	Peroxidase
RAS	Renin-angiotensin system
RAAS	Renin-angiotensin-aldosterone system
RO·	Alkoxyl radical
ROO	Peroxyl radical
ROS	Reactive oxygen species
RP-HPLC	Reversed phase high performance liquid chromatography
SBP	Systolic blood pressure
SET	Single electron transfer
SFE	Supercritical fluid extraction
SHRs	Spontaneously hypertensive rats
SM	*Solanum macrocarpon*
SOD	Superoxide dismutase
SRSA	Superoxide radical scavenging activity
STZ	Streptozotocin
T2DM	Type 2 diabetes mellitus
TAC	Total antioxidant capacity
TBARS	Thiobarbituric acid
TEAC	Trolox equivalent antioxidant activity
TFC	Total flavonoid content
TNF-α	Tumour necrosis factor -α
TO	*Telfairia occidentalis*
TPC	Total phenol content
TPTZ	2,4,6, -tripyridyyl-triazine

TRAP	Total radical-trapping antioxidant assay
UAE	Ultrasound assisted extraction
VLDL	Very low-density lipoprotein
VV	Vegetable variety
WHO	World health organization

CHAPTER ONE

1.0 GENERAL INTRODUCTION

Obesity, diabetes, Alzheimer's and other degenerative diseases are of major concern because they constitute a critical economic burden on human health due to their increasing global prevalence and rate of occurrence among populations around the world("2020 Alzheimer's Disease Facts and Figures," 2020; Dementia Prevention, Intervention, and Care: 2020 Report of the Lancet Commission - The Lancet, n.d.; WHO | Global Health and Ageing, n.d.; DeTure & Dickson, 2019; Guevara et al., 2019; Hernández-Ledesma et al., 2011; Hruby & Hu, 2015; Livingston et al., 2020). The etiology of these diseases is associated with oxidative stress that results from excessive production of reactive oxygen species (ROS) or free radicals. Free radicals are molecules with one or more unpaired electrons, which make them unstable and highly reactive (Poprac et al., 2017). Some of the highly ROS include hydroxyl radical (•OH), nitric oxide (NO), superoxide radical (•O2) and peroxyl radicals (ROO•). Free radicals are either beneficial or deleterious depending on their balance with antioxidants present in living systems. The beneficial effects of ROS come in to play in that they take part in certain activity and cellular signaling pathways in living organisms. When there is an overproduction of ROS and a reduction in the antioxidant species, an imbalance occurs thus causing damage to biological systems in the body (Phaniendra et al., 2015a; Poprac et al., 2017; Prescott & Bottle, 2017). ROS damage macromolecules such as protein, lipids, DNA, and cell membranes thus giving rise to some physiological and pathophysiological processes such as inflammation, carcinogenesis, ageing, atherosclerosis and other degenerative disease (García-Sánchez et al., 2020; Liguori et al., 2018; Sies & Jones, 2020; Xu et al., 2017). The presence of antioxidant defense mechanism in the body such as superoxide dismutase (SOD), catalases (CAT), glutathione (GSH) and peroxidases counter the activities of ROS thus exerting a protective role (Poprac et al., 2017).

Excessive activities of some enzymes such as renin, angiotensin-I-converting enzyme (ACE), α-amylase, pancreatic lipase, α-glucosidase, and trypsin have been implicated in disease generation within biological systems. For example, excessive activities of renin and ACE in the renin-angiotensin system (RAS) lead to the production of angiotensin II from angiotensin I, which causes vasoconstriction of blood vessels, especially in the heart thus leading to high blood pressure and eventually the pathogenic condition known as hypertension (Pihlanto & Mäkinen, 2013). Other enzymes such as α-glucosidase, α-amylase, trypsin, and pancreatic lipase catalyze reactions that give rise to the production of glucose and lipids, which can become detrimental to health when excessive amounts are present within the blood circulatory system. When these conditions of excessive nutrient levels in the blood and high blood pressure are left untreated, they invariably lead to diseases such as hypertension, diabetes, and obesity. Therefore, inhibition of activities of these enzymes is important in reducing or preventing certain chronic diseases within the human body. In addition to enzyme inhibition, good dietary habit and lifestyle modifications are fundamental in the management of these diseases (Ülger et al., 2018). Studies have shown that dietary modifications or nutritional intervention can ameliorate the effect of these diseases thus reduce the global health care burden.

Over the years, drug therapy and some synthetic antioxidants have been used for the treatment and management of these diseases. However, there are safety concerns and high health care cost arising from prolonged use of these drugs, ranging from drug toxicity to negative side effects. Therefore, there is need for alternative strategies such as the use of natural antioxidants derived from plants that are safer and cheap (Buchholz & Melzig, 2016; Lourenço et al., 2019; Sellami et al., 2017). These alternatives derived from plants are not as potent as pharmaceutical drugs, but they have health benefits with little or no side effects. They are natural products that

can be easily absorbed by the body without exerting much burden on the activity of the kidney and liver. Since most diseases occur because of free radical damage in the body and because the current mode of management is not enough to treat these diseases, different studies have been done on how to delay or prevent the onset of these diseases. The most effective way in the fight against these diseases is by improving the antioxidant status of the body, which can be achieved by consuming fruits and vegetables (Goñi & Hernández-Galiot, 2019; Guevara et al., 2019; Hrelia & Angeloni, 2020; Slavin & Lloyd, 2012) Foods of plant origin are rich sources of antioxidants that can scavenge free radicals and chelate metal ion. Plants are one of the sources of natural compounds used in traditional medicine for the treatment of certain diseases and it is estimated that about 17% of flowering plants around the world have some beneficial value in terms of health promotion. The use of plants as therapy for some diseases have dated back to 1870, which involve the use of whole plant parts or some parts of the plants (Capasso et al., 2000). This means that certain bioactive properties found in one plant species can also be found in other plant species in the same family, resulting in a diverse range of plants that can be used for medicinal purposes and provide health benefit. Reliance on energy dense food have resulted into the developing of various diseases such as obesity, diabetes, and hypertension. Studies have shown that consumption of fruits and vegetables can help prevent these diseases (Capasso et al., 2000).

Plants during their own metabolism, converts soil minerals into nutrients that benefit the human body when ingested. These nutrients are active macromolecules that are produced during the metabolic activities within the plant. Plant macromolecules support organ function to perform specific functions in tissues and increase the body's defense against certain diseases (Liguori et al., 2018). In the fight against certain diseases such as diabetes, jaundice and

shortness of breath, plants have played a major role in managing these diseases. According to the World Health Organization (WHO), about 80% of the world's population depend on plants for their health care needs (Ekor, 2014; Mahomoodally, 2013; Özaslan & Oguzkan, 2018). They also reported that about 11% of the 252 drugs considered as basic and essential are mainly of flowering plants origin (Veeresham, 2012). Different substances (phytochemical) obtained from plants used by humans for the treatment of certain diseases have less adverse effects compared to their synthetic counterparts. The plant-derived compounds have found much importance in clinical use because of their safety, efficacy, patient's tolerance, and acceptance. Aside from their use as new drug entities, they can also serve as chemical models or templates for the design, synthesis and semi synthesis of novel substances for treating human diseases (Forni et al., 2019; Zhang et al., 2015). Plant-derived compounds have been reported to have anticancer, antimicrobial, antioxidant, anti-inflammatory and antidiarrheal properties (Greenwell & Rahman, 2015; Özaslan & Oguzkan, 2018). Therefore, this work is dedicated to elucidation of the structural characteristics of antioxidant and antihypertensive properties of locally grown vegetables, using in vitro and in vivo evaluation methods.

1.1 Hypotheses

1. The level of fertilizer micro-dosing will influence in vitro and in vivo activities of aqueous polyphenolic extracts of AV, SM and TO.
2. The vegetable leaf polyphenolic extracts will inhibit in vitro activities of some of the gastrointestinal digestive enzymes (α-amylase, α-glucosidase, pancreatic lipase, trypsin, and chymotrypsin) through competitive and non-competitive modes.
3. Inhibitory activity will be correlated with ability of the polyphenolic extracts to change the secondary and tertiary conformations of target enzymes.

4. Oral administration of the vegetable extracts will reduce systolic blood pressure of spontaneously hypertensive rats (SHRs) during short-term feeding experiments.

5. The chlorophyll and non-chlorophyll polyphenolic compounds will differ in their antioxidant and enzyme inhibition properties.

1.2 Objectives

The overall aim of this research is to determine the in vitro and in vivo antioxidant and enzyme inhibition properties of aqueous extracts of leafy vegetables (AV, SM, TO).

The specific objectives are to determine the;

1. Effects of fertilizer micro-dosing and time of application on bioactive properties of aqueous polyphenolics extracts obtained from AV, TO and SO leaves.
2. Antioxidant properties and anticholinesterase activities of the vegetable extracts
3. Mode and kinetics of inhibition of digestive enzymes (α-amylase, α-glucosidase, pancreatic lipase, trypsin, and chymotrypsin).
4. Antihypertensive properties of the polyphenol extract *in vitro* and *in vivo* using spontaneously hypertensive rats (SHR).
5. Modulation of secondary and tertiary structures of enzymes by the polyphenolic extracts.
6. Differences between the in vitro bioactive properties of chlorophyll and non-chlorophyll fractions.

Therefore, findings from this work will provide mechanistic information on the mode of interactions between polyphenols and enzymes that are implicated in various metabolic

disorders. The polyphenol extracts can be incorporated into food systems for the preservation of food materials or as functional ingredients in the formulation of therapeutic diets.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Vegetables and their nutritional importance

2.1.1 Vegetables

Over the years, especially in developing countries, plants and their extracts have become effective tools used in managing various degenerative diseases. This is mainly because of the phenolic compound; which plants use to adapt to stress conditions and microorganism infections. Vegetables constitute a small part of our daily caloric intake, but their health benefits surpass their caloric contributions. These health benefits are due to the presence of vitamins and proteins but mainly the phenolic compounds, which are plant secondary metabolite with strong antioxidant properties (Gomez-Gomez et al., 2018; GutiErrez-Grijalva et al., 2016; Swallah et al., 2020).

African eggplant (*Solanum macrocarpon*), slender amaranth (*Amaranthus viridis*) and fluted pumpkin (*Telfairia occidentalis*) are green leafy vegetables that are used for food and for medicinal purposes in Nigeria (Onuminya et al., 2018). These vegetables are harvested at all stages in the growth process and consumed whether as fresh, processed, or semi-processed forms. In most countries, plant phytochemicals such as flavonoids, phenolic acids and proanthocyanins have received much attention due to their disease protective effect and their ability to scavenge free radicals (Aiyegoro & Okoh, 2009; Larbie et al., 2015). Due to the increased understanding of vegetables as sources of minerals and vitamins in human diets, production of vegetables has increased especially among local farmers and individuals cultivating it in personal and market gardens for local sales (Egun, 2007; Enujiugha, 2014).

2.1.1.1 Slender amaranth *(Amaranthus viridis)*

Amaranthus viridis (AV) known as slender amaranth is an annual crop belonging to the Amaranthaceae family. It is one of the 70 species of the Amaranthaceae family that produce edible leaves and it is majorly found and cultivated in Africa, Asia, America, Australia, Europe, and other tropical countries. *Amaranthus viridis* is a short-lived perennial herb up to 1 m tall with a slender stem and alternate leaves with petiole, which can be up to 10 cm long. The leaves and succulent stem are cheap and widely available. It is an excellent source of proteins and essential amino acids such as lysine, and methionine (Sarker & Oba, 2019). It is also a good source of dietary fiber, carotenoids, and minerals such as phosphorus, potassium, calcium, iron, zinc, copper, manganese, and calcium. It is an excellent source of antioxidants and some pigments such as anthocyanin, beta carotene, vitamin C, phenolics, flavonoids and chlorophylls (Sarker & Oba, 2019). The leaves are usually sold in local markets and eaten either as cooked, steamed, or fried. The plant has been reported to have antioxidant, anti-inflammatory, antidiabetic, antimicrobial and hepatoprotective properties (Ferdous et al., 2015).

2.1.1.2 African eggplant (*Solanum macrocarpon*)

Eggplant, *Solanum macrocarpon* (SM) known as gboma or igbagba (Yoruba) is a fruit that is grown and commonly consumed throughout the tropical and subtropical region of the African continent (Eletta et al., 2017). It belongs to the Solanaceae family like tomato and pepper. They are herbaceous plants or shrubs with 2-7 flowers that are about 3-8 cm in diameter. The flowers are hermaphrodite having both female and male plant parts with round fruits that have grooved portions at the top and bottom (Dougnon et al., 2012). There are about 25 species of eggplants commonly grown in Nigeria, but SM is one of the most widely cultivated (Eletta et al., 2017). The fruits are served usually during ceremonial occasions with kola as a token of goodwill to represent blessings and fruitfulness while the leaves are used to prepare African salad or added to soups, and stew delicacies (Eletta et al., 2017). They are also used in folklore medicine to treat diseases such as constipation, ulcers, toothache, asthma, skin infections rheumatic disease, joint pains, and snake bite (Majesty, 2013; Ogunsuyi et al., 2020). The fruit of this plant is rich in nutrients such as amino acids and phenolic compounds with high antioxidant properties that can scavenge free radicals (Eletta et al., 2017). Studies have shown that the SM extracts have anti-inflammatory properties, chelate ferrous ion, prevent the generation of hydroxyl radicals, in addition to antihypertensive, antidiabetic and antioxidant activities

(Nwanna et al., 2016; Oluwagunwa et al., 2019). Existing literature shows that SM plant parts and their extracts can positively influence human health via various important nutritional and functional effects. Ogunsuyi et al., (2018) found that scopolamine-induced cognitive and antioxidant status impairment in rats were reversed when pretreated with dietary inclusions of SM. Other studies have reported that the aqueous extract of SM abate hyperglycaemia in alloxan-induced diabetic rats (Ajiboye et al., 2021), the leave extract inhibit acetylcholinesterase activities in *Drosophila melanogaster in-vitro* (Ogunsuyi et al., 2020).

2.1.1.3 Fluted pumpkin (*Telfairia occidentalis*)

Fluted pumpkin is a tropical plant belonging to the cucurbitaceae family. It is popularly grown in West and Central Africa and a common vegetable consumed in Nigeria as part of the diet (Ayoola et al., 2018; Salman et al., 2020). It is a high climbing perennial homestead garden crop cultivated mainly for its succulent leaves (Ademiluyi et al., 2019; Nwidu & Oboma, 2019). It is grown close to fences, trees, and walls on which the shoots climb. The shoots also creep on the floor or staked for easy cultivation of the leaves and pods (Aworunse et al., 2018). The leaves are dark green 18 cm wide and 35 cm long with nutrients such as carbohydrates, proteins, minerals, vitamins, and fiber. The leaves are a relish used in soup preparation such as egusi and edikang ikong. The leaves are also rich in essential and non-essential amino acids, antioxidants,

and phenols (Njoku et al., 2018; Emmanuel et al., 2018). Fluted pumpkin is prominent for healing purposes and used as blood tonic to treat anaemia especially among the Igbo tribe in Nigeria. The seeds are rich sources of protein, fat, and vitamins (Aworunse et al., 2018; Osukoya et al., 2016). The plant has been reported to have anti-diabetic, antioxidant, antimicrobial and hepatoprotective properties (Eseyin et al., 2018; Salman et al., 2018). It is widely grown for its tasty and nutritious leaves, which are often eaten as vegetables. In Nigeria, the plant's herbal preparation has been used in the treatment of anaemia, chronic fatigue, and diabetes. The leaves' infusion is used as a blood tonic for convalescents, as well as for the treatment of malaria and loss of appetite (Cyril-Olutayo et al., 2018). Several studies have shown its benefits in ameliorating different diseases such as sickle cell, erectile dysfunction, and hepatotoxicity (Ademiluyi et al., 2019; Atabo et al., 2016; Cyril-Olutayo et al., 2018; Salman et al., 2018).

2.1.2 Nutritional importance of vegetables

Vegetables are edible plant parts which are important for human health. They contain vitamins, minerals, phytochemical compounds, and dietary fiber content which play important role in preventing human diseases (Ülger et al., 2018). Different studies have been able to establish the nutritional importance of vegetables and it has been established that adequate consumption of fruits and vegetables can prevent the development of certain diseases such as diabetes, Alzheimer, obesity, cancers, hypertension aging and inflammation due to the presence of some phytochemicals which act as an antioxidant in them (Hossain et al., 2015; Nahak et al., 2014).

Over the last three decades, the use of herbal medicinal products and supplements has increased, with more people relying on them for their healthcare. Leafy vegetables and indigenous foods are part of Africa's cultural heritage and play an important role in the customs,

traditions, and food culture of various households. Despite the potential health benefits and medicinal values of these vegetables, little research has been done on their use in Africa. These may be due to lack of consumer awareness about their benefits, cultural beliefs of some people and human preferences (Akinola et al., 2020; Kansiime et al., 2018; Mbhenyane, 2017). Most of these indigenous vegetables have been marginalised in favour of exotic varieties which may be nutritionally inferior. This may be due to lack of knowledge on their level of use and importance in rural economies, their economic values, and a lack of world markets. They are underutilized and indigenous knowledge regarding the use of these species is not uniformly distributed among the people (Akinola et al., 2020; Onuminya et al., 2018). Though they have good taste and high nutritional value, most household remove them from their household diets as the financial status improve. Since traditional vegetables are associated with poor rural lifestyles and low social status, cultural changes and urbanizations have resulted in increased neglect in response to the decline in indigenous vegetable production, consumption, and diversity (Onuminya et al., 2018). Despite their promising potential in the treatment of some diseases, and their effectiveness proven, many of them remain untested, and their use is either poorly controlled or not monitored at all (Ekor, 2014). Due to limited understanding of their mode of actions, their interactions with other existing drugs and potential adverse effect, their misuse is inevitable. Therefore, further research in this area is important to create more consumer awareness about the health benefits of these vegetables, contribute new knowledge to database and to identify gaps where more research is urgently needed.

Diabetes, obesity, Alzheimer's, CVD are leading causes of death in both developing and developed countries and most techniques employed in the treatment of these diseases are raising some health concerns due to the negative side effects of the synthetic drugs used (Alzheimer's

disease facts and figures, 2020; DeTure & Dickson, 2019; Guevara et al., 2019; Hernández-Ledesma et al., 2011). Globally, there is an increase demand for natural plant-derived products as alternatives to these synthetic drugs. Several studies have reported the health benefits of *Telfairia occidentalis* (TO), *Amaranthus viridis* (AV) and *Solanum macrocarpon* (SM) leaf extracts in combating these diseases both *in vitro* and *in vivo* using animal studies. The protective effect of these vegetables is largely attributed to their phenolic contents, which have some biological activities (Aworunse et al., 2018). Hyperlipidemia, a condition characterized by abnormal levels of lipid in the plasma, is a chronic inflammatory disorder implicated in major CVD. A study showed that administering TO supplemented-diets significantly reduced cholesterol-induced heart enlargement in Wistar rats and led to reduced lipid peroxidation and blood cholesterol levels (Eseyin et al., 2007). Methanolic extracts of TO, SM and AV have also been reported to lower plasma glucose levels and inhibit the activities of α-glucosidase and α-amylase in Wistar rats (Adaramoye et al., 2007, Aderibigbe et al., 1999; (Eseyin et al., 2010; Oboh et al., 2012; Salman et al., 2008). The antioxidant properties of these leaf extracts have been attributed to the presence of bioactive compounds. According to (Oboh et al., 2010), aqueous extracts of TO have significantly higher free radical scavenging potential more than its ethanolic extracts. Other studies have reported the ability of TO-fortified diets to prevent lipid peroxidation and tissue damage in Wistar rats and elevate GSH, CAT and SOD activities (Akang et al., 2015; Jimoh, 2018; Kayode et al., 2010; Kayode et al., 2009; Nwanna & Oboh, 2007). Other therapeutic properties of these vegetables include antimicrobial and anticancer effects (Aworunse et al., 2018). All these properties both *in vitro* and *in vivo* have validated their use as herbal medicine in developing countries. Chemical compositions, and bioactivity of these leaf extracts vary due to

source and method of cultivation. Therefore, there is need for a unified method of cultivation to minimize variations that may arise in the number of secondary metabolites in these vegetables.

2.2 Fertilizer Micro-dosing

Increase in the world's population from 6.1 billion in mid-2001 to an estimated value of 9.3 billion by 2050 calls for a significant increase in food production to ensure food security and prevent malnutrition especially in the developing countries (Debez et al., 2010). According to the World Food Summit, there is a gradual decline of food production especially in West Africa due to loss of soil fertility and drought. This in turn affects rural household in terms of decreased income and shortage of food (INCRISAT, 2009). Also, in areas with high amount of rainfall, soil erosion affects so many farmlands leading to depletion of soil acidity thus making the soil infertile. Due to the loss of soil fertility, different soil management techniques ranging from the use of organic and inorganic fertilizers have been utilized. The high cost of inorganic fertilizers to smallholder farmers and low availability of organic fertilizers has resulted in low food productivity. However, advances in agriculture have brought about a new technology with low-input and high crop productivity that can improve soil nutrient and organic matter (Bindraban et al., 2015; Okebalama et al., 2016). In Africa, production of vegetables involves the use of fertilizer as a means of enhancing the growth and development of the leaves. Micro-Veg is a project funded by the Government of Canada to train local vegetable farmers in West Africa on how to limit the amount of fertilizer applied to their farm without compromising nutrient supply. The old method of applying fertilizer called the broadcasting method is being discouraged due to its negative effect on the environment and the underground water system (Olarewaju et al., 2018). A new fertilizer application method called the micro-dosing method is a farming technique developed by scientists at the International Crop Research Institute for the Semi-Arid

Tropics (ICRISAT) to help small-scale farmers in the semi-arid Sahel region and Africa boost their crop yields. This will boost crop yields and bring about different crop varieties to alleviate hunger and poverty among the local farmers. Fertilizer micro-dosing is a precision method involving the application of small amounts of fertilizer in the planting hole at the time of planting or as top dressing 2-4 weeks after seed emergence. This is based on the efficient use of fertilizer through the 4R Nutrient Stewardship principle, which involve the use of fertilizers from the right source, at the right rate and at the right time with the right placement (Bindraban et al., 2015). Farmers measure out fertilizers with the aid of coca-cola or beer bottle cap or with three-finger pinch (ICRISAT, 2008; ICRISAT, 2009; (Okebalama et al., 2016). This method reduces the amount of fertilizer farmers will apply to their farm and when combined with organic manure, produce enough nutrients in the soil to support the growth of vegetables. The use of mineral fertilisers is one of the key factors driving the increase in global agricultural production needed to feed the growing human population, especially in developing countries.

2.3 Free Radicals, Oxidative Stress and Chronic Disease

2.3.1 Reactive oxygen species (ROS)

Oxygen is essential to life and its reaction with biological molecules in the body can lead to the generation of highly reactive compounds that have been implicated in various human diseases and aging thus affecting human health (Friedman, 2011; Tan et al., 2018). These compounds generated are highly reactive oxygen radicals and non-radicals of partially reduced oxygen products that are produced in the chloroplast, mitochondria, peroxisome, and other aerobic cells. The electron structure of molecular oxygen makes it susceptible to radical formation. Due to the lone pair of electrons in its outer electron shell, oxygen is reduced through the addition of electrons thus leading to the formation of ROS and damage of DNA, proteins, and

lipids (Held, 2012). The main oxygen radicals are the superoxide anion (•O2), hydroxyl radical (HO•), alkoxyl and peroxyl radicals (RO• and RO2•) while hypochlorous acid (HOCl), ozone (O3), singlet oxygen (1O2), peroxynitrite (ONOO-) and hydrogen peroxide (H_2O_2) are non-radicals but can easily lead to free-radical reactions in living organisms (Das & Roychoudhury, 2014; Ozcan, Ayla and Ogun, 2015; Phaniendra et al., 2015; Pizzino et al., 2017). Figure 2.1 shows the production of ROS, their role in cellular damage and the importance of endogenous antioxidant system in maintaining normal homeostasis. ROS can also be generated by exposure of the human body to certain external toxic compounds that cause some physiological imbalances and interfere with the normal functioning of the body system. These radicals are by-products of normal cellular and enzymatic processes that are naturally present in the human body. They can be regulated by maintaining a balance between the antioxidant defense system within the body and free radical production using both endogenous and exogenous antioxidants (Kurutas, 2016; Pizzino et al., 2017). Upon exposure of the human body to environmental stress such as UV radiation, heat, and ionizing radiation, generation of ROS/free radical increases rapidly, which may result in significant damage to cell structures such as lipids, proteins, carbohydrates, and nucleic acids (Brieger et al., 2012; Phaniendra et al., 2015; Sharifi-Rad et al., 2020). Other sources of ROS/free radicals could be from diets and exposure to microbial pathogens. Various enzymes such as nicotinamide adenine dinucleotide phosphate [NADPH] oxidases, xanthine oxidase, nitric oxide synthetase, p450 cytochromes are some of the enzymes involved in the generation of ROS and serve as a signaling function in the cell (Pizzino et al., 2017).

In defense of the host cell, ROS have some beneficial effects. They are used by the host defense system to fight pathogens such as termination of microbes, stimulation of growth factors

and control of inflammatory responses and perform other vascular functions such as oxidative phosphorylation, redox signaling pathway and mobilization of electron transport system (Phaniendra et al., 2015; Pizzino et al., 2017). One of its key roles in the immune system is well exemplified by patients with granulomatous disease. Since they are unable to generate O2• due to a malfunctioning NADPH oxidase, they are vulnerable to numerous and, in most cases, recurrent infections (Forrester et al., 2018; Pizzino et al., 2017).

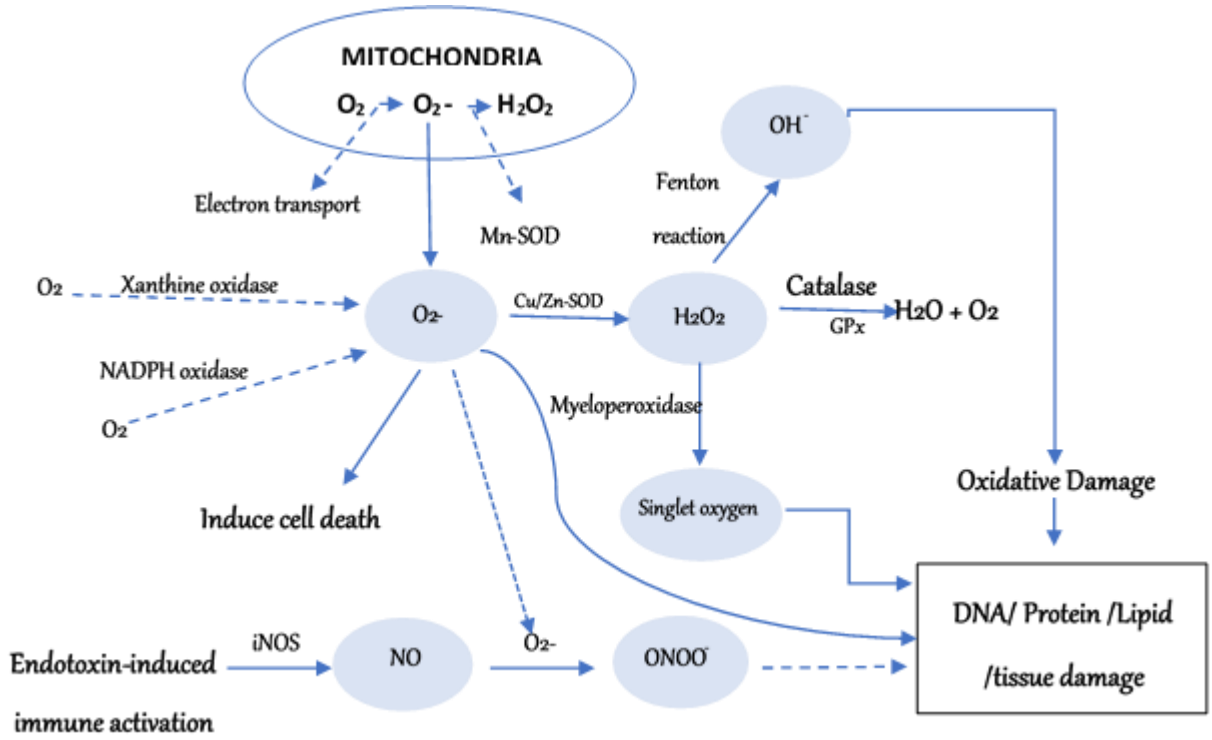


Figure 2. 1. Production of ROS/free radicals and oxidative damage to biological macromolecules.

Alterations in the redox balance of the body and overproduction of ROS/free radicals have been associated with various human diseases (atherosclerosis, diabetes mellitus, and stroke) and metabolic dysfunction thus causing oxidative stress. Excessive production of these radicals can cause damage to cells thus increasing the risk of inflammation and the development of diseases such as cancer, diabetes, hypertension, neurodegenerative disease, and age-related

functional decline (Brieger et al., 2012; Forrester et al., 2018; Goncharov et al., 2015; Phaniendra et al., 2015; Pizzino et al., 2017; Sharifi-Rad et al., 2020). This section will further explain the role of ROS/free radical in diabetes, obesity, hypertension, and Alzheimer's disease which is the focus of this research.

2.3.2. Free radicals in human health conditions

Chronic disorders such as cardiovascular disease, diabetes, neurodegenerative diseases, and cancer all have oxidative stress as a key factor in their pathogenesis. Long-term exposure to high levels of pro-oxidant factors causes structural defects of the DNA, as well as functional changes in several enzymes and cellular structures, leading to gene expression abnormalities. The western lifestyle of consuming processed foods, exposure to a wide variety of chemicals, and a lack of exercise, plays a significant role in the production of oxidative stress (Kurutas, 2016; Sharifi-Rad et al., 2020).

2.3.2.1 Oxidative stress and neurodegenerative disease

Alzheimer's disease (AD) is one of the most prevalent neurodegenerative diseases affecting the older adult's population accounting for 60-80% of all dementia cases (DeTure & Dickson, 2019; Livingston et al., 2020; Manoharan et al., 2016; Marasco, 2020). According to Alzheimer's Society of Canada, about 747,000 Canadians are living with cognitive impairment based on a 2012 study and it is a disease affecting people aged 45 yrs and older and is the most common disease among people living in long term care facilities (Wong et al., 2016). It is characterised with confusion, loss of vision, impaired reasoning and personality changes resulting in high health burden and death (Fiest et al., 2016; Manoharan et al., 2016). AD is the world's fourth leading cause of death, affecting about 16 million people worldwide, and the incidence is expected to rise due to an ageing population. (Ferri et al., 2005). Many factors have

been implicated in the pathogenesis of AD but the pathway underlying the neurodegeneration in AD has not yet been determined. The cause of AD can be attributed to genetic and environmental factors such as age, family history and gender (Manoharan et al., 2016; Prasansuklab & Tewin, 2013) but the role of ROS cannot be overemphasized.

Oxidative stress is one of the major factors responsible for AD. The development of AD occurs due to loss of the brain's antioxidant defense mechanism and mitochondrial dysfunction. The reliance of the brain on oxidative phosphorylation and the amount of ATP required to maintain the neuronal homeostasis makes it highly susceptible to oxidative stress (Friedman, 2011; Tan 2018). Other factors responsible for the pathogenesis of AD are accumulation of the by-products of lipid and protein oxidation in the brain and amyloid beta (Aβ) peptides. Amyloid-beta peptides are protein fragments produced from amyloid protein precursors deposited abnormally in various cells and organs. They can generate ROS in the presence of metal ions and accumulation of these Aβ between the neurons in the brain causes amyloid plaques, which is a hallmark of AD (Aisen et al., 2017; Buffington et al., 2013; DeTure & Dickson, 2019; Prasansuklab & Tewin, 2013). Aβ are misfolded proteins that cause amyloid plaques in the brain. Amyloid plaques are hard, fibrous-like, and insoluble protein aggregates that block nerve cells in the brain thus inducing oxidative stress and interfering with normal functioning of the brain (DeTure & Dickson, 2019; Manoharan et al., 2016). Accumulation of Aβ contributes to neurodegeneration by interfering with neuron-to-neuron synaptic communication, thus blocking the transport of nutrients and other essential molecules within neurons (Marasco, 2020).

Iron induced oxidative stress is another mechanism implicated in the pathogenesis of AD. This oxidative stress occurs due to the build-up of iron in the brain. Iron is a transition metal that can produce free radicals such as hydroxyl radical through Fenton reaction (Choi et al., 2012;

Kim et al., 2015). These metals, such as zinc, aluminium, iron, and copper, have a direct effect on the precursor of the amyloid protein and bind to it, promoting its aggregation (Liguori et al., 2018). Microglia, one of the most important defense systems of the brain, accounts for about 15% of all the cells in the central nervous system (CNS). It functions as macrophages and when activated, produces proinflammatory cytokines such as TNF-α, IL-1β, interferon-γ and superoxide anions through NADPH oxidase (Bachiller et al., 2018; Borrajo et al., 2021; Choi et al., 2012; Li et al., 2018; Shalavadi et al., 2012). Activation of glial cells by inducible nitric oxide synthase (iNOS) produce nitric oxide (NO), which reacts with superoxide to generate a highly reactive peroxynitrite (Choi et al., 2012). Another cause of AD is the activity of acetylcholinesterase, an enzyme responsible for the breakdown of acetylcholine (ACh) into acetate and choline. ACh is a neurotransmitter that is released from ACh receptor involved in cholinergic neurotransmission through the activation of nicotinic receptor to modify postsynaptic cell functions. When the concentration of ACh is low, ACh receptor release more ACh into the synaptic cleft and this can cause an accumulation of ACh in the synaptic cleft thus affecting neurotransmission. AChE is an enzyme that plays an important role in maintaining ACh in the body by clearing free or excess ACh from the synapse. It breaks down ACh into choline and acetate thus affecting its signaling function (Frahm et al., 2015; Trang & Khandhar, 2021). The excessive clearing of ACh from the synaptic cleft by AChE limits the amount of ACh needed for neurotransmission thus causing memory loss and other dementias. Also, as the body ages, there is a decline in the amount of ACh produced and the activity of AChE continues with the little ACh produced. This, therefore, affects cholinergic functions and lead to progressive memory loss, which is a major pathogenesis of AD. Inhibition of AChE is very important in order to maintain the amount of ACh needed for neurotransmission (Čolović et al., 2013). Overall,

oxidative stress and inflammation in the neurons are the main factors in the etiology of neurodegenerative diseases. It is, therefore, important to develop neuroprotective strategies that can reduce oxidative stress, inhibit the production of ROS, and decrease the levels of proinflammatory cytokines released during microglial activation.

2.3.2.1.1 Anti-neurodegenerative effect of plant and their derived compounds

Rivastigmine, galantamine, demecarium, donepezil, tacrine, edrophonium, ladostigil, ungeremine, and lactucopicrin are commercial reversible AChE inhibitors that have therapeutic uses in cognitive health (Alzheimer's Disease Fact Sheet, 2020; Consumer Reports, 2012; Nwidu et al., 2017). Their use has been reported to be effective in treating myasthenia gravis (orally) and reversing neuromuscular block (intravenously) (Consumer Reports, 2012). Due to their synthetic nature and several adverse effects associated to them, their therapeutic use has been limited in AD patients. Based on these negative side effects, research has delved into the finding alternatives with less side effects from plant derived bioactive component. Plant polyphenols have useful and beneficial effect in the treatment of different human diseases (Balkrishna et al., 2019; Cherniack, 2012). Plant secondary metabolites such as flavonoids, lignans and alkaloids have shown promising inhibitory activity against acetylcholinesterase. The ability of alkaloids to act as an anti-cholinesterase was attributed to its similarity with ACh. Due to its positively charged nitrogen ions, it can bind at the active site of AChE (Balkrishna et al., 2019). The areas of ethno-pharmaceutical research in AD management indicate promising potential of polyphenolic compounds as active inhibitors of AD. The ability of plant polyphenols to improve cognition in AD patients was reported when the aqueous and methanolic extracts of *Withania somnifera* (with inhibition of 24.26% and 30.03%, respectively) and *Tinospora cordifolia* (26.01% and 40.58% respectively) at 100 µg/mL inhibited AChE at a higher rate (Balkrishna et

al., 2019). The use of polyphenolic extract EGb 761 from Ginkgo biloba in human clinical studies, produce a neuroprotective effect, enhance cognition, and maintain the neurotransmitter ACh in cognitively affected adult patients (Tan et al., 2015). Oboh et al. (2014) reported that 70% ethanol extracts of *Amaranthus cruentus* and *Telfairia occidentalis* leaves inhibited AChE at a higher rate with EC50 of 97.9 ug/mL and 52.7 ug/mL respectively. Olarewaju et al. (2018) also reported that aqueous extracts of *Telfairia occidentalis*, *Solanum macrocarpon* and *Amaranthus viridis* inhibited AChE *in-vitro* 19.52%, 13.88% and 12.90% respectively. Other vegetables and fruits with anticholinesterase activity include *Thymus vulgaris* (Kindl et al., 2015), *Aristotelia chilensis* (Cespedes et al., 2017), chia seeds extracts (Kobus-Cisowska et al., 2019) and grapefruit and leave extracts (Fouad & Rizk, 2019). There is growing evidence that foods or supplements rich in plant-derived flavonoids may delay the onset and/or slow the development of AD and associated neurodegenerative disorders. Consumption of plant polyphenols from vegetables, fruit juices and red wine has been shown to delay the onset of the disease with respect to AD (Williams & Spencer, 2012). Polyphenols are important and beneficial in AD development due to their several biological mechanisms which include inactivation of free radicals, prevention of vascular inflammation through the production of adipocyte generated inflammatory cytokines, interaction with transition metals, activate ACh on neurons that enables neuronal and microglial growth gene and modulation in the activity of different enzymes (Cherniack, 2012; Colizzi, 2019). It has been reported that they modulate the breakdown and removal of beta amyloids that are responsible for the development of AD in animal models (Williams & Spencer, 2012).

2.3.2.2 Hypertension and the renin-angiotensin system (RAS)

High blood pressure, also called hypertension, affects a wide range of the world's population and is responsible for about 45% of all deaths worldwide. It is the risk factor for myocardial infarction, heart failure and stroke (WHO 2013). Hypertension can be defined as the force that the blood exerts on the wall of the blood vessel. A normal blood pressure is when the systolic blood pressure (SBP) is less than 120 mmHg, and the diastolic blood pressure (DBP) is less than 80 mmHg. Blood pressure is high when SBP and DBP are higher than 140 and 90 mmHg, respectively (Campbell et al., 2016). According to world hypertension league, hypertension is a public health epidemic affecting four in ten adults over the age of 25 years (Campbell et al., 2016; Campbell et al., 2014). It is the second leading cause of global death and disability (Institute for Health Metrics and Evaluation, 2015) and a risk factor for other diseases such as stroke, heart failure, dementia, and diabetes. It is a disease that has a great impact on health care with 10% of treatment costs directly related to high blood pressure and its complications (Campbell et al., 2014). One major factor causing this disease is unhealthy food choices and lifestyle practices such as high salt intake, saturated fats, sedentary lifestyle, and alcohol consumption (Campbell et al., 2016). The RAS and kinin-nitric oxide system (KNOS) play a key role in regulating the systemic volume and vascular resistance; thus, influencing cardiac output and blood pressure (Irigoyen et al., 2016). The cleavage of angiotensinogen by the action of renin gives rise to the production of angiotensin I (AT-I), an inactive decapeptide, which is a rate-limiting step in the RAS pathway. AT-1 is converted to angiotensin II (AT-II), a powerful vasoconstrictor by the action of angiotensin converting enzyme (ACE). Excessive production of AT-II causes severe blood vessel contractions and limited relaxation thus leading to high blood pressure. The KNOS produces bradykinin, a vasodilator that is responsible for the

relaxation of blood vessels (Irigoyen et al., 2016; Kim et al., 2020; Ra et al., 2020). Bradykinin exerts its antihypertensive effect by generating reactions that increase the intracellular concentrations of Ca^{2+} leading to the activation of nitric oxide synthase (NOS) to produce NO, a potent vasodilator that relaxes blood vessels (Ibrahim, 2006; Su, 2017). AT-II acts on the receptors of the smooth muscle cell thus inducing a vasoconstriction effect as well as cellular proliferation and hypertrophy (Brozovich et al., 2016; Yaghini et al., 2010). ACE degrades bradykinin thus creating a vasoconstriction effect that leads to salt retention in the macula densa triggering a high blood pressure. AT-II acts as a radical responsible for several cardiovascular disorders and is important in the pathophysiology and the development of diseases and other disease risk factors. Inhibiting the activity of ACE, will increase bradykinin activity thus lowering blood pressure and preventing generation of AT-II. Inhibition of renin is also important in the management of hypertension as this will prevent the synthesis of AT-I to limit AT-II production (Segall et al., 2007; Tikellis & Thomas, 2012). To manage hypertension and protect other organs of the body from damage, it is therefore important to inhibit the activity of the RAS system.

2.3.2.2.1 Antihypertensive effect of plants and their derived compounds

Wide range of phytochemicals exhibit antioxidant and antihypertensive properties, and total biological effect of plant extracts is usually attributed to the additive and synergistic effects of the complex mixture of phytochemicals present in plants. Several ACE-inhibiting compounds have been reported from an enormous list of plants. The ACE-inhibitory activity has been reported for some plants such as *Ocimum gratissimum, Mesona procumbens, Hordeum vulgare,* Korean red pine, and quercetin to mention a few. The inhibitory concentration that reduced ACE activity by 50% (IC_{50}) was reported to be 56.3 µg/mL for *Ocimum gratissimum* and the oral

administration of the extract to spontaneously hypertensive rats (SHRs) shows that the systolic blood pressure (SBP), ACE levels in plasma and lung, and plasma endothelin-1 reduced significantly (Kim et al., 2020; Ra et al., 2020; Shaw et al., 2017; Yeh et al., 2009). Phytochemicals derived from plants such as quercetin was able to induce a significant reduction in systolic (–18%), diastolic (–23%) and mean (–21%) arterial blood pressure and heart rate (–12%) in SHRs at a single oral daily dose of 10 mg/kg for five weeks (Perez-Vizcaino et al., 2009). Studies also showed that *Hibiscus Sabdariffa* lowers SBP and diastolic blood pressure in salt induced hypertensive and in the normotensive rats (Verma et al., 2021). Ra et al (2020) also reported that all the compounds isolated from *Hordeum vulgare* (barley seed) such as feruloylquinic acid, lutonarin, saponarin, isoorientin, orientin, isovitexin, isoorientin-7-O-[6-sinapoyl]-glucoside, isoorientin-7-O-[6-feruloyl]-glucoside, isovitexin-7-O-[6-sinapoyl]-glucoside, and isovitexin-7-O-[6-feruloyl]-glucoside exhibited excellent ACE inhibition activities, with the IC_{50} values ranging between 7.3 and 43.8 µM.

The mechanism of ACE inhibition by some plant extracts has been studied using both extract and isolated compounds. Using the Lineweaver-Burk plots for inhibition kinetics studies, most ACE inhibitors from plants and their isolated compounds exerted their activities by non-competitive mode of inhibition (Ra et al., 2020), indicating that the extracts and compounds have affinity for both the free ACE and the ACE–substrate complex. Moreover, some plant polyphenolic extracts (*Vernonia amygdalina* and *Gongronema latifolium*) have also exhibited mixed type of inhibition and uncompetitive modes of inhibition for ACE and renin (Ajibola et al., 2011). A competitive mode of inhibition was reported for *Punica granatum*, Quercetin-3-O-glucoside, epicatechin and quercetin-3-O-glucuronic acid (Balasuriya & Rupasinghe, 2012;

Khan & Kumar, 2018). These antihypertensive effects may be due to the antioxidant capacity of these plant extracts and various polyphenolics compounds isolated from them.

2.3.2.3 Diabetes and carbohydrate digesting enzymes

Diabetes mellitus (DM) is a metabolic syndrome often characterized by hyperglycemia and hyperlipidemia. It is a chronic debilitating disease due to dysfunction in the metabolism of lipid, carbohydrate, and proteins. It is attributed to abnormal secretion of insulin by the pancreatic beta cells or insulin resistance and due to high glucose production in the liver. Insulin is very important in the breakdown of sugars to metabolizable product in the body and the inability to perform this function will result in the accumulation of sugar in blood with excess by-products in the liver. Insulin function is impaired when the beta cells in the pancreas do not produce enough insulin or when the body is resistant to the insulin produced thus causing long-term damages and failure of various body organs (Buowari, 2013; Folorunso & Oguntibeju, 2013; Krzymien & Ladyzynski, 2019; Salehi et al., 2019; Zaccardi et al., 2016). DM occurs in two forms: type 1 and type 2 DM. Type 1 DM is insulin dependent due to the damage of the islet beta cell in the pancreas. This occurs when the insulin-secreting β-cells are destroyed due to genetic or autoimmune disorder of the pancreas. It occurs mostly in children at their early years and affect adults aged 30-40 years. It occurs in about 0.5 percent of the general population, with an unprecedented annual increase of 3%, raising serious concerns among healthcare professionals around the world. This can be controlled by daily administration of insulin to normalise the level of glucose within the blood stream (Farooq et al., 2019; Krzymien & Ladyzynski, 2019; Sharif et al., 2018; Stamatouli et al., 2018; Zaccardi et al., 2016). Type 2 DM (T2DM) is not insulin dependent and this results from the inability of the body to efficiently utilize the insulin produced. Between the two categories, type 2 diabetes is the most common

accounting for more than 90% of diabetes cases due to excess glucose in the blood and physical inactivity. It a disease of public health concern and its global prevalence has been increasing due to urbanization and lifestyle changes. T2DM is a risk factor for other diseases such as hypertension, stroke, cancer, heart failure, and CVD (Lehrke & Marx, 2017; Salehi et al., 2019).

Obesity, a condition characterized by the accumulation of fat in the body has been found to be associated with T2DM. Obesity or overweight is a major contributing factor in the development of DM because it releases pro-inflammatory chemicals by the fat cells that makes the body less sensitive to the activity of insulin. This makes insulin-sensitive cells and tissues to be less responsive thus increasing insulin resistance, which is the major hallmark of T2DM (Al-Goblan et al., 2014; Siddiqui, 2018; Zheng et al., 2018). Obesity and T2DM are interrelated because they both have common risk factors such as genetics, family history, physical inactivity, environmental conditions, and dietary intake in their development (Butland et al., 2007; Khawandanah, 2019; Kolb & Martin, 2017; Lau et al., 2007; Zheng et al., 2018). According to WHO statistics, about 13% of the world's population majorly adults were obese in 2014 while the prevalence of diabetes in adults aged 18 yrs and above was estimated to be 8.5% with 1.6 million deaths (WHO, 2016; 2017b).

In Canada, about 5.3 million adults were reported to be obese in 2014 and about 2.1 million Canadians have diabetes (Statistics Canada, 2017). The global diabetes prevalence in 2019 is estimated to be 9.3% (463 million people), rising to 10.2% (578 million) by 2030 and 10.9% (700 million) by 2045 (Saeedi et al., 2019). T2DM is a disease known only in the developed countries that affects only adults but is now prevalent in developing countries affecting both adolescents and children (Folorunso & Oguntibeju, 2013). The prevalence of diabetes is increasing daily more in developing countries than in developed countries. According

to World Health Organisation (WHO), it is projected that DM will be the seventh leading cause of death in 2030 with increasing number accounting for more than 82 million people in developing countries (Kakkar, 2016; Levitt, 2008; Narayan et al., 2004). The management strategies for obesity and diabetes involve having good dietary intake, maintaining normal body weight and regular physical activities (WHO, 2016; WHO, 2017a). It has been established that a 5-10% reduction in weight is beneficial to the health of obese individuals and increase insulin sensitivity in diabetic patients, which is critical in the management of these diseases (Gaal & Scheen, 2015; Kelly et al., 2020; Khawandanah, 2019; Wing et al., 2011). Based on different strategies used in the management of diabetes and obesity, lifestyle modification remains the most effective because it aims at reducing energy intake and increasing energy expenditure (Khawandanah, 2019). The main approach to the reduction of energy intake is by inhibiting digestive enzymes thus reducing the amount of glucose that can be absorbed in the body (Lacroix & Li-Chan, 2013).

α-amylase and α-glucosidase are carbohydrate-hydrolyzing enzymes involved in the breakdown of polysaccharides into smaller units that can be absorbed into the blood stream. The human diet relies mainly on starches as a source of glucose for energy metabolism. Starches are hydrolyzed into glucose through the actions of carbohydrate digesting enzymes. α-amylase (salivary and pancreatic α-amylase) are enzymes that breakdown complex carbohydrates into oligosaccharides and disaccharides by cleaving α-1,4 glycosidic bonds of amylose and amylopectin and the resultant product is hydrolysed into glucose by the brush-border enzyme α-glucosidase into glucose, which is then absorbed into the blood stream (Tysoe et al., 2016b; Visvanathan et al., 2020). Inhibition of α-glucosidase is very critical in the management of diabetes because it is the key enzyme responsible to produce glucose, which is the end-product

of carbohydrate digestion (Uraipong and Zhao et al., 2016; Ercan and El, 2016). Other key enzymes important in the digestion and absorption of nutrients in the body include pancreatic lipase, pepsin, chymotrypsin, and trypsin. These enzymes are vital in the digestion of fat and proteins but increase in their catalytic activity may contribute to the development of some symptoms of disease conditions. Pancreatic lipase is the enzyme involved in the breakdown of lipids into simple units (monoglycerides and free fatty acids) that can be absorbed into the body. Inhibition of pancreatic lipase is very important because this leads to reductions in the breakdown of triglycerides and the absorption of fat in the small intestine thus initiating long-term losses in body weight. This approach is a key factor in the management of obesity and in the development of anti-obesity drugs (Siow et al., 2016). Dietary proteins are partially broken down in the stomach into polypeptides, small oligopeptides, and free amino acids through the activity of pepsin. The product of pepsin digestion is further hydrolysed in the duodenum by trypsin and chymotrypsin into smaller oligopeptides and free amino acids, which can then be absorbed by the body (Gurina & Mohiuddin, 2021).

2.3.2.3.1 Antidiabetic effect of plant and their derived compounds

Different antidiabetic drugs such as biguanides, sulfonylureas, meglitinides, thiazolidinediones, dipeptidyl peptidase IV (DPP-4) inhibitors, and α-glucosidase inhibitors (acarbose and miglitol) exert their biological activity by reducing blood glucose, increasing the release of insulin from the beta cells and inhibit enzymes involved in the breakdown of carbohydrates in the body (DiNicolantonio et al., 2015; Ganesan et al., 2021; Meneses et al., 2015). Although all these synthetic drugs could control the progression of DM, complications such as toxicity and drug resistance persist with some negative side effects such as dizziness, nausea, vomiting, drowsiness, heartburn, and frequent urination (Chaudhury et al., 2017;

Meneses et al., 2015). Other problems such as loss of effectiveness after a period pose some challenges to the continuous use of these synthetic drugs. Therefore, there is need to discover alternative compounds from natural sources with no toxicity effect and that are well tolerated within the body.

Natural products of plant origin are promising lead in new drug development due to ease of accessibility, low cost, safety, and less side effects (Sharifi-Rad et al., 2018; Salehi et al., 2018). More than 800 plant materials such as slender amaranth leaves and seeds, rosemary leaves, finger millet, ocimum, and citrus fruits (Ali et al., 2014; Choi et al., 2012; Cruz and Andrade-Cetto, 2015; Lans, 2006; Salehi et al., 2019) possess hypoglycemic and anti-obesity activities. Plants have rich contents of bioactive compounds, which are natural and have desirable biological activity with most drugs been developed from them either directly or indirectly. The major therapeutic effect of plants on the treatment of diabetes have been attributed to their ability to increase insulin secretion or inhibit the absorption of glucose in the gastrointestinal tract (Arumugam et al., 2013). Studies have also reported that many plants have biological effects such as anti-inflammatory, antidiabetic, and antihyperglycemic properties as well as digestive enzyme inhibition (Salehi et al., 2019).

Several *in vitro* and *in vivo* studies have shown that dietary phenolic compounds have a variety of health-promoting properties. Studies have shown that phenolic compounds are effective at inhibiting alpha-amylase, alpha-glucosidase, and other enzymes. Different *in vivo* studies have shown the effectiveness of different plant extracts in inhibiting α-amylase and α-glucosidase due to their polyphenol content. Asante et al. (2016) reported that ethanolic extract of *Vernonia amygdalina* have antihyperglycemic effect and at a dose of 300 mg/kg, there was a significant reduction in low density lipoprotein (LDL), very low-density lipoprotein (VLDL) and

an increase in high density lipoprotein (HDL) in streptozotocin (STZ) induced diabetic rat for four weeks. It was also reported that the plant extract was able to regenerate the beta cells of the islets of Langerhans in treated rats. Airaodion et al. (2019) reported that the methanolic extract of *Telfairia occidentalis* leaves administered at 200, 400 and 600 mg/Kg B.W of extract showed significant decrease ($P<0.05$) in blood glucose level in alloxan-induced diabetic rats. Antidiabetic effects of other plants such as *Amaranthus viridis* (Uddin et al., 2016), *Solanum nigrum* (Umamageswari et al., 2017), *Telfairia occidentalis* (James et al., 2016; Okonkwo et al., 2018), *Vernonia amygdalina* (Yazid et al., 2020), *Calpurnia aurea* (Belayneh & Birru, 2018), *Terminalia brownii* (Alema et al., 2020), and *Sedum adenotrichum* (Naz et al., 2019) have also been established. The antidiabetic effect of these plant extracts is based on their ability to enhance insulin activity, protect the pancreatic beta cells, and regulate carbohydrate metabolism. These natural dietary polyphenols are safe and cost-effective than their synthetic counterparts (Dias et al., 2017).

2.4 Polyphenols as Plants Secondary Metabolites

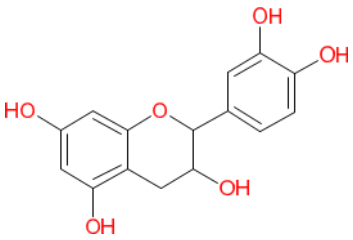
Polyphenols have gained a lot of significance in the research industry basically due to their antioxidant activities and their ability to prevent the progression or development of certain diseases such as diabetes, cancer and obesity and have gained a lot of attention in drug discovery ((Elnour & Abdelsalam, 2018; Khan et al., 2017). Polyphenols and their metabolites have been investigated extensively for a variety of biological functions, including antimicrobial, antiproliferative, antioxidant, and anti-inflammatory properties (Bernardi et al., 2020). They are the most common sources of antioxidants metabolized from phenylalanine and tyrosine (Shahidi, 2000) and due to their chemical structure, they can scavenge free radicals. Polyphenol is a word used to describe a group of plant constituents, which are named based on their chemical structure

and functional group. The word “poly” is derived from a Greek word ðïëýò which means many or much while phenol represent the chemical structure with hydroxyl (-OH) functional group found in alcohols attached to a phenyl ring hence the suffix “ol”. Polyphenols are defined according to White-Bate-Smith-Swain-Haslam as a group of compounds that are soluble in water with molecular weight ranging from 500-4000 Da and having 12-16 phenolic hydroxyl groups attached to 5-7 aromatic rings per 1000 Da (Cosme et al., 2020; Quideau et al., 2011). They are secondary metabolites present in plants and are the most abundant dietary bioactive compounds ((Tressera-Rimbau et al., 2017; Wang et al., 2020). Polyphenols have unique physical and chemical characteristics based on their molecular weights and structures and recently plant polyphenols have received great recognition not only among the scientific community but among the general public due to their presence in vegetables, fruits, beverages and derived foodstuffs whose daily consumption has been claimed to have beneficial health effect and to reduce some age-related degenerative diseases (Quideau et al., 2011). More than 100,000 polyphenols exist in different range of plant foods and their derivatives such as wines, coffee, grains, teas, fruits and vegetables and are responsible for taste, astringency and color of plants (Pandey & Rizvi, 2009) as well as a protective agent for plants against certain pathogens, radiation, drought, UV radiation, and extreme temperatures. They are ubiquitous in the plant kingdom with around 8000 different phenolic structures. They are present as both free form in plants and may be bound to sugars or proteins (Mojzer et al., 2016; Cosme et al., 2020; Goszcz et al., 2017). The number of polyphenols found in food vary differently among different classes and their bioavailability also varies based on the efficiency of absorption and the rate at which they are excreted (Manach et al., 2004). Some are present in all plants while others are specific to certain plant species. These variations in the number of polyphenols present in certain plant foods are based on some

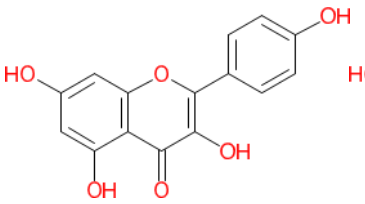
environmental factors, storage, and ripeness (Pandey & Rizvi, 2009). Polyphenols are broken down into four main groups based on their structure such as phenolic acids, flavonoids, stilbenes and lignans with different subclasses (Cosme et al., 2020).

2.4.1. Flavonoids

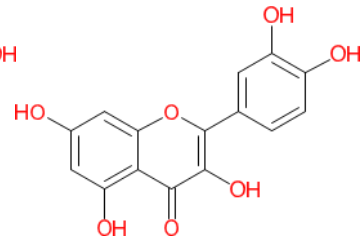
Flavonoids are the most common polyphenols usually abundant in foods, which together with carotenoids and chlorophylls give colour to many fruits and vegetables (Brglez Mojzer et al., 2016; Cosme et al., 2020). This group is further subdivided into six subclasses including flavonols, flavanols, flavanones, flavonones, flavones, isoflavones and anthocyanins. The differences between these subclasses are due to variations in the number and positions of the hydroxyl groups as well as in their range of alkylation and glycosylation. Flavonoids consist of a 15-carbon structure with two benzene rings connected by three carbon atoms and can occur as aglycones or conjugated to sugars or organic acids (Brglez Mojzer et al., 2016; Cosme et al., 2020; Durazzo et al., 2019). Flavones represent the main basic structure of most flavonoids with a ketone functional group. This basic structure can then be built upon or changed with the addition of phenyl group, hydroxyl group and double bonds to form other flavonoid structures. Among all the six subclasses of flavonoids, flavonols are the most common in foods such as onions, kale, tomatoes, red wine, tea, broccoli, and blueberries but their concentration can be reduced when exposed to sunlight. Their antioxidant and biological activities depend on the presence, number, and position of hydroxyl groups in the chemical structure of these compounds (Cosme et al., 2020). Examples include catechin, epicatechin, quercetin, kaempferol, daidzein and genistein.



Catechin



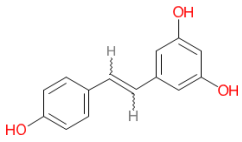
Kaempferol



Quercetin

2.4.2. Stilbenes

They are found less frequently in plants when compared to flavonoids and phenolic acids. The structure is basically made up of two benzene rings connected to an ethylene group on each side (Chong et al., 2009) and are mostly found in berries, peanuts, plum and wine (Błaszczyk et al., 2019; Chang et al., 2016; Hassan et al., 2018; Shrikanta et al., 2015). They occur either as trans or cis isomers thus giving rise to different reactivities and chemical properties. Consumption of stilbenes have been associated with a reduced risk of diabetes, obesity, and the onset of hypertension (Grosso et al., 2017; Miranda et al., 2016; Tresserra-Rimbau et al., 2014). Examples include resveratrol found in wines.

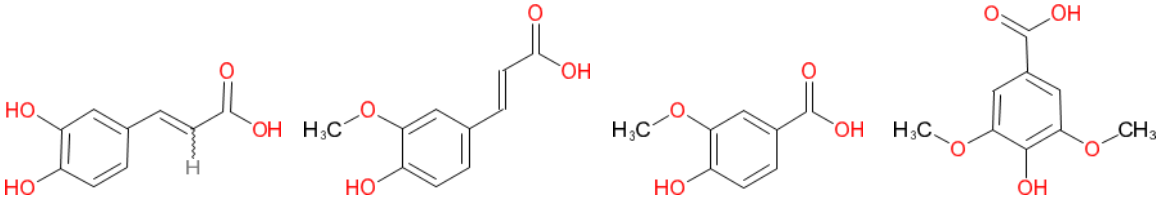


Resveratrol

2.4.3. Phenolic acids

Phenolic acids are the next most abundant polyphenols found in foods. They are non-flavonoid polyphenolic compounds consisting of two subclasses, which include hydroxycinnamic (C3-C6) acid and hydroxybenzoic acids (C1-C6). Hydroxycinnamic acids are cinnamic acid derivatives and are more common in foods than hydroxybenzoic acids, which are

benzoic acid derivative. Phenolic acids are mostly found in grains, fruits, vegetables, and seeds. Most of these phenolic acids are found in a free form (aglycones) in fruits and vegetables and in bound forms in the hulls, seeds, and brans of grains (Abbas et al., 2017; Durazzo et al., 2019; Khoddami et al., 2013) and can only be available (free) by acid, alkali, or enzyme hydrolysis. Caffeic acid is the most common phenolic acid found in fruits. Other examples include ferulic acid found in wheat sinapinic acid, vanillic acid, syringic and p-coumaric acid (Cosme et al., 2020).

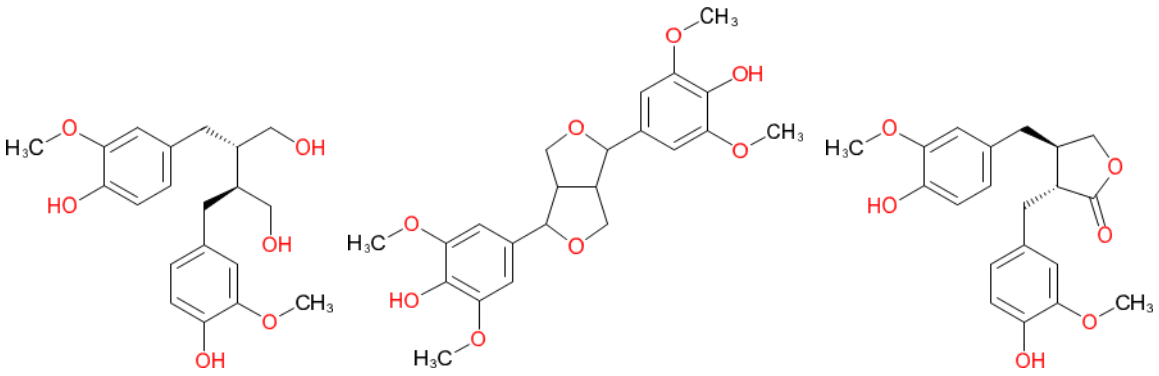


Caffeic acid Ferulic acid Vanillic acid Syringic acid

2.4.4. Lignans

The presence of lignans in foods are less common than the other polyphenol groups. Lignans are present in small amounts in various seeds, grains, fruits, and vegetables, but in higher amounts in sesame and flax seeds. As a result, lignan digestion and thus lignan bioavailability, is highly variable and depends on the form of diet consumed (Cosme et al., 2020; Durazzo et al., 2018; Rodríguez-García et al., 2019; Yeung et al., 2020). They are formed from two phenylpropane units joined by at least one carbon-carbon bond between the two central β -carbons of the C3 chains (Durazzo et al., 2018; Gnabre et al., 2015). Linseed (flaxseed) has the largest percentage of lignan called secoisolariciresinol with amount as high as 3.0 g/kg dry weight in addition to low quantities of matairesinol and syringaresinol. Other minor sources of lignans include algae, lentils, triticale, garlic, asparagus, carrots, pears, and prunes (Durazzo et al., 2018). Lignans have many biological activities, showing antiviral, anticancer, anti-

inflammatory, antimicrobial, antioxidant, immunosuppressive properties and hepatoprotective and osteoporosis prevention (Durazzo et al., 2018; Gnabre et al., 2015; Rodríguez-García et al., 2019).



Secoisolariciresinol Syringaresinol Matairesinol

2.5. Antioxidant Defense System of Plant Polyphenols

Antioxidants are essential to humans due to the benefit they proffer within the human body, which is based on the type, location, and the disease to be treated. They have different beneficial role in different cells and in some cases act as pro-oxidant causing more damage than cure. When antioxidants are ingested, they can be degraded, or their structure affected in the gastrointestinal tract thus affecting their effectiveness. This can affect their bioavailability and bio-accessibility (Pizzino et al., 2017; Rahal et al., 2014; Sharifi-Rad et al., 2020). The ability of an antioxidant compound to prevent oxidative reactions *in vitro* does not determine its ability to perform the same action *in vivo*. The endogenous antioxidant defense system is not enough to prevent oxidative reaction within the body, thus the need to supplement with exogenous antioxidants. Exogenous antioxidants can act as anti-inflammatory, anticancer and antiradical agents but their supplementation with endogenous antioxidants has posed more research problems due to their pro-oxidative capabilities (Rice-Evans and Diplock, 1993, Halliwell et al., 2005; Craft et al., 2012). Antioxidants have also been used in the food industry to extend product

shelf-life, but their efficacy depends on the food environmental conditions such as the pH of the food, solubility, and interactions with other constituents present in the food (Grgić et al., 2020; Hassoun et al., 2020; Lourenço et al., 2019).

Since it has been established that oxidative stress plays a major role in the etiology of human clinical conditions, different studies have found that the therapeutic approach to combat the effect of oxidative stress is by using antioxidant therapy (Liguori et al., 2018). Antioxidants are exogenous or endogenous molecules, which act to detoxify the activities of free radicals and its associated effects in the cellular system. They neutralize ROS/free radicals to inhibit oxidative stress. Natural foods such as plants are good sources of antioxidants such as flavonoids, other phenolic compounds, vitamin C and carotene. These antioxidants prevent protein oxidation, lipid peroxidation and production of free radicals thus acting as therapeutic agents against oxidative stress (Chen et al., 2012). An antioxidant is a molecule that is stable enough to donate an electron to a free radical to neutralize and make it stable. They can terminate chain reaction involved in cellular damage by interacting with free radicals. They act as hydrogen donors, singlet oxygen quencher, radical scavenger, and metal chelating agent.

The enzymatic antioxidants are the primary endogenous antioxidant defense mechanism in the human body that breakdown toxic and highly reactive oxidative species and intermediates into less active and more stable form. They are responsible for the inhibition of lipid peroxidation and maintain structure and function of cell membranes (Balasaheb Nimse & Pal, 2015; Hasanuzzaman et al., 2020; Kurutas, 2016; Santos-Sánchez et al., 2019). These enzymatic antioxidants include SOD, CAT, peroxidases (POD) and glutathione peroxidase (GPx). SOD is primarily found in the mitochondria and cytosol where it converts superoxide into hydrogen peroxide using cofactors such as manganese, copper, or zinc. CAT is an enzyme found in most

plant and animal cells especially in the peroxisome and it is responsible for the breakdown of hydrogen peroxide to water and molecular oxygen using either iron or manganese as cofactor while GPx enzymes reduces peroxides to water thus eliminating peroxides as potential substrates for Fenton reaction. All these enzymes work synergistically together to scavenge free radicals within the human body. The non-enzymatic antioxidant systems such as reduced glutathione, ascorbic acid, Vitamin E, carotenoids, and flavonoids all occur in plant tissues and are also responsible for the alleviation of deleterious effects of ROS. Ascorbic acid is one of the most powerful ROS with the ability to donate electrons and can scavenge free radicals such as singlet oxygen, oxygen, and hydroxyl radicals. It also can regenerate tocopherol for membrane protection (Das & Roychoudhury, 2014; He et al., 2017; Huang et al., 2019; Mehta & Gowder, 2015; Racchi, 2013). Glutathione occurs in plants in reduced form in the cytosol, endoplasmic reticulum, mitochondria, chloroplasts, and peroxisomes (Racchi, 2013). It regulates several physiological processes such as signal transduction and detoxification of xenobiotics. It protects the cell against oxidative stress induced by the action of ROS. It scavenges free radicals such as hydrogen peroxide, oxygen, and hydroxyl radicals (Das & Roychoudhury, 2014; Huang et al., 2019). All these actions constitute antioxidant defense against free radicals and the resulting oxidative stress.

2.6 Extraction and Characterization of Plant Polyphenols

Plants especially those of medicinal value are important sources of phytochemicals with therapeutic value and can be used in the development of new drugs in the management of some diseases. Extraction of active compounds from plants is a critical step in their utilization for other food and drug formulations. These bioactive compounds can be extracted from fresh, frozen, or dried plant materials. Solvent extraction is the most used method for the extraction of active

compounds from plant materials and the type of solvent used will affect the overall yield. Plant materials contain various phenolic compounds such as polymerized substances (tannins), carbohydrates, sugars, phenolic acids, anthocyanins, and proteins; therefore, there is no universal method of extraction for all plant phenolics (Andres et al., 2020; Rajbhar et al., 2014; Zhang et al., 2018). Extraction is a very critical step in the isolation and characterization of phenolic compounds in plants due to their complex nature.

Different methods have been used in the isolation of phenolic compounds in plants and this ranges from liquid-liquid and solid-liquid extractions. Hydrophilic polyphenols can be extracted using water and other polar organic solvents such as ethanol, methanol, acetone, ethyl acetate and chloroform or mixture of these solvents with water at different ratios (Khoddami et al., 2013). The choice of solvent used for extraction will determine the rate at which the biologically active compounds present in the plant material can be extracted. In order to prevent environmental impact, reduce solvent consumption and protect consumers, methods such as supercritical fluid (SFE), microwave extraction, ultrasound-assisted extraction and pressurized fluid extraction are some of the green extraction concepts introduced (Andres et al., 2020; Brglez Mojzer et al., 2016; Chaves et al., 2020; Khoddami et al., 2013; Rajbhar et al., 2014) as other methods for polyphenol extraction. The SFE method is fast, uses small amounts of solvent and can be automated. The bioactive compounds extracted with SFE are less susceptible to degradation due to the absence of light and air during extraction. The most widely used critical fluid is the supercritical carbon dioxide (SC-CO2) due to its low toxicity, compatibility with processed foods and environmentally friendly. It is an inexpensive method that could be used for the extraction of natural products with a wide range of polarity (Brglez Mojzer et al., 2016; Chaves et al., 2020; Nahar & Sarker, 2012). Ultrasound assisted extraction (UAE) is a method

that work based on the implosion of cavitation bubbles when acoustic waves in kHz is propagated by a shear force. This technique of extraction is based on sound waves and frequencies that stimulate the breakdown of the cell wall thus allowing the release of active compounds present in the cell. The collapse of the bubbles can bring about physical, chemical, and mechanical effects that causes the breakdown of the cell wall of plant materials. This process allows the solvent to penetrate the biological membranes and cause the release of active compounds into the solvent. It is a relatively low-cost method used both for small- and large-scale bioactive compounds extraction processes in both food and pharmaceutical industry (Chaves et al., 2020; Khoddami et al., 2013; Um et al., 2018). It is fast and cause less degradation of plant phenolics. The microwave-assisted extraction (MAE) method uses the microwave energy to facilitate the separation of active components from the sample matrix into the solvent. This method reduces extraction time and the volume of solvent used (Chaves et al., 2020). For the identification and quantification of phenolic compounds and their metabolites in plant extracts, techniques such as high-performance liquid chromatography (HPLC), mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) are regularly used. HPLC is one of the techniques popularly used for the analysis of polyphenols based on its ability to analyze all compounds of interest and their derivatives (Cheynier, 2012; Khoddami et al., 2013; Kumar, 2017; López-Fernández et al., 2020).

2.7. Effect of Extraction Parameters on the Stability of Polyphenols

The extraction of phenolic compounds in plant material is influenced by their chemical nature, the sample size, extraction time and storage conditions as well as the presence of interfering substances (Ahmed et al., 2020). Different parameters such as extraction time, type of solvent, extraction method, solid-to-solvent ratio and extraction temperature affect the efficiency

of an extraction process (Ahmed et al., 2020; Andres et al., 2020). These parameters play a significant role in the extraction process, which can invariably affect the antioxidant activity of the extracted compound. Polarity of the solvent used will affect the type of flavonoid compounds that can be extracted while the solid-to-solvent ratio determine the quantity and antioxidant capabilities of the compounds extracted (Andres et al., 2020; Brglez Mojzer et al., 2016; Thouri et al., 2017). The extraction time, which is a factor of how long the extraction process occur is very important in minimizing cost and energy usage. All these will enhance the extraction process and thus provide information on how viable these parameters can favor industrial process (Tay et al., 2014).

2.7.1. Influence of extraction solvent

Solvents such as ethanol, acetone, ethyl acetate, methanol, and the combination of these solvents with each other or with water have been used for the extraction of phenolics from plant materials. Solvents such as methanol and aqueous acetone have been used to extract lower and higher molecular weight polyphenols, respectively (Andres et al., 2020; Cvjetko Bubalo et al., 2018). The choice of a solvent to effectively extract biologically active compounds will depend on the following properties such as low toxicity, preservation action, and inability to form complex with extract or dissolve it. Other factors for solvent selection depend on the quantity of plant metabolite to be extracted, the extraction rate, its potential health hazard, ability to extract different types of plant metabolites, the intended end use of the extract and its ability in not interfering with bioassay process (Andres et al., 2020; Guido & Moreira, 2017; Zhang et al., 2018). It has been discovered that the rate of extraction can be influenced by temperature at which the extraction process occurs. Phenolic extracts of plants are always a mixture of different classes of phenols, which are selectively soluble in the solvents. The use of "green extraction

processes" and "green solvents" has recently gotten a lot of attention. In this case, water is the "cleanest solvent." Water is readily available, cheap, easily treated, non-toxic, and non-flammable, and its use as a solvent can satisfy both environmental and economic requirements (Andres et al., 2020). Water is also a polar solvent that can be used to dissolve polar compounds such as phenols (Cvjetko Bubalo et al., 2018). Solvent extraction is frequently used to extract phenolic compounds from their plant sources due to their ease of use, efficiency, and wide applicability (Rajbhar et al., 2014).

The use of water as extraction solvent at high temperature can allow other compounds such as proteins and polysaccharides to be extracted when compared with other solvents. Therefore, the use of organic solvent and water mixtures could be a better option of extracting solvent in terms of yield and extract purity. The ratio of organic solvent to water and solid also affects the extraction yield. Due to the volatility of organic solvents, using them at high temperature can result in evaporation thus changing the organic solvent to water ratio (Zhang et al., 2018). Based on the work of Wissam et al. (2012) on the influence of solvent on the extraction of proanthocyanidins and total polyphenol, it was discovered that the water extracts had the highest polyphenols and proanthocyanidin yield followed by 50% ethanol and no significant difference between the 75% and 25% ethanol extracts. Oxygen is one of the most important compounds responsible for the degradation of polyphenols. The lower values reported for the ethanol-water mixture solvents could be based on the ability of oxygen to dissolve more easily in ethanol than in water (Wissam et al., 2012). According to Thouri et al. (2017), extraction solvent significantly affected the phytochemical content (total polyphenols, flavonoids, and condensed tannins), antioxidant activity, and the inhibition capacity of date seeds extract. It was found that the phenolic, flavonoid, and condensed tannins content of water

extracts of date seeds was higher than that of acetones and methanol extracts. Water is the most suitable solvent for polyphenol extraction for use in food and pharmaceutical industries since there is no toxic effect and gives the highest yield. Water at higher temperatures and pressures, may be an efficient green extractant due to its relatively high H-bonding donor and accepting capacity (Li & Guo, 2016; Nandasiri et al., 2019).

2.7.2. Influence of extraction temperature

Different studies have shown the effects of extraction temperature on the stability of polyphenols with some having positive effect and others having a negative effect. Based on findings, extraction of polyphenols at 50^{o}C or above significantly increases the amount concentration of important metabolites such as catechin and epicatechin in the extracts of grape seed. High extraction temperature can also breakdown the plant tissues and affect the interactions between phenol, protein and polysaccharides resulting in more polyphenols migrating to the solvent (Bucić-Kojić et al., 2009; Jiménez-Moreno et al., 2019). High extraction temperature enhances the efficiency of the extraction process by breaking down the cell wall of the compounds to be extracted thus making them accessible for the solvent to permeate. Increase in extraction temperature also increases the solubility and the rate of diffusion of the compounds of interest thereby decreasing the viscosity of the solvent and allowing the solvent to pass through the test compound and increasing the number of polyphenols to be extracted (Andres et al., 2020; Kankara et al., 2014). Different studies have shown that, extraction temperature higher than 60^{o}C reduces the phenolic content of the test compound and causes their degradation (Dent et al., 2013; Wissam et al., 2012). Due to the susceptibility of phenolic compounds to oxidation, the use of temperatures above 70^{o}C and longer extraction time is discouraged because beyond this temperature, degradation of the phenolic compounds could occur thus affecting the final yield of

extract and loss of antioxidant properties (Brglez Mojzer et al., 2016). However, high temperatures are not always appropriate for extracting all types of phenolic compounds since less soluble antioxidants extracted at low temperatures decompose before thermo stable antioxidants are extracted, potentially resulting in antioxidant activity loss. Also, increased solvent losses at high temperatures were also reported (Dent et al., 2013; Kankara et al., 2014). Therefore, only phenolic compounds that can withstand higher extraction temperatures should extracted at higher temperatures.

2.7.3. Influence of extraction time

Extraction time is very important in enhancing the yield of polyphenols and their antioxidant abilities. Extraction of polyphenols at a longer time can decrease the total number of phenolics that can be extracted because some of the phenolics might be lost due to exposure to higher temperature and oxidation thus, they polymerize into insoluble compounds (Dent et al., 2013). It should also be noted that different bioactive compound with diverse structures require different time of extraction (Kankara et al., 2014). Based on the findings of Shi et al. (2003) for the extraction of grape seeds, longer period of extraction time produces little effect on the phenolic yield. At a third stage of extraction, the yield of phenolics obtained was between 2.5-5% when compared to the first two stages of extraction where about 94-98% yield was obtained. Therefore, for proper efficiency and to lower the cost of solvent removal, a two-stage extraction process is recommended (Shi et al., 2003). Bajkacz et al. (2018) observed that using more than one extraction cycles improve the extraction efficiency of polyphenol compounds from plant materials when compared to one extraction cycle with the same solvent. Prolonged extraction can destroy phenolic compounds and reduce the yield due to enzymatic degradation because the enzymes present in the plants to be extracted could breakdown phenolic compounds (Bajkacz et

al., 2018; Che Sulaiman et al., 2017). Increased extraction time potentially increases the loss of solvent by vaporization, it is therefore suggested that extraction time of three hour be employed (Dent et al., 2013)

2.7.4. Influence of solvent-to-solid ratio

This parameter determines how much solute that can be dissolved in the solvent thus affecting the number of polyphenolic compounds that can be extracted. Solid-to-solvent ratio affects the antioxidant capacity of extracts. Different studies have shown that increase in the solid-to-solvent ratio increases yield while some stated otherwise. In the study of Tay et al. (2014), it was found that increases in the solid-to-solvent ratio from 1:10 to ratio 1:20 improved the yield of *Aquilaria crassna* with an insignificant increase up to ratio 1:60. The little increases observed at higher ratios suggests that at ratio 1:20, the solvent becomes saturated, and an equilibrium occurs between the solvent and the solute thus making the solute less soluble in the solvent. Kankara et al. (2014) observed that a solid-to-solvent ratio of 1:10 appeared to be the best for extracting the antioxidant activity of *Guiera senegalensis* although there was no significant difference with that observed at ratio 1:25. This may be clarified by the fact that as the interaction between bioactive compounds coming into contact with the extracting solvent increases as a result of the strong extraction solvent, more phenolic components are leached out. According to the mass transfer principle, a high concentration gradient resulting from a high solid-solvent ratio increases diffusion rate, which facilitates greater solid-solvent extraction (Kankara et al., 2014).

2.8. Methods for the Analysis of Antioxidant Activity of Phenolic Compounds

Antioxidant assays can be based on their reaction mechanisms such as the ability to transfer hydrogen atom (HAT) or transfer of a single electron (SET); or the combination of both

mechanisms (Liang & Kitts, 2014; Siddeeg et al., 2021). Although the result of the two mechanisms is the same, the kinetics and reaction rate differs. The hydrogen atom transfer method is based on the ability of an antioxidant to quench free radicals by donating hydrogen atom to form a stable compound. It involves the use of a synthetic free radical generator, an antioxidant, and a molecular probe. The bond dissociation enthalpy (BDE) is a critical parameter in evaluating the antioxidant action in this process. The lower the BDE of the H-donating group in a possible antioxidant, the faster the free radical inactivation reaction would be. This method is not dependent on pH and solvent, and it require little time to quantify (Liang & Kitts, 2014).

The single electron transfer method is based on the ability of an antioxidant to transfer an electron to reduce a compound, which can either be a metal, carbonyl and radical to give a colour change. In this process, the antioxidant's ionisation potential (IP) is the most critical factor in determining the antioxidant's effectiveness. The lower the ionization potential, the easier is the electron abstraction (Liang & Kitts, 2014; Siddeeg et al., 2021). The degree of the colour change is correlated with the antioxidant activity of the sample measured. Unlike the HAT method, SET is both dependent on pH and solvent and requires long time for evaluation (Liang & Kitts, 2014; Siddeeg et al., 2021).

Some of the assays that utilize the HAT method include DPPH and oxygen radical absorbance capacity (ORAC) while the assays that utilize SET method include ferric reducing antioxidant assay, (FRAP) 2,2`-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and total phenol content (TPC). Different *in vitro* chemical methods are used to quantify the antioxidant activity of phenolic extracts. Prominent among these methods are 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging, total antioxidant capacity (TAC), ferric reducing antioxidant capacity (FRAP), and superoxide radical scavenging activity (SRSA).

2.8.1. Ferric reducing antioxidant capacity (FRAP)

The FRAP is a colorimetric method based on SET whereby Fe (III)-TPTZ (a yellow ferric tripyridyltriazine complex) is reduced to iron II ferrous complex (a blue colour) (Apak et al., 2016; Liang & Kitts, 2014; Siddeeg et al., 2021). It is a simple, rapid, and inexpensive assay that requires acidic conditions to maintain iron solubility and it is measured spectrophotometrically at 593 nm (Apak et al., 2016). High absorption rate indicates a high reducing power of the phenolic compound and can be expressed as Fe2+ equivalent by using a standard compound (Santos-Sanchez et al., 2019).

2.8.2. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method

This is the most common method used for the evaluation of free radical scavenging activity of plant phenolic extracts. The reaction mechanism is based on both HAT and SET from a phenolic compound to the DPPH radical (Liang & Kitts, 2014; Siddeeg et al., 2021). DPPH is a stable form of free radicals that can accept an electron to become a stable diamagnetic molecule (Balasaheb Nimse & Pal, 2015). This is based on the concentration of the sample to be tested, reaction time and temperature (Liang & Kitts, 2014). DPPH radical, a purple-coloured compound interacts with phenolic compounds to form a yellow or colourless compound as the concentration of the antioxidant increases (Balasaheb Nimse & Pal, 2015; Santos-Sanchez et al., 2019; Miguel-Chavez, 2017) and this can be quantified spectrophotometrically at 517 nm (Vladimir-Knezevic et al., 2012). The antioxidant activity of the plant phenolic extracts can be measured based on the number of DPPH molecules that is reduced by one molecule of antioxidants or by the concentration needed to reduce 50% of DPPH (Vucic et al., 2013). One of the advantages of this method is that it is an easy, economic, and rapid method to evaluate the radical scavenging activity of non-enzymatic antioxidants (Siddeeg et al., 2021)

2.8.3. Total phenolic content (TPC)

This method is also called the Folin-Ciocalteu (FC) method. This method is based on single electron transfer mechanism, and it is used to quantify total phenolic contents of plant extracts using gallic acid as standard. It is an oxidation-reduction reaction that involves the reduction of molybdenum into phosphotungstic/phosphomolybdic complex in an alkaline medium with a Folin reagent to yield a colored product with a broad band having an absorbance maximum between 750 and 765 nm. Interaction of Folin reagents with phenolic compounds can be measured spectrophotometrically at 765 nm (Apak et al., 2016).

2.8.4. Metal chelating ability

The principle of this method relies on the ability of the sample to compete for ferrozine, which is a chemical that can bind to Fe^{2+} and produces a color change. The decrease in the color change measured at 562 nm indicates a Fe^{2+} chelating potential of the sample (Siddeeg et al., 2021).

2.8.5. Hydroxyl radical scavenging ability

The hydroxyl (HO•) radicals are the most damaging and highly reactive free radicals in the cells. It is produced through a Fenton reaction mediated by the interaction of H_2O_2 with Fe (II) (Liang & Kitts, 2014; Siddeeg et al., 2021). The presence of Fe (II)/H_2O_2 conjugate is not ideal for the scavenging assay ass some antioxidants can chelate Fe (II). The percentage of hydroxyl radical scavenging activity of test sample is determined in comparison with a negative control. The limitation of this assay is that since most samples can chelate transition metal ions, such as Fe^{2+}, this can interfere with the Fenton reaction, which normally generates the hydroxyl radical. Therefore, it is difficult to characterize the antioxidant activity because it cannot be

established whether antioxidant activity of the sample is scavenging the hydroxyl radical directly or acting indirectly by chelating Fe^{2+} (Liang & Kitts, 2014; Siddeeg et al., 2021).

Other methods include oxygen radical absorbance capacity (ORAC), Trolox equivalent antioxidant capacity (TEAC), 2,2'- azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid (ABTS), total radical-trapping antioxidant assay (TRAP), ferric thiocyanate assay, thiobarbituric acid method (TBARS) and superoxide radical scavenging activity (Liang & Kitts, 2014; Siddeeg et al., 2021). Although these antioxidant assays are critical in evaluating the ability of a compound to act as an antioxidant, it is important to note that no one antioxidant assay will reflect the total antioxidant activity of a sample. Therefore, it is not possible to use only one assay to evaluate the antioxidant activity of a sample, thus the need to use different assays.

2.9 References

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CHAPTER THREE

MANUSCRIPT ONE

INFLUENCE OF NITROGEN FERTILIZER MICRO-DOSING ON PHENOLIC CONTENT, ANTIOXIDANT AND ANTICHOLINESTERASE PROPERTIES OF AQUEOUS EXTRACTS OF THREE TROPICAL VEGETABLES

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Abstract

This work investigated the effect of fertilizer micro-dosing on *in vitro* antioxidant and anti-acetylcholinesterase (AChE) properties of aqueous extracts of leaves of three vegetables (*Solanum macrocarpon* L., *Amaranthus viridis* L., and *Telfairia occidentalis* f. Hooke). Urea was combined (0, 20, 40 and 60 kg/ha) with cow manure (5 t/ha) or without cow manure (80 kg/ha) to grow the leafy vegetables. Significantly higher ($p < 0.05$) polyphenol extract yield was obtained with 60 kg N/ha when compared to other fertilizer doses. Total polyphenol (510.70-521.50 mg gallic acid equivalent/g) and total flavonoid (609.51-742.50 µg rutin equivalent/g) contents were reduced as fertilizer dose increased. 2,2 diphenyl-1 picrylhydrazyl radical scavenging and iron reducing properties were enhanced by organic manure while metal ion chelation and anti-AChE activity was highest at the 60 kg N/ha. We conclude that the combined use of organic manure with urea fertilizer led to enhanced antioxidant and anti-AChE activities of the leaf polyphenolic extracts.

Practical applications

The search for natural scavengers of free radicals as food preservatives and nutraceutical agents has intensified in the past decade because of increasing negative reactions to synthetic compounds by consumers. Vegetable leaf polyphenolic compounds used in this work showed free radical scavenging and other antioxidant properties that are comparable to those of butylated hydroxytoluene, a synthetic antioxidant agent. The polyphenol extracts also showed acetylcholinesterase (AChE)-inhibitory property that is similar to that of galanthamine, a drug used for the treatment of Alzheimer's disease. Since oxidative stress is also associated with the development of chronic diseases, the vegetable leaf extracts are potential agents that can be used both as effective food preservatives and bioactive agents against neurodegenerative diseases.

Keywords: vegetable leaves; polyphenols; antioxidant; acetylcholinesterase; free radical scavenging; nitrogen fertilizer

3.0 Introduction

The body produces reactive oxygen species such as hydroxyl, superoxide, peroxide and hydrogen peroxide for normal functioning and metabolism (Halliwell & Gutteridge, 1999). They are also responsible for normal gene expression and molecular signaling. However, excessive production of these free radicals can lead to oxidative stress and cell damage, which promotes the development of diseases such as cardiovascular disease (CVD), diabetes, cancer, Alzheimer's, and Parkinson's diseases (Aiyegoro & Okoh, 2010; Sen et al., 2010). Phenols as antioxidants have been found to be effective in the prevention of oxidative stress by scavenging free radicals and delaying the onset of free radical production (Hasnat et al., 2013). In the food processing industry, artificial antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene (BHT), propyl gallate, and tert-butyl hydroquinone have been used as antioxidant food additives but their prolonged use may be toxic (Hasnat et al., 2013; Wanasundara & Shahidi, 1998). Consumer health awareness has led to a high demand for natural antioxidants in contrast to synthetic antioxidants that could negatively affect human health (Sasidharan et al., 2011). Substituting these synthetic antioxidants with naturally occurring plant antioxidants could promote the production of safer food systems.

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that usually occurs among the elderly with symptoms that include memory loss and cognitive deficit (Otaegui-Arrazola, Amiano, Elbusto, Urdaneta, & Martinez-Lage, 2014). High oxidative stress in the brain can lead to neuron damage and contribute to AD pathogenesis (Resende et al., 2008; Zhou et al., 2014). However, AD development is also characterized by low acetylcholine (a neurotransmitter) concentration in the brain, which arises due to excessive activities of acetylcholinesterase (AChE). This is because AChE catalyzes rapid hydrolysis of acetylcholine

to choline in the brain (Singh et al., 2013). Some drugs such as galanthamine and rivastigmine are AChE inhibitors that have been used in AD treatment but their uses have been limited because they do not actually slow or reverse the disease progression (P. Williams & Howes, 2011). Research is now focused on developing naturally occurring compounds from plants as potential AChE inhibitors that could reduce AD progression by boosting acetylcholine concentration and preventing oxidative stress.

Amaranthus viridis L. leaves (*AV*), *Telfairia occidentalis* f. Hooke (*TO*) and *Solanum macrocarpon* L. (*SM*) leaves are popular leafy vegetables that are widely consumed in the West African region for various health benefits (Khandaker et al., 2008). Several studies have associated the antioxidant properties of vegetables with their ability to neutralize toxic free radicals, thereby reducing the risk of chronic diseases (Dasgupta & De, 2007). Several health benefits of *TO* such as its ability to treat diabetes and anemia, reduce fatigue, and prevent the production of free radicals have been reported (Aderibigbe, Lawal, & Oluwagbemi, 1999(W. Zheng & Wang, 2001) (Yang et al., 2002); Zheng & Wang, 2001). *AV* is rich in betalain identified as rutin, quercetin, amaranthin, amaricin and hydrohydroxycinnamates. Betalain in amaranth has been found to have anticancer, antiviral and antioxidant properties (Hussain et al., 2008). The hemolytic and anti-inflammatory properties of *SM* have also been reported in literature (Ng, Zainal Abidin, Shuib, & Israf Ali, 2015; Oboh, Ekperigin, & Kazeem, 2005)

Phenolic compounds are the most abundant naturally occurring antioxidants because of their presence in most plant products. They have multifunctional antioxidant properties because of their ability to scavenge free radicals, donate hydrogen, chelate metal ions, break radical chain reactions, and quench singlet oxygen (Hasnat et al., 2013). Plant polyphenols play a key role in the prevention of free radical-mediated lipid oxidation, nucleic acid degradation, DNA alteration

and damage of platelet functions. Consumption of vegetables has been associated with reduced incidence of degenerative diseases such as cancer, cardiovascular diseases, Alzheimer disease, diabetes, and hypercholesterolemia as well as improving the immune system, all which are attributed to their antioxidant activities (Sasidharan et al., 2011). Research has shown that synthetic antioxidants have limited use due to the possibility of causing adverse effects on human health, though they are very effective and stable (Nakatani, 1996; Pokorny, 2007). Therefore, recent research interests have been geared towards the use of naturally occurring plant antioxidants instead of the synthetic antioxidants. According to the World Health Organization (WHO), a large percentage of people in developing countries depend mainly on traditional medicine for their health care needs and natural products such as plant extracts provide a wide range of alternatives to drug use (Sasidharan et al., 2011).

Improved vegetable production is associated with fertilizer use as a means of enhancing vegetative growth and healthy development of the leaves. However, the traditional method of fertilizer application involves broad spreading (broadcast) over the soil without consideration for environmental consequences. Therefore, the Micro-Veg Project, funded by the Government of Canada is training vegetable farmers in West Africa on precision application of fertilizer at optimum level, termed “micro-dosing” to supply just the right amount of fertilizer needed by the plant to optimize leaf yields and reduce the potential run-off of excessive nitrogen into the underground water system. To the best of our knowledge, the effects of fertilizer micro-dosing on the potential bioactive properties of the vegetable leaf polyphenols remain unknown. Therefore, the aim of this work was to determine the *in vitro* antioxidant and anti-AChE activities of polyphenolic extracts from *AV*, *SM* and *TO* leaves that were cultivated under nitrogen fertilizer micro-dosing.

3.2 Materials and Methods

3.2.1 Materials

DPPH (2, 2 diphenyl-1 picrylhydrazyl radical), BHT, 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt (Ferrozine), TPTZ (2,4,6-tripyridyl-s-triazine), galanthamine, AChE (electric eel), Folin-Ciocalteu phenol reagent, gallic acid, catechin, myricetin, caffeic acid and rutin were purchased form Sigma Aldrich (Sigma Chemicals, St. Louis, MO, USA). All other reagents were of analytical grade and purchased from Fisher Scientific (Oakville, ON, Canada). *AV, TO* and *SM* were produced at the Micro-Veg Project experimental location, in Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria. The three vegetable species were produced with application of mineral fertilizer according to fertilizer micro-dosing technology using a randomized complete block design with five nitrogen (urea) fertilizer doses replicated four times as follows: 0, 20, 40, 60 and 80 kg of urea/ha. Basal incorporation of manure (cow manure) at 5 t/ha was done on experimental units (2 m x 3 m) that received urea doses at 20, 40 and 60 kg/ha. The 80 kg/ha contained only urea while 0 kg/ha contain only cow manure. Fertilizer was applied to each plot at planting (T1) or two weeks after seedling emergence (T2) to obtain the following samples: 0T1, 0T2, 20T1, 20T2, 40T1, 40T2, 60T1, 60T2, 80T1, and 80T2. Leaves were harvested 25 days after emergence, rinsed in potable water, destalked, dried in a hot air cabinet at 60 °C for 8 h, milled to fine powder using a Marlex Excella dry mill (Marlex Appliances PVT, Daman, India) and stored at -20°C.

3.2.2 Extraction of free polyphenolic compounds

Extraction of the free water soluble polyphenols was carried out according to the method of (Alu'datt et al., 2010) with slight modifications. Samples were extracted using distilled water at 1:20 ratio (leaf powder:water) at 60°C for 2 h in a 500 mL beaker under continuous stirring. The

extracts were allowed to cool to room temperature and centrifuged at 10,000 x g for 30 min. The supernatants were filtered through a cheese cloth and the process repeated to obtain a second supernatant. Both supernatants were pooled and concentrated under vacuum in a rotatory evaporator at 60°C. The concentrated extracts were freeze-dried and stored at -20°C. Due to low yield, I was unable to extract the bound polyphenols.

3.2.3 Total phenolic content (TPC)

The TPC of each extract was determined using the Folin-Ciocalteu method (J. Hoff & Singleton, 1977) with some modifications. A standard calibration curve was prepared using 25-350 µg/mL gallic acid concentration in 50% (v/v) methanol. The polyphenol extracts were also diluted with 50% methanol to a concentration range of 600-1400 µg/mL. A 0.25 mL aliquot of Folin-Ciocalteu reagent was added to 0.25 mL of gallic acid solution or the sample and then mixed. After standing in the dark at room temperature for 5 min, 0.5 mL of 20% sodium carbonate solution was added followed by 4 mL of double distilled water. The contents were mixed and incubated in the dark for 1 h. The intensity of the green colour was then measured at 725 nm using an Ultospec UV-visible spectrophotometer (GE Healthcare, Montreal, PQ, Canada). TPC was expressed as milligrams gallic acid equivalents (GAE) per gram of dry leaf powder (mg GAE/g).

3.2.4 Total flavonoid content (TFC)

TFC was determined colorimetrically according to the method described by (Nabavi et al., 2013a) with slight modifications. An aliquot of 30 µL extracts in methanol was sequentially mixed with 90 µL of methanol, 6 µL of 10% aluminum chloride, 6 µL of 1 M potassium acetate and 168 µL of double distilled water, followed by incubation in the dark at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm in a Synergy H4 multi-

mode microplate reader (Biotek Instruments, Winooski, Vermont, USA). TFC was calculated as rutin equivalent from a rutin calibration curve (0.05, 0.1, 0.125, 0.25, 0.5 and 1 µg/mL in methanol).

3.2.5 High-Performance liquid chromatography

The polyphenolic profile of each leaf extract was determined using a 5-micron C18 analytical (250 x 4.6 mm) reverse-phase HPLC column (Phenomenex Inc., Torrance, CA, USA) fitted on a Varian 920/940-LC system (Agilent Technologies, Santa Clara, CA, USA). Polyphenol standards (gallic acid, catechin, rutin, myricetin and caffeic acid) were dissolved in ethanol at 0.5 mg/mL while 10 mg/mL of the extracts were prepared using 1% (v/v) acetic acid. A 100 µL aliquot of each standard or sample was injected onto the column, which was heated to 37°C. An isocratic gradient was used with 1% acetic acid as elution buffer. The elution time of the peaks obtained from the standards were compared to those of the samples to identify specific polyphenolic compounds.

3.2.6 DPPH radical scavenging assay

The scavenging activity of vegetable leaf extracts against DPPH radical was determined using a previously described method (Aluko & Monu, 2003), which was modified for a 96-well clear flat-bottom plate. The leaf extracts were dissolved in 0.1 M sodium phosphate buffer, pH 7.0 containing 1% (w/v) Triton X-100 to give 0.0625, 0.125, 0.25, 0.5, and 0.625 mg phenolics/mL final assay concentrations. DPPH was dissolved in methanol to a final concentration of 100 µM. A 100 µL aliquot of the plant extract solution was mixed with 100 µL of the DPPH solution in the 96-well plate and incubated at room temperature in the dark for 30 min. The absorbance values of the blank and samples were measured at 517 nm using the Synergy H4 multi-mode microplate reader. The blank consisted of buffer only instead of the plant extract while BHT was

used as a positive control. The percentage DPPH radical scavenging activity of the extracts was determined using the following equation.

$$DPPH(\%) = \frac{Abs(blank) - Abs\ (samples)}{Abs\ (blank)} \times 100$$

Effective concentration that scavenged 50% of DPH radicals (EC_{50}, mg/mL) was calculated by non-linear regression from a plot of extract concentration versus percent DPPH scavenged.

3.2.7 Metal ion chelation

The metal ion chelating activity (MCA) was measured using a modified method described by (Z. Xie et al., 2008a). Briefly, a 1 mL solution of leaf extracts, BHT (final assay concentration of 0.125, 0.25, 0.5, and 1 mg phenolics/mL) or distilled water (blank) was added to 925 mL of water and 0.05 mL of 2 mM $FeCl_2$ in a reaction tube. After mixing, 25 µL of 5 mM Ferrozine solution was added and vortexed thoroughly. The mixture was then allowed to stand at room temperature for 10 min and an aliquot of 200 µL pipetted into a clear bottom 96-well plate. The absorbance of blank and samples was measured at 562 nm using the Synergy H4 multi-mode microplate reader. Percentage metal chelating effect was calculated using the following equation:

$$Metal\ chelating\ activity\ (\%) = \frac{Abs(blank) - Abs\ (samples)}{Abs\ (blank)} \times 100$$

Inhibitor concentration that chelated 50% of ferrous ion (IC_{50}, mg/mL) was calculated by non-linear regression from a plot of extract concentration versus percent MCA.

3.2.8 Ferric Reducing Antioxidant Power (FRAP)

FRAP activity was measured using a previously described protocol (Benzie & Strain, 1998), which was slightly modified as follows. The FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) prepared in 40 mM HCl and 20 mM $FeCl_3 \cdot 6H_2O$ in the ratio of 10:1:1. The leaf extracts (40 µL) were mixed with 200 µL of

working FRAP reagent in a 96-clear well microplate to give 0.0625-1.0 mg phenolics/mL final assay concentration followed by absorbance measurement at 593 nm in the Synergy H4 multi-mode microplate reader. A standard curve for $FeSO_4 \cdot 7H_2O$ was prepared using a concentration of 25-150 mM. The results were expressed in mM of Fe^{2+} reduced per gram of extract using the calibration curve of the $FeSO_4$ standard.

3.2.9 Total antioxidant capacity (TAC)

TAC of the leaf extracts was evaluated using the phosphomolybdenum method as previously described ((Prieto et al., 1999) with slight modifications. A 0.25 mL aqueous aliquot of the leaf extract was combined with 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) to give final concentrations in the 2.5-10 mg phenolics/mL range. The tubes containing the reaction solution were incubated at 95 °C for 90 min, allowed to cool to room temperature and absorbance measured at 695 nm using the Synergy H4 multi-mode microplate reader with distilled water as blank. A calibration curve was prepared using 2.0 - 4.5 mg/mL gallic acid and the absorbance of each sample was used to determine the gallic acid equivalent using the standard curve.

3.2.10 *In-vitro* acetylcholinesterase inhibition assay

The assay for AChE activity was conducted using Ellman's colorimetric method with acetylthiocholine iodide (ATCI) as substrate (Khan and Ghani, 2012). Briefly, 25 µL of 15 mM ATCI, 75 µL of 3 mM DTNB and 50 µL of 50 mM Tris HCl, pH 8.0 containing 2% bovine serum albumin (BSA) were mixed with 25 µL of each extract or galanthamine (10-50 µg/mL) in a microplate. The blank reaction contained all these reagents except the extract or galanthamine. This was followed by addition of 25 µL AChE (0.26 U/mL) dissolved in buffer containing 1% BSA and the reaction samples were incubated at room temperature for 30 min. The absorbance

was then measured in the Synergy H4 multi-mode microplate reader at 405 nm for 20 min at 37 °C. Percentage inhibition of AChE activity was calculated as follows.

AChE inhibition (%) = $\frac{Ab(B)-Ab(S)}{Ab(B)} \times 100$

Where Ab (B) is the absorbance of the blank (uninhibited reaction) and Ab (S) is the absorbance of the sample (inhibited reaction).

3.2.11 Statistical Analysis

Triplicate determinations were used to obtain mean values and standard deviations. For statistical analysis, 3-way analysis of variance (ANOVA) using a model that included vegetable variety (VV), fertilizer dose (FD) and fertilizer application time (FAT) as fixed variables was performed. Duncan's multiple-range test was used to determine the mean treatment differences and significant differences taken at ($p < 0.05$). IBM SPSS Statistical package (version 24) was used for all statistical analyses.

3.3 Results and Discussion

3.3.1 Extract yield, total phenolic content (TPC) and total flavonoid content (TFC)

Aqueous extraction was preferred because initial extraction with solvents or solvent/water mixtures produced insoluble extracts that did not dissolve properly in the aqueous media used for the antioxidant and AChE assays. Fertilizer dose (FD) and vegetable variety (VV) had effects ($p < 0.05$) on the polyphenol extract yield while there was no significant ($p > 0.05$) effect of the time of the fertilizer application (Table 3.1). The polyphenol yields of *TO* and *AV* (32.7 and 32.2) did not differ significantly but were significantly ($p < 0.05$) higher than that of the *SM* (30.1). Results showed that polyphenol extract yield was significantly higher at 60 kg N/ha compared to other fertilizer doses. The results suggest that the optimum fertilizer dose for optimum polyphenol yield is 60 kg urea N/ha thus implying that precision application of fertilizer may be

a useful technique to enhance accumulation of water-soluble compounds, but this effect was highly dependent on the type of vegetable. The polyphenolic extracts yield obtained for leaves from plants grown with fertilizer combination (20, 40 and 60 kg urea N/ha containing organic fertilizer) did not differ significantly ($p > 0.05$) when compared to the extract of leaves from plants raised with only the organic fertilizer (0 kg urea N/ha), except the 60T, which had the highest yields. Leaves harvested from plants grown with only urea fertilizer (80 kg urea N/ha) had the lowest polyphenol extract yield, which suggests that combined use of inorganic and organic fertilizers led to improved content of free leaf polyphenols. It is noteworthy that *TO* had significantly higher ($p < 0.05$) amounts of TPC and TFC when compared to *SM* and *AV*, especially with the TFC being double and TPC being ~27% higher in *TO*. Table 3.1 also shows that TPC was influenced by fertilizer dosing rates ($p < 0.05$) with significant decreases when compared to the control (0 kg N/ha). Total phenolics were significantly different between fertilizer treatments with decreases as urea fertilizer level increased to 60 kg N/ha. The results are in contrast to the work of Nguyen and Niemeyer (2008) who observed a higher phenolic content in basil leaves from plants grown under lower nitrogen fertilizer application. This contrast in data trend may be due to the differences in leaf type. However, the current results agree with the work of Onyango et al. (2012) who reported that the TPC tends to decrease with increasing nitrogen applications, and that the extent of this decrease depends on the level and source of nitrogen. The results are also similar to those reported for artichoke where nitrogen fertilizer application led to reduced TPC (Lombardo et al., 2017). Therefore, nitrogen fertilizer application did not promote polyphenol accumulation, which is consistent with the Carbon Nitrogen balance hypothesis which states that synthesis of carbon-based secondary metabolites

such as polyphenols is not promoted in the presence of high nitrogen availability (Chapin & Klein, 1983).

Table 3. 1. Results from 3-way ANOVA and Duncan's test of the effects of vegetable variety (VV), fertilizer dose (FD), and fertilizer application time (FAT) on antioxidant and anti-acetylcholinesterase properties of aqueous extracts of Telfairia occidentalis, (TO), Solanum macrocarpon (SM), and Amaranthus viridis (AV) leaves1

Parameters	**Source of variation (F values)**			**Mean intensity for VV[2]**			**Mean intensity for FD (kg urea N/ha)[2]**					**Mean intensity for FAT[2]**	
	VV	FD	FAT	SM	AV	TO	20	40	60	80	0	Before planting	After planting
DPPH	34.35*	21.5*	6.39*	0.161^{b}	0.173^{c}	0.156^{a}	0.157^{b}	0.173^{c}	0.162^{b}	0.173^{c}	0.150^{a}	0.161^{a}	0.166^{b}
				(0.002)	(0.002)	(0.002)	(0.002)	(0.002)	(0.002)	(0.002)	(0.002)	(0.001)	(0.001)
FRAP	2149*	45.5*	62.89*	0.571^{b}	0.527^{a}	0.851^{c}	0.659^{bc}	0.595^{b}	0.669^{cd}	0.647^{b}	0.680^{d}	0.633^{a}	0.667^{b}
				(0.004)	(0.004)	(0.004)	(0.005)	(0.005)	(0.005)	(0.005)	(0.005)	(0.003)	(0.003)
MCA	1704*	274.1*	4.15*	0.55^{c}	0.19^{a}	0.46^{b}	0.55^{e}	0.34^{b}	0.29^{a}	0.39^{c}	0.41^{d}	0.40^{b}	0.39^{a}
				(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.00)	(0.00)
TAC	1807*	203.2*	2.06	16.29^{a}	36.27^{c}	32.29^{b}	30.25^{d}	28.40^{c}	34.53^{e}	22.37^{a}	25.86^{b}	28.07^{a}	28.49^{a}
				(0.249)	(0.249)	(0.249)	(0.321)	(0.321)	(0.321)	(0.321)	(0.321)	(0.203)	(0.203)
TFC	3083*	29.37*	0.93	354.16^{a}	506.25^{b}	1137.71^{c}	742.50^{c}	625.14^{a}	680.35^{b}	609.51^{a}	672.71^{b}	670.21^{a}	661.88^{a}
				(7.48)	(7.48)	(7.48)	(9.66)	(9.66)	(9.66)	(9.66)	(9.66)	(6.11)	(6.11)
TPC	5810.35*	24.19*	13.12	501.88^{b}	463.15^{a}	585.16^{c}	518.37^{b}	512.31^{a}	510.21^{a}	521.50^{b}	521.26^{c}	515.02^{a}	518.44^{a}
				(0.82)	(0.82)	(0.82)	(1.06)	(1.06)	(1.06)	(1.06)	(1.06)	(0.69)	(0.69)
AChE	190.35*	14.26*	9.43*	13.88^{b}	12.90^{a}	19.52^{c}	15.65^{b}	15.13^{b}	17.23^{c}	13.7^{a}	15.46^{b}	14.97^{a}	15.89^{b}
				(0.259)	(0.259)	(0.259)	(0.334)	(0.334)	(0.334)	(0.334)	(0.334)	(0.211)	(0.211)
Yield	37.90*	19.21*	8.64	30.10^{a}	32.73^{b}	32.44^{b}	31.43^{b}	31.89^{b}	33.86^{c}	30.22^{a}	31.38^{b}	31.38^{a}	32.15^{a}
				(0.235)	(0.235)	(0.235)	(0.303)	(0.303)	(0.303)	(0.303)	(0.303)	(0.192)	(0.192)

1 DPPH (2, 2 diphenyl-1 picrylhydrazyl radical); FRAP (Ferric reducing antioxidant power); MCA (metal ion chelating ability); TAC (total antioxidant capacity); TFC (total flavonoid content); TPC (total phenolic content); AChE (acetylcholinesterase activity inhibition); Yield (gross yield of aqueous extracts). Mean intensity values (standard error of the mean) with different letters in the same row (parameter) are significantly different ($p < 0.05$).

2Units: DPPH (Effective concentration that scavenged 50%, EC50 mg/mL); FRAP (mmol Fe2+); MCA (Concentration that chelated 50%, IC50 mg/mL); TAC (mg/g gallic acid); TFC (µg/g rutin equivalent); TPC (mg gallic acid equivalent/g); AChE (%); Yield (%).

*significant at $p < 0.05$.

Figure 3.1 shows that the fertilizer treatments resulted in only minor variations in TPC content of the *SM* extracts whereas wider variations were observed for the *AV* and *TO* extracts. For example, at the 0 and 20 kg N/ha dose, TPC for *AV* extract increased when the fertilizer was applied after planting whereas the opposite effect was observed at 40 kg N/ha. There was no significant difference between 60 and 80 kg N/ha doses at both fertilizer application time. The TPC of *TO* also showed significantly ($p < 0.05$) higher value at 80 kg N/ha when urea was applied after planting. From the *TO* results, it was observed that fertilizer application two weeks after planting enhanced TPC in 20, 40 and 80 kg/ha samples while application of fertilizer during planting favored TPC production in 0 and 60 kg/ha. The results are similar to those reported for *Arabidopsis thaliana*, *Lycopersicon esculentum* and several fruit species (Stewart et al., 2001) where nitrogen fertilizer application produced varying effects on polyphenol accumulation depending on dose and time of application.

Flavonoids are considered the most abundant polyphenolic compounds with catechin, genistein, quercetin, epicatechin, luteolin (or epigenin), butein and naringenin as typical examples (Quideau et al., 2011). Therefore, flavonoid content is an important parameter to estimate polyphenol accumulation by plants in response to fertilizer treatments. Generally, variations in TFC content (µg rutin/mg) in response to fertilizer dose was less in *SM* extracts (320.63-495.63) when compared to *AV* (164.38-783.13) and *TO* (939.38-1376.88) extracts (Figure 3.2). The results suggest that in addition to chlorophylls, some of the polyphenolic compounds in the vegetable leaf extracts are probably flavonoids with some of them identified in Figure 3.3 The extracts had similar polyphenol profile, except the higher levels of myricetin in *AV* and *TO* when compared to *SM*. As shown in Table 3.1, TFC was influenced by vegetable

variety and dose but not fertilizer application time. Reductions in the TFC as the fertilizer dose increased (Table 3.1) could be attributed to the

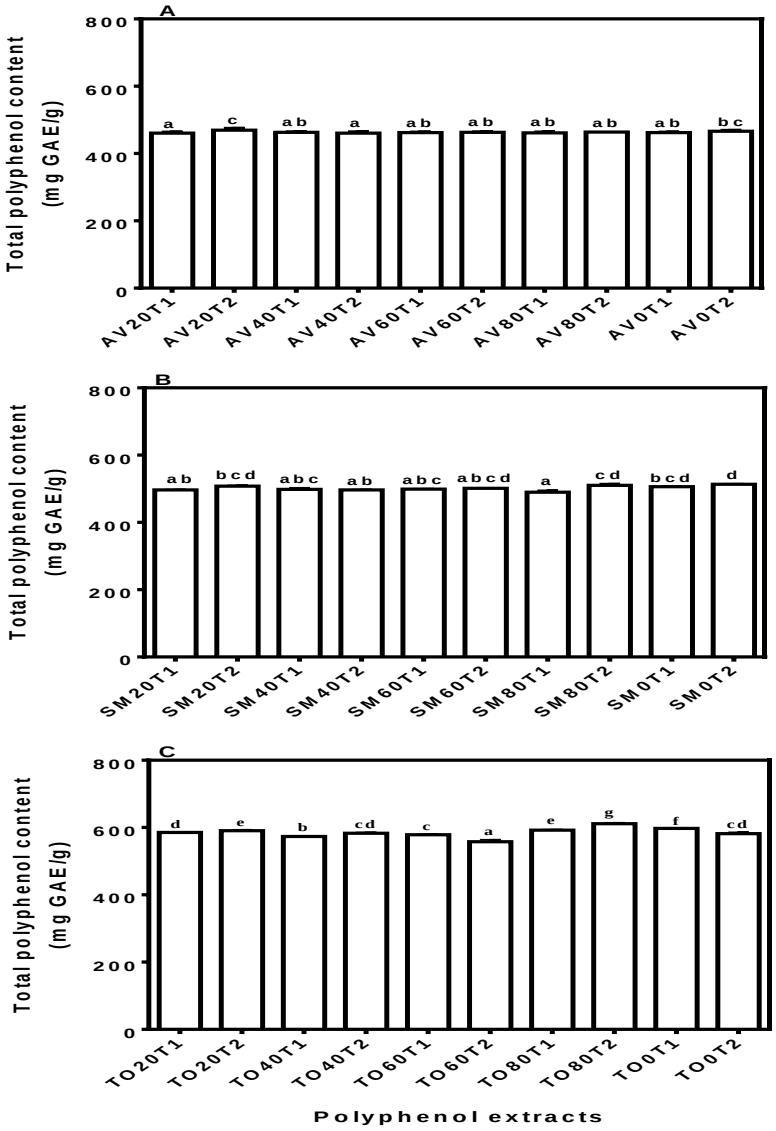

Figure 3. 1. Total polyphenol content (expressed as gallic acid equivalent, GAE) of aqueous extracts from the dried leaves of A—Amaranthus viridis leaves (AV), B—Solanum macrocarpon (SM), and C —Telfairia occidentalis (TO). Bars with different letters have significantly ($p<.05$) different mean values. Vegetables were grown under different nitrogen fertilizer doses (0, 20, 40, 60, and 80 kg/ ha), that were applied before (T1) or after (T2) planting

competition for phenylalanine which is either used to synthesize phenolic compounds or proteins (Jones & Hartley, 1999). As protein accumulates due to higher concentration of nitrogen, the level of phenolic compounds are reduced for a certain amount of phenylalanine (Stewart et al., 2001). The correlation between the nitrogen fertilization and the content of phenolic compounds in these vegetables can be explained by the study carried out by Ibrahim and Jaafar (2011). The study shows that at zero or 90 kg N/ha fertilization dose, phenolics and especially flavonoids accumulate more in leaves than in other plant parts when compared to a higher dose of 270 kg N/ha. This correlation can also be explained by the protein competition model hypothesis which predicts that at high dose of fertilization, there will be an increase in biomass (a consequence of vegetative growth). The increased biomass then leads to reduced synthesis of secondary metabolites such as the polyphenolic compounds (Jones & Hartley, 1999). TFC was highest (742.5) at a lower urea dose of 20 kg N/ha, followed by 60 kg N/ha (680.35), 0 kg N/ha (672.71) and 40 kg N/ha (625.14). The 80 kg N/ha (609.51) has the least value, which suggests that TFC was enhanced by the combined use of urea fertilizer treatment (20-60 kg N/ha) with organic fertilizer or application of organic fertilizer alone (0 kg N/ha) when compared to the urea fertilizer treatment (80 kg N/ha) alone. The study of Ibrahim et al. (2013) also showed that application of organic fertilizer influenced TFC of *Labisia pumila* by 22 % when compared to the inorganic fertilizer.

3.3.2 Total antioxidant capacity (TAC)

TAC reflects the synergistic interactions of compounds present in the vegetable leaf extracts and is an index that describes ability of the antioxidants to neutralize preformed free radicals (van Boekel et al., 2010). The TAC of the extracts increased dose-dependently for all the vegetable varieties, but *SM* extracts were generally the weakest while *AV* extracts were the strongest

(Figure 3. 4). TAC of *TO, SM and AV* polyphenolic extracts had maximum values at 60 kg N/ha using 10 mg/mL

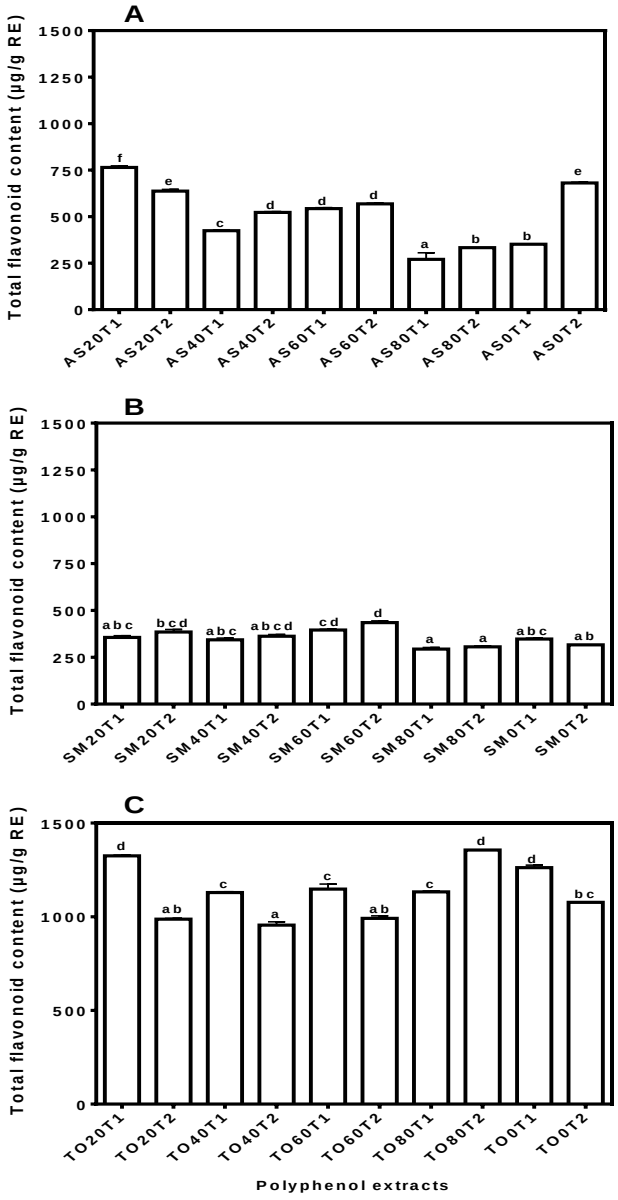

Figure 3. 2. Total flavonoid content (expressed as rutin equivalent, RE) of aqueous extracts from the dried leaves of A - *Amaranthus viridis* leaves (AV), B - *Solanum macrocarpon* (SM) and C - *Telfairia occidentalis* (TO). Bars with different letters have significantly ($p < 0.05$) different mean values. Vegetables were grown under different nitrogen fertilizer doses (0, 20, 40, 60, and 80 kg/ha), that were applied before (T1) or after (T2) planting.

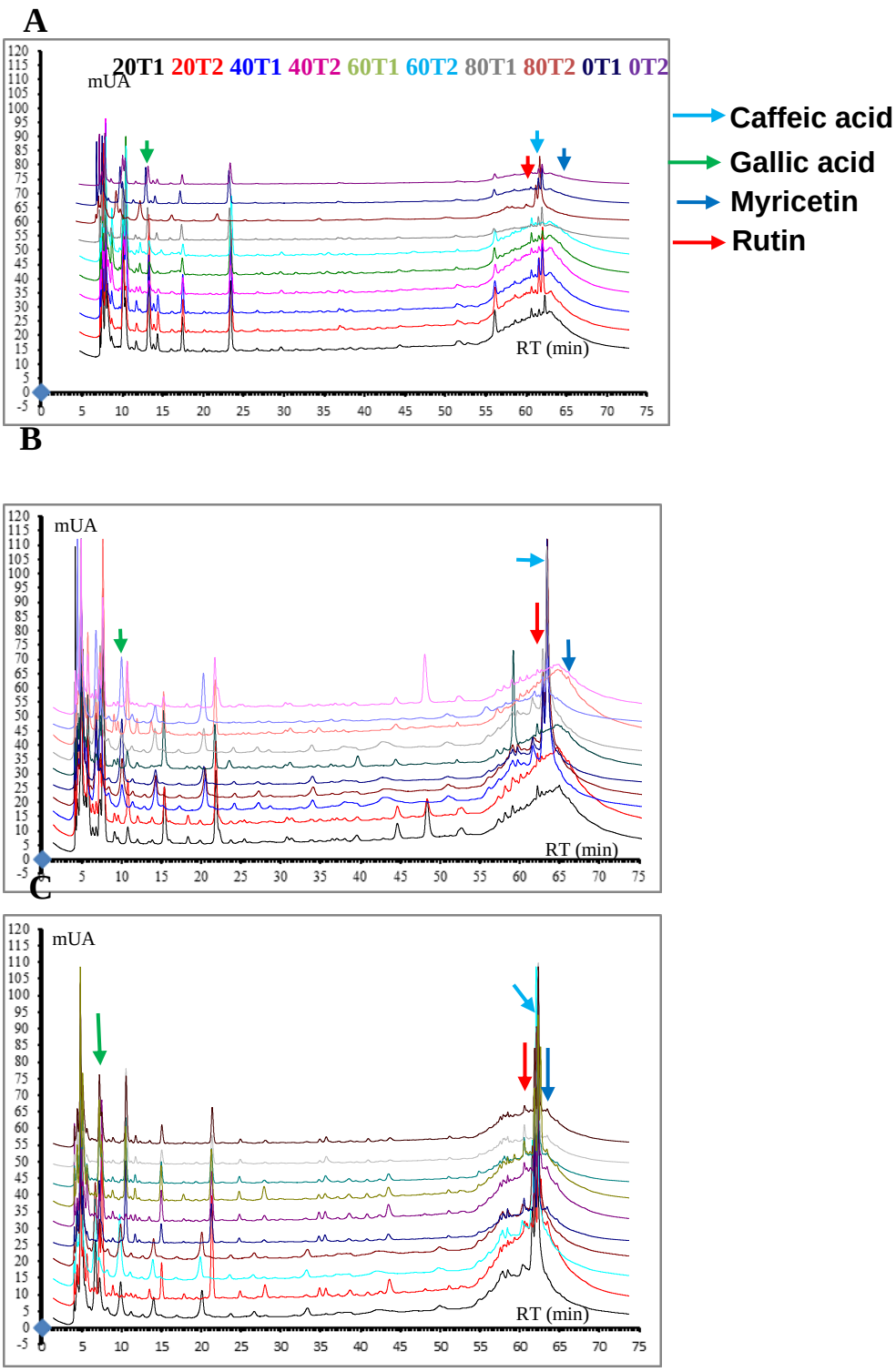

Figure 3. 3. HPLC profile of aqueous extracts of dried leaves: A, *Solanum macrocarpon*; B, Amaranthus viridis; and C, Telfairia occidentalis. Vegetables were grown under different nitrogen fertilizer doses (0, 20, 40, 60, and 80 kg/ha), that were applied before (T1) or after (T2) planting.

sample concentration. TAC was not dependent on fertilizer application time, but vegetable variety and fertilizer dose had significant ($p < 0.05$) influences (Table 3.1). *AV* had the highest TAC when compared to *SM* and *TO*. TAC was significantly ($p < 0.05$) increased (10-34%) by combined application of nitrogen and organic fertilizer (20T-60T) when compared to individual application of inorganic (80T) or organic (0T) fertilizers. Studies have shown that the TAC of plant is basically due to their flavonoids and other polyphenol contents of plants (Potential et al., 2016). However, the TAC showed an opposite trend to those of TPC and TFC, which suggests that the activity of individual compounds may be more important than the synergistic effects of several polyphenols present in the extracts.

3.3.3 DPPH radical scavenging assay

DPPH is a stable synthetic compound that has been used in the determination of free radical scavenging activity of various antioxidants (Li et al., 2009). In the presence of a molecule consisting of a stable free radical (DPPH), an antioxidant with the ability to donate a hydrogen atom will quench the stable free radical, a process that is associated with a change in the absorption and it is measured spectrophotometrically. DPPH radical scavenging activity of the vegetable leaf extracts generally increased up to 0.25 mg/mL polyphenol concentration but declined when concentration increased to 0.5 and 0.625 mg/mL (Figure 3.5). The initial increases in DPPH radical scavenging activity as polyphenol concentration increased from 0.0625 mg/mL to 0.25 mg/mL reflects a higher number of hydrogen atoms and electrons that could be donated. However, at 0.5 and 0.625 mg/mL, there may have been increased polyphenol-polyphenol interactions to form oligomers with reduced radical scavenging activity as previously reported for other polyphenolic samples (Cheynier, & Williamson, 1998; Saint-Cricq de Gaulejac, & Bourgeois, 1999).

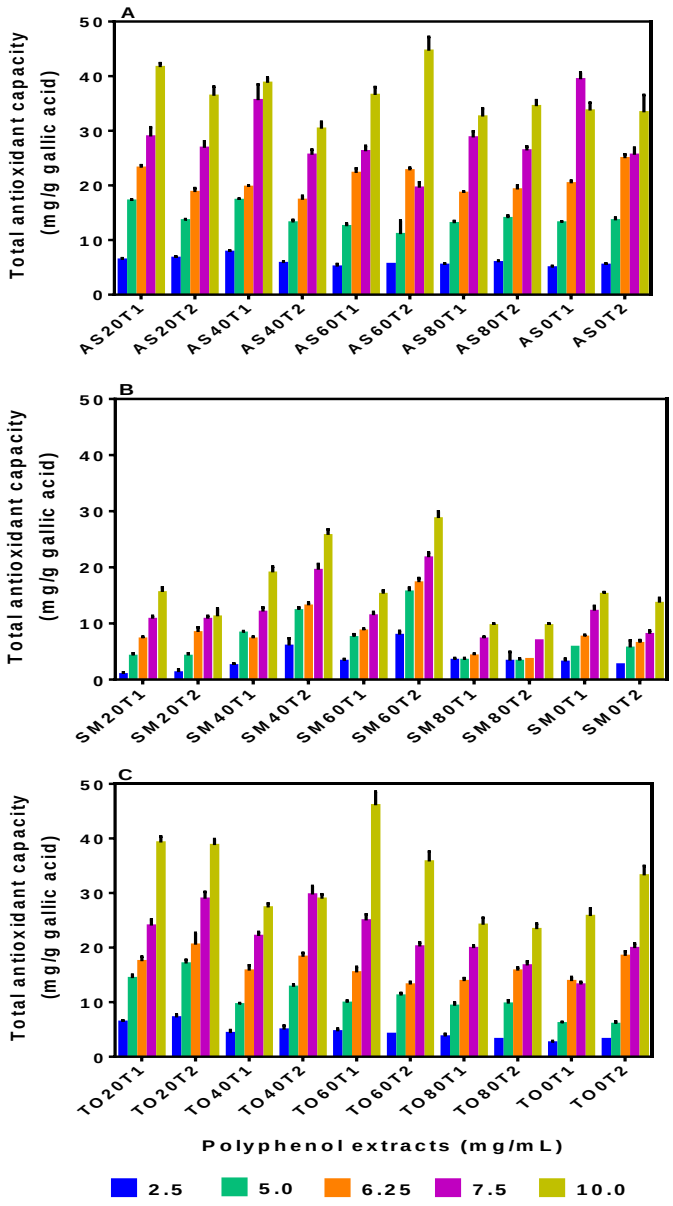

Figure 3. 4. Total antioxidant capacity of aqueous extracts from the dried leaves of A - *Amaranthus viridis* leaves (AV), B - *Solanum macrocarpon* (SM) and C - *Telfairia occidentalis* (TO). BHT, butylated hydroxytoluene. Vegetables were grown under different nitrogen fertilizer doses (0, 20, 40, 60, and 80 kg/ha), that were applied before (T1) or after (T2) planting.

Moreover, hydrogen atom transfer from a polyphenol normally yields a phenolic radical, which is then stabilized by electronic delocalization; an unstabilized phenolic radical essentially

becomes a free radical (Quideau et al., 2011). Therefore, the results suggest low stability of the polyphenol radicals, which may have reacted with the reduced form of DPPH to revert to the radical form, hence reduced antioxidant efficiency at 0.5 and 0.625 mg/mL. Overall, the vegetable extracts had significantly ($p < 0.05$) lower DPPH scavenging ability than that of BHT, a standard antioxidant compound (Figure 3.5). The polyphenol extract's effective concentration that scavenged 50% of the DPPH radical (EC_{50}) was used to determine the statistical data shown in Table 3.1. The DPPH radical scavenging activity of the plant extracts were influenced by vegetable variety, fertilizer dose and fertilizer application time. The DPPH radical scavenging activity was attributed to fertilizer source and rates of nitrogen levels ($p < 0.05$; Figure 5). At 0.25 mg/mL, the percentage DPPH radical scavenging activity had the highest value for all three vegetable varieties. The EC_{50} results shows that *TO* have the highest DPPH radical scavenging activity value (EC_{50} = 0.156 mg/mL) followed by *SM* (EC_{50} = 0.161 mg/mL) and *AV* (EC_{50} = 0.173 mg/mL). Based on the fertilizer doses, 0 kg N/ha (EC_{50} = 0.15 mg/mL) had the highest DPPH radical scavenging activity followed by 20 kg N/ha (EC_{50} = 0.157 mg/mL), 60 kg N/ha (EC_{50} = 0.162 mg/mL), and the least at 40 kg N/ha and 80 kg/ha treatment (EC_{50} = 0.173 mg/mL). The results suggest that the usage of organic fertilizer may enhance the DPPH radical scavenging activity of *TO, SM and AV* leaf aqueous extracts and when compared to high urea fertilizer rates. The results also suggest that the combination of urea fertilizer at lower doses with organic fertilizer favored DPPH radical scavenging activity better than high urea dose (80 kg N/ha) only. DPPH radical scavenging assay measures the activity of water-soluble antioxidants (Frankel et al., 1994). Results obtained from this study do not agree with a previous work that suggested high inorganic

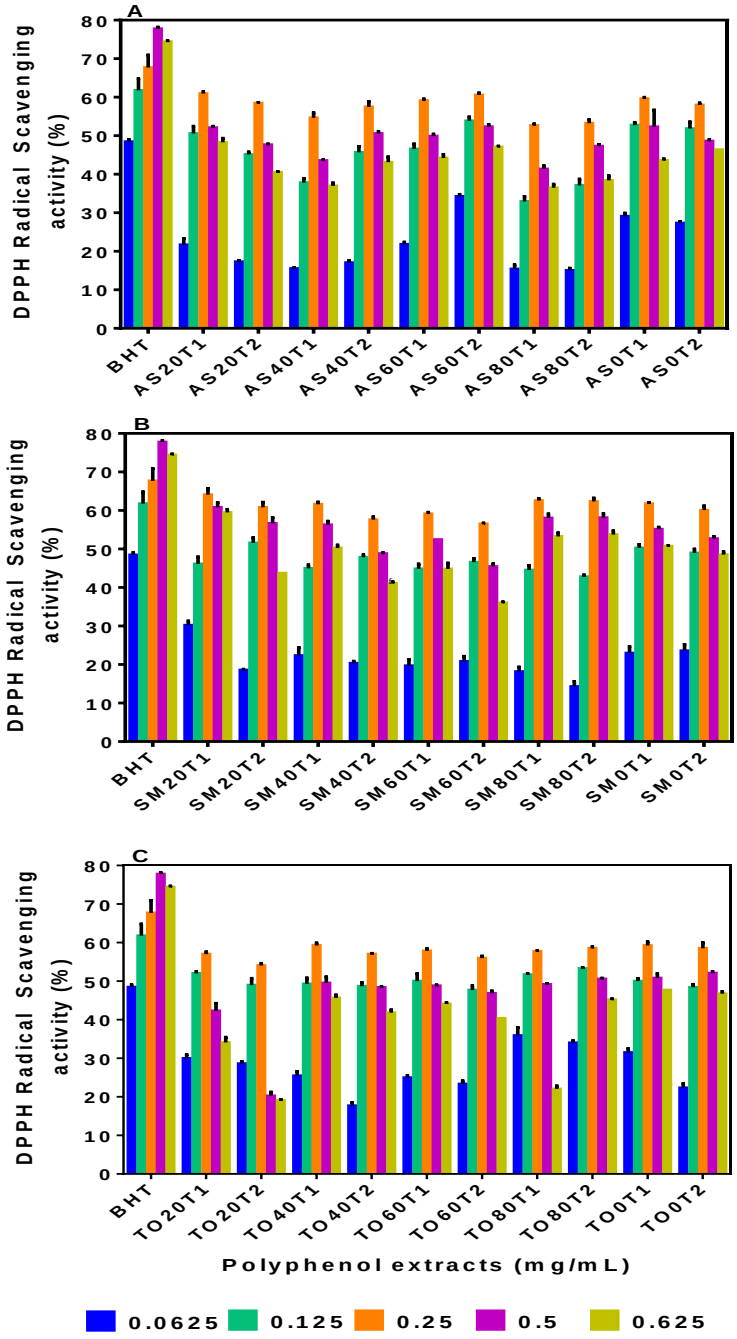

Figure 3. 5. DPPH radical scavenging activity of aqueous extracts from the dried leaves of A - *Amaranthus viridis* leaves (AV), B - *Solanum macrocarpon* (SM) and C - *Telfairia occidentalis* (TO)). BHT, butylated hydroxytoluene. Vegetables were grown under different nitrogen fertilizer doses (0, 20, 40, 60, and 80 kg/ha), that were applied before (T1) or after (T2) planting.

N fertilizer application rate (90 kg N/ha) improved the DPPH radical scavenging activity of *Labisia pumila* extracts (Ibrahim et al., 2013). This may be attributed to differences in the vegetable variety used as well as the extraction medium, which was water in this work while *Labisia pumila* was extracted with an aqueous ethanol solution.

3.3.4 Chelation of metal ions

The presence of transition metals in food or tissues is responsible for the formation of free radicals; therefore, metal chelating agents could reduce the risk of radical-induced damage in the body by stabilizing transition metals (Jayakumar et al., 2009). Ferrozine forms a stable-colored complex with ferrous ion (Fe^{2+}) but these complexes are affected when other chelating agents are present thereby reducing color intensity. The metal chelating activity of the vegetable extracts was enhanced by increased extract concentration up to 0.75 mg/mL, but overall effect was not dose-dependent (Figure 3.6). Metal chelating activity of *SM* and *TO* was increased from 0.125 to 0.75 mg/mL but decreased at 1.0 mg/mL. For *AV* extracts, metal chelating activity increased only up to 0.5 mg/mL while lower values were obtained at 0.75 and 1.0 mg/mL. The lower metal chelating efficiency at high concentrations may be due to polyphenol aggregation through hydrophobic interactions, which would have reduced availability of binding sites (Delimont & Lindshield, 2017). It is also possible that such polyphenol aggregates become less soluble, which would have minimized interactions with the Fe^{2+}. The metal chelating EC_{50} values were used to determine sources of variation as shown in Table 3.1. Vegetable variety, fertilizer dose, and fertilizer application time all influenced the metal chelating ability of the polyphenolic extracts. The *AV* extract had significantly ($p < 0.05$) higher (lowest IC_{50} value) metal chelating ability than the *SM* and *TO* extracts. Therefore, there was no direct relationship between TPC and metal chelating ability, which may be because other factors such as phenolic structure

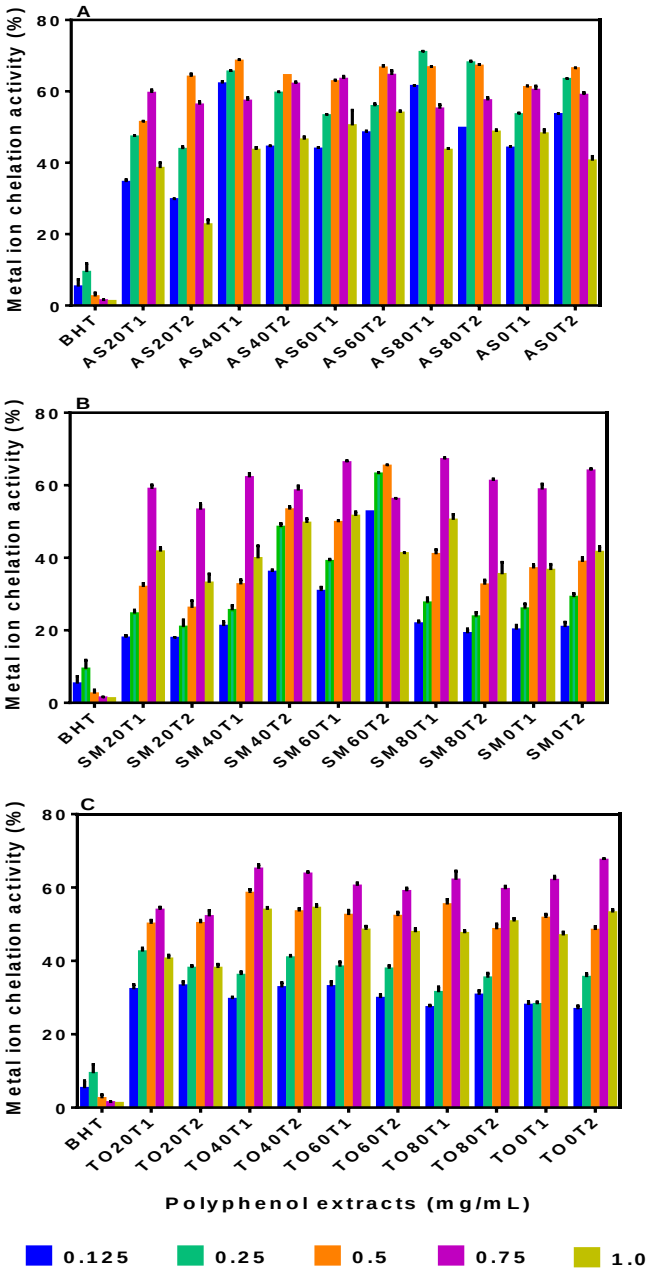

Figure 3. 6. Metal ion chelation activity of aqueous extracts from the dried leaves of A - *Amaranthus viridis* leaves (AV), B - *Solanum macrocarpon* (SM) and C - *Telfairia occidentalis* (TO). BHT, butylated hydroxytoluene. Vegetables were grown under different nitrogen fertilizer doses (0, 20, 40, 60, and 80 kg/ha), that were applied before (T1) or after (T2) planting.

and position of the hydroxyl groups are also important determinants of metal binding (Santoso et al., 2004). Nitrogen application at 40-80 kg N/ha led to significantly ($p < 0.05$) enhanced metal chelating ability (lower EC_{50} values) when compared to the plants that had no nitrogen fertilizer. Application of inorganic fertilizer enhanced the metal chelating ability of the vegetable leaf extracts as shown by the decreases in IC_{50} values for the 20T-60T samples. This is also supported by the lower IC_{50} value for 80T extract (inorganic fertilizer alone) when compared to 0T (organic fertilizer alone). The results suggest that the 40-80 kg N/ha doses enhanced formation of polyphenols with synergistic effects that led to increased metal binding capacity. Overall, the chelating ability of the three vegetable extracts was significantly ($p < 0.05$) higher than that of BHT. The higher metal chelating ability of the extracts may be due to synergistic interactions between the various polyphenolic compounds when compared to the BHT that is a single compound.

3.3.5 Ferric reducing antioxidant power (FRAP)

FRAP assay is based on the reduction of Fe^{3+} to Fe^{2+} and the values obtained are expressed as the concentration of electron-donating antioxidants. Figure 3.7 shows dose-dependent increases in FRAP for the three vegetable varieties, which indicate concentration-enhanced electron donating ability of the extracts. FRAP was influenced by the vegetable variety, fertilizer dose and the fertilizer application time (Table 3.1). The *TO* extract had a significantly higher FRAP value than *SM* and *AV* extracts, which is consistent with the TFC and TPC values. The results are similar to those reported for peanut skins where TFC, TPC and FRAP had direct correlations (Shem-Tov et al, 2012). The vegetable extracts had significantly ($p < 0.05$) less FRAP values when compared to BHT; maximum values were achieved at 1.0 mg/mL for vegetables in contrast to 0.5 mg/mL

for the BHT. FRAP was influenced by fertilizer source and rates ($p \leq 0.05$), and followed the same

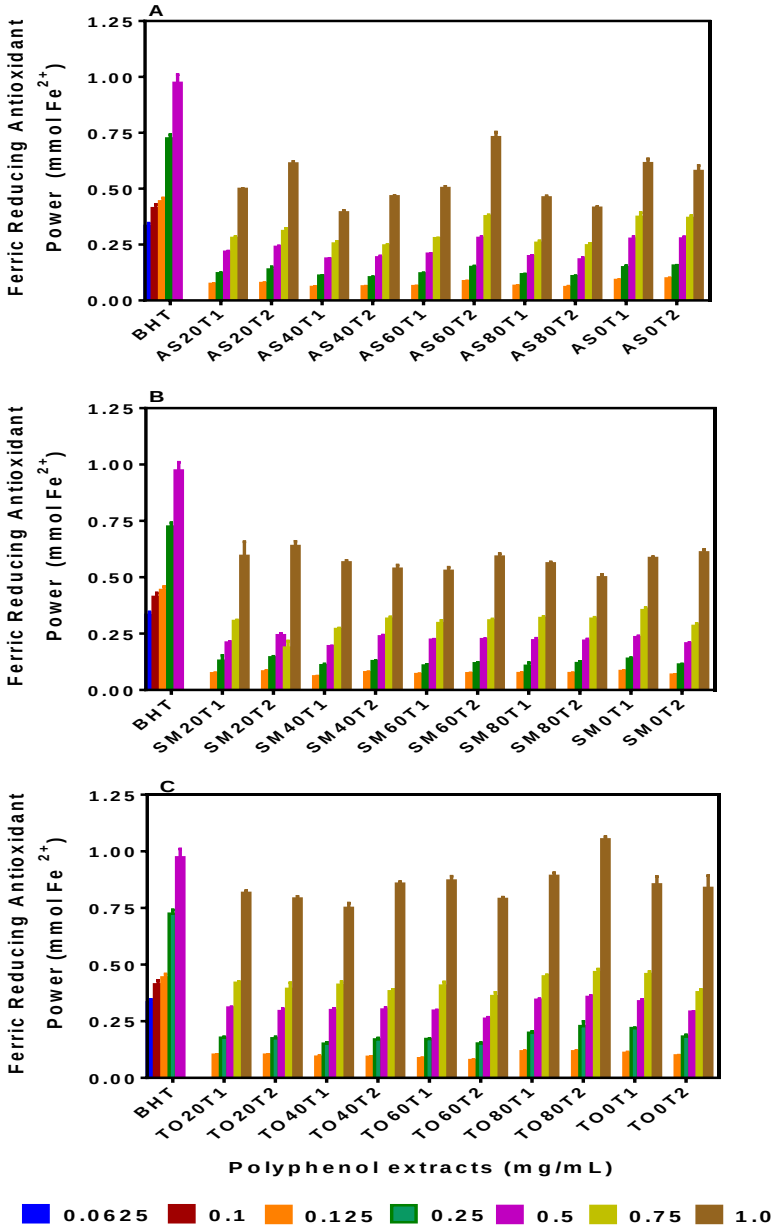

Figure 3. 7. Ferric reducing antioxidant power of aqueous extracts from the dried leaves of A - *Amaranthus viridis* leaves (AV), B - *Solanum macrocarpon* (SM) and C - *Telfairia occidentalis* (TO). BHT, butylated hydroxytoluene. Vegetables were grown under different nitrogen fertilizer doses (0, 20, 40, 60, and 80 kg/ha), that were applied before (T1) or after (T2) planting.

trend with DPPH, where the reducing ability was highest under organic fertilization with the highest activity at 0 kg N/ha, and the lowest activity at 40 kg N/ha. The results are consistent with the findings of Ibrahim et al. (2013) on the impact of nitrogen fertilizer on the antioxidant activity of *Labisia pumila*. The results indicate that as fertilization dose increased, the abilities of the vegetable extracts to reduce ferric ions also increased except for 40 kg N/ha, which has the lowest value and was not significantly different from the 80 kg N/ha. The use of organic fertilizer alone favours the total antioxidant power of these vegetables followed by the combined organic fertilizer treatment with 60 kg urea N/ha. The positive impact of organic fertilizer may be due to increased metabolic activity under these conditions (Woese et al., 1997). It has also been reported that the use of organic fertilizer improves soil properties by increasing soil physical, chemical and biological properties and prevents soil erosion (Ibrahim et al., 2013). Higher rate of antioxidant properties obtained with organic fertilizer might be due to the presence of other major and minor elements present in the organic fertilizer since the inorganic fertilizer used in this study supplied only nitrogen. Application of fertilizer two weeks after planting enhanced the rate of ferric ion reduction than those applied during planting, which suggests positive effect on the synthesis of compounds with strong iron-reducing properties.

3.3.6 *In vitro* inhibition of acetylcholinesterase (AChE) activity

Some neurodegenerative disorders such as dementia and Alzheimer's disease have been suggested to occur as a result of excessive activity of acetylcholinesterase, an enzyme that breaks down the neurotransmitter acetylcholine into choline and an acetate group (Saravanan and Ponmurugan, 2013; (Pervin et al., 2014). This is due to the reduced availability of acetylcholine, which leads to decreased nerve signal transmission. Therefore, compounds that inhibit acetylcholinesterase activity could boost acetylcholine concentration to facilitate normal nerve

functions (Saravanan and Ponmurugan, 2013). All the three vegetable extracts exhibited acetylcholine inhibitory activity in a pattern that was not dose-dependent (Figure 3.8). AChE inhibition increased from 0.01 to 0.02 mg/mL mostly for the *TO* extracts whereas some of the *SM* extracts had inhibitory values that increased up to 0.03 and 0.04 mg/mL. For the *AV* extracts, AChE inhibition was either highest at 0.01 and 0.02 mg/mL followed by decreases at higher concentrations or highest inhibition occurred at only 0.01 mg/mL. The lack of a dose-dependent inhibition at the concentrations used in this work suggests polyphenol aggregation, which could have reduced ability to interact with AChE protein. Table 3.1 shows that AChE inhibition by the polyphenol extracts was influenced by vegetable variety, fertilizer dose and fertilizer application time. Generally, the *TO* extracts had significantly ($p < 0.05$) higher AChE-inhibitory properties than *SM* and *AV* extracts. AChE inhibition by all the polyphenol extracts was very similar from zero N fertilizer application up to 40 kg N/ha dose but then decreased at the 80 kg N/ha dose. The maximum activity occurred at 60 kg urea N/ha in combination with organic fertilizer while the least inhibition occurred at 80 kg urea N/ha (no organic fertilizer). There was no significant difference between the organic fertilizer alone (0 kg urea N/ha) treatment and those of the combined treatments (20 and 40 kg urea N/ha). The result suggests that inhibition of AChE activity may be better achieved with the use of organic fertilizer alone or in combination with urea. The AChE-inhibitory activity of the extracts was more effective when the fertilizer was applied two weeks after planting, which suggests enhanced synthesis of active compounds. The vegetable extracts had significantly ($p < 0.05$) higher AChE inhibition than galanthamine, the standard drug used for comparison. Therefore, depending on bioavailability, the vegetable extracts could be important sources of compounds that exert neuroprotective effects similar to galanthamine. The AChE-inhibitory values obtained in this work are higher than those reported

for the aqueous extract of *Thymus vulgaris*, which was assayed at 0.05 mg/mL concentration but lower than the 37, 37 and 62% for *Corydalis cava* at 0.025, 0.05 and 0.1 mg/mL, respectively (Hasnat et al., 2013).

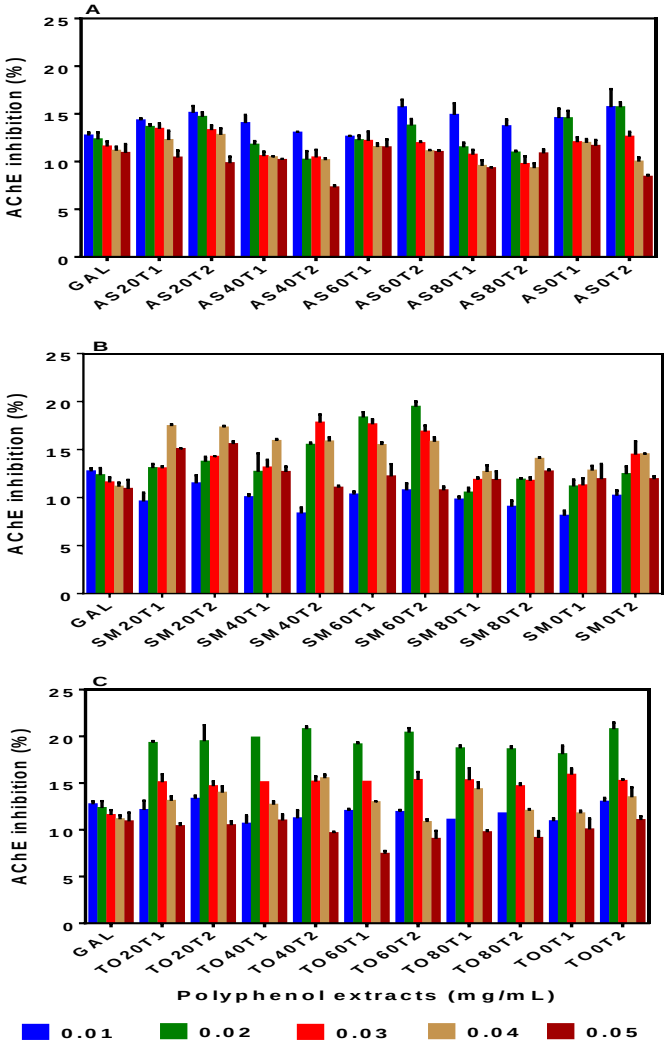

Figure 3. 8. Inhibition of acetylcholinesterase (AChE) activity by aqueous extracts from the dried leaves of A - *Amaranthus viridis* leaves (AV), B - *Solanum macrocarpon* (SM) and C - *Telfairia occidentalis* (TO). GAL, galanthamine. Vegetables were grown under different nitrogen fertilizer doses (0, 20, 40, 60, and 80 kg/ha), that were applied before (T1) or after (T2) planting.

3.4 Conclusions

Aqueous extracts of the three vegetables had *in vitro* antioxidant properties that could provide a basis for reducing the damaging effects of free radicals in foods or in human tissues. Nitrogen fertilizer application had a negative influence on the accumulation of polyphenolic compounds, which suggests that the plants channeled excess nutrients towards vegetative growth. However, TAC, metal ion chelation and AChE inhibition were increased up to 60 kg N/ha application when combined with organic fertilizer; this dose may serve as the optimum level for enhanced bioactive properties of the vegetable leaves**.** The results suggest that the *TO* extracts have the highest antioxidant and anti-AChE potential when compared to *AV* and *SM*. Therefore, the *TO* extracts could serve as suitable ingredients to formulate functional foods and nutraceuticals against oxidative stress and AChE-dependent neurodegenerative disorders. The ability of these vegetables to act as an antioxidant could be attributed to the presence of phenolic compounds such as gallic acid, rutin, myricetin and caffeic acid. Future studies that use model food systems or appropriate diseased animal models will be required to confirm the potential benefits of these polyphenolic extracts.

3.5 Acknowledgment

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3.6 Conflict of interest

The authors declare no conflict of interest in this study.

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3.8 TRANSITION STATEMENT ONE

In the preceding chapter, the antioxidant properties of the three vegetables were determined using different antioxidant assays and their ability to inhibit acetylcholinesterase was also investigated. The three vegetables had effective *in vitro* antioxidant properties with the ability to scavenge DPPH radicals, reduce ferric iron, and chelate metal iron an indication of their potential use to manage degenerative disorders arising from damage impacted by excessive production of ROS/free radicals. They were able to inhibit acetylcholinesterase, an enzyme implicated in Alzheimer's disease with *Telfaria occidentalis* having the highest result. Additionally, the study found that caffeic acid, rutin, gallic acid and myricetin were the phenolic compounds present in the three vegetable extracts. Application of organic fertilizer enhanced some of the antioxidant and anti-cholinesterase properties of these vegetables up to 60 kg N/ha which may serve as the optimum level for enhanced bioactive properties of the vegetable leaves. Since their *in vitro* antioxidant properties have now been established in the preceding chapter, the next chapter (Manuscript 2) addressed the *in vitro* antihypertensive effect of *Amaranthus viridis* and *Telfairia occidentalis* as well as *in vivo* lowering of systolic blood pressure in spontaneously hypertensive rats. It was determined that the vegetable leaf extracts have potential antihypertensive properties and thus could be used as therapeutic agents for the prevention, amelioration, treatment, or management of chronic cardiovascular diseases.

CHAPTER FOUR

MANUSCRIPT TWO

ANTIHYPERTENSIVE EFFECT OF AQUEOUS POLYPHENOL EXTRACTS OF *AMARANTHUS VIRIDIS* AND *TELFAIRIA OCCIDENTALIS* LEAVES IN SPONTANEOUSLY HYPERTENSIVE RATS

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AUTHOR CONTRIBUTIONS

REA: Conceptualization, funding acquisition, and project administration. REA, AMA and OAO methodology. REA: resources. OAO: writing—original draft preparation. REA and AMA: writing—review and editing. OAO: formal analysis. REA and AMA: supervision. All authors have read and agreed to the submitted version of the manuscript

Reprinted from Antihypertensive effect of aqueous polyphenol extracts of *Amaranthus viridis* and *Telfairia occidentalis* leaves in spontaneously hypertensive rats. *J. of Food Bioactives.* 2018; 1:166-173.

Abstract

The antihypertensive effects of aqueous polyphenol-rich extracts of *Amaranthus viridis* (AV) and *Telfairia occidentalis* (TO) leaves in spontaneously hypertensive rats (SHR) were investigated. The dried vegetable leaves were extracted using 1:20 (leaves:water, w/v) ratio for 4 h at 60^{o}C. Results showed significantly ($P<0.05$) higher polyphenol contents in TO extracts (80-88 mg gallic acid equivalents, GAE/100 mg) when compared with the AV (62-67 mg GAE/100 mg). Caffeic acid, rutin and myricetin were the main polyphenols found in the extracts. The TO extracts had significantly ($P<0.05$) higher *in vitro* inhibition of angiotensin I-converting enzyme (ACE) activity while AV extracts had better renin inhibition. Oral administration (100 mg/kg body weight) to SHR led to significant ($P<0.05$) reductions in systolic blood pressure for the AV (-39 mmHg after 8 h) and TO (-24 mmHg after 4 and 8 h). The vegetable extracts also produced significant ($P<0.05$) reductions in diastolic blood pressure, mean arterial blood pressure and heart rate when compared to the untreated rats. Thus, both the AV and TO leaf extracts have the potential to be used as antihypertensive agents, especially the AV, which produced persistent long-lasting effect over a 24 h period.

Keywords: Renin; Angiotensin converting enzyme; Antihypertensive properties; Spontaneously hypertensive rats; Blood pressure; Polyphenol extract

4.1. Introduction

Hypertension is responsible for 9.4 million deaths world-wide, which is equivalent to about 18% of all annual deaths (WHO, 2013). Hypertension is also known as high blood pressure and is a chronic disease that afflicts millions of people worldwide. High blood pressure occurs when the systolic blood pressure is above 140 mmHg and the diastolic blood pressure is above 90 mmHg (Pickering et al., 2005). Hypertension can also lead to other negative health outcomes such as stroke, coronary heart disease, physical disability and kidney dysfunction if left untreated (Chen et al., 2009). However, the disease intensity of hypertension can be ameliorated with the use of different drugs such as diuretics, angiotensin converting enzyme (ACE) inhibitors, calcium channel blockers, renin inhibitors and vasodilators (nitrates). ACE and renin are targets for hypertension therapies because they form a key component of the renin-angiotensin system (RAS) that controls mammalian blood pressure. When there is a fall in blood pressure, renin breaks down plasma angiotensinogen to give rise to angiotensin I, which is then converted to angiotensin II (a vasoconstrictor) by the action of ACE (Chen et al., 2009; Girgih et al., 2011). However, under disease conditions, there is excessive formation of angiotensin II, which leads to insufficient relaxation of blood vessels and hence high blood pressure develops. Therefore, inhibition of ACE and renin activities is important in lowering plasma level of angiotensin II, which facilitates blood pressure reductions. However, the use of antihypertensive drugs has been associated with negative side effects such as rashes, edema, dry coughing and erectile dysfunction (Abassi et al., 2009; Blumentals et al., 2003; Chen et al., 2009; Fogari et al., 2001; Girgih et al., 2011; Gunkel et al., 1996; Tenenbaum et al., 2000). Therefore, natural alternative therapies such as peptides (Aluko, 2015) and polyphenols (Hellstrom et al., 2010;

Kivimaki et al., 2013; Shaw et al., 2017) with potential fewer negative side effects have been suggested as effective antihypertensive agents.

Traditionally, green leafy vegetables have been used as spice in foods or for culinary purposes while vegetable extracts have formed part of folk medicine agents used to treat or manage different human diseases (Farombi & Owoeye, 2011). Moreover, consumption of vegetable-rich diets has been reported to improve or attenuate some human diseases due to their antioxidant properties (Oboh & Rocha, 2007). This is because plants are rich sources of phenolic compounds, which have anti-diabetic, anti-carcinogenic, anti-inflammatory, anti-hypertensive and hepatoprotective properties (Farombi & Owoeye, 2011).

Amaranthus viridis (AV) and *Telfaria occidentalis* (TO) leaves are common vegetables found in West Africa. The leaf extracts of these vegetables have been reported to have various pharmacological effects in addition to antimicrobial, antidiabetic, and antihyperlipidemic activities (Nwanna et al., 2016; Oboh et al., 2010). AV contains betalains and saponins (Hussain et al., 2008), while the leaves of TO are rich in vitamins and potassium (Oboh et al., 2010). These leafy vegetables are also rich in polyphenols (gallic acid, chlorogenic acid, quercetin, kaempferol, caffeic acid, rutin, myricetin), which are a group of compounds that have been demonstrated to inhibit ACE activity and lead to blood pressure reductions (Dong et al., 2011; Kivimaki et al., 2013; Shaw et al., 2017). The vegetables are cheap, readily available and their consumption may provide health benefits by functioning as antihypertensive agents. However, there is scant information on the ability of polyphenolic extracts from these vegetables (AV and TO) to inhibit ACE and renin activities or reduce blood pressure. Therefore, the aim of this work was to determine the inhibition of *in vitro* activities of ACE and renin by aqueous extracts of AV

and TO leaves. Ability of the leaf polyphenolic extracts to reduce blood pressure and heart rate was then measured after oral administration to spontaneously hypertensive rats (SHR).

4.2. Materials and Methods

4.2.1 Materials

Ground leaf powders of the two vegetables were obtained from vegetable plants cultivated at the Teaching and Research Farm of the Obafemi Awolowo University, Ile-Ife, Nigeria. Urea fertilizer doses (0, 20, 40, 60 and 80 kg N/ha) were applied to the vegetables on plot-by-plot basis in randomized complete block design. Fertilizer was applied to each plot either at planting (T1) or one week after seedling emergence (T2) to obtain the following samples: 0T1, 0T2, 20T1, 20T2, 40T1, 40T2, 60T1, 60T2, 80T1, and 80T2. Samples were harvested by plucking, cut into small pieces, dried in a hot air cabinet at ~60^{o}C for 8 h and then milled to fine powder using a Marlex Excella dry mill (Marlex Appliances PVT, Daman, India). The ground powders were then stored at -20^{o}C prior to analysis. N-(3-[2-furyl] acryloyl)-phenylalanylglycylglycine (FAPGG), and ACE from rabbit lung (E.C.3.4.15.1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A Human Recombinant Renin Inhibitor Screening Assay Kit was purchased from Cayman Chemicals (Ann Arbor, MI, USA). All other reagents are of analytical grade and purchased from Fisher Scientific (Oakville, ON, Canada).

4.2.2 Polyphenol extraction

Extraction of the free water-soluble polyphenols was carried out according to the method of (Alu'datt et al., 2010) with slight modifications. It should be noted that this method does not measure the content of bound polyphenols. Samples were extracted using distilled water at ratios of 1:20 (w/v) ground leaf powder to water for 2 h in a beaker with continuous stirring at 60^{o}C. The extract was centrifuged (10000 x g, 30 min, 24^{o}C), the supernatant saved while the residue

was re-extracted with 20 volumes of water under same conditions as the first extraction. After the second centrifugation, the two supernatants were combined, filtered through cheese cloth, and concentrated under vacuum using a rotatory evaporator at 60^{o}C. The concentrated extracts were freeze dried and stored at -20^{o}C for further analysis. The total polyphenolic content (TPC) of each extract was determined using the Folin–Ciocalteu method, (Hoff & Singleton, 1977) which was modified as follows. A standard calibration curve was prepared using 25-350 µg/mL gallic acid concentration in 50% (v/v) aqueous methanol. The polyphenol extracts were also diluted with the 50% methanol to 600-1400 µg/mL. A 250 µL aliquot of Folin–Ciocalteu reagent was added to 250 µL of gallic acid solution or the diluted sample and then mixed on a vortex. After standing in the dark at room temperature for 5 min, 0.5 mL of 20% (w/v) sodium carbonate solution was added followed by 4 mL of double distilled water. The contents were vortexed and incubated again in the dark for 1 h. Intensity of the green colour was then measured at 725 nm using an Ultospec UV-visible spectrophotometer (GE Healthcare, Montreal, PQ, Canada). TPC was expressed as milligrams gallic acid equivalents (GAE) per 100 mg dry leaf extract (mg GAE/100 mg).

4.2.3. Estimation of main polyphenolic compounds

The major polyphenol constituents of the AV and TO leaf extracts were quantified using the Varian 900-LC series analytical HPLC (Phenomenex Inc., Torrance, CA, USA) using a Phenomenex C18 HPLC column (250 mm × 4.6 mm, 5 µm) heated to 30°C. The mobile phases used were 1% acetic acid (solvent A) and methanol (solvent B) with the following gradients 95% A to 5% B for 35 min; 80% A to 20% B for 15 min; 40% A to 60% B for 10 min and 70% A and 30% B for 15 min. The flow rate was fixed at 1.0 mL/min and standards (caffeic acid, catechin, gallic acid, rutin, myricetin and quercetin) and samples were injected using 20 µL solutions

while detection wavelengths were at 280 and 320 nm. The level of each polyphenol was estimated using the calibration curve generated from the linear plot of elution time versus peak area of each standard polyphenol compound and the results expressed as mg/g of dry leaf extracts.

4.2.4 Renin inhibition assay

In vitro inhibition of human recombinant renin activity was conducted according to the method described by Li and Aluko (2010) using the Renin Inhibitor Screening Assay Kit. Prior to the assay, renin buffer was diluted with 50 mM Tris–HCl (pH 8.0) that contained 100 mM NaCl. The renin protein solution was diluted 20 times with assay buffer before use, and the assay buffer was pre-warmed to 37^{o}C before the reaction was initiated in a fluorometric microplate reader (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA, USA) maintained at 37^{o}C. Before the reaction, (1) 20 µL substrate, 160 µL assay buffer, and 10 µL Milli-Q water were added to the background wells; (2) 20 µL substrate, 150 µL assay buffer, and 10 µL Milli- Q water was added to the control wells; and (3) 20 µL substrate, 150 µL assay buffer, and 10 µL sample (0.25 mg GAE/mL final assay concentration) were added to the inhibitor wells. The reaction was initiated by adding 10 µL renin to the control and sample wells. The microplate was shaken for 10 s to mix and incubated at 37^{o}C for 15 min, and the fluorescence intensity (FI) was then recorded using an excitation and emission wavelengths of 340 and 490 nm, respectively. The percentage inhibition was calculated as follows:

$$Inhibition\ (\%) = \frac{\text{FI of control wells} - \text{FI of sample wells}}{\text{FI of control wells}} \times 100$$

4.2.5 ACE inhibition assay

Ability of the polyphenolic extracts to inhibit *in vitro* activity of ACE was measured according to a spectrophotometric method using synthetic N-[3-(2-Furyl) acryloyl]-L-

phenylalanyl-glycyl-glycine (FAPGG) as the substrate (Holmquist et al., 1979). Briefly, 20 µL of sample dissolved in 50 mM Tris-HCl buffer containing 0.3 M NaCl, pH 7.5 (0.5 mg GAE/mL final assay concentration) was mixed with 10 µL of ACE (20 mU final reaction activity) and 170 µL of 0.5 mM FAPGG dissolved in the same buffer in a 96-well microplate. The rate of decrease in absorbance at 345 nm was recorded for 30 min at 37ºC using a microplate reader. The buffer was used instead of sample solutions in the blank experiment.

4.2.6 Blood pressure measurement

SHRs at 6 weeks were purchased from Charles River Laboratories (Montreal, PQ, Canada) and implanted with telemetry sensors after acclimatization (under 12-h day and night cycle at 21ºC) for 2 weeks. Rats were provided with ad libitum access to regular chow feed and tap water. Telemetry sensors were surgically implanted into the rats according to our previously described protocols (O'Keeffe et al., 2017). After a 2-week recovery from the surgery, the groups were assigned 4 rats each followed by oral administration (1 mL) of test agents, which were all dissolved in phosphate buffered saline (PBS): PBS only (negative control); 20 mg/kg body wt captopril (antihypertensive drug as a positive control); and 100 mg GAE/kg body wt polyphenol extract. Real time changes in heart functions were measured as systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rates (HR) in a quiet room with each rat cage placed on the respective receiver (Model RPC-1, DSI instruments, St. Paul, MN, USA). All the rat protocols were approved by the University of Manitoba Animal Care Committee. Data were recorded continuously at 10 min intervals for 24 h using the Ponemah 6.1 data acquisition software (DSI instruments). The system was linked to an APR-1 atmospheric-pressure monitor (DSI instruments), which normalized the transmitted pressure values to produce blood pressure signals independent of changes in atmospheric pressure.

Results are reported as the SBP, DBP, MAP and HR values at 2, 4, 6, 8, 12 and 24 h minus their baseline measurements at time zero.

4.2.7 Statistical Analysis

Analyses were conducted in replicates as indicated above and analyzed by 3-way analysis of variance (ANOVA) for *in vitro* assay using a model that included vegetable variety (VV), fertilizer dose (FD) and fertilizer application time (FT) as fixed variables and one-way analysis of variance for *in vivo* assay. Data were reported as mean ± standard deviation. Statistical significance of differences was evaluated by Duncan's multiple range test ($P<0.05$) using the IBM SPSS Statistical package (version 24).

4.3. Results and Discussion

4.3.1 TPC, polyphenol contents and the inhibitions of *in vitro* ACE and renin activities

In this work, water extraction was used because initial trials with solvents or mixtures of solvents and water yielded insoluble extracts after freeze-drying. In contrast, the freeze-dried water extracts were readily solubilized by the aqueous reagents used for various analytical and *in vivo* determinations. The TPC of the samples are shown in Table 4.1 and the data indicate that the extracts were mainly composed of polyphenols with values up to 469 and 611 mg GAE/g of dried extracts for AV and TO, respectively. However, all the TO extracts contained significantly ($P<0.05$) higher polyphenol contents than similar AV extracts. The results suggest the presence of more free water-soluble polyphenols in the TO leaves than the AV leaves. Table 4.2 shows that caffeic acid, rutin and myricetin were the main polyphenols detected in the leaf extracts. Caffeic acid was the polyphenol in the TO extracts while rutin and caffeic acid were present in the AV extracts at similar

Table 4. 1. Total phenolic content (mg gallic acid equivalent/g) of *Amaranthus viridis* (AV) and *Telfairia occidentalis* (TO) leaf extracts

Samples	AV	TO
0T1	462.09 ± 1.42^{ab}	597.57 ± 1.50^{f}
0T2	466.18 ± 2.45^{bc}	581.94 ± 3.97^{cd}
20T1	460.46 ± 2.83^{a}	585.42 ± 0.00^{d}
20T2	469.44 ± 5.10^{c}	590.63 ± 2.60^{e}
40T1	462.91 ± 1.42^{ab}	573.26 ± 1.50^{b}
40T2	460.46 ± 3.74^{a}	582.81 ± 2.60^{cd}
60T1	462.09 ± 1.42^{ab}	578.47 ± 1.50^{c}
60T2	462.91 ± 1.42^{ab}	557.64 ± 6.01^{a}
80T1	461.27 ± 2.45^{ab}	592.36 ± 1.50^{e}
80T2	463.73 ± 0.00^{ab}	611.46 ± 2.60^{g}

*Mean + standard deviation. For each column, values with different letters are significantly different ($P<0.05$)

levels. Myricetin was the least abundant in both extracts while the levels of caffeic acid was higher in the TO extracts than the AV extracts.

Since there were differences in the TPC values, samples were equalized to contain same amount of polyphenols (GAE) for all the *in vitro* and *in vivo* experiments. Apart from TO 20T1 and irrespective of the fertilizer dosage and time of application, the TO extracts had significantly higher ($P<0.05$) ACE-inhibitory activities than the AV extracts (Fig. 4.1). In addition to variety effect, the fertilizer dose also had a significant effect on ACE inhibition by the polyphenol extracts (Table 4.2). However, fertilizer application time (FT) had no significant effect on ACE inhibition. The results are consistent with previous reports that showed ACE activity inhibition by phenolic-rich plant extracts (Lacaille-Dubois et al., 2001; Oboh et al., 2013). ACE inhibitors as treatment for hypertension has achieved great feat in the field of medicine. This is because

these inhibitors promote endothelia function and increased activity of bradykinin, which is a vasodilator (Ghiadoni et al., 2003). Since the same polyphenol concentration was used, the better ACE-inhibitory activity of TO suggests that the higher contents of caffeic acid may have been a contributing factor.

Fig. 4.2 shows that all the AV extracts inhibited renin activity while only the TO extracts from the 20-60 kg N fertilizer application had inhibitory activities. This trend of renin inhibition is opposite that of ACE-inhibition where TO extracts had superior effects than the AV extracts. The results obtained in this work agree with those reported by Udenigwe et al. (2009) and Malomo et al. (2015) who found no direct correlation between ACE and renin inhibitions because the mode of activity of individual enzyme is different. In contrast to the AV, the absence of detectable renin inhibition or loss of inhibition by TO extracts at 0, 60T2, and 80 suggests possible antagonistic effects between the polyphenolic compounds. The results also suggest that unlike ACE inhibition the high contents of caffeic acid in TO extracts may not be an important factor for renin inhibition. Overall, renin inhibition was influenced by all the experimental factors, including sample variety, nitrogen fertilizer dose and time of fertilizer application. Table 4.3 shows that ACE and renin inhibitions were significantly affected by vegetable variety and the urea fertilizer dose as well as the interactions between these two variables. In contrast fertilizer application time influenced ACE-inhibitory properties but not renin. The statistical analysis also confirmed that the TO extracts had significantly ($P<0.05$) higher ACE inhibitory properties than AV extracts, but the reverse was obtained for renin inhibition.

Table 4. 2. Estimated phenolic compounds in *Amaranthus viridis* (AV) and *Telfairia occidentalis* (TO) leaf aqueous extract (mg/g dry weight) *

Samples	Caffeic acid		Rutin		Myricetin	
	AV	TO	AV	TO	AV	TO
0T1	1.57 ± 0.08	6.22 ± 0.04	1.55 ± 1.34	0.05 ± 0.00	0.17 ± 0.15	0.01 ± 0.00
0T2	1.55 ± 0.04	4.19 ± 0.02	1.24 ± 0.92	0.01 ± 0.00	0.17 ± 0.05	0.01 ± 0.00
20T1	1.70 ± 0.04	5.23 ± 0.11	1.18 ± 0.15	0.06 ± 0.00	0.15 ± 0.01	0.01 ± 0.00
20T2	1.62 ± 0.01	3.64 ± 0.26	0.94 ± 0.04	0.05 ± 0.02	0.13 ± 0.01	0.01 ± 0.00
40T1	0.30 ± 0.04	7.33 ± 0.02	0.33 ± 0.02	0.07 ± 0.00	0.11 ± 0.01	0.01 ± 0.00
40T2	1.14 ± 0.94	6.66 ± 0.43	1.16 ± 0.77	0.32 ± 0.14	0.14 ± 0.03	0.01 ± 0.00
60T1	1.62 ± 0.05	7.73 ± 0.10	1.04 ± 0.54	0.07 ± 0.00	0.17 ± 0.02	0.01 ± 0.00
60T2	1.54 ± 0.02	5.24 ± 0.29	0.94 ± 0.05	0.03 ± 0.00	0.17 ± 0.02	0.01 ± 0.00
80T1	1.49 ± 0.02	4.64 ± 0.15	0.89 ± 0.01	0.03 ± 0.00	0.08 ± 0.06	0.00 ± 0.00
80T2	1.44 ± 0.06	6.88 ± 0.02	0.80 ± 0.07	0.06 ± 0.00	0.05 ± 0.00	0.01 ± 0.00

*0, 20, 40, 60, 80 indicate amount of applied urea fertilizer (kg/ha); T1 and T2 represents fertilizer applied at planting and two weeks after seedling emergence, respectively.

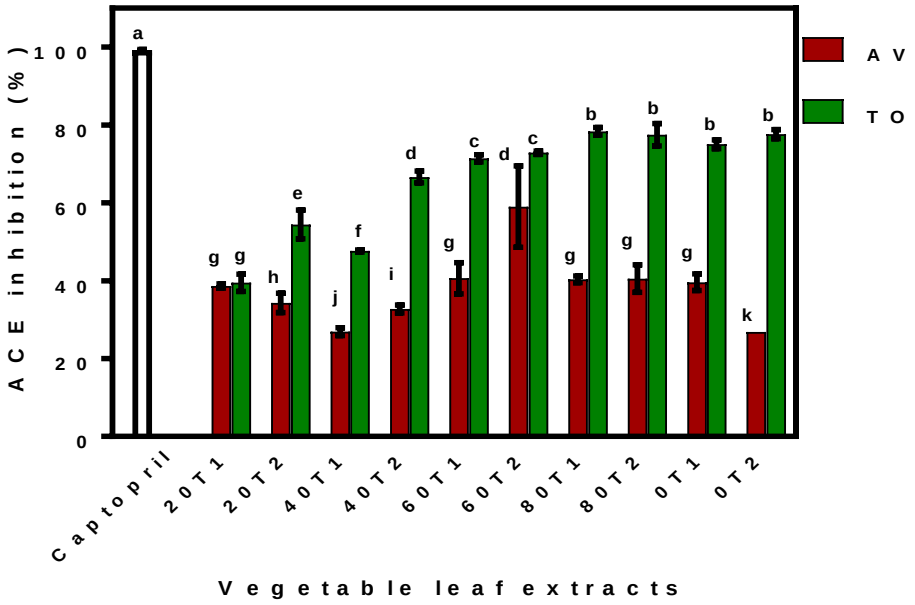

Figure 4. 1. ACE-inhibitory activity of *Amaranthus viridis* (AV) and *Telfaria occidentalis* (TO) polyphenol concentrates at 0.5 mg/mL. 0-80 represent applied nitrogen fertilizer at planting (T1) or 1 week after seedling emergence (T2)

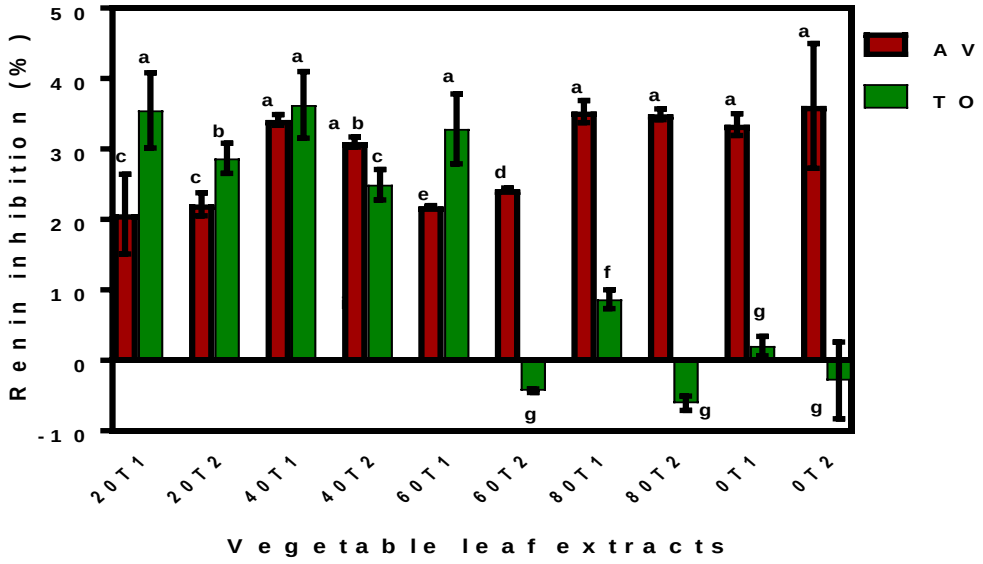

Figure 4. 2. Renin-inhibitory activity of *Amaranthus viridis* (AV) and *Telfaria occidentalis* (TO) polyphenol concentrates at 0.5 mg/mL. 0-80 represent applied nitrogen fertilizer at planting (T1) or 1 week after seedling emergence (T2)

Table 4. 3. Results from 3-way ANOVA and Duncan's test of the effects of vegetable variety, VV; fertilizer dose, D; fertilizer application time, FT; *Telfairia occidentalis*, TO; *Amaranthus viridis*, AV; (0T-80T as fertilizer dosing treatments) on ACE and renin inhibitory activities of vegetable extracts.

Parameters	Source of variation (F value)							Mean inhibition of FD				Mean inhibition of VV		Mean inhibition for application time	
	VV	D	FT	VV*D	VV*FT	D*FT	VV*D*FT	20T	40T	60T	80T	0T	AV	TO	Before	After
ACE	183.56*	12.86*	4.87*	7.31*	0.36	0.90	3.62*	41.71^{a}	43.49^{a}	60.98^{b}	59.19^{b}	56.39^{b}	38.33^{a}	66.17^{b}	54.52^{b}	49.98^{a}
								(2.39)	(2.34)	(2.34)	(2.39)	(2.34)	(1047)	(1.43)	(1.41)	(1.49)
Renin	23.43*	3.18*	0.48	10.29*	0.38	1.43	0.80	26.77^{bc}	31.58^{c}	18.67^{ab}	18.22^{ab}	17.20^{a}	30.07^{b}	15.80^{a}	23.96^{a}	21.92^{a}
								(3.43)	(3.21)	(3.21)	(3.43)	(3.21)	(2.11)	(2.06)	(2.03)	(2.14)

* Significant at $P < 0.05$; Mean inhibition values (followed in brackets by the standard error of the mean) within the same variable (FD, VV, FT) with different alphabets within the same row (parameter) are significantly different ($P < 0.05$).

4.3.2 *In vivo* reduction of blood pressure by vegetable extracts

In order to test potential antihypertensive effects, two extracts from each plant variety (AV 40T1 and AV 80T1 or TO 40T1 and TO 60T2) were orally administered to SHR. The choices were based on the need to determine whether *in vitro* ACE inhibition is a better prediction of antihypertensive effect than renin inhibition and vice versa. Thus, AV 40T1 and 80T1 had two of the highest *in vitro* renin inhibitions but low ACE inhibition. In contrast, TO 40T1 and 60T2 both had high (43-70%) ACE inhibition but TO 60T2 had no detectable renin inhibition. Fig. 4.3A shows the SBP-lowering effects of AV and TO vegetable extracts in SHRs when compared to the antihypertensive drug, captopril. Single oral administration (100 mg/kg body weight) of the vegetable extracts to SHRs resulted in varying but significant ($P<0.05$) changes in SBP up to the 24 h period when compared to the negative control (saline). A maximum SBP

decrease (-39.02 ± 3.23 mmHg) was obtained for AV 40T1 after 8 h. In contrast the AV 80T1 and TO extracts had lower SBP reductions with maximum values of approx. -24 mmHg after 4 or 8 h. For all the samples, the SBP-lowering effect gradually but significantly ($P<0.05$) diminished 24 h post-administration. The AV 40T1 had a significantly ($P<0.05$) stronger persistent effect with up to ~11 mmHg SBP reduction after 24 h when compared to the zero value for other samples. The results suggest that the AV 40T1 extract had a better resistance to enzymatic clearance from the blood circulatory system when compared to the remaining extracts. This pattern suggests that the extract was rapidly absorbed to produce maximum SBP decreases after 8 h. Interestingly, the SBP-reducing effects did not have a strict, direct relationship with observed *in vitro* ACE and renin inhibitions. However, the AV 40T1 extract with reduced *in vitro* ACE-inhibitory activity but increased renin inhibition produced most significant ($P<0.05$) SBP reductions, especially after 8 h. Disparity between the *in vivo* and *in vitro* activities of these vegetable extracts could be due to the degradation of the structure of the compounds responsible for the inhibition of ACE and renin during passage along the digestive tract. It is also possible that the polyphenols are metabolized into compounds with weaker activities in the case of TO extracts or strong inhibitory compounds for the AV 40T1. Changes in the DBP were similar to those observed for the SBP, except that there were no significant differences up to 8 h after oral administration (Fig. 4.3B). However, the AV 40T1 extract showed significantly ($P<0.05$) longer persistence with DBP values of -18 mmHg after 12 and 24 h when compared to a range of -3 to -12 mmHg for the other extracts. The results obtained in this study correlate with several reports that indicate polyphenolic-rich extracts are able to delay the onset of hypertension and also regulate blood pressure (Sarr et al., 2006). Taubert et al. (2003) reported that polyphenols from cocoa reduced blood pressure in elderly humans with mild isolated hypertension while Negishi et al. (2004) showed that black tea

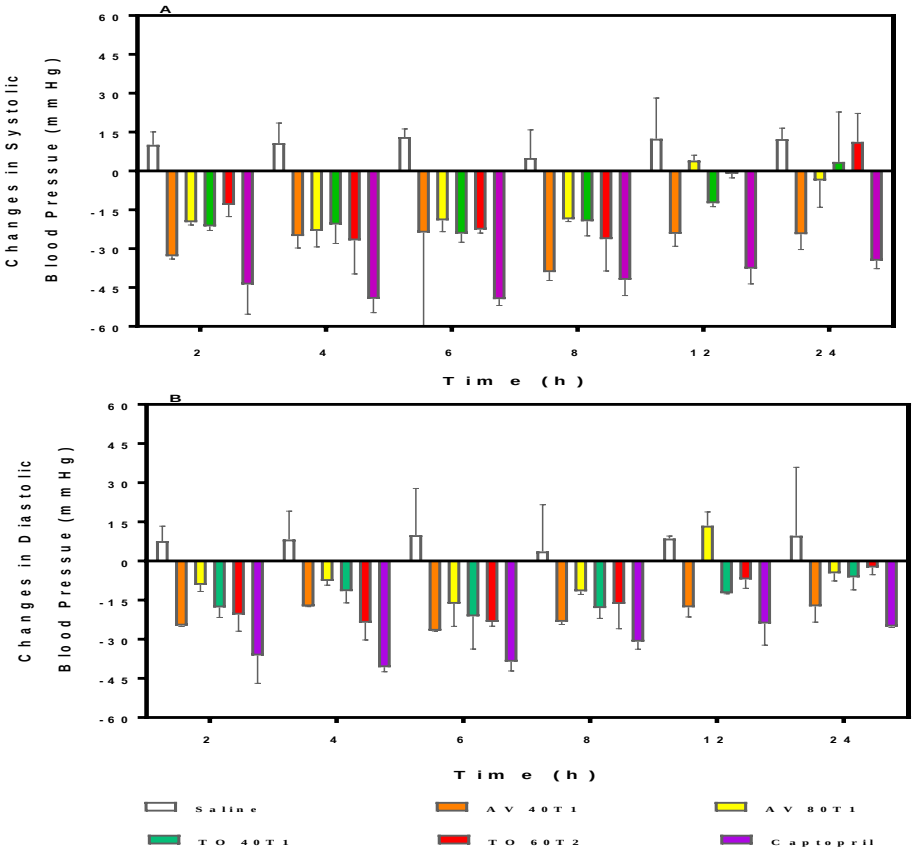

Figure 4. 3. (A) Changes in Systolic and (B) Diastolic blood pressure (mmHg) of spontaneously hypertensive rats after oral administration of 100 mg/kg body weight polyphenol concentrates from *Amaranthus viridis* (AV 40T1and AV 80T2), and *Telfairia occidentalis* f. (TO 40T1 and TO 60T2). 40, 60, and 80 represent applied nitrogen fertilizer at planting (T1) or 1 week after seedling emergence (T2)

polyphenols reduced blood pressure in stroke-prone spontaneously hypertensive rats. Recently, a polyphenol concentrates from *Ocimum gratissimum* leaves had blood pressure-reducing effects after oral administration of 500 mg/kg body wt to SHR (Shaw et al. 2017). Since the antihypertensive effects of the AV and TO extracts were evident at 100 mg/kg bw, they can be considered to be more potent than *O. gratissimum* leaf extracts.

4.3.3 Effect of vegetable extracts on MAP and HR

Mean arterial pressure (MAP) is how well the body can process oxygenated blood that is delivered to the tissues and organs. It represents the mean blood pressure of an individual and is a measure of how well the heart can pump blood and the rate of flow of blood from the arteries (Henry et al., 2002). Therefore, high MAP values (>140 mmHg) indicate a high workload for the heart, and this may lead to heart attack, stroke and heart disease. When MAP is low (<60 mmHg), this signifies that blood circulations to the necessary organs of the body are hindered and this can lead to shock. As shown in Fig. 4.4, single oral administration of the vegetable extracts to SHRs induced a reduction in mean arterial blood pressure (MAP). The AV 40T1 produced significantly better reductions in MAP especially after 6-8 h of oral administration and had the most persistent effect when compared to the other samples.

Heart rate (HR) is an important factor in the assessment of hypertension, and it is the number of times the heart beats per minute. The vegetable extracts reduced HR of the SHRs with persistence of up 12 and 24 h for AV samples. The AV 80T1 showed the most reductions with 107 ppm after 8 h and -73 after 24 h. An increase in heartbeat rate is associated with increased blood pressure and possible risk of cardiovascular disease (James & Friday, 2011; Reule & PE, 2012). The result obtained revealed that the vegetable extracts reduce MAP and HR with AV and TO extracts having long lasting effects. The long-lasting effects of AV and TO on heart rate indicate that they may be effective antihypertensive agents or could function as ACE and/or renin inhibitors in hypertensive patients. The results are consistent with a recent report that showed significant heart rate lowering effect after consumption of an anthocyanin-rich beverage by human volunteers over a 24-h test period (Igwe et al., 2017).

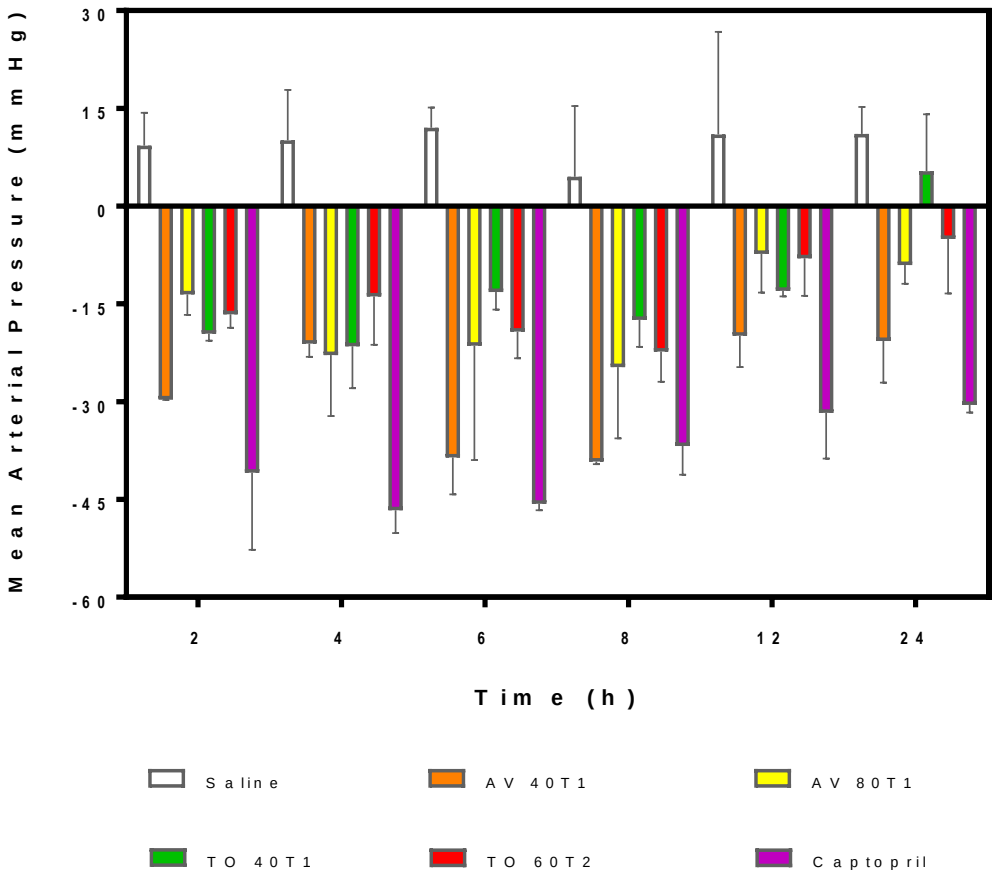

Figure 4. 4. Changes in mean arterial blood pressure (mmHg) of spontaneously hypertensive rats after oral administration of 100 mg/kg body weight polyphenol concentrates from *Amaranthus viridis* (AV 40T1and AV 80T2), and *Telfairia occidentalis* f. treatments (TO 40T1 and TO 60T2). 40, 60, and 80 represent applied nitrogen fertilizer at planting (T1) or 1 week after seedling emergence (T2)

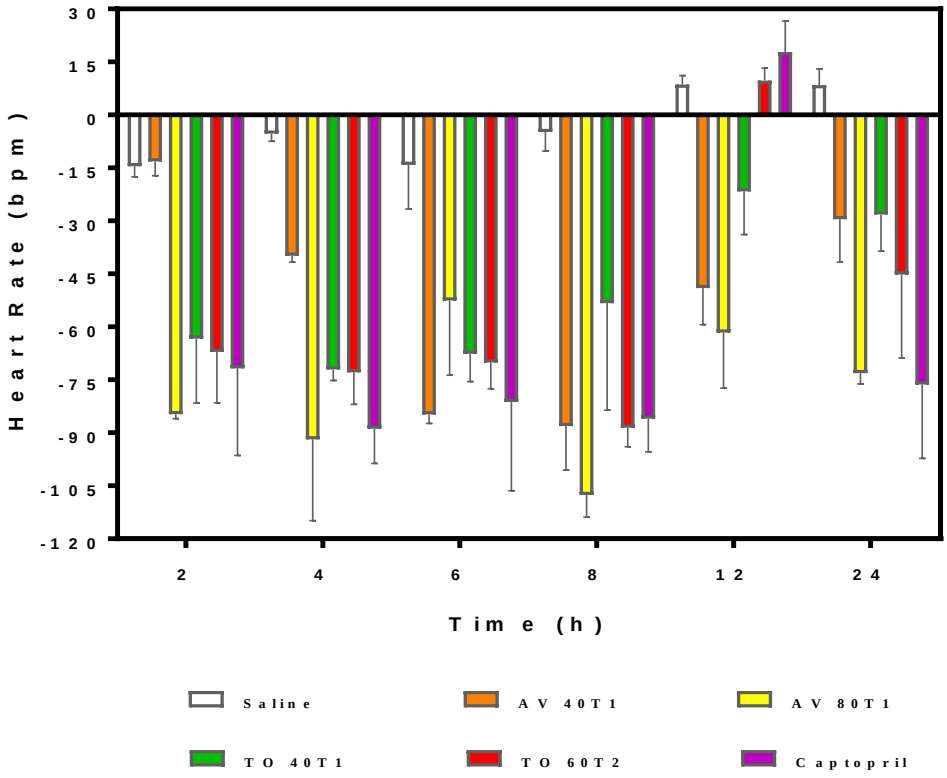

Figure 4. 5. Changes in heart rate (bpm) of spontaneously hypertensive rats after oral administration of 100 mg/kg body weight polyphenol concentrates from *Amaranthus viridis* (AV 40T1and AV 80T2), and *Telfairia occidentalis* f. treatments (TO 40T1 and TO 60T2). 40, 60, and 80 represent applied nitrogen fertilizer at planting (T1) or 1 week after seedling emergence (T2)

4.4. Conclusion

The aqueous extracts of AV and TO leaves were composed mainly of polyphenols and possessed inhibitory activities against ACE and renin under *in vitro* conditions with TO extracts having higher ACE inhibitory activity than the AV. In contrast, the AV extracts had better renin inhibition than the TO. Oral administration to SHRs showed greater reductions in blood pressure and heart rate by the AV extract. There was no direct relationship between antihypertensive effects and *in vitro* enzyme (ACE and renin) inhibition patterns, which suggest that *in vivo* mode of action, may be different. Overall results suggest that these vegetable extracts could be used as functional food ingredients with therapeutic potential in the treatment or prevention of

hypertension. However, future works that orally administer the main phenolic compounds (especially caffeic acid and rutin) to the SHRs will be required to confirm the blood pressure-reducing effects of the leaf polyphenol extracts.

4.5 Acknowledgement

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4.7 TRANSITION STATEMENT TWO

The previous chapter established the antihypertensive properties of *Amaranthus viridis* and *Telfaria occidentalis*, which can be used as therapeutic agent in treating human disease conditions, including hypertension and oxidative stress-induced diseases. The next manuscript addressed the use of the third vegetable *Solanum macrocarpon* as an antihypertensive agent through the *in vitro* inhibition of ACE and renin activities as well as *in vivo* lowering of blood pressure and heart rate in spontaneously hypertensive rats. The study also quantified the major phenolic compounds that were present in the extract as well as the chlorophyll content. It was established that the vegetable extracts inhibited *in vitro* activities of renin and ACE to varying extents and also reduced heart rate, systolic and diastolic blood pressure. Rutin, caffeic acid and myricetin were the phenolic compounds present in the extract. The vegetable extract could be used to formulate functional foods that could help ameliorate cardiovascular disease conditions.

CHAPTER FIVE

MANUSCRIPT THREE

SOLANUM MACROCARPON LEAF EXTRACTS REDUCED BLOOD PRESSURE AND HEART RATE AFTER ORAL ADMINISTRATION TO SPONTANEOUSLY HYPERTENSIVE RATS

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REA: Conceptualization, funding acquisition, and project administration. REA, AMA and OAO methodology. REA: resources. OAO: writing—original draft preparation. REA and AMA: writing—review and editing. OAO: formal analysis. REA and AMA: supervision. All authors have read and agreed to the submitted version of the manuscript

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Abstract

The aim of this work was to determine the ability of polyphenol-enriched aqueous vegetable leaf extracts to ameliorate blood pressure and heart rate dysfunctions in spontaneously hypertensive rats (SHRs). *Solanum macrocarpon* (SM), a common edible vegetable leaf in West Africa was cultivated using varying levels (0-80 kg/ha) of urea that was applied at planting (T1) or 2 weeks after planting (T2). *In vitro* analysis showed that total polyphenolic compounds varied from 489.87 $\pm$ 5.66 to 513.56 $\pm$ 1.42 gallic acid equivalent/g dry wt of extract. Rutin, caffeic acid and myricetin were the major polyphenols quantified in the extracts. All the extracts inhibited *in vitro* activities of renin and angiotensin converting enzyme to varying extents. Two extracts (SM40T1 and SM80T2) were orally administered to SHRs at 100 or 500 mg/kg body wt. Results showed that systolic blood pressure was reduced to a similar level by SM40T1 (10.8 mmHg) and SM80T2 (10.5 mmHg) for the 100 mg/kg body wt dose; the 500 mg/kg body wt dose produced similar values. Diastolic blood pressure and heart rate were also depressed by the two extracts with similar effects observed for both doses. We conclude that the leaf extracts could serve as potential ingredients to formulate antihypertensive functional foods and nutraceuticals.

Keywords: *Solanum macrocarpon*; polyphenols; renin; angiotensin converting enzyme; blood pressure, heart rate; spontaneously hypertensive rats; telemetry

5.1 Introduction

Hypertension is a lifestyle-related disease of public health concern that affects about 25% of the world's population and it is likely to increase to about 30% in 2025 (Collaboration, 2017; Domanski et al., 2002; Whelton et al., 2002). Hypertension is characterized by elevated blood pressure (systolic and diastolic blood pressures >140 and 90 mmHg, respectively), which makes it difficult for the heart to pump blood (Carretero et al., 2000; Pickering et al., 2005; Sundström et al., 2015). Excessive high blood pressure is a primary risk factor for heart disease, myocardial infarction, stroke, pulmonary embolism, and renal failure (Pickering et al., 2005; Beevers et al., 2007; Franklin et al., 2012; WHO, 2013). Dietary modifications such as low body weight, low sodium intake, consumption of foods rich in fruits and vegetables, low alcohol consumption and low-fat foods have been proven for the management of hypertension. Development of hypertension is linked to the renin-angiotensin-aldosterone system (RAAS) in which renin and angiotensin-converting enzyme (ACE) play significant roles (Acharya et al., 2003; Aluko, 2015). The rate-determining step involves renin, which converts angiotensinogen into angiotensin I; ACE then hydrolyzes angiotensin I to form angiotensin II, a powerful vasoconstrictor whose excessive level is one of the factors responsible for maintaining high blood pressure (Acharya et al., 2003; Allen et al., 2012; Aluko, 2015). Therefore, inhibition of renin and ACE activities is key to controlling excessive rise in blood pressure. Several antihypertensive drugs such as captopril, lisinopril and enalapril (ACE inhibitors) or aliskiren (renin inhibitor) have been used to treat hypertension and to suppress its symptoms (Chen et al., 2013). However, the efficiency of these drugs is only 40 to 60% and in some cases, two or more antihypertensive drugs are required to be combined to get the desirable results thereby increasing treatment cost and side effects (Bangalore et al., 2007; Otari et al., 2012; Sultana & Asif, 2017).

In addition to the treatment cost, several negative side effects such as cough, diarrhea, loss of appetite, dizziness, angioedema, erectile dysfunction, and congenital deformations have been associated with antihypertensive drug therapy (Israili, 1992; Yesil et al., 1994; Blumentals et al., 2003; Cooper et al., 2006; Abassi et al., 2009). Therefore, there is need to search for alternative antihypertensive compounds from natural sources with reduced potential for toxicity (Otari et al., 2012). For example, it has been estimated that 75 to 80% of the world's population depend on traditional medicines for their health care needs due to lesser negative side effects and high level of tolerance within the human systems (Cunningham, 1993; Tabgoto and Townson, 2001). Polyphenols are a class of phytochemicals that have been shown to have potential utilization as antihypertensive agents because of their ability to inhibit ACE and renin activities (Shaw et al., 2017; Xie and Zhang, 2012). In fact, blood pressure-reducing effects have been demonstrated for various polyphenol-rich foods (Alashi et al., 2018; Gunathilake et al., 2013; Hellstrom et al., 2010; Hobbs et al., 2012; Igwe et al., 2017; Kivimaki et al., 2013) and polyphenol concentrates (Olarewaju et al., 2018; Shaw et al., 2017).

Solanum macrocarpon (SM) also known as gboma eggplant is a vegetable crop grown in the non-arid parts of Africa mainly for its edible leaves and fruits (Bukenya-Ziraba and Bonsu, 2004). It is a small tropical perennial plant found in several African countries, especially Nigeria, Ethiopia, Cameroon, Sierra Leone and Zimbabwe. It can also be found in some parts of Southeast Asia, Brazil and Southern Europe (Yamaguchi, 1983). The leaf of this plant is nutritionally dense with a protein content of 4.3% fresh weight in addition to 32.6 and 8.2 mg/kg for Ca and Zn contents, respectively (Oboh et al., 2005). The leaves are consumed as vegetable soup while the fruits are edible only when cooked. This plant is used in traditional medicine for the management of various diseases such as bronchitis, aches, asthma, inflammatory tumors, rheumatism, and Parkinson's disease (Stoker, 1995). The extracts of this vegetable have been

reported to have various pharmacological effects, including antimicrobial, antidiabetic, antihyperlipidemic and wound healing properties (Nwanna et al., 2016; Oboh et al., 2010). They are also rich in phenolic compounds, which could be used to enhance blood pressure. Since the vegetable is cheap and readily available, its consumption may provide health benefits by functioning as RAAS modulators (Actis-Goretta et al., 2006). Therefore, the aim of this study was to investigate the *in vitro* renin and ACE-inhibitory activities of SM leaf aqueous extracts. The most active extracts were subsequently tested for blood pressure-reducing effects in spontaneously hypertensive rats (SHRs).

5.2 Materials and Methods

5.2.1 Materials

Vegetable plants were cultivated at the Obafemi Awolowo University, research farm located in Ile-Ife, Osun State, Nigeria. Nitrogen (urea) fertilizer treatments (kg/ha) were administered to the vegetable plants at different doses and denoted as 0T, 20T, 40T, 60T and 80T. Two trials of each fertilizer application were carried out and the samples harvested SM0T1 SM0T2, SM20T1, SM20T2, SM40T1, SM40T2, SM60T1, SM60T2, SM80T1, and SM80T2. Fertilizer dose was applied to the vegetables at planting time (T1) or 2 weeks after planting (T2). Leaves were harvested by plucking, dried in hot air cabinet at 60°C and milled in a Marlex Excella dry mill (Marlex Appliances PVT, Daman, India) to obtain the powder. The ground powders were stored at -20°C prior to analysis. N-(3-[2-furyl] acryloyl)-phenylalanylglycylglycine (FAPGG), and ACE from rabbit lung (E.C.3.4.15.1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A Human Recombinant Renin Inhibitor Screening Assay Kit was purchased from Cayman Chemicals (Ann Arbor, MI, USA). All other reagents were of analytical grade and purchased from Fisher Scientific (Oakville, ON, Canada), except stated otherwise.

5.2.2 Polyphenol extraction

Extraction of the water-soluble polyphenols was carried out according to the method of Olarewaju et al. (2018). Briefly, each sample was extracted using distilled water at 1:20 ratio of dried leaf powder to water for 2 h in a beaker with continuous stirring at 60°C. The extract was centrifuged at 10000*g* for 30 min, the supernatant filtered through cheesecloth and the process repeated to obtain a second supernatant. Both supernatants were combined, concentrated under vacuum using a rotatory evaporator at 60°C, freeze-dried and stored at -20°C. The total polyphenolic content (TPC) of each extract was determined using the Folin–Ciocalteu method as previously described (Olarewaju et al., 2018) and expressed as milligrams gallic acid equivalent (GAE) per gram dry leaf extract (mg GAE/g).

5.2.3 Renin inhibition assay

In vitro inhibition of the activity of human recombinant renin assay was conducted according to the method described by Li and Aluko (2010) using the Renin Inhibitor Screening Assay Kit. Prior to the assay, renin buffer was diluted with 50 mM Tris–HCl, pH 8.0, containing 100 mM NaCl. The renin protein solution was diluted 20 times with assay buffer before use, and the assay buffer was pre-warmed to 37ºC before initiating the reaction in a fluorometric microplate reader (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA) maintained at 37ºC. The reaction mixture contained: (1) 20 µL substrate, 160 µL assay buffer, and 10 µL Milli-Q water for the background wells; (2) 20 µL substrate, 150 µL assay buffer, and 10 µL Milli- Q water for control (uninhibited) wells; and (3) 20 µL substrate, 150 µL assay buffer, and 10 µL polyphenol extract sample (0.25 mg GAE/mL final assay concentration) for the inhibited wells. The reaction was initiated by adding 10 µL renin to the inhibited and uninhibited wells only. The microplate was shaken for 10 s to mix and incubated at 37ºC for 15 min followed by recording fluorescence intensity (FI) using an excitation wavelength of 340 nm and emission wavelength

of 490 nm. FI of the background wells were subtracted from those of the control and sample wells and percentage inhibition calculated as follows:

$$\text{Renin inhibition (\%)} = \frac{\text{FI of control well} - \text{FI of sample well}}{\text{FI of control well}} \times 100$$

5.2.4 ACE inhibition assay

Ability of the polyphenolic extracts to inhibit *in vitro* activity of ACE was measured according to a spectrophotometric method using FAPGG as the substrate (Holmquist et al., 1979). Briefly, 20 µL of dried leaf extract sample dissolved in 50 mM Tris-HCl buffer containing 0.3 M NaCl, pH 7.5 (0.5 mg GAE/mL final assay concentration) was mixed with 10 µL of ACE (20 mU final reaction activity) and 170 µL of 0.5 mM FAPGG (dissolved in the same buffer) in a 96-well microplate. The rate of decrease in absorbance (ΔAbs) at 345 nm was recorded for 30 min at 37^{o}C using a microplate reader. The buffer was used instead of sample solution in the blank (uninhibited) reaction mixture.

$$\text{ACE inhibition (\%)} = \frac{\Delta\text{Abs of blank} - \Delta\text{Abs of sample}}{\Delta\text{Abs of blank}} \times 100$$

5.2.5 Determination of major polyphenolic compounds contents

The major polyphenol constituents of SM leaf extracts were quantified on a Varian 900-LC series analytical HPLC (Phenomenex Inc., Torrance, CA, USA) using Phenomenex C18 HPLC column (250 mm × 4.6 mm, 5 µm) heated to 30°C. The mobile phases consisted of 1% acetic acid as solvent A and methanol as solvent B. Gradient elution was performed using: 95% A to 5% B for 35 min; 80% A to 20% B for 15 min; 40% A to 60% B for 10 min and 70% A and 30% B for 15 min at 1 mL/min flow rate. Caffeic acid, catechin, gallic acid, rutin, myricetin and quercetin were used as standards and various concentrations of each were injected using 20 µL solutions. Eluted polyphenols were detected at wavelengths of 280 and 320 nm. The level of

each sample polyphenolic peak was estimated using the calibration curve generated from the linear plot of elution time versus peak area of each standard polyphenol compound and the results expressed as mg/g of dry leaf extract.

5.2.6 Estimation of chlorophyll content

Dried leaf powders (0.1 g) were extracted with 40 mL of 80% acetone according to previous methods (Hiscox and Israelstam, 1979; Rajalakshmi and Banu, 2015) and the extract centrifuged at 7500*g* for 5 min. The supernatant was transferred into a glass container and the procedure was repeated until the residue became colorless. Absorbance of the extract was read at 645 nm (A645) and 663 nm (A663) against the solvent (80% acetone) blank. The concentrations of chlorophyll a, chlorophyll b and total chlorophyll were calculated using the following equation (Rajalakshmi and Banu, 2015):

Total chlorophyll: 20.2(A645) + 8.02(A663)
Chlorophyll a: 12.7(A663) – 2.69(A645)
Chlorophyll b: 22.9(A645) – 4.68(A663)

5.2.7 Blood Pressure Measurements

SHRs at 6 weeks of age were purchased from Charles River Laboratories (Montreal, PQ, Canada) and maintained on ad libitum access to regular chow feed and tap water under 12-h day and night cycle at 21 °C for 2 weeks. Telemetry sensors were then surgically implanted into the rats according to our previously described protocols (O'Keeffe et al., 2017) followed by a 2-week recovery period. The experimental groups were assigned 4 rats each, which were subjected to oral administration (1 mL) of the following test agents dissolved in phosphate buffered saline (PBS): PBS only (negative control); 20 mg/kg body weight captopril (antihypertensive drug as a positive control); 100 or 500 mg GAE/kg body weight of polyphenol extract. After placing each rat cage on the respective receiver (Model RPC-1, DSI instruments, St. Paul, MN, USA), the real-time changes in heart functions were then continuously measured as systolic blood pressure

(SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rates (HR). All the rat protocols were approved by the University of Manitoba Animal Care Committee. Data were recorded at 10 min intervals for 24 h using the Ponemah 6.1 data acquisition software (DSI instruments) that was linked to an APR-1 atmospheric-pressure monitor (DSI instruments). Attachment of the APR-1 enabled normalization of the transmitted pressure values to produce blood pressure signals independent of atmospheric pressure variations. Changes in SBP, DBP, MAP and HR were reported using values obtained at 2, 4, 6, 8, 12 and 24 h.

5.2.8 Statistical Analysis

Analyses were conducted in replicates and data analyzed by one-way analysis of variance (ANOVA) for *in vitro* and *in vivo* assay. Data were reported as mean ± standard deviation. Statistical significance of differences was evaluated by Duncan's multiple range test ($p < 0.05$) using the IBM SPSS Statistical package (version 24).

5.3 Results and Discussion

5.3.1 TPC, polyphenol and chlorophyll contents

The TPC and polyphenol composition of the samples are shown in Table 5.1 and the data indicate that the extracts were mainly composed of polyphenols with values ranging from 496 to 513 mg GAE/g of dried extracts for SM 20T1 and SM 0T2, respectively. The values are similar to those reported for the leaf extracts of *Amaranthus viridis* and *Telfairia occidentalis* (Olarewaju et al., 2018). The HPLC analysis revealed the presence of three major compounds (caffeic acid, rutin, and myricetin) in the *Solanum macrocarpon* extracts. These flavonoid compounds are potent antioxidants with strong radical scavenging activity (Ibrahim et al., 2013; Seal, 2016). All the vegetable extracts at the different fertilizer doses contain all the identified compounds with rutin present in the highest concentration for SM 60T1 (6.95 µg/mg dry wt) while SM 20T2 had significantly the highest level of caffeic acid at 2.60 µg/mg dry wt. In

contrast, the SM 20T2 and SM 80T2 had highest ($p < 0.05$) levels of myricetin at 0.35 and 0.34 µg/mg dry wt, respectively. In general, there was a trend towards higher content of polyphenolic compounds in extracts from plants with fertilizer dose applied two weeks after planting (T2) when compared to T1. The results suggest there may be more nitrogen available for polyphenol synthesis after the plants have already emerged from the soil. But with respect to each of the 3 major polyphenolic compounds, there was no observable trend in terms of effect of time of fertilizer application. However, myricetin was present at the lowest concentration, which suggests it is a minor component when compared to caffeic acid and rutin. The caffeic acid contents obtained in this work are lower than those previously reported for the aqueous extracts of *Telfairia occidentalis* (Olarewaju et al., 2018). In contrast, the SM extracts had higher rutin and myricetin levels when compared to the *T. occidentalis* extracts.

Chlorophyll is a greenish pigment found in many higher plants and algae. It occurs in two forms as chlorophyll *a* and chlorophyll *b*. Chlorophyll *a* has a green-blue color while chlorophyll *b* has a green-yellow color (Arnon, 1949). Chlorophyll content of the leaf extracts (Table 5.2) was determined, and the highest total chlorophyll content was detected in SM 40T1 (108.5 mg/g) followed by SM 80T1 (106.4 mg/g). Chlorophyll has a good antioxidant property that could nullify the effect of toxic free radicals in the body. Chlorophylls also function therapeutically to purify the blood from toxins, prevent cancer, inhibit formation of kidney stone, normalize blood pressure, detoxify the kidney as well as prevent bad breath and body odour (Mishra et al., 2011). Studies have also shown chlorophyll to prevent the interaction of carcinogen and DNA in target cells by inhibiting the carcinogen enzyme activator (Mishra et al., 2011).

Table 5. 1. Total phenolic content (TPC, mg gallic acid equivalent/g dry weight) and estimated major phenolic compounds (µg/mg dry weight) in *Solanum macrocarpon* leaf aqueous extract. For each column, values (mean ± standard deviation) with different letters are significantly different ($p < 0.05$).

Samples	**Rutin**	**Caffeic**	**Myricetin**	**TPC**
SM20T1	1.23 ± 0.15^{ab}	1.97 ± 0.06^{c}	0.21 ± 0.02^{cd}	496.41 ± 1.42^{ab}
SM20T2	1.83 ± 0.98^{abc}	2.60 ± 0.09^{f}	0.35 ± 0.05^{f}	507.84 ± 2.45^{bcd}
SM40T1	2.36 ± 0.99^{bcd}	2.21 ± 0.08^{d}	0.23 ± 0.04^{d}	498.04 ± 4.90^{abc}
SM40T2	0.20 ± 0.01^{a}	1.50 ± 0.00^{a}	0.10 ± 0.01^{a}	496.41 ± 1.42^{ab}
SM60T1	6.95 ± 2.36^{e}	1.70 ± 0.06^{ab}	0.22 ± 0.03^{d}	498.86 ± 1.42^{abc}
SM60T2	3.60 ± 0.03^{cd}	1.50 ± 0.01^{a}	0.12 ± 0.01^{b}	501.31 ± 1.42^{abcd}
SM80T1	2.50 ± 1.29^{bcd}	2.38 ± 0.19^{de}	0.32 ± 0.09^{ef}	489.87 ± 5.66^{a}
SM80T2	3.85 ± 0.06^{d}	2.25 ± 0.01^{d}	0.34 ± 0.01^{f}	510.29 ± 5.20^{cd}
SM0T1	1.06 ± 0.32^{ab}	2.53 ± 0.30^{ef}	0.26 ± 0.05^{de}	506.21 ± 1.42^{bcd}
SM0T2	1.10 ± 0.58^{ab}	1.89 ± 0.03^{bc}	0.15 ± 0.02^{bc}	513.56 ± 1.42^{d}

Table 5. 2. Chlorophyll content (mg/g) of *Solanum macrocarpon* leaf extracts. Values (mean ± standard deviation) with different letters within the same column are significantly different ($p < 0.05$).

Samples	Chlorophyll a	Chlorophyll b	Total chlorophyll
SM20T1	42.4 ± 0.03	20.3 ± 0.01	62.8 ± 0.04
SM20T2	51.6 ± 0.00	29.6 ± 0.04	81.4 ± 0.04
SM40T1	62.9 ± 0.04	46.2 ± 0.10	108.5 ± 0.14
SM40T2	69.4 ± 0.01	32.1 ± 0.01	101.5 ± 0.02
SM60T1	64.7 ± 0.02	32.1 ± 0.03	96.9 ± 0.01
SM60T2	66.7 ± 0.01	30.4 ± 0.02	97.2 ± 0.03
SM80T1	72.9 ± 0.01	33.6 ± 0.01	106.4 ± 0.01
SM80T2	52.9 ± 0.01	25.8 ± 0.02	78.7 ± 0.02
SM0T1	45.8 ± 0.01	20.8 ± 0.00	66.6 ± 0.01
SM0T2	71.2 ± 0.01	31.1 ± 0.04	102.5 ± 0.05

Therefore, depending on bioavailability, the total chlorophyll content could contribute to the ameliorative effect of leaf extracts against cardiac disorder in the SHR model of hypertension used in this work.

5.3.2 *In vitro* ACE and renin inhibition

Since the leaf extracts had varied TPC values, ability to inhibit *in vitro* activities of ACE and renin was evaluated using 0.5 mg/mL and 0.25 mg GAE/mL, respectively in order to ensure the presence of same amount of polyphenolic compounds in each enzyme assay mixture. As shown in Fig. 5.1, all leaf extracts had the capacity to inhibit ACE activity, though to varying degrees. ACE-inhibitory activity ranged from 75.4% for 80T1 to 35.5% for SM60T2 with significant ($p < 0.05$) differences between the samples. Apart from the 80 kg/ha urea dose (SM80T1 and SM 80T2), application of nitrogen fertilizer led to decreases in ACE-inhibitory efficiency of the leaf extracts. The results contrast with the work of Olarewaju et al. (2018) who reported stronger ACE inhibition by aqueous leaf extracts of *T. occidentalis*. The differences may be due to

variations in the major polyphenol compounds present in the leaf extracts. This is because the *S. macrocarpon* extracts had similar contents of caffeic acid and rutin while *T. occidentalis* extracts were dominated by caffeic acid only. The time of fertilizer application also significantly influenced the rate at which ACE was inhibited. The *S. macrocarpon* extracts with fertilizer dose applied at the time of planting (T1) had higher ACE inhibition than those harvested from plants treated with fertilizer two weeks after planting (T2). In contrast, the *T. occidentalis* and *Amaranthus viridis* leaves T1 extracts had weaker ACE-inhibitory activity than the T2 extracts (Olarewaju et al., 2018). The results obtained in this work also suggest stronger ACE-inhibitory activity of the *S. macrocarpon* leaf extracts when compared to guava leaf extracts that were also assayed at 0.5 mg/mL (Ademiluyi et al., 2016). Differences in contents of polyphenolic compounds may have contributed to the observed variations in the effect of fertilizer application time on ACE activity inhibition by the leaf extracts.

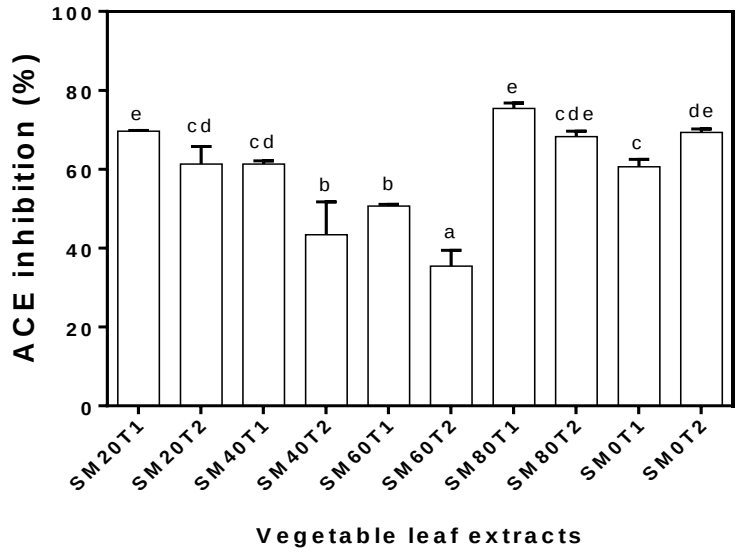

Figure 5. 1. ACE-inhibitory activity of aqueous leaf extracts of *Solanum macrocarpon* (SM) at 5 mg gallic acid equivalent/mL assay concentration: 0-80 represent urea fertilizer doses applied at planting (T1) or 2 weeks after planting (T2). Bars having different letters have significantly ($p < 0.05$) mean values.

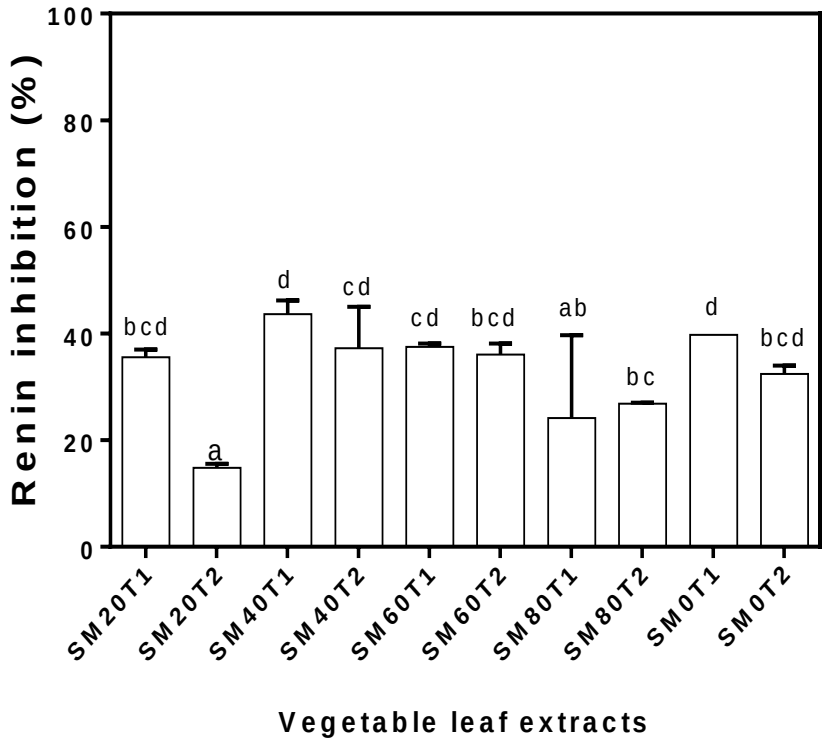

Figure 5. 2. Renin-inhibitory activity of aqueous leaf extracts of *Solanum macrocarpon* (SM) at 0.25 mg gallic acid equivalent/mL assay concentration: 0-80 represent urea fertilizer doses applied at planting (T1) or 2 weeks after planting (T2). Bars having different letters have significantly ($p < 0.05$) mean values.

Unlike ACE inhibition, ability of the *S. macrocarpon* to inhibit renin activity was fairly consistent across the samples, with the exception of SM20T2, which had the least inhibition (Fig. 5.2). The results suggest that differences in major polyphenolic compounds as shown in Table 5.1 did not have any significant effect on the renin-inhibitory ability of the polyphenolic extracts. Therefore, the mode of ACE inhibition is different from that of renin, which is consistent with previous reports showing that the impact of polyphenolic inhibitors differs for the two enzymes. For example, *Gongronema latifolium* polyphenolic extract inhibited ACE activity through a mixed-type mode, indicating binding to the free enzyme in addition to the enzyme-substrate complex (Ajibola et al., 2011). In contrast, the *G. latifolium* polyphenolic extract showed uncompetitive inhibition of renin activity, which suggests binding only to the enzyme-substrate

complex. ACE and renin are key enzymes responsible for maintaining vascular tension in the mammalian body and their inhibition is, therefore, an important therapeutic tool in the treatment of high blood pressure (Aluko, 2019). Based on the *in vitro* ACE and renin inhibitory patterns, SM40T1 and SM80T2 extracts were selected for *in vivo* antihypertensive studies because the two samples had strong inhibitions of these enzymes.

5.3.3 Blood pressure changes

Table 5.3 shows the SBP-lowering effects of SM leaf extracts in SHRs when compared to the antihypertensive drug, captopril. Single oral administration of the vegetable extracts to SHRs resulted in varying but significant ($p < 0.05$) changes in SBP up to the 24 h period when compared to the negative control (saline). The maximum SBP decrease (175.3 ± 3.23 mmHg) was obtained 8 h after oral administration of SM 40T1 using a 100 mg/kg dose; this represented a 10.8 mmHg reduction in SBP. A similar 10.5 mmHg decrease in SBP was obtained for SM80T2 at the same dosage. However, the antihypertensive effect of SMT2 was achieved after 4 h, which is 50% faster than the effect of SM40T1. The faster activity of SM80T2 suggests that the polyphenolic compounds were absorbed quicker than those present in SM40T1. This may be due to the higher concentration of rutin in SM80T2 extract when compared to SM40T1. Rutin is a precursor of quercetin, which has been shown to be bioavailable with potential blood pressure-reducing ability (Serban et al., 2016). Increasing the polyphenol dose to 500 mg/kg rat body wt produced similar SBP changes as the lower 100 mg/kg dose, which suggests that absorption of additional amounts of polyphenolic compounds did not occur despite the higher dose.

Table 5. 3. Changes in systolic blood pressure (SBP) after oral administration of *Solanum macrocarpon* leaf extracts to spontaneously hypertensive rats: 40 and 80 represent urea fertilizer dose (kg/ha) applied at planting (T1) or 2 weeks after planting (T2) while 100 and 500 are the leaf extract doses (mg/kg rat body wt). Values (mean ± standard deviation) with the different letters within the same column are significantly different ($p < 0.05$).

	SBP (mmHg)					
Time (h)	**Saline**	**Captopril**	**$40T1_{100}$**	**$80T2_{100}$**	**$40T1_{500}$**	**$80T2_{500}$**
0	184.2 ± 8.5^{a}	220.1 ± 19.1^{c}	186.1 ± 4.8^{bc}	198.4 ± 1.3^{b}	186.1 ± 4.8^{bc}	198.4 ± 1.3^{b}
2	183.8 ± 15.2^{a}	186.9 ± 1.3^{a}	192.97 ± 1.4^{cd}	195.6 ± 1.7^{b}	185.81 ± 5.1^{bc}	199.3 ± 4.4^{b}
4	184.9 ± 1.7^{a}	180.9 ± 3.2^{a}	190.3 ± 1.7^{bcd}	187.9 ± 0.8^{a}	186.4 ± 1.1^{bc}	210.5 ± 4.1^{bc}
6	199.7 ± 14.8^{ab}	183.6 ± 7.8^{a}	183.0 ± 1.1^{b}	195.1 ± 3.4^{b}	185.1 ± 1.3^{b}	215.0 ± 13.5^{c}
8	191.1 ± 7.4^{a}	192.8 ± 17.2^{ab}	175.3 ± 6.5^{a}	194.8 ± 5.3^{b}	181.0 ± 1.4^{ab}	187.4 ± 1.6^{a}
12	222.4 ± 5.1^{b}	196.3 ± 7.7^{ab}	196.1 ± 0.5^{d}	212.7 ± 0.8^{c}	177.8 ± 1.8^{a}	216.8 ± 1.9^{c}
24	204.1 ± 21.4^{ab}	206.0 ± 10.0^{b}	209.9 ± 1.1^{e}	224.1 ± 1.2^{d}	192.6 ± 0.7^{c}	196.5 ± 0.1^{b}

For example, there were 8.3 and 11.0 mmHg maximum decreases in SBP for SM40T1 and SMT2 extracts, respectively. Thus, increasing the polyphenolic dose did not enhance bioavailability. In fact, the SBP-reducing effects at 500 mg/kg were delayed as the maximum reductions were achieved at 12 h for SM40T1 and 8 h for SM80T2, when compared to 8 and 4 h, respectively at the 100 mg/kg dose. The delay in SBP-reducing effect at the 500 mg/kg dose could be attributed to increased viscosity of the sample solution. This is because for the oral administration to SHRs, both doses were dissolved in the same volume (1 mL) of PBS and the higher dose was clearly more viscous than the lower dose. Thus, it is possible that the increased viscosity at the 500 mg/kg dose delayed absorption, hence the SBP-reducing effect was also delayed. Overall, the SBP-reducing effect of the *S. macroacarpon* leaf polyphenolic extracts was weaker than captopril (antihypertensive drug), which produced a maximum decrease of 36.6 mmHg after 6 h. The *S. macroacarpon* extracts are also weaker antihypertensive agents than

similar extracts of *A. viridis* and *T. occidentalis*, which reduced SBP of SHRs by 39 and 24 mmHg, respectively at 100 mg/kg dose (Olarewaju et al., 2018). However, the results obtained in this work are consistent with previous reports showing the SBP-reducing ability of plant polyphenols in SHRs (Negishi et al., 2004; Sarr et al., 2006; Kivimaki et al., 2013; Shaw et al., 2017; Alashi et al., 2018). The similarity in the maximum SBP-reducing effect of SM40T1 and SM80T2 may be due to their almost equal ability to inhibit renin and ACE activities as shown in the *in vitro* studies (Fig. 5.1 and 5.2).

For the DBP, only the SM80T2 extract had significant effect with 12.2 and 8.9 mmHg reductions at the 100 and 500 mg/kg doses, respectively (Table 5.4). These values are lower than the maximum 18 mmHg reported for *A. viridis* but similar to the 3-12 mmHg reductions for *T. occidentalis* extract (Olarewaju et al., 2018). As expected, there was no noticeable effect of the saline in reducing DBP while captopril was most effective with 32.1 mmHg reduction after 4 h. In contrast, the SM40T1 did not produce any reduction in DBP throughout the 24 h study period. As indicated above, the stronger DBP-reducing ability of the SM80T2 may be attributed to the higher content of rutin; however, determination of polyphenolic content of blood is required to confirm this hypothesis. Similar to the delayed SBP-lowering effect observed at the 500 mg/kg dose, the maximum DBP depression was achieved after 8 h of oral administration when compared to 4 h for the 100 mg/kg dose. The increased solution viscosity at 500 mg/kg may have reduced absorption rate of the polyphenols, hence the delayed DBP-lowering effect. These decreases in SBP and DBP are important because of the reported direct relationship between the cardiac factors and risk of cardiovascular diseases (Antikainen et al., 1998; McInnes, 2005). In fact, it has been established that the risk of stroke, coronary heart disease, and all-cause mortality can be reduced by 40%, 16%, and 13%, respectively through lowering of DBP by 5-6 mmHg and SBP by 10-12 mmHg maintained over 2.5 years (Collins and MacMahon, 1994). While

direct translation from animal to human effect cannot be accurately made, the current level of SBP and DBP reductions are within these beneficial values, which suggest use of the *S. macrocarpon* leaf extracts as potential antihypertensive agents.

MAP is the average blood pressure throughout the arterial circulatory system in each cardiac cycle. It is the regulated pressure necessary to maintain end-organ tissue perfusion as required for adequate cellular oxygenation (Bradshaw, 2012). Organs such as brain, heart, and kidneys depend on MAP to maintain control of tissue blood flow and delivery of blood to the required tissue (McGhee and Bridges 2002). Therefore, maintenance of normal MAP levels is important during times of illness. Low MAP can cause hypotension, loss of consciousness and shock due to an inadequate supply of blood to the necessary organs. On the other hand, higher MAP could give rise to increased oxygen demand by the heart, and cause blood vessel damage, stroke, as well as changes in the size, shape, structure, and the way heart functions (Wehrwein & Joyner, 2013). As shown in Table 5.5, single oral administration (100 and 500 mg/kg body wt) of the vegetable extracts to SHRs induced significant ($p < 0.05$) reductions in MAP when compared to the saline treatment.

Table 5. 4. Changes in diastolic blood pressure (DBP) after oral administration of *Solanum macrocarpon* leaf extracts to spontaneously hypertensive rats: 40 and 80 represent urea fertilizer dose (kg/ha) applied at planting (T1) or 2 weeks after planting (T2) while 100 and 500 are the leaf extract doses (mg/kg rat body weight). Values (mean ± standard deviation) with the different letters within the same column are significantly different ($p < 0.05$).

	DBP (mmHg)					
Time (h)	**Saline**	**Captopril**	**$40T1_{100}$**	**$80T2_{100}$**	**$40T1_{500}$**	**$80T2_{500}$**
0	121.3 ± 5.3^{a}	145.0 ± 12.0^{e}	110.6 ± 6.2^{a}	133.4 ± 2.6^{c}	110.6 ± 6.2^{a}	133.4 ± 2.6^{b}
2	120.7 ± 8.8^{a}	118.8 ± 0.7^{ab}	123.3 ± 0.8^{abc}	127.2 ± 0.8^{b}	124.1 ± 3.9^{bc}	133.3 ± 5.2^{b}
4	120.8 ± 3.35^{a}	112.9 ± 3.2^{a}	123.6 ± 1.6^{abc}	121.2 ± 0.1^{a}	126.1 ± 1.7^{bc}	136.4 ± 0.6^{b}
6	132.6 ± 5.5^{ab}	116.5 ± 7.5^{ab}	119.8 ± 0.3^{ab}	127.7 ± 1.7^{b}	128.8 ± 1.4^{c}	138.3 ± 0.5^{b}
8	127.9 ± 3.1^{a}	125.6 ± 12.7^{bc}	135.3 ± 16.8^{bc}	129.9 ± 3.0^{bc}	127.2 ± 4.5^{c}	124.5 ± 0.8^{a}
12	131.0 ± 1.1^{b}	127.6 ± 4.3^{bc}	138.6 ± 3.0^{c}	144.8 ± 0.8^{d}	117.0 ± 2.8^{ab}	148.7 ± 0.4^{c}
24	135.2 ± 15.6^{ab}	135.9 ± 7.2^{d}	140.4 ± 2.4^{c}	154.4 ± 0.1^{e}	128.5 ± 3.7^{c}	134.9 ± 0.1^{b}

Captopril produced the most reduction of 55.4 mmHg followed by SM40T1 100 and 500 mg/kg doses with 15.4 and 14.3 mmHg, respectively. SM80T2 was less effective as it reduced MAP by 8.9 and 10.4 mmHg for the 100 and 500 mg/kg doses, respectively. The MAP-lowering ability of the *S. macrocarpon* leaf extracts is weaker than those of *A. viridis* and *T. occidentalis*, which produced 18-37 mmHg reductions (Olarewaju et al., 2018). While the effects of the *S. macrocarpon* leaf extracts are weaker, the levels of MAP reduction suggest potential beneficial effects on heart health.

5.3.4 Changes in heart rate (HR)

HR is an important parameter of cardiac health since it reflects the workload on heart muscles. For example, lower HR indicates the health of the heart that largely affects longevity and lowers the risk of heart attack (Mishra & Rath, 2011). Therefore, HR is an important tool to measure the overall wellness and normal functioning of the cardiovascular system. Table 6

shows significant reductions of HR after oral administration of both the 100 and 500 mg/kg doses of the leaf extracts. At the 100 mg/kg dose, the reductions were similar for both SM40T1 and SM80T2 extracts with approx. 28 reductions in beats per minute (bpm). Increasing the dose to 500 mg/kg did not enhance HR-lowering ability and the values decreased to 21 and 16 bpm for SM40T1 and SM80T2, respectively. In contrast, captopril had the strongest HR-reducing ability with up to 87 bpm reductions. The effect of *S. macrocarpon* extracts are less than those reported for similar leaf extracts that reduced HR of SHR by up to 107 bpm (Olarewaju et al., 2018).

Table 5. 5. Changes in mean arterial blood pressure (MAP) after oral administration of *Solanum macrocarpon* leaf extracts to spontaneously hypertensive rats: 40 and 80 represent urea fertilizer dose (kg/ha) applied at planting (T1) or 2 weeks after planting (T2) while 100 and 500 are the leaf extract doses (mg/kg rat body weight). Values (mean ± standard

	MAP (mmHg)					
Time (h)	**Saline**	**Captopril**	**40T1$_{100}$**	**80T2$_{100}$**	**40T1$_{500}$**	**80T2$_{500}$**
0	151.7 ± 6.8^{a}	199.9 ± 12.3^{d}	160.2 ± 7.3^{c}	165.0 ± 0.7^{b}	160.2 ± 7.3^{a}	165.0 ± 0.7^{b}
2	150.4 ± 12.0^{a}	150.4 ± 0.8^{a}	158.1 ± 2.3^{bc}	158.1 ± 2.3^{a}	153.4 ± 4.5^{bc}	165.2 ± 4.7^{b}
4	151.6 ± 2.3^{a}	144.5 ± 3.6^{a}	156.3 ± 2.7^{bc}	154.6 ± 1.9^{a}	155.0 ± 1.4^{bc}	174.2 ± 5.9^{bc}
6	164.6 ± 9.9^{ab}	147.6 ± 8.1^{a}	149.6 ± 0.4^{ab}	157.4 ± 2.6^{a}	153.4 ± 3.2^{bc}	179.9 ± 12.2^{c}
8	158.0 ± 5.5^{a}	157.6 ± 15.3^{b}	144.8 ± 5.7^{a}	156.1 ± 3.0^{a}	149.9 ± 2.7^{b}	154.6 ± 0.3^{a}
12	165.9 ± 3.0^{b}	159.6 ± 6.1^{b}	162.8 ± 0.9^{c}	178.0 ± 0.2^{c}	145.9 ± 2.3^{ab}	181.7 ± 1.4^{c}
24	168.1 ± 19.4^{ab}	169.4 ± 8.5^{c}	174.2 ± 2.4^{d}	188.5 ± 0.6^{d}	159.8 ± 1.2^{c}	162.0 ± 0.2^{b}

Since excessive HR has been linked to hypertension and associated cardiovascular diseases (James and Friday, 2011; Reule and Pe, 2012), the observed reductions in this work suggest that the *S. macrocarpon* extracts could be considered as potential agents against these

diseases. This is based on a recent report that showed HR-reducing effect and associated cardiovascular benefits when an anthocyanin-rich juice was consumed by human subjects (Igwe et al., 2017). The results obtained in this work are similar to the 27 bpm reductions in heart rate reported for lingonberry juice after oral administration to SHRs (Kivimaki et al., 2013). In contrast Shaw et al. (2017) reported lack of effect on HR after oral administration of another type of vegetable leaf extract to SHRs.

Table 5. 6. Changes in heart rate after oral administration of *Solanum macrocarpon* leaf extracts to spontaneously hypertensive rats: 40 and 80 represent urea fertilizer dose (kg/ha) applied at planting (T1) or 2 weeks after planting (T2) while 100 and 500 are the leaf extract doses (mg/kg rat body weight). Values (mean ± standard deviation) with the different letters within the same column are significantly different ($p < 0.05$).

Heart rate (beats per minute)						
Time (h)	**Saline**	**Captopril**	**40T1$_{100}$**	**80T2$_{100}$**	**40T1$_{500}$**	**80T2$_{500}$**
0	284.8 ± 16.2^{a}	262.4 ± 74.7^{a}	284.7 ± 4.2^{c}	296.89 ± 1.32^{c}	280.5 ± 1.8^{cd}	295.5 ± 0.6^{bc}
2	270.27 ± 13.2^{a}	296.4 ± 31.3^{a}	274.3 ± 4.3^{b}	288.63 ± 0.88^{b}	273.3 ± 0.6^{bc}	296.3 ± 3.5^{bc}
4	279.5 ± 14.0^{a}	280.0 ± 22.2^{a}	276.0 ± 0.5^{bc}	269.33 ± 2.49^{a}	263.9 ± 2.1^{ab}	279.0 ± 10.7^{a}
6	270.6 ± 3.7^{a}	273.5 ± 8.0^{a}	257.1 ± 2.8^{a}	269.22 ± 0.21^{a}	259.7 ± 0.1^{a}	309.3 ± 10.4^{c}
8	280.0 ± 21.6^{a}	313.9 ± 26.7^{a}	271.8 ± 4.8^{b}	285.39 ± 3.47^{b}	267.9 ± 5.4^{ab}	280.4 ± 11.2^{a}
12	363.3 ± 6.4^{b}	342.3 ± 16.5^{a}	354.7 ± 2.9^{e}	384.01 ± 5.32^{e}	284.9 ± 6.0^{d}	400.2 ± 5.4^{d}
24	293.2 ± 38.4^{a}	299.0 ± 29.3^{a}	302.0 ± 5.6^{d}	334.70 ± 1.49^{d}	279.1 ± 7.5^{cd}	300.4 ± 9.1^{bc}

This work has shown that *S. macrocarpon* polyphenol-rich leaf extracts can modulate various cardiac functions after oral administration to rats, which suggest bioavailability of active polyphenolic compounds. *In vitro* reductions in ACE and renin activities provided the basis for the SHR oral feeding tests, which confirmed blood pressure and heart rate-reducing effects. Results showed no advantage of the higher 500 mg/kg dose, which suggest that the 100 mg/kg

dose provided enough bioavailability that ensured *in vivo* disease ameliorating effects. However, increased viscosity of the 500 mg/kg dose solution may have contributed to the observed lack of improved cardiovascular benefits over that of the 100 mg/kg dose. Due to the stronger DBP-reducing ability when compared to SM40T1, the SM80T2 leaf extract will be the preferred sample for future human intervention tests needed to confirm utility as an effective antihypertensive agent.

5.4 Acknowledgments

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5.5 Conflict of Interest

The authors have no conflict of interest.

5.6 References

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5.7 TRANSITION STATEMENT THREE

Digestion is very important in the release of nutrients and bioactive compounds into the body system. The interactions between food and digestive enzymes can alter the digestion rate. Understanding this reaction rate is of benefit to the food and nutrition industry in the production of functional foods and nutraceuticals suitable for the maintaining human health. However, excessive activity of digestive enzymes can also have deleterious effects on human health. In the preceding chapters, the antioxidant and anticholinesterase as well as renin and ACE inhibitory properties were determined as a measure of potential impact of the vegetable extracts on human health. In this chapter, additional potential health benefits were examined using ability of the leaf extracts to inhibit trypsin activity. Inhibiting trypsin activity could contribute to the prevention and/or delay of chronic diseases that arise from excessive activity of this enzyme in the body. The study also determined the kinetics and mode of trypsin inhibition by the selected vegetable extracts. It was established that these extracts inhibited trypsin activity through a competitive mode, suggesting that the polyphenolic compounds in the extracts attach at the active site of the enzyme thus blocking substrate entry.

CHAPTER SIX

MANUSCRIPT FOUR

TRYPSIN INHIBITORY ACTIVITY OF AQUEOUS EXTRACTS FROM *AMARANTHUS VIRIDIS, SOLANUM MACROCARPON,* AND *TELFAIRIA OCCIDENTALIS* VEGETABLE LEAVES.

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AUTHOR CONTRIBUTIONS

REA: Conceptualization, funding acquisition, and project administration. REA, AMA and OAO methodology. REA: resources. OAO: writing—original draft preparation. REA and AMA: writing—review and editing. OAO: formal analysis. REA and AMA: supervision. All authors have read and agreed to the submitted version of the manuscript

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Abstract

Trypsin is one of the most widely studied serine proteases because it is responsible for the normal physiological functioning of cells such as digestion, coagulation of blood, immune responses, and blood pressure regulation. Phenolic compounds are ubiquitous and abundant vegetable secondary metabolites in the human diet and are capable of inhibiting major digestive enzymes. Polyphenols were extracted from the leaves of three Nigerian vegetables, *Amaranthus viridis* (AV), *Solanum macrocarpon* (SM) *and Telfairia occidentalis* (TO) using 1:20 ground leaf powder to water ratio at 60 ^{o}C. The vegetables were cultivated using different doses (0, 20, 40, 60 and 80 kg/ha) of urea fertilizer applied either at the time of planting or two weeks after planting. Effects of fertilizer micro-dosing, time of application and vegetable variety on the inhibition of trypsin by polyphenol extracts from these vegetable leaves and mode of inhibition of trypsin activities via kinetic studies in the absence and presence of the vegetable extracts was also investigated. In general, trypsin inhibition increased with increases in concentration of the polyphenolic-rich extract. It was also observed that AV (44.25 %) significantly ($p<0.05$) exhibited higher inhibitory activities than TO (6.30 %) and SM (19.21 %). Lineweaver-Burk plot results indicate that the polyphenolic compounds interacted with the enzyme active site to reduce substrate binding as evident in the competitive mode of action. We conclude that AV polyphenols have stronger binding affinity for trypsin and are better inhibitors of this enzyme when compared to TO and SM.

Keywords: Trypsin inhibition, leafy vegetables, fertilizer micro-dosing, polyphenol extracts, Lineweaver-Burk

6.1 Introduction

Digestion plays an important role in the release of nutrients and bioactive substances in the body. The interactions between food components and digestive enzymes can alter the rate of digestion thereby affecting human health. Knowledge of this interaction is an important tool in the pharmaceutical industry and in the development of functional foods that have health benefits (Cirkovic Velickovic & Stanic-Vucinic, 2017). Polyphenols are the most abundant and prevalent plant metabolites and are major components of human and animal diets but the current research focus on polyphenols is due to their bioactivity and associated health benefits (Sources & Significance, 1998). Generally, the absorption rate of polyphenols from the gastrointestinal tract (GIT) is low and most of the consumed polyphenols have been found to form complexes with digestive enzymes (Cirkovic Velickovic & Stanic-Vucinic, 2017). Studies have shown that polyphenolic compounds are capable of inhibiting the catalytic activity of major enzymes responsible for starch, lipid and protein digestion such as α-amylase, α-glucosidase, pancreatic lipase, pepsin and trypsin (Boath et al., 2012; He et al., 2006; Lochocka et al., 2015; Sakulnarmrat et al., 2014). For example, tea polyphenols were found to have a strong complexing activity with enzymes and proteins (Huang et al., 2004). Polyphenols such as tannic acids, catechin, and proanthocyanidin can also form a complex by reacting with proteins and enzymes to alter protein bioactivity (Huang et al., 2004; Xie et al., 2017). The interactions of polyphenols with proteins have been relevant in food processing operations such as clarification and tannin precipitation as well as for beverage haze stability and food taste enhancement (Siebert, 1999).

Trypsin is one of the most widely studied serine proteases because it catalyzes several reactions that maintain normal physiological functioning of cells such as digestion, blood coagulation, immune responses, and blood pressure regulation. Excessive activity of this

proteolytic enzyme can lead to various physiological disorders such as cancer, arthritis, and pancreatitis. Moreover, trypsin inhibitors have been used as important anti-viral agents to prevent various forms of infectious diseases such as diarrhea and acquired immune deficiency syndrome (Shahwar et al., 2012). Therefore, the use of protease inhibitors from natural sources is a key approach in ameliorating various pathological disorders and chronic diseases (Shahwar et al., 2012). The objective of the present study was to evaluate the efficacy of aqueous leaf extracts obtained from three commercially grown Nigerian vegetables, *Amaranthus viridis* (AV), *Solanum macrocarpon* (SM) and *Telfairia occidentalis* (TO) as trypsin inhibitors.

6.2 Materials and Methods

6.2.1 Plant materials and reagents

Freshly harvested leaves of AV, SM and TO were obtained from the MicroVeg Project 107983 site located at the Teaching and Research Farm, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria. Urea fertilizer doses (0, 20, 40, 60 and 80 kg N/ha) were applied to the vegetables on plot-by-plot basis in randomized complete block design. The urea fertilizer was applied to each plot either at planting (T1) or one week after seedling emergence (T2) to obtain the following samples: 0T1, 0T2, 20T1, 20T2, 40T1, 40T2, 60T1, 60T2, 80T1, and 80T2. Samples were harvested by plucking, dried in a cabinet dryer at ~60 ^{o}C for 8 h and then milled into powder using a laboratory blender. Each powder was sieved through a 315-micron mesh size (Laboratory Test Sieve, Endecotts Limited IS033-10, London) and stored at -20 °C prior to analysis. Trypsin, Dimethyl Sulfoxide (DMSO), Tris-HCl and Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride (BApNA) were purchased from Sigma Aldrich (Sigma Chemicals, St. Louis, MO). All other reagents were of analytical grade and purchased from Fisher Scientific (Oakville, ON, Canada).

6.2.2 Extraction and yield of extracts

Extraction of the polyphenol was carried out according to the method of (Alu'datt et al., 2010) with slight modifications. Samples were extracted using distilled water at ratio 1:20 ground leaf powder to water for 2 h in a beaker with continuous stirring at 60 °C; the extracts were centrifuged at 10,000*g* for 30 min. The supernatants were filtered through cheese cloth and the process repeated to obtain a second batch of supernatant. Both supernatants were mixed, concentrated in a rotatory evaporator at 60 °C, freeze-dried as the crude polyphenol extracts, and stored at -20 °C.

6.2.3 Trypsin inhibitor assay

Inhibition of trypsin activity was determined using the method of (Souza et al., 2016) with slight modifications. Inhibition of trypsin activity was determined by measuring the residual enzyme activity of the substrate BApNA, at pH 7.5 in the presence of the polyphenol extracts. Trypsin (200 µL, at 60 µg·mL^{-1} final concentration), dissolved in Tris buffer (pH 7.5) containing 0.02 M $CaCl_2$, was pre-incubated for 5 min at 37 °C with 200 µL of the buffer (full enzyme activity) or with 200 µL of polyphenol extracts (inhibited reaction). Enzyme catalysis was initiated by adding 500 µL of 1 mM BApNA prepared with 1% (v/v) DMSO in Tris buffer. After 10 min at 37 °C, the reaction was stopped by adding 100 µL of 30% (v/v) aqueous acetic acid. The extent of the enzymatic hydrolysis of the substrate was evaluated by recording the increase in absorbance at 410 nm.

6.2.4 Kinetics of enzyme inhibition

To determine inhibitory pattern of the polyphenol extracts, enzyme activities were analyzed using different substrate concentrations (0.25 – 1.75 mM) at a fixed concentration of polyphenol extracts (0, 2.5, 5, 7.5 or 10 µg mL^{-1}). Lineweaver–Burk plots were created by plotting the reciprocal of the enzyme reaction velocity (1/V) against the reciprocal of the

substrate concentration (1/[S]). The maximum reaction velocity (Vmax) and the Michael's constant (Km) were obtained from the reciprocal of the intercept on the 1/V axis and from the negative reciprocal of the intercept with the 1/[S] axis, respectively. All samples were analyzed in triplicate.

6.2.5 Statistical Analysis

Triplicate determinations were used to obtain mean values and standard deviations. For statistical analysis, 3-way analysis of variance (ANOVA) was performed using a model that included vegetable variety (VV), fertilizer dose (FD) and fertilizer application time (FT) as fixed variables. Duncan's multiple-range test was used to determine the differences in the mean treatment and significant differences at $p < 0.05$. IBM SPSS Statistical package (version 24) was used for all the statistical analyses.

6.3 Results and Discussion

6.3.1 In vitro inhibition of enzyme activity by polyphenol extracts

6.3.1.1 Dose-dependent inhibition of trypsin

The inhibition of trypsin activity by the three vegetable extracts at different concentrations is given in Figure 6.1. The data indicate that the inhibition of trypsin activity varied with polyphenol extract concentration, specifically a dose-dependent effect was observed for all the three polyphenol extracts evaluated in this study. Pre-incubation of the enzyme with vegetable extracts at a higher concentration (100 µg mL^{-1}) produced stronger inhibition (19.28-61.63% for AV extracts, -1.88 to11.51% for TO extracts and 6.91-43.66 % for SM extracts) than the values (2.34-16.12 %, -0.25 to 21.08 % and -0.71 to 19.09 % for AV, TO and SM extracts, respectively) obtained at a lower concentration of 10 µg mL^{-1}. In general, at each concentration tested, the inhibitory activities of AV polyphenolic extracts were higher than those of SM and TO extracts. It was also observed that for AV, the extracts from 80 kg urea ha^{-1} (61.05 %) and 0

kg ha^{-1} (51.81 %) fertilizer doses had higher trypsin inhibition than the other fertilizer levels. In contrast, for SM, the extracts from 40 kg ha^{-1} (45.23 %) and 60 kg ha^{-1} (39.61 %) fertilizer doses exhibited the highest degree of trypsin inhibition when compared to other fertilizer treatments. TO extracts had the lowest trypsin inhibitory activity and the degree of inhibition was not significantly ($p>0.05$) affected by fertilizer dose. The results suggest that extracts from plants grown with urea fertilizer (40, 60 and 80 kg/ha) had better trypsin inhibition ability than those from plants that did not receive this fertilizer treatment. The results obtained also reveal that extracts from 80 kg ha^{-1} urea fertilizer dose have the highest inhibitory activity. Enzyme inhibitors have received increasing attention due to their reactivity and for their potential use in pharmaceutical industry. Enzyme inhibitors have proven to have a higher affinity to inactivate target proteases in the development of human diseases such as high blood pressure, arthritis, pancreatitis, and cancer. They are also used for pest control and to improve the nutritional quality of food (Bijina et al., 2011). Shahwar et al. (2012) reported a higher degree of trypsin inhibition for caffeic acid (84 %), and Ferulic acid (60 %) than those obtained in this study. Similarly, Huang et al. (2013) reported that hot water extracts from Chinese tea deactivated trypsin enzyme. The rate of trypsin inhibition also depends on the type of enzyme used for the assay. For example, Pando et al. (1999) showed that human trypsin activity was inhibited (2.55 mg g^{-1}) more than bovine (1.79 mg g^{-1}) and porcine (1.12 mg g^{-1}) trypsin by *Crotalaria paulina seed* crude extracts.

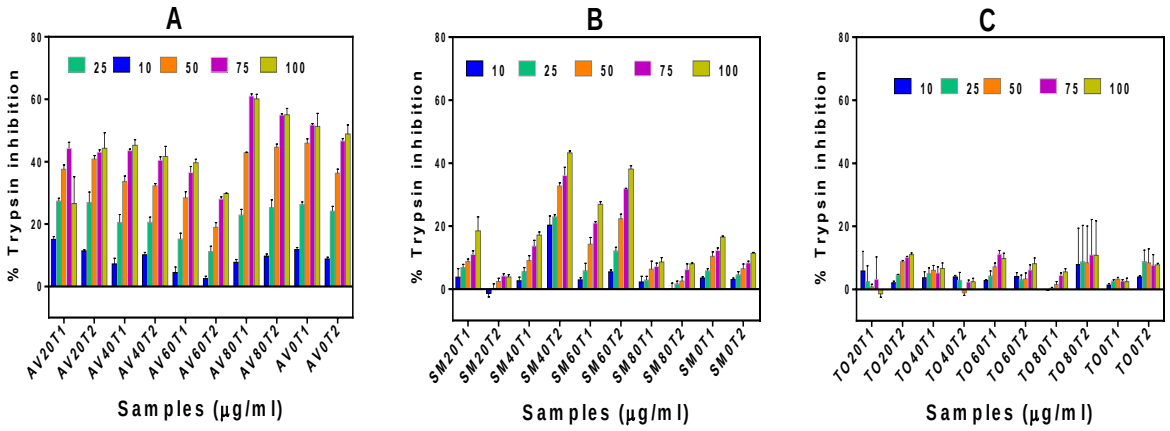

Figure 6. 1. Dose-dependent trypsin inhibitory activity of extract from *Amaranthus viridis* (A), *Solanum macrocarpon* (B) and *Telfairia occidentalis* (C)

6.3.1.2 Effect of vegetable variety, fertilizer dose and time of application on trypsin inhibitory activity

A three-way AVONA was conducted for trypsin inhibition using the three vegetable extracts at the highest inhibition concentration (100 µg/mL) and to determine the effects of VV, FD and FT on trypsin inhibition (Table 6.1). Based on the results, it was observed that AV (44.25 %) significantly ($p<0.05$) exhibited higher inhibitory activity than TO (6.30 %) and SM (19.21 %). The results suggest that AV is a better trypsin inhibitor with a higher binding affinity for trypsin when compared to the other two vegetable varieties. The FT also significantly ($p<0.005$) affected the level of trypsin inhibition by the polyphenol extracts. Results obtained showed that extracts from plants that received fertilizer treatment two weeks after planting had better trypsin inhibition when compared to those from plants treated with fertilizer at the time of planting. From the fertilizer doses applied to the vegetables, 40 kg/ha extracts had the highest trypsin inhibition. There was no significant difference ($p>0.05$) between extracts when the fertilizer dose was 40 kg/ha or 60 kg/ha; however, extracts from 20 kg/ha-treated plants had the lowest trypsin inhibition. Except for VV x FT, the trypsin inhibitory activity of the polyphenolic

extracts was positively influenced by vegetable variety, fertilizer dose, fertilizer application time and their interactions.

6.3.2 Trypsin inhibition kinetics

Based on the inhibitory results obtained in this experiment, the kinetics of inhibition of trypsin activity was measured in the absence and presence of the vegetable extracts. Kinetics of inhibition gives a guideline on the binding affinity and effectiveness of the extracts as enzyme inhibitors. Kinetics plots also determine the amount of substrate or extracts (inhibitor) needed to induce the reaction or inhibit the activities of the enzymes as reflected by the affinity to bind to the active site of the enzyme. Ki is the dissociation or inhibition constant and provides an indication of the binding affinity of the inhibitor or the ability of an enzyme to form the enzyme-inhibitor complex. Fig. 6.2a–c. shows Lineweaver–Burk plots of the trypsin reaction with and without inhibitors at two concentrations of each extract. The pattern of inhibition displayed was competitive inhibition for all the vegetable extracts, which means that the inhibitor and substrate compete for the active site of the enzyme. This also implies that the polyphenolic compounds in the extracts were bound to the active site of the enzyme to form enzyme-inhibitor complexes, which prevented substrate binding. The competitive mode of inhibition would have reduced the formation of enzyme-substrate complexes and hence decreases in catalysis rate, which is evident in the higher slopes of the inhibited reactions when compared to the uninhibited reaction. In this competitive inhibition mode, the maximum velocity (Vmax) of the reaction is unaffected but affinity of the substrate binding to the active site (Km) will decrease. Therefore, in the presence of vegetable extracts, the Vmax for trypsin remained unchanged as evident in the lines intersecting the Y-axis at the same point while the Km value increased (different intersections on the X-axis).

The activity of an enzyme depends on its characteristic structure and changes will lead to variations in catalytic efficiency (Wu et al., 2013). Enzyme catalysis depends on the covalent and noncovalent interactions of the amino acid components of the enzyme with the substrate. The vegetable extracts considerably decreased the enzyme activity by binding to its active site thereby changing the ability of the catalytic amino acid residues to interact effectively with the substrate. Competition between the inhibitor and substrate can be decreased by addition of more substrates thereby reducing the binding ability of the inhibitor (Bjelakovi et al., 2002). The mode of trypsin inhibition reported in this study is different from the mode of inhibition reported for tea polyphenols. Huang et al (2004) reported a non-competitive

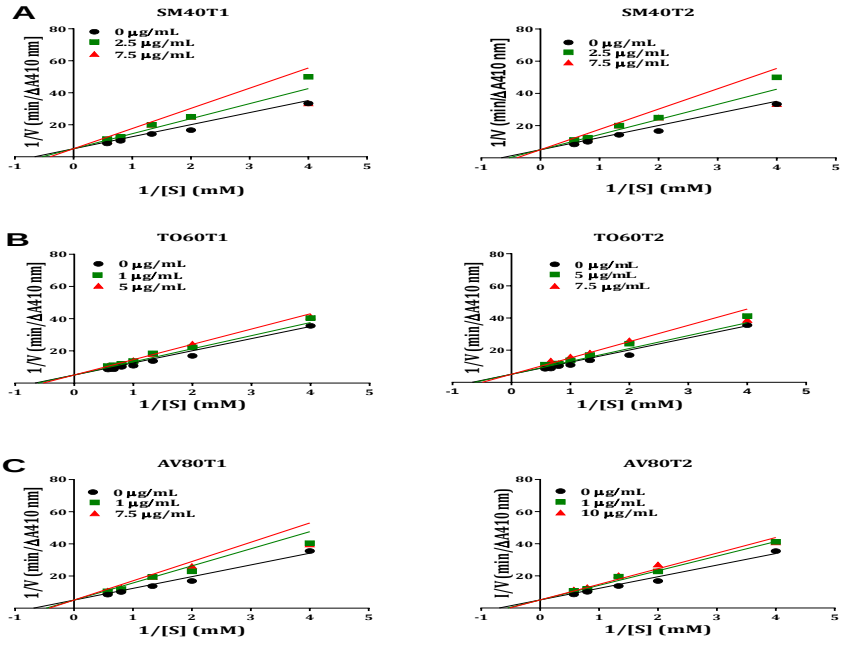

Figure 6. 2. Lineweaver-Burk plots for the inhibition of trypsin activity by vegetable extracts on trypsin (A*) Solanum macrocarpon*; (B) *Telfairia occidentalis*; and (C) *Amaranthus viridis* in the hydrolysis of BApNA at different concentrations

Table 6. 1. Results from 3-way ANOVA and Duncan's test of the effects of vegetable variety, (VV); fertilizer dose, (FD); fertilizer application time, (FT); *Telfairia occidentalis*, (TO); *Solanum marcrocarpon*, (SM); *Amaranthus viridis*, (AV); (0T-80T as fertilizer dosing treatments) on the trypsin inhibitory activity of vegetable extracts at 100 µg mL^{-1}.

Parameters	Source of variation (F -values)							Mean intensity for VV			Mean intensity for FD					Mean intensity for FT	
	VV	FD	FT	VV x FD	VV x FT	FD x FT	VV x FD x FT	SM	AV	TO	20T	40T	60T	80T	0T	Before planting	After planting
Trypsin	1.09 x 10^3*	22.81*	9.41*	54.95*	4.01	4.76*	27.20*	19.21^b	44.25^c	6.30^a	17.13^a	26.05^c	25.38^c	24.68^{bc}	23.02^b	22.22^a	24.29^b
								(0.584)	(0.584)	(0.584)	(0.755)	(0.755)	(0.755)	(0.755)	(0.755)	(0.477)	(0.477)

a,b,c,d Mean intensity values (followed in brackets by the standard error of the mean) within the same variable ''vegetable variety'', ''fertilizer dose'' and ''fertilizer application time'' with the same letter within the same row (parameter) are not significantly different ($p < 0.05$).*significant at $p < 0.05$.

inhibition of trypsin for tea polyphenols in an assay that also used BApNA as a substrate. Wu et al. (2013) also reported a non-competitive inhibition for trypsin by phenylpropanoid glycoside in Kudingcha leaves from *Ligustrum purpurascens.* Differences in the inhibition modes may be due to differences in the polyphenolic components of vegetable leaf and tea extracts.

6.4 Conclusion

All the polyphenolic-rich extracts obtained from the three vegetables (AV, SM and TO) inhibited trypsin enzyme activity but AV extracts exhibited significantly ($p<0.05$) higher effects. The polyphenol extracts also had a high affinity to bind trypsin as revealed by their competitive mode of inhibition, which altered enzyme catalytic activities. Most of the pharmaceutical agents known are based on the concept of competitive inhibition, therefore, these vegetables especially AV could serve as an effective enzyme inhibitor, which may be of therapeutic importance in the management of human health and diseases.

6.5 Acknowledgement

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6.7 TRANSITION STATEMENT FOUR

The preceding chapter has provided additional information on potential health benefits of the vegetable leaf extracts but the effects on the twin metabolic disorders of obesity and diabetes have not been investigated. Therefore, in this chapter, the vegetable leaf extracts were evaluated for their ability to inhibit α-amylase and pancreatic lipase, which are two of the key enzymes responsible for calorie release from diets. The results showed that all the leaf extracts inhibited α-amylase and lipase in a dose dependent manner. The study also determined the overall protein conformation as well as secondary and tertiary structures of the enzymes in the presence of polyphenolic extracts using intrinsic fluorescence and circular dichroism.

CHAPTER SEVEN

MANUSCRIPT FIVE

INHIBITION OF THE *IN VITRO* ACTIVITI 0-ES OF α-AMYLASE AND PANCREATIC LIPASE BY AQUEOUS EXTRACTS OF *AMARANTHUS VIRIDIS, SOLANUM MACROCARPON* AND *TELFAIRIA OCCIDENTALIS* LEAVES.

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Abstract

Inhibition of digestive enzymes such as α-amylase and pancreatic lipase (PL) is a promising therapeutic strategy for the treatment and management of chronic health conditions such as diabetes and obesity. Therefore, the aim of this work was to determine the enzyme inhibitory activity of polyphenol-rich aqueous extracts of *Amaranthus viridis* (AV), *Solanum macrocarpon* (SM) and *Telfairia occidentalis* (TO) leaves, which were harvested from plants produced using multiple urea fertilizer doses (0-80 kg/ha). Fertilizer application was applied at two time points (at planting or 2 weeks after seedling emergence). Leaf extracts were obtained using aqueous extraction (1:20, leaves:water) for 4 h at 60°C followed by centrifugation and freeze-drying of the supernatant. Results showed that the extracts inhibited α-amylase, and pancreatic lipase dose-dependently with TO extracts having significantly ($p < 0.05$) higher inhibitory activities for both enzymes. Fluorescence intensity and circular dichroism spectra in the presence and absence of leaf extracts indicate significant changes to the enzyme protein secondary and tertiary conformations. We conclude that the leaf extracts, especially from TO are potential agents for reducing calorie intake as a preventive or treatment tool against chronic diseases such as diabetes and obesity.

Keywords: Leaf extracts; polyphenolic compounds; α-amylase; pancreatic lipase; enzyme inhibition; fluorescence intensity; circular dichroism

7.1. Introduction

Polyphenol-rich plant foods have been reported to induce insulin-like effects and can act as good inhibitors of enzymes such as α-amylase and pancreatic lipase associated with type 2 diabetes, obesity and lipid peroxidation p. In order to control the function of these enzymes, larger plants, animals and microorganisms have been found to produce large number of different enzyme protein inhibitors of these enzymes. These enzyme inhibitors block the enzyme's active center thus preventing the rate at which polysaccharides are digested (2–4). Different *in vitro* and *in vivo* studies have shown that dietary phenolic compounds have many beneficial properties in maintaining human health (5). Studies have found that phenolic compounds are good inhibitors of α-amylase, and pancreatic lipase (6–9). The ability of plant-derived products such as from oats (10), berry (11) and tea (12) to inhibit these enzymes are associated with the phenolic content and other flavonoid components (13). One of the major ways of controlling diabetes is by inhibiting a carbohydrate-hydrolyzing enzyme such as α-amylase, which reduces the amount of glucose available for absorption into the body from the small intestine (14,15). α-amylase cleaves α-1,4 glycosidic bonds to convert complex dietary carbohydrates like starch into oligosaccharides and disaccharides, which are further broken down into absorbable monosaccharides such as glucose and fructose by glucosidases (16,17). Another enzyme of utmost importance in diabetes is pancreatic lipase (PL), which digests lipids, mainly dietary triacyl-glycerides, which are broken down into monoglycerides and free fatty acids that can be readily absorbed into the blood circulatory system (18,19). PL inhibition reduces the absorption of fat in the small intestine thus contributing to reduced calorie intake and prevention of excessive body weight gain (20). Therefore, inhibition of α-amylase and PL activities is a known strategy to prevent the breakdown of dietary polysaccharides and fats, which leads to reductions

in the absorption of simple sugars and lipids (21,22). With the global increase in the occurrence of diabetes and obesity, inhibition of these enzymes is of utmost importance in disease management (23).

Fluorescence emission spectroscopy is a useful tool to measure minute structural changes in protein structure, especially upon binding to small molecule ligands such as enzyme inhibitors. This is because aromatic amino acids like Trp, Tyr, and Phe can emit fluorescence spectra with maximum values at 350, 303 and 280 nm, respectively when they are excited in the UV region (24). However, the emission wavelengths reflect conformational changes in the protein, which are dependent on exposure of the aromatic amino acids to the hydrophilic environment (25). For example, decreases in fluorescence intensity are indications of protein unfolding and increased exposure of aromatic amino acids to a more polar environment while increases suggest shift to non-polar environments (24,26). Circular dichroism (CD) measures the secondary and tertiary conformations of proteins, which can be used to evaluate relationships of enzyme protein structure to catalytic activity especially in the presence of inhibitors. The CD signal of each protein depends on the number and proximity of the aromatic amino acid residues to each other, degree of H-bonding and presence of disulfide bonds (27).

Foods are well known sources of enzyme inhibitory compounds, but their levels could be dependent on agronomic practices, which are critical for defining crop productivity, nutrient composition, and food availability. Therefore, in order to ensure food supply and increase crop productivity, the positive effect of mineral fertilizers cannot be overemphasized (28,29). The use of fertilizer micro-dosing gives promising results in terms of crop productivity and farmers' income when compared to the traditional fertilizer broadcasting method. Fertilizer micro-dosing is a method of fertilizer application in small quantities at an optimal depth and distance around

the target crop such as leafy vegetables (29,30) and maize (31) either during the time of planting or some weeks after planting. Water is a universal solvent with numerous advantages as a green extraction solvent because it is cheap, non-flammable, nontoxic, environmentally friendly, and prevent pollution when compared to organic solvents (32). Moreover, the use of water enhances solubility of the extracted polyphenolic compounds within the mainly aqueous-based in vitro assay reagents and also in the aqueous gastrointestinal tract when ingested, which ensures bioavailability. Although the organic leaf extract fluted pumpkin (33), and eggplant (34) have been reported to inhibit α-amylase, there is paucity of information in literature on the inhibition of α-amylase and PL by aqueous extracts of these plants as well as the consequence of phenolic interactions on enzyme structural conformation. Therefore, the aim of this work was to determine the effect of fertilizer micro-dosing on the *in vitro* inhibitory activities of aqueous extracts of *Amaranthus viridis* (AV), *Telfairia occidentalis* (TO) and *Solanum marcrocarpon* (SM) leaves against α-amylase, and PL. The effects of these leaf extracts on the structural conformations of α-amylase and PL were also measured using circular dichroism (CD) and intrinsic fluorescence to determine possible means by which the phenolic compounds attenuate catalysis rate. AV, SM and TO were chosen for this study because they are one of the most under-utilized indigenous culinary herbs in Nigeria, popularly grown among local farmers. They are commonly consumed by indigenous people as blood boosters, to treat infertility, as anti-inflammatory, antidiabetic and antiviral agents. Moreover, AV, SM and TO produce a range of polyphenolic compounds including caffeic acid, rutin and myricetin (30,35,36)

7.2 MATERIALS AND METHODS

7.2.1 Materials and Chemicals

Porcine pancreas PL (26.9 units/mg protein) and α-amylase Type VI-B (25 units/mg solid) were purchased from Alfa Aesar (Tewksbury, MA, USA) and Sigma Aldrich (St. Louis, MO, USA), respectively while other analytical grade reagents were from Fisher Scientific (Oakville, ON, Canada). The plants (AV, TO, and SM) were produced at the Micro-Veg Project experimental farm, located at the Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria. The plants were grown using mineral fertilizer application according to the fertilizer micro-dosing technology (~0.1 – 0.5 cm deep) with a randomized complete block design and five nitrogen (urea) fertilizer doses, which were replicated four times as follows: 0, 20, 40, 60, and 80 kg of urea/ha. Cow manure was used as a base organic fertilizer at 5 t/ha on experimental plots (2 m X 3 m) that received urea at 20, 40, and 60 kg N/ha. In contrast, the plots that received 80 kg/ha urea did not contain organic fertilizer while the 0 kg/ha plots received only the organic fertilizer but not urea. The urea fertilizer was applied to each plot during planting (T1) or two weeks after emergence of seedlings (T2) to obtain the following samples: 0-T1, 0-T2, 20-T1, 20-T2, 40-T1, 40-T2, 60-T1, 60-T2, 80-T1, and 80-T2. Twenty-five days after seedlings emerged, the leaves were harvested, rinsed in potable water, destalked, and dried using an air cabinet at 60°C for 8 hr. The dried leaves were milled into fine powder using a Marlex Excella dry mill (Marlex Appliances PVT, Daman, India) followed by storage at -20°C.

7.2.2. Preparation of Aqueous Extracts Containing Free Polyphenols

Free water-soluble polyphenolic compounds were extracted using the method of Olarewaju et al. (26). Dried leaf powders were mixed with 20 volumes of distilled water at 60°C for 2 h under constant stirring. After cooling to 25°C, the mixture was centrifuged at 10,000 x g for 30 min

and supernatant passed through a cheese cloth. The residue was re-extracted and centrifuged using same conditions to obtain a second supernatant. The two supernatants were pooled together, concentrated using a vacuum rotatory evaporator at 60°C, freeze-dried and the extract powder stored at -20°C. We have previously reported that the dried leaf extracts are composed mainly of polyphenols with total polyphenolic content values in the range of 460-611 mg gallic acid equivalent/g where rutin, myricetin, and caffeic acid were detected as the major compounds (30).

7.2.3. α-Amylase Inhibition Assay

The α-amylase inhibitory activity of leaf extracts was determined using the method described by Karakaya et al. (37) with slight modifications. The enzyme substrate was prepared by bringing to boil 100 mL of distilled water in a 250 mL beaker on a hot plate and then added to a smooth paste of potato starch followed by stirring until it is dissolved. The starch solution was then allowed to cool down to room temperature before it is used for the enzyme assay. The dried leaf extracts were dissolved in 0.02 M sodium phosphate buffer containing 0.006 M NaCl, pH 6.9. A 100 µL aliquot of each sample (assay concentrations of 1.1-2.3 mg/mL) and 100 µL of α-amylase enzyme solution (1 mg/mL) were added to test tubes and allowed to incubate at 37 ^{o}C for 10 min. A sample blank was prepared with the enzyme omitted. After incubation, 100 µL of 1% (w/v) starch solution was added to test tubes and the reaction mixture incubated at 37^{o}C for 10 min. The reaction was terminated by adding 200 µL of 3,5-dinitro-salicylic acid (DNS) color reagent (96 mM DNSA, 2 M sodium potassium tartrate tetrahydrate and 2 M NaOH) followed by incubation in a boiling water bath at 100^{o}C for 5 min. The reaction mixture was allowed to cool to room temperature, after which 3 mL of MilliQ water was added. A 200 µL aliquot of the reaction mixture was then transferred to a 96-well microplate and the absorbance read at 540 nm

using a Synergy™ H4 microplate reader (Biotek™, Vermont, USA) set at 37ºC. Acarbose (α-amylase inhibitory drug) at 10 µg/mL was used as the positive control. Percentage inhibitions of all samples were calculated using the equation:

Inhibition (%) = [Ac − (As − Asb) / Ac] x 100

Ac = Absorbance of the negative control (uninhibited reaction), As = Absorbance of the sample (inhibited reaction), and Asb = Absorbance of the sample blank (enzyme omitted)

7.2.4. Pancreatic Lipase Inhibition

PL inhibitory activity of the extracts was determined according to protocols described in previous methods (38,39) with slight modifications. PL activity was measured using the release of 4-methylumbelliferone (4-MU) from the substrate, which is 4-methylumbelliferyl oleate (4-MU oleate). A 25 µL aliquot of samples (assay activity of 0.5-2.5 mg/mL) dissolved in Tris-buffer (13 mM Tris-HCl, 150 mM NaCl and 1.3 mM $CaCl_2$, pH = 8) and 225 µL of a 0.5 mM 4-MU oleate solution were mixed in a 96-well microplate and incubated for 15 min at 37°C. An enzyme blank was prepared with the substrate omitted. After incubation, 25 µL of PL solution (assay concentration = 3.125 U/mL) was added to start the enzyme reaction and then incubated at 37ºC for 1 hr. After incubation, the amount of 4-methylumbelliferone released by the lipase was measured with a fluorimeter at an excitation wavelength of 340 nm and emission wavelength of 450 nm. Orlistat (PL inhibitory drug at 0.25 mg/mL) served as a positive control and was analyzed using same protocol. The PL inhibitory activity (%) was calculated using the equation:

Inhibition (%) = [(Ac – Aeb) − As] /(Ac – Aeb) x 100

Ac = Absorbance of the negative control (uninhibited reaction), As = Absorbance of the sample (inhibited reaction) and Aeb = Absorbance of the enzyme blank (substrate omitted). The concentration of extract that reduced enzyme activity by 50% (IC_{50}) was obtained by non-linear regression analysis of a plot of PL inhibition (%) versus the sample concentrations using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA).

7.2.5. Intrinsic Fluorescence Emission

The method described by Li and Aluko (40) was used to record intrinsic fluorescence spectra on the Jasco FP-6300 spectrofluorometer (JASCO, Tokyo, Japan) at 25^{o}C with a 1 cm path length cuvette. Sample (leaf extracts) stock solutions (10 mg/mL) and enzymes (PL or α-amylase) were prepared in 13 mM Tris-HCl buffer containing 150 mM NaCl and 1.3 mM $CaCl_2$, pH 8 for PL or 20 mM sodium phosphate, containing 6 mM NaCl, pH 6.9 for amylase. The enzyme and sample solutions were then mixed to obtain assay concentrations of 1 mg/mL and 6.25-50 µg/mL, respectively, which were then used for fluorescence emission measurement. The fluorescence spectra were recorded at an excitation wavelength of 275 nm and emission wavelength range of 280 to 450 nm. Buffer emission spectrum was subtracted from those of the respective samples to obtain reported spectrum of each enzyme/extract mixture.

7.2.6. Measurement of Circular Dichroism (CD) Spectra

The CD spectra of enzyme/leaf extract mixtures (α-amylase and PL) complexes were measured at 25^{o}C in a J-810 spectropolarimeter (JASCO, Tokyo, Japan) using the spectral range of 190-240 nm (far-UV) for secondary structure determinations and 250-320 nm (near-UV) for tertiary structure (41). Stock solutions of extract and that of the enzymes (PL and α- amylase) were prepared as described above for intrinsic fluorescence. The extract and enzyme solutions were mixed to give assay concentrations of 1-3 mg/mL and 1 mg/mL, respectively. The far-UV

and near-UV spectra were acquired using 0.05 and 0.1 cm cuvette path lengths, respectively. The reported enzyme spectra were obtained after subtraction of the respective buffer spectrum.

7.3. Statistical Analysis

Triplicate determinations were used to obtain mean values and standard deviations. For statistical analysis, one-way analysis of variance (ANOVA) was carried out. Significant differences ($p<0.05$) between mean values were determined using the Duncan's multiple-range test. Statistical analyses were performed with the IBM SPSS Statistical package (version 24).

7.4. RESULTS AND DISCUSSION

7.4.1. α-Amylase Inhibition

α-amylase is one of the main enzymes involved in the breakdown of dietary starch, giving rise to oligosaccharides that can be further broken down to absorbable monosaccharides in the brush border of the intestine. Inhibition of this enzyme is therefore considered an active strategy for managing diabetes. The inhibitory activity of the polyphenolic extracts increased with increasing concentration, indicating a dose-dependent effect (Fig. 1a-c). This corresponds with the report of Sachan et al. (4), which also reported dose-dependent inhibitory activities for the organic extracts of the medicinal plants *Pluchea lanceolata*, *Alhagi pseudalhagi*, and *Caesalpinia bonduc*. Among the three vegetable extracts tested, TO-40-T2 (100.00% at 1.8 mg/mL) had the highest inhibition followed by SM-20-T1 (75.74 %) and AV-20-T1 (68.45 %) at 2.3 mg/mL, respectively. However, there was no direct relationship between fertilizer dose and inhibition of α-amylase activity. The inhibitory activity of the extracts was lower than that of the standard acarbose (86.64% at 10 µg/mL). When comparing the three vegetable extracts, it was

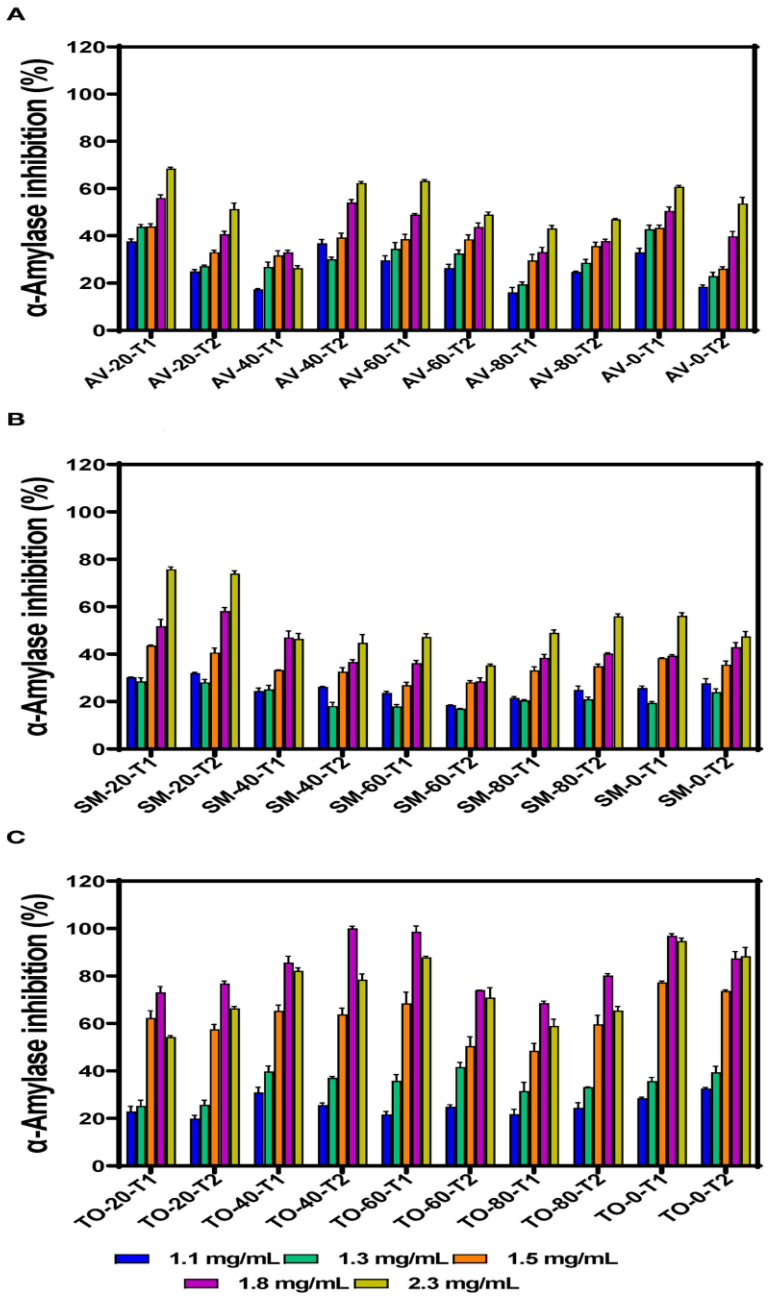

Figure 7 1: α-amylase inhibition by aqueous extracts from the dried leaves of (A) Amaranthus viridis (AV), (B) Solanum macrocarpon (SM) and (C) Telfairia occidentalis (TO). Plants were produced with different urea fertilizer doses (0, 20, 40, 60, and 80 kg/ha), which were applied at (T1) or after (T2) planting. Each bar is the mean of 3 determinations while the error bar represents standard deviation.

observed that all the TO extracts showed highest inhibitory activity at 1.8 mg/mL assay concentration after which the activity decreased. This indicate that at concentrations >1.8 mg/mL, there may have been antagonistic interactions between the extract constituents, which led to reduced interactions with the enzyme. This antagonistic effect could be due to increased polyphenol-polyphenol interactions or binding of a compound occupies a space on the enzyme surface or active site, which prevents attachment of other compounds. The results obtained for SM extracts also show weaker inhibitory effects when compared to *Solanum melongena* (40.11%) and *Solanum macrocarpon* (42.66%) at 80 µg/mL as previously reported (34). Differences in activities may be due to variations in cultivar and agronomic practices as well as the extraction media (aqueous versus organic), which could affect the type and ratio of polyphenolic compounds present in the extracts. Our previous work has showed that the main polyphenolic compounds in the AV, SM, and TO vegetable leaf extracts are caffeic acid, rutin and myricetin (35,36). However, the work of Nwanna (34) with SM extracts did not indicate the dominant polyphenols.

The effects of fertilizer dose, time of fertilizer application and vegetable variety differences on the inhibitory activity of the extracts were analyzed using three-way ANOVA (Table 1). The results show that there are significant differences ($p<0.05$) among the fertilizer dose treatments. The leaf extract from urea fertilizer dose of 80 kg N/ha (53.99%) had the lowest α-amylase inhibition while that of the control treatment (0 kg N/ha – with only organic fertilizer) had the highest inhibition (66.85%). The rate of α-amylase inhibition decreased as the rate of urea fertilizer dose increased indicating a negative effect, which could not be explained by the differences in content of polyphenolic compounds that we previously reported (35,36). Nitrogen is an important nutrient required for plant growth and metabolism of cellular compounds such as

Table 7. 1. Results from 3-way ANOVA and Duncan's test of the effects of vegetable variety, (VV); fertilizer dose, (FD); fertilizer application time, (FAT); *Telfairia occidentalis*, TO; *Solanum marcrocarpon*, SM; *Amaranthus viridis*, AV; (0T-80T as fertilizer dosing treatments) on α-amylase inhibitory activity of vegetable extracts.

Parameters	**Mean intensity for VV (%)**			**Mean intensity for FD (%)**					**Mean intensity for FAT (%)**	
	SM	AV	TO	20T	40T	60T	80T	0T	T1	T2
α-amylase	53.19[a]	52.59[a]	74.92[b]	64.99[d]	56.44[b]	58.912	53.99[a]	66.85[e]	61.06[a]	59.42[b]
	(0.360)	(0.360)	(0.360)	(0.464)	(0.464)	(0.464)	(0.464)	(0.464)	(0.294)	(0.294)

a,b,c,d Mean intensity values (followed in brackets by the standard error of the mean) within the same variable ''vegetable variety'', ''fertilizer dose'' and ''fertilizer application time'' with the same letter within the same row (parameter) are not significantly different ($p < 0.05$).

proteins, nucleic acids, ATP, chlorophyll, pigments, and for production of secondary metabolites (42). A study conducted using green and red lettuce indicate that low nitrogen availability increased the concentration of phenolic compounds (43) while in basil leaves (44-46), *Sesamum indicum* (47) and *Hypericum pruinatum* (48), higher nitrogen availability led to a decrease in rosmarinic acid, the main phenolic compounds, which may explain the results obtained in this study. The reduction in α-amylase inhibition could also be explained by the study done by Olarewaju et al (30), which observed that increase in fertilizer dose led to a decrease in polyphenol content of leaf extracts of AV, SM and TO. Therefore, the results suggest that the synthesis of polyphenolic compounds is not necessarily supported by higher concentration of nitrogen fertilizer. Thus, precise or optimized targeted use of nitrogen fertilizer could be an effective strategy for enhancing bioactive properties of plants. The time of urea fertilizer application also significantly ($p<0.05$) affected the rate of α-amylase inhibition by the leaf extracts because application at the time of planting led to significantly ($p<0.05$) higher (61.01%)

value than when applied two weeks after planting (59.42%). Among the three vegetable extracts, TO leaf extracts had significantly ($p<0.05$) higher inhibition of α-amylase activity when compared to SM and AV extracts. The stronger inhibitory effect of TO extracts may be due to the higher contents of caffeic acid as previously reported (35,36). The results are consistent with findings that plants contain some chemical substances which are potential inhibitors of *α*-amylase and due to this they can be used as therapeutic agents or as functional foods in the management of diseases associated with carbohydrate uptake (49).

7.4.2. Pancreatic Lipase (PL) Inhibition

The IC_{50} values of samples, which reflect the concentration of the extracts at which 50% of enzyme activity is inhibited (compared to the uninhibited reaction) are presented in Figures 2a-c. Throughout the investigation, the aqueous extracts of TO show the highest PL inhibitory effects but TO-0-T2, AV-0-T1and SM-0-T2 (zero nitrogen fertilizer) had the highest inhibitory activity with IC_{50} values of 1.000, 1.006 and 1.038 mg/mL respectively when compared to the other TO, SM and AV samples. The results show very similar values for the TO samples (T1 and T2) while the SM and AV samples had more variations. The results indicate a negative effect of nitrogen fertilizer application on the PL-inhibitory activity of extracts. Therefore, application of urea fertilizer may not be compatible with producing AV, SM, and TO leaves that contain polyphenolic compounds with strong high enzyme-inhibitory potency. The PL inhibition efficacy obtained in this study is lower than those obtained for aqueous extracts of *Vitis vinifera* and *Rhus coriaria* with IC_{50} values of 14.14 and 19.95 µg/mL, respectively (50). The variation in these results may be attributed to the type of method used in determining PL activity and the effect of different bioactive compounds present in the extracts. Other studies have identified natural products for their PL inhibition with profound inhibition effect on fat digestion. Some of

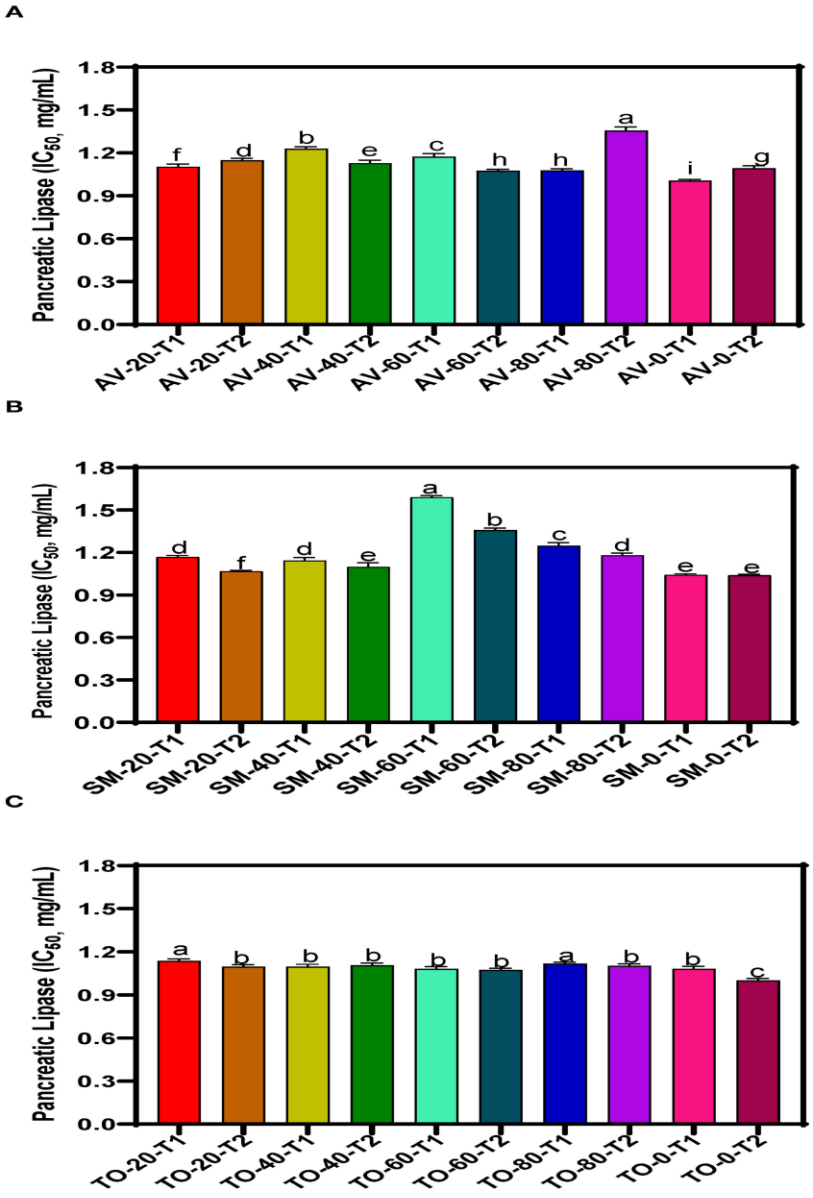

Figure 7 2: Pancreatic lipase inhibition of aqueous extracts from the dried leaves of (A) *Amaranthus viridis* leaves (AV), (B) *Solanum macrocarpon* (SM) and (C) *Telfairia occidentalis* (TO). Plants were produced with different urea fertilizer doses (0, 20, 40, 60, and 80 kg/ha), which were applied at (T1) or after (T2) planting. Each bar is the mean of 3 determinations while the error bar represents standard deviation. Bars with different letters (a-g) have mean values that are significantly ($p < 0.05$) different.

these plants include *Ononis natrix* (IC_{50} 167 μg/mL), *Fagonia arabica* ($IC_{50,}$ 204.1 μg/mL), *Origanum syriaca* ($IC_{50,}$ 234 μg/mL), and *Hypericum triquetrifolium* ($IC_{50,}$ 236.2 μg/mL) (51). Teixeira et al. (52) also found that *Passiflora nitida* extract inhibited PL with an IC_{50} value of 21.2 ± 0.8 μg/mL. Although transferring *in vitro* experiment to *in vivo* experiment might not bring out the same outcome but with the high inhibition rate of the leaf extract of AV, SM and TO, conducting an *in vivo* study using this leaf extracts might pave way for a better understanding of their mechanism of action, possible side effect and their optimal dose. Establishing this will give rise to a more effective and safer strategy in the management or treatment of obesity.

The three-way ANOVA results (Table 2) revealed a significant difference in the inhibitory activity of the extracts of AV, SM and TO. Extracts with fertilizer dose of 60 kg N/ha had the lowest PL inhibitory activity while the control treatment (0 kg N/ha) had the highest inhibitory effect among all the samples. This shows that extracts from the control treatment (no nitrogen fertilizer) are better inhibitors of PL when compared with extracts that contain both organic and urea fertilizers (20, 40 and 60 kg N/ha). Among the three leaf extracts studied, TO have the best inhibitory effect when compared with the other two vegetables (SM and AV). The time of fertilizer application significantly affected the rate at which the extracts inhibited PL activity. Leaf extracts from plants that received fertilizer treatment at the time of planting (T1) were better inhibitors of PL than those from plants fertilized two weeks after planting (T2). The reason is not clear but could be that early fertilizer application ensured faster synthesis and accumulation of inhibitory polyphenolic compounds than the late treatment.

Table 7. 2. Results from 3-way ANOVA and Duncan's test of the effects of vegetable variety, (VV); fertilizer dose, (FD); fertilizer application time, (FAT); *Telfairia occidentalis*, (TO); *Solanum marcrocarpon*, (SM); *Amaranthus viridis*, (AV); (0T-80T as fertilizer dosing treatments) on pancreatic lipase inhibitory activity of vegetable extracts.

Parameters	**Mean intensity for VV (%)**			**Mean intensity for FD (%)**					**Mean intensity for FAT (%)**	
	SM	AV	TO	20T	40T	60T	80T	0T	T1	T2
Pancreatic lipase	76.125^{b} (0.275)	74.57^{a} (0.275)	77.71^{c} (0.275)	74.51^{b} (0.355)	76.77^{c} (0.355)	71.28^{a} (0.355)	75.97^{c} (0.355)	82.14^{d} (0.355)	76.59^{a} (0.225)	75.92^{b} (0.225)

a,b,c,d Mean intensity values (followed in brackets by the standard error of the mean) within the same variable ''vegetable variety'', ''fertilizer dose'' and ''fertilizer application time'' with the same letter within the same row (parameter) are not significantly different ($p < 0.05$).

7.4.3. Intrinsic Fluorescence Emission

7.4.3.1. α-Amylase

The effects of leaf extracts on α-amylase conformational changes were evaluated by intrinsic fluorescence intensity measurements using samples with same nitrogen fertilizer treatment (20 kg/ha). Changes in the emission spectra of tryptophan are common in response to protein conformational transitions, subunit association, substrate binding, or denaturation (53). The results show varied outcomes but mostly increases in the fluorescent intensity (FI) at higher concentration of leaf extracts (Fig. 3a-f). Decreases in the FI represent the unfolding of the protein structure and exposure of tryptophan residues to a more polar environment while increases suggest shift to non-polar environments (24,26). The results suggest that at some concentrations, addition of the extracts led to a loose α-amylase protein conformation with greater exposure to the hydrophilic environment, hence FI quenching. However, for all the three leaf varieties at the highest concentration of 50 µg/mL, the α-amylase aromatic amino acids may have interacted with the polyphenolic compounds in the extract, which reduced exposure to the hydrophilic environment, hence increased FI. α-amylase alone had wavelength of maximum FI

(λmax) at 344 nm, which was red shifted after addition of the extracts. The red-shift indicates changes to the tryptophan microenvironment with greater exposure to hydrophilic residues upon binding with polyphenols in the extracts (24). At 6.25 and 12.5 µg/mL, a shift from 344 to 346 nm occurred for all the extracts except for AV-20-T2, which instead shifted to 348 nm. However, at 25 and 50 µg/mL, there was increased red shift to 350 nm for all the extracts. This suggests that the tryptophan residues inside the protein molecules were more exposed to the protein surfaces thus making the tryptophan environment more polar (54). Results showing changes in FI and λmax indicate that the α-amylase inhibitory activity of the AV, SM, and TO leaf extracts as shown in Fig. 1 is due to ability of the extracts to change the enzyme protein structure from a conformation that facilitated substrate catalysis into those not favorable for optimum enzyme activity. Interaction of polyphenols with enzymes could bring about changes in the emission spectra of the enzymes due to polyphenol binding. The reaction of polyphenol compounds such as epigallocatechin-G, epigallocatechin, epicatechin-G, naringenin, kaempferol-glu, caffeic acid, rosmarinic acid and p-coumaric acid with bovine serum albumin (BSA) shows that, these polyphenols can quench the intrinsic fluorescence of BSA. Titration of epigallocatechin-G, epigallocatechin, epicatechin-G with BSA showed a higher shift in the emission spectra indicating a more polar environment for tryptophan while the emission spectra of BSA was not significantly affected by other polyphenols (55). This suggest that the result obtained in our study could be attributed to activity of the polyphenol compounds (rutin, caffeic acid and myricetin) present in the AV, SM, and TO leaf extracts. Therefore, the changes observed in the FI at different concentrations of the AV, SM, and TO leaf extract may be due to the direct quenching or as a result of enzyme conformational changes induced by the reaction of polyphenol compounds with α-amylase protein (55). It is also possible that hydrogen bonding

occurred between the polyphenols of the AV, SM, and TO leaf extracts and α-amylase thus altering the microenvironment of the intrinsic chromophore groups of the enzyme (56,57).

7.4.3.2. Pancreatic Lipase

FI was also used to evaluate PL structural changes as a result of addition of various leaf extract concentrations. The results show that increases in the concentration of AV, SM, and TO leaf extracts led to decreased FI, which indicate conformational changes that exposed the aromatic amino acids to a more polar environment (Fig. 4a-f). PL exhibited a single fluorescence emission peak at 346 nm, which is due to the presence of tryptophan amino acid residue. Therefore, decreases in FI suggest unfolding of the PL protein molecule, which led to increased tryptophan interactions with the hydrophilic environment. Addition of SM-40-T1 and TO-40-T1 resulted in a slight blue shift of λmax to 344 nm and SM-40-T2 exhibited a red shift to 348 nm at 6.25 µg/mL. A similar result was reported for the interaction between caffeic acid and PL (58). The blue shift obtained upon addition of the AV, SM, and TO leaf extracts indicates changes that moved the tryptophan into a more hydrophobic environment (24,59). This suggests that there was structural reorganization that led to unfolding of enzyme molecule accompanied by changes in the microenvironment of tryptophan and tyrosine residues in the protein (54,59). The lower FI values observed for the enzyme interaction with 50 µg/mL of AV, SM, and TO leaf extracts show that the tryptophan residues were most exposed to the polar environment, which represented the loosest conformation when compared to the lower concentrations (60-62). A previous study reported similar results, which showed that the fluorescence intensity of pancreatic lipase decreased gradually at increased concentrations of quercetin, isoquercetin, and rutin with a slight blue shift from 354 to 351 nm (63). This suggests that flavonoids could expose

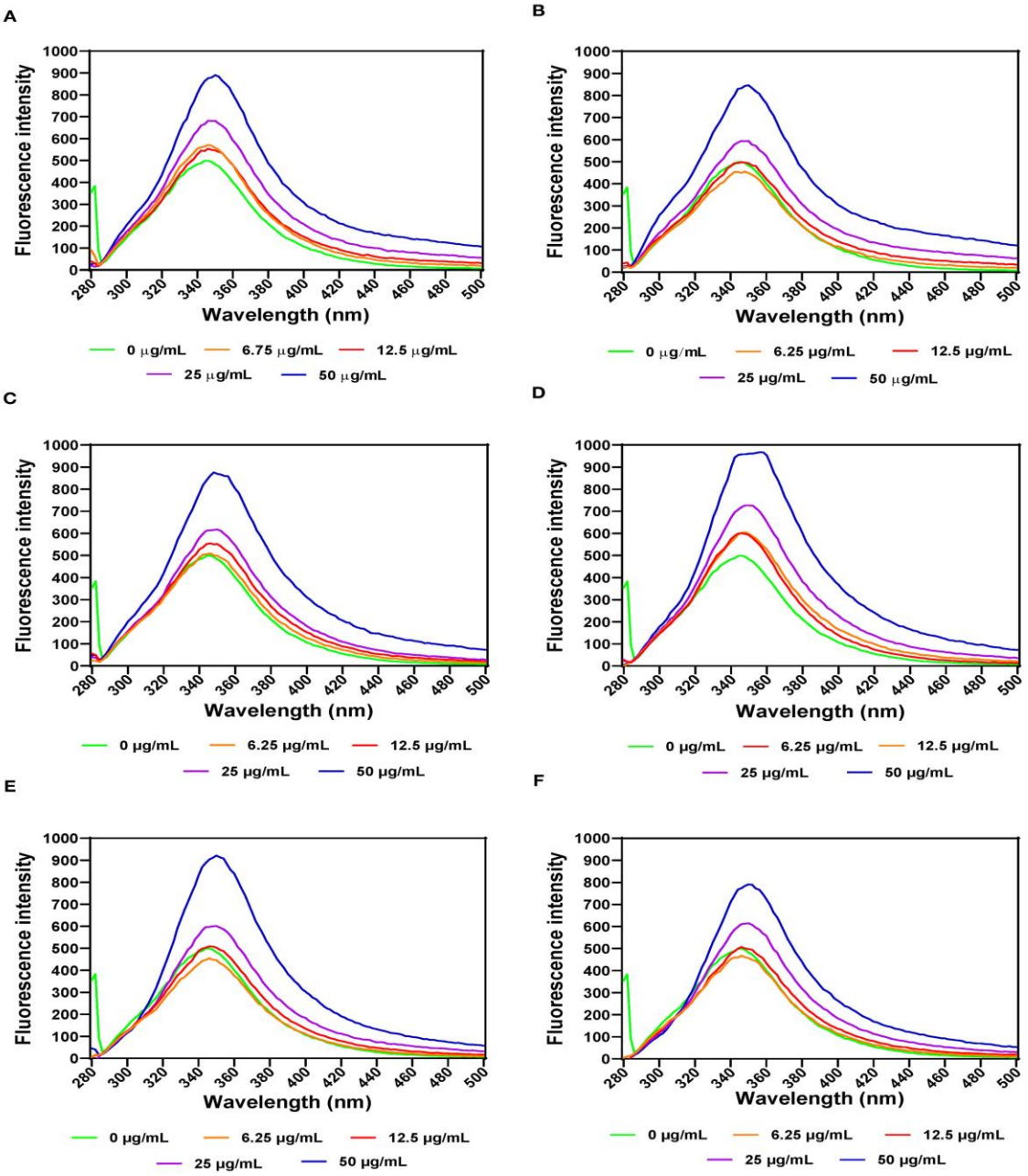

Figure 7 3: Intrinsic fluorescence intensity of α- amylase in the presence of varied concentrations of leaf extracts from: (A) AV-20-T1, (B) AV-20-T2, (C) SM-20-T1, (D) SM-20-T2, (E) TO-20-T1, and (F) TO-20-T2. Plants were produced with nitrogen fertilizer (20 kg/ha), which was applied at (T1) or after (T2) planting.

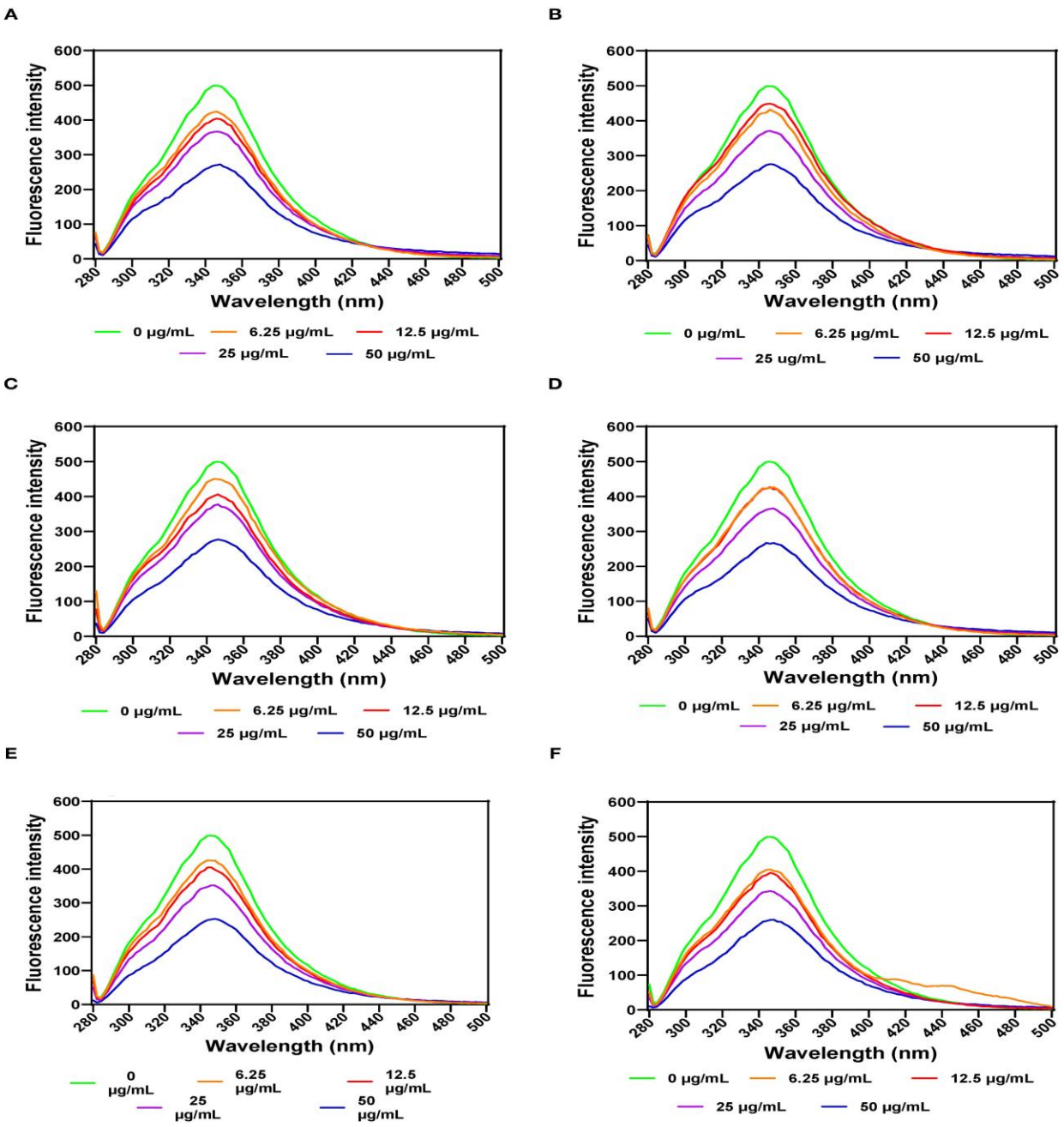

Figure 7 4: Intrinsic fluorescent intensity of pancreatic lipase in the presence of varied concentrations of leaf extracts from: (A) AV-40-T1, (B) AV-40-T2, (C) SM-40-T1, (D) SM-40-T2, (E) TO-40-T1, and (F) TO-40-T2. Plants were produced with nitrogen fertilizer (40 kg/ha), which was applied at (T1) or after (T2) planting.

tryptophan residue to a more hydrophobic environment and lead to attenuated fluorescence intensity of lipase. Gonçalves et al. (64) also reported a decrease in the FI of PL upon interactions with grape seed procyanidins but with no changes to the structure of PL. In another study, a different result was obtained, which reported an increased FI for the interactions of PL with galangin, kaempferol, quercetin, and myricetin (65). The results obtained in this study suggests that the fluorescence of PL was quenched by vegetable extracts as a results of protein structural unfolding, which is consistent with the observed reductions in PL activity.

7.4.4. Far-UV Circular Dichroism (CD) Spectra

7.4.4.1. α-Amylase

The secondary structure of α-amylase in the presence of AV, SM, and TO leaf extracts was investigated using CD measurements between 190 and 240 nm at extract concentrations of 1, 2, and 3 mg/mL. The CD spectrum of α-amylase alone indicate a typical signal of a protein containing both α-helix and β-sheet as evident by the negative peaks at about 206-208 and 229-231 nm, and a positive peak in the 193-200 nm region (Fig. 5a-f), which is similar to a previous report (66). The α-amylase spectrum showed that the 206-208 and 229-231 nm peaks were reduced upon addition of the AV, SM, and TO leaf extracts, which suggest conformational changes that led to altered secondary structures. However, the intensity in the 193-200 nm region increased upon addition of the SM-20-T1, TO-20-T1, and TO-20-T2 extracts. The result is similar to data from a previous work which showed increased intensity of 193-200 nm region of α-amylase CD spectra upon addition of a diosgenin from *Dioscorea bulbifera* (66). This shows that interaction of the leaf extracts with enzyme resulted in changes in the secondary structure conformation of α-amylase. As observed for the intrinsic fluorescence data, addition of 50 µg/mL of leaf extracts resulted in the most change in ellipticity, especially for AV and SM samples. In contrast, changes in the a-amylase CD spectra were minimal upon addition of TO.

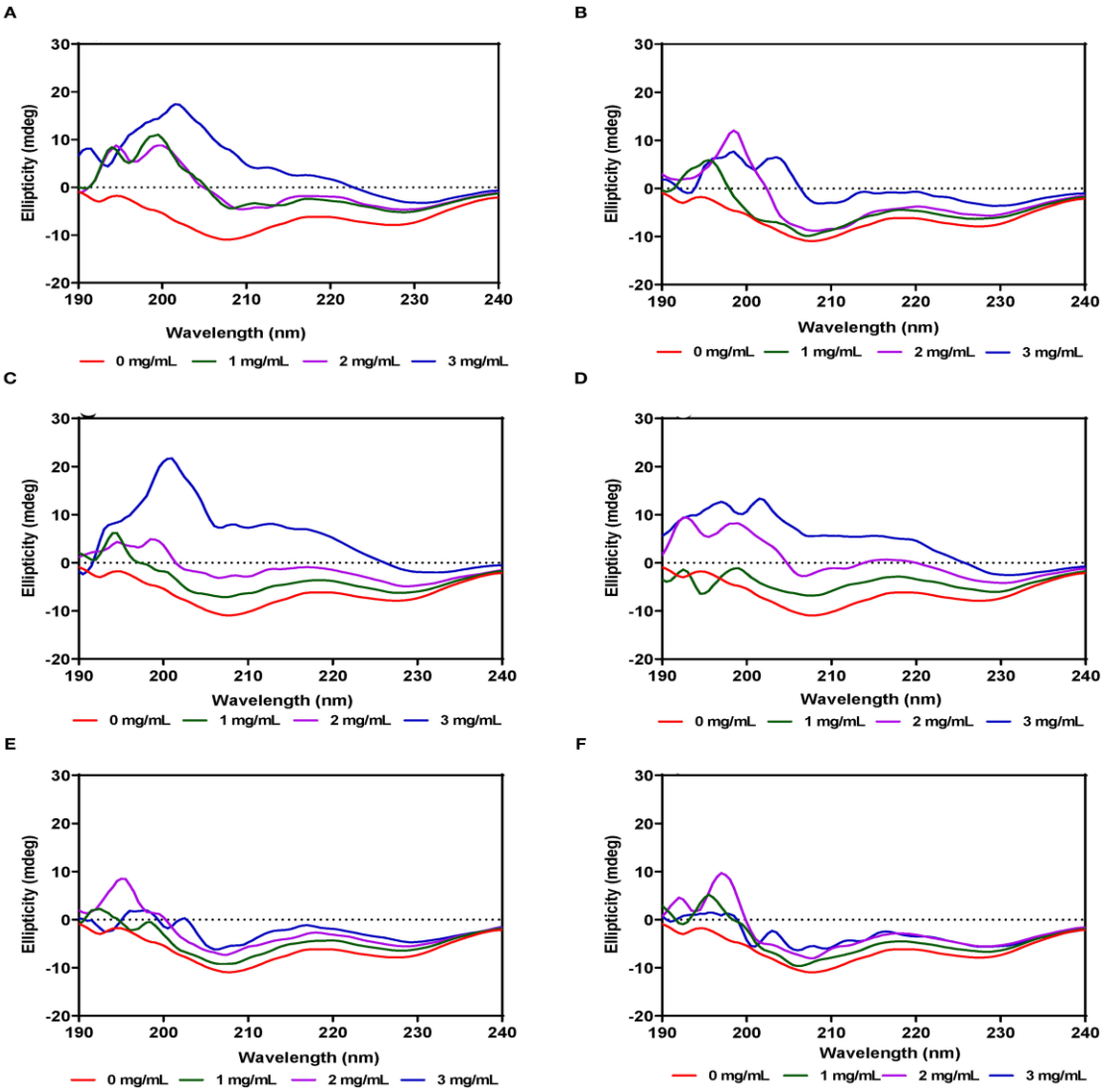

Figure 7 5: Far-UV CD of a-amylase in the presence of varied concentrations of leaf extracts from: (A) AV-20-T1, (B) AV-20-T2; (C) SM-20-T1, (D) SM-20-T2, (E) TO-20-T1, and (F) TO-20-T2. Plants were produced with nitrogen fertilizer (20 kg/ha), which was applied at (T1) or after (T2) planting.

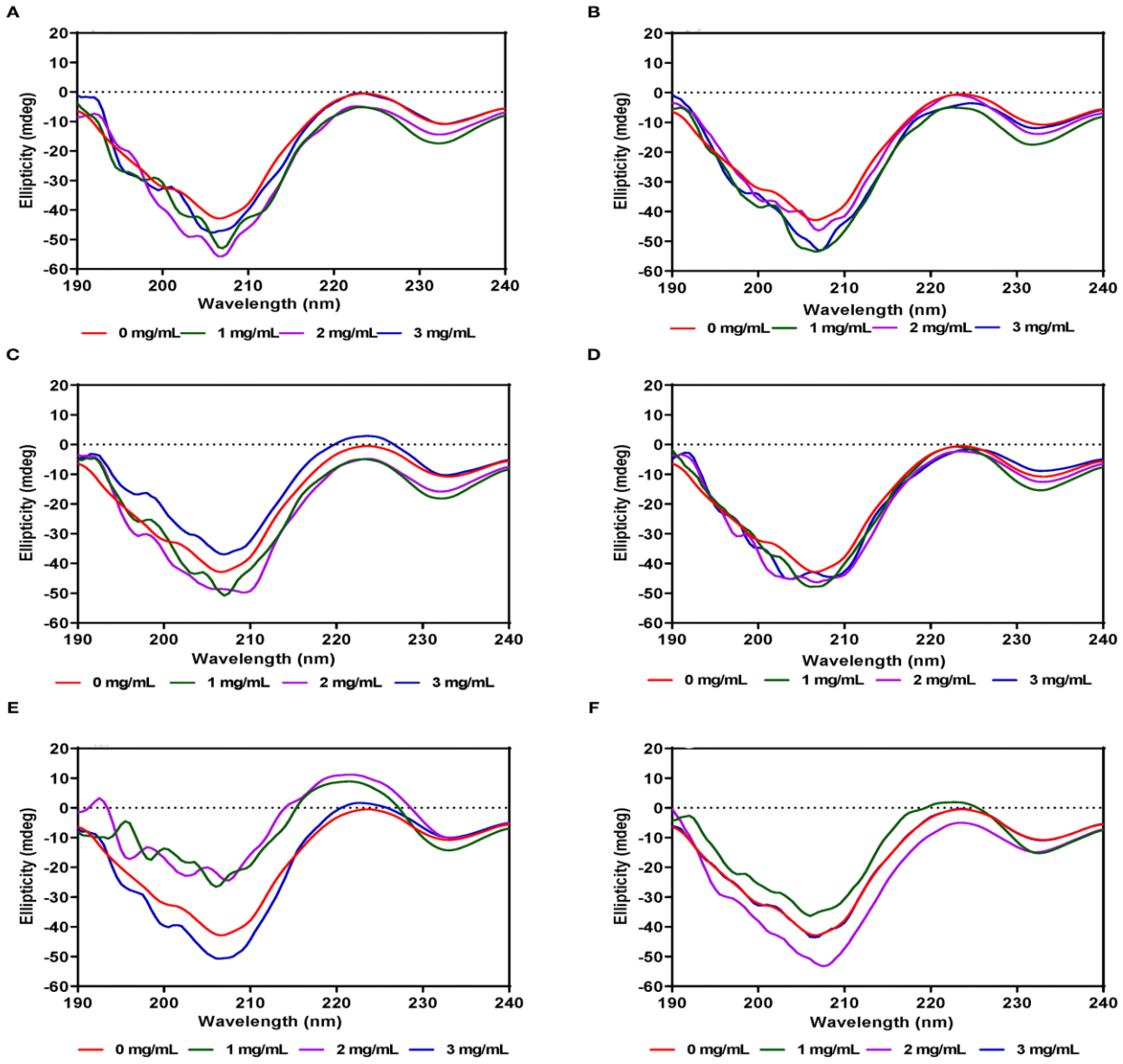

Figure 7 6:| Far-UV CD of pancreatic lipase in the presence of varied concentrations of leaf extracts from: (A) AV-40-T1, (B) AV-40-T2, (C) SM-40-T1, (D) SM-40-T2, (E) TO-40-T1, and (F) TO-40-T2. Plants were produced with nitrogen fertilizer (40 kg/ha), which was applied at (T1) or after (T2) planting.

7.4.4.2. Pancreatic Lipase (PL)

Unlike α-amylase, secondary structural changes to PL conformation in the presence of the AV, SM, and TO leaf extracts were modest with very minimal changes for AV-40-T1, AV-40-T2, SM-40-T1 and SM-40-T2 (Fig. 6a-f). However, addition of the TO-40-T1 and TO-40-T2 extracts led to greater changes in ellipticity at 206-208 and 229-231 nm, which suggest ability to modulate enzyme secondary structure. The ability of TO extracts to produce greater modification of PL secondary structure is consistent with the observed stronger enzyme activity inhibition when compared to the AV and SM extracts. Results obtained in this work are consistent with previous reports that showed changes in α-glucosidase (66), bovine serum albumin (67) and α-lactalbumin (68) proteins secondary structure in the presence of polyphenolic compounds. For example, in the presence of tea epigallocatechin-3-gallate, there was a significant increase in the 190-200 nm peak of α-lactalbumin when compared to the same protein alone.

7.4.5. Near-UV CD Spectra

7.4.5.1. α-Amylase

The tertiary structure of the α-amylase in the presence of AV, SM, and TO leaf extracts was evaluated using near-UV CD. The CD spectra show that the structural conformation of the enzymes was significantly affected at different extract concentrations of AV, SM, and TO. The spectra for α-amylase showed a prominent positive peak ellipticity at 269-270 nm and weak negative peak ellipticity at 296-298 nm (Fig. 7a-f). Addition of 1 and 2 mg/mL AV-20-T1 did not change α-amylase conformation but the 3 mg/mL produced an increased ellipticity peak in the 269-270 nm, which indicate structural changes that moved the aromatic groups away from the hydrophilic surface into more asymmetric environments when compared to the native enzyme. Addition of diosgenin was also shown not to change the near-UV spectra of α-amylase (66). In contrast, addition of 1 mg/mL AV-20-T2 led to enzyme conformation changes that exposed the

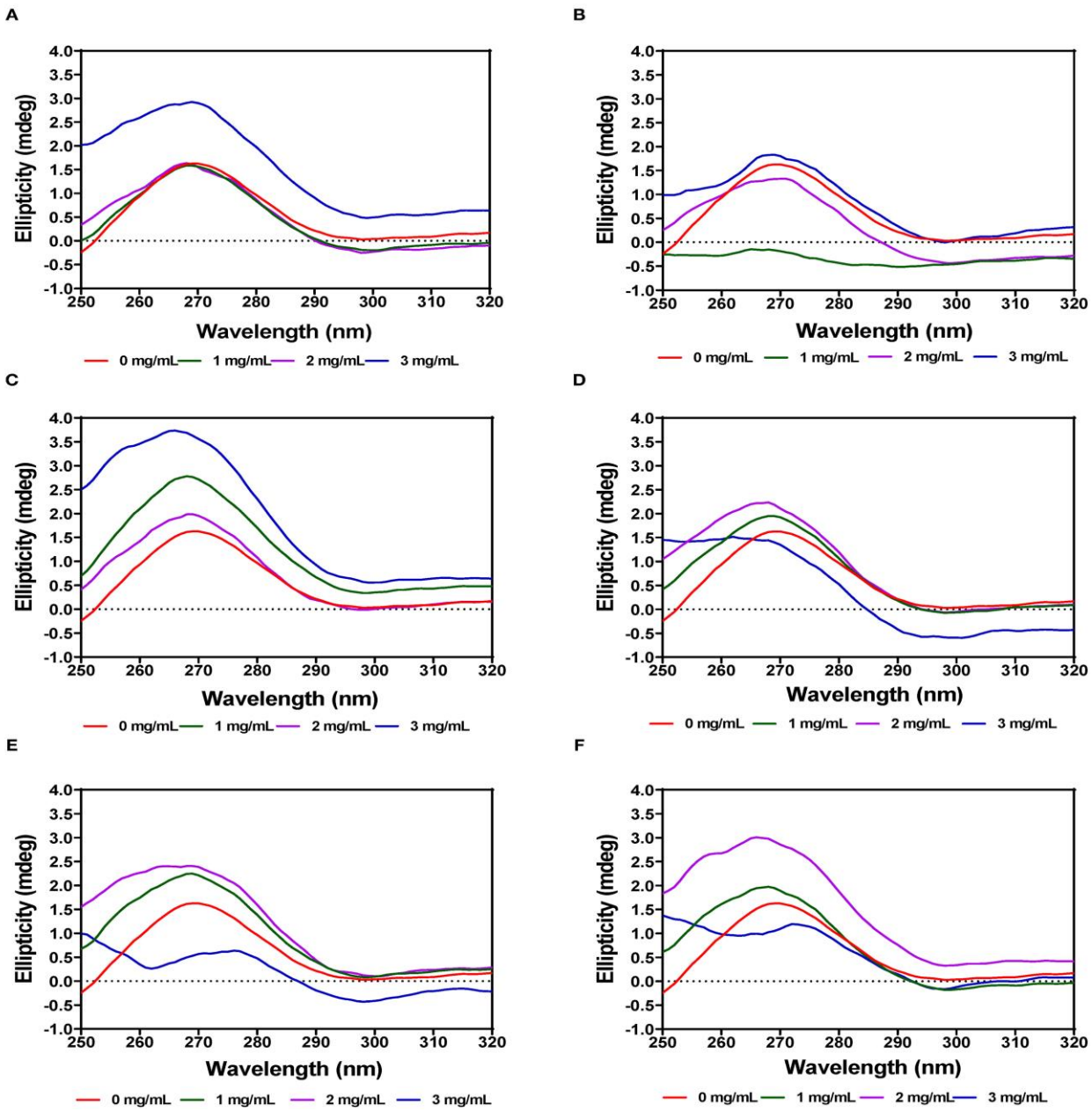

Figure 7 7: Near-UV CD of a-amylase in the presence of varied concentrations of leaf extracts from: (A) AV-20-T1, (B) AV-20-T2, (C) SM-20-T1, (D) SM-20-T2, (E) TO-20-T1, and (F) TO-20-T2. Plants were produced with nitrogen fertilizer (20 kg/ha), which was applied at (T1) or after (T2) planting.

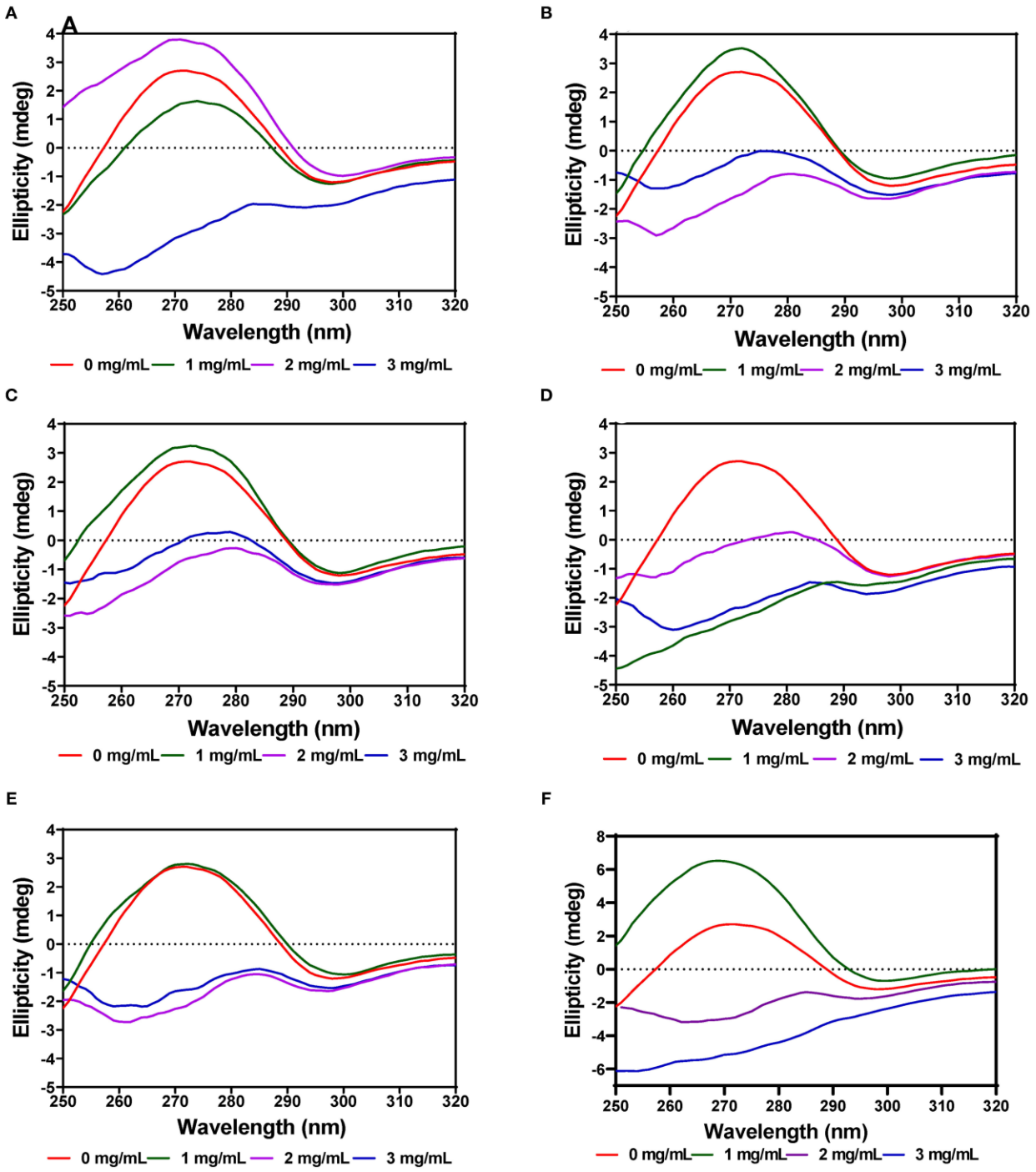

Figure 7 8: Near-UV CD of pancreatic lipase in the presence of varied concentrations of leaf extracts from: (A) AV-40-T1, (B) AV-40-T2, (C) SM-40-T1, (D) SM-40-T2, (E) TO-40-T1, and (F) TO-40-T2. Plants were produced with nitrogen fertilizer (40 kg/ha), which was applied at (T1) or after (T2) planting

aromatic amino acids into less asymmetric environments as evident in the near-zero ellipticity. Increased concentrations (2-3 mg/mL) of AV-20-T2 produced no substantial changes in α-amylase conformation. The SM-20-T1, SM-20-T2, TO-20-T1, and TO-20-T2 extracts also produced changes in enzyme structure as shown by the increases or decreases in ellipticity peak at 269-270 nm. Addition of varying levels of the AV, SM, and TO leaf extracts led to significant reductions in the 269-270 nm peak, suggesting a concentration-dependent modification of enzyme protein conformation. The observed larger losses of structural rigidity and asymmetric environments of α-amylase in the presence of SM and TO extracts are consistent with the stronger enzyme inhibitory effects when compared to the AV extracts.

7.4.5.2. Pancreatic Lipase (PL)

The near-UV CD spectra for PL showed a positive peak ellipticity at 272-274 nm and negative peak ellipticity at 298-300 nm, which indicate that tyrosine and tryptophan are the main contributors to the observed structure (Fig. 8a-f). Addition of AV-40-T1 led to disappearance of the 272-274 nm and 298-300 nm peaks, which indicate relocation of the aromatic amino acid residues to a more hydrophilic or less asymmetric environment. At 2-3 mg/mL, all the extracts (AV, SM, and TO) showed this type of behavior of significant reductions in the two peaks present in the native enzyme, which also suggest significant modifications to PL enzyme structure. The results are consistent with previous studies that have also shown interactions of polyphenols with enzymes can result in conformational changes to the tertiary structure of enzymes (69-72).

7.5. CONCLUSIONS

The aqueous extracts of AV, SM and TO leaves had strong inhibitory activities against α-amylase and PL. Fluorescence intensity analysis coupled with CD indicate that in the presence of

leaf extracts, there were changes to enzyme structure that could have been responsible for the observed enzyme activity modulation. Based on fluorescence emission patterns, α-amylase interactions with the extracts indicated shifting of aromatic groups to non-polar environments, which contrasted that of PL where the groups became exposed to hydrophilic environments. Therefore, mechanism of catalysis inhibition by the polyphenolic-rich leaf extracts was enzyme specific, but nitrogen fertilizer application led to decreased inhibitory efficacy of the leaf extracts against α-amylase and PL. Hence the application of urea fertilizer may not be an efficient way of enhancing the enzyme-inhibitory activities of AV, SM and TO leaf polyphenolic extracts. Overall, the TO extracts had the strongest inhibitory effects, which were reflected in more extensive changes to the secondary and tertiary structures of the enzymes. Inhibitions against these two enzymes by the vegetable extracts suggest their potential use as agents that could down regulate blood glucose through reduced intestinal digestion of nutrient polysaccharides and an additional suppression of calorie intake by reducing fat digestion. Therefore, consumption of these leaf extracts, especially TO may interfere with digestive functions that lead to reduced blood glucose and lipids, which could enable body weight control. However, animal and human feeding experiments are required to confirm these *in vitro* data. Moreover, additional studies are required to determine inhibition of glucose transporters 2, because of the potential competition between polyphenolic compounds and glucose for these bioreceptors.

7.6. DATA AVAILABILITY STATEMENT

Data is contained within the article.

7.7. CONFLICT OF INTEREST

The authors declare no conflict of interest.

7.8. AUTHOR CONTRIBUTIONS

REA: Conceptualization, funding acquisition, and project administration. REA, AMA and OAO methodology. REA: resources. OAO: writing—original draft preparation. REA and AMA: writing—review and editing. OAO: formal analysis. REA and AMA: supervision. All authors have read and agreed to the submitted version of the manuscript.

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7.12. TRANSITION STATEMENT FIVE

Overall, the *Telfaria occidentalis* (TO) leaf extracts showed the effective properties as antioxidant, antihypertensive and enzyme inhibition agents both *in vitro* and *in vivo*. Therefore, in this chapter, the TO extract obtained at 60 kg/ha nitrogen fertilizer dose was separated into chlorophyll and non-chlorophyll fractions in order to establish the role of chlorophyll in the observed physiological effects. The chlorophyll and non-chlorophyll fractions were obtained after partitioning by column chromatography and then tested for bioactive properties. The active compounds present in the fractions were estimated using tandem mass spectrometry and it was found that quercetin O-rutinoside (rutin) and kaempferol O-rutinoside were dominant. The rate at which the secondary and tertiary structures of enzymes were modulated by the fractions was evaluated. It was established from the study that there were differences in the bioactive properties of chlorophyll and non-chlorophyll fractions.

CHAPTER EIGHT

MANUSCRIPT SIX

IN VITRO BIOACTIVE AND STRUCTURAL CHARACTERIZATION OF CHLOROPHYLL-ENRICHED AND CHLOROPHYLL-DEPLETED POLYPHENOLIC FRACTIONS FROM LEAVES OF *TELFAIRIA OCCIDENTALIS*

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AUTHORS CONTRIBUTION

REA: Conceptualization, funding acquisition, and project administration. REA, AMA and OAO methodology. REA: resources. OAO: writing—original draft preparation. REA and AMA: writing—review and editing. OAO: formal analysis. REA and AMA: supervision. All authors have read and agreed to the submitted version of the manuscript.

Abstract

This study investigated the fractionation of *Telfairia occidentalis* (TO) leaf aqueous extract into chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions and the differences between their *in vitro* bioactive properties were determined. The extract was fractionated on a silicic acid-packed column using 20% ethanol as the eluting solvent. CH (unbound polyphenolic compounds) was collected until the solvent became clear while NC (bound polyphenolic compounds) was removed by washing the column with two column volumes of the 20% ethanol. Antioxidant, antihypertensive, and antidiabetic properties of CH and NC were determined and compared to those of TO extract. Results showed that fractionation enhanced the antioxidant activities of CH and NC, especially superoxide and hydroxyl radical scavenging ability. The CH and NC fractions also inhibited activity of enzymes such as DPP-IV, α-amylase, pancreatic lipase, α-glucosidase, trypsin, and chymotrypsin that are implicated in various metabolic disorders. Generally, activity of the CH and NC fractions differed depending on the assay. The secondary and tertiary structures of enzymes were modulated by the CH and NC fractions, which suggest binding of the polyphenolic compounds to the enzyme protein.

Keywords: Polyphenols, chlorophyll, *Telfairia occidentalis*, antioxidant, enzyme inhibition, silicic acid, circular dichroism, intrinsic fluorescence

8.1 Introduction

Changes in the environment, pollution, toxins, chemicals, and radiation have led to generation of different kinds of free radicals that induce oxidative stress and tissue injury (Aryal et al., 2019; Baba & Malik, 2014). Reactive oxygen species (ROS) such as hydrogen peroxides, hydroxyl ion, and superoxide ion are highly reactive toxic molecules produced during normal cell metabolism in the body. They are responsible for the damage to vital cells such as DNA, nucleic acids, lipids and enzymes and their activities can cause tissue injury. These free radicals are responsible for various diseases in the human body such as cancer, inflammation and neurodegenerative conditions thus affecting the body's natural antioxidant defense (Aryal et al., 2019; Baba and Malik, 2014). Although the body has some endogenous antioxidant enzymes such as glutathione, superoxide dismutase and catalase that could counter the effect of free radicals, these antioxidants may not be sufficient to perform this function. Therefore, exogenous sources of antioxidant that could perform this function are needed. Natural antioxidants from dietary sources rich in flavonoids and other phenolic compounds can play a vital role in reducing the destructive activity of free radicals.

Due to the evidence from different studies that natural products or compounds from plants could suppress the activity of free radicals and associated disease conditions, a lot of attention has been focused on plants (Sudan et al 2014). Phytochemicals such as flavonoids and other phenolic compounds present in plants play a crucial role as natural antioxidants to counter the oxidative stress induced by free radicals in the human body. Different synthetic drugs have been used to improve the body's antioxidant defense and to counter the activity of free radicals, but their use is associated with some harmful side effects. Due to these undesirable effects, a lot of focus has been on natural products from plants because they are cheap, safe, and easily

tolerated in the body system with little or no side effects. Therefore, the phenolic compounds can provide alternatives to synthetic drugs for the treatment of various diseases that arise from the activities of toxic free radicals. Moreover, epidemiological studies have shown that consumption of leafy vegetables with high antioxidant properties could lower the development of diseases such as cancer, neurodegeneration, and cardiovascular impairment.

Telfairia occidentalis, commonly known as fluted pumpkin is a plant that is native to Nigeria and other parts of West Africa. It is locally referred to as ugu and planted basically for its leaves and edible seeds (Osukoya et al., 2016). It belongs to the Cucurbitaceae family and is used in soups and herbal medicines (Nwanna & Oboh, 2007b; Okoli & Mgbeogu, 1983; Osukoya et al., 2016). Traditionally, the leaves have been used to treat inflammation, convulsion, pain, malaria, and anaemia (Osukoya et al., 2016). The leaf is a rich source of protein, oils, carbohydrates, and minerals (Oboh et al., 2012). Chlorophyll is the green pigment present in plants for photosynthesis process but also has a good antioxidant property that could quench the effect of free radicals and function therapeutically to purify the blood from toxins, prevent cancer and other types of diseases (Oluwagunwa et al., 2019). Chlorophyll can contribute to the antioxidant properties of plant extracts. Therefore, this work aims to determine the effect of chlorophyll removal on the antioxidant, antihypertensive and enzyme inhibition properties of *Telfairia occidentalis* polyphenolic fractions.

8.2 Materials and Methods

8.2.1. Materials

The 2,2-diphenyl-1 picrylhydrazyl radical (DPPH), butylated hydroxytoluene (BHT), 3-(2-Pyridyl)- 5,6-diphenyl-1,2,4-triazine-40,400-disulfonic acid sodium salt (Ferrozine), 2,4,6-tripyridyl-s-triazine (TPTZ), galanthamine, AChE (electric eel), and Folin–Ciocalteu phenol

reagent were purchased form Sigma Aldrich (Sigma Chemicals, St. Louis, MO). All other reagents were of analytical grade and purchased from Fisher Scientific (Oakville, ON, Canada). *Telfairia occidentalis* leaves (TO) were produced at the Micro-Veg Project experimental location, in Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria. The vegetable was produced with application of mineral fertilizer according to fertilizer micro-dosing technology using a randomized complete block design with 60 kg of urea/ha. Fertilizer was applied to each plot at planting (T1) or two weeks after seedling emergence (T2) to obtain 60T1 and 60T2. The leaves obtained at both fertilizer application (T1 and T2) was mixed and used for this study. Leaves were harvested 25 days after emergence, rinsed in potable water, destalked, dried in a hot air cabinet at 60 ^{o}C for 8 hr, milled to fine powder using a Marlex Excella dry mill (Marlex Appliances PVT, Daman, India) and stored at -20 ^{o}C.

8.2.2 Extraction of free polyphenolic compounds

Extraction of the free water-soluble polyphenols was carried out according to the method of Olarewaju et al. (2018). Samples were extracted using distilled water at 1:20 ratio (leaf powder:water) at 60 ^{o}C for 2 hr in a 500 mL beaker under continuous stirring. The extracts were allowed to cool to room temperature and centrifuged at 10,000 x *g* for 30 min. The supernatants were filtered through a cheesecloth and the process repeated to obtain a second supernatant. Both supernatants were pooled and concentrated under vacuum in a rotatory evaporator at 60 ^{o}C. The concentrated extracts were freeze-dried and stored at -20 ^{o}C.

8.2.3. Fractionation of leaf extracts

The dried extract obtained from the aqueous extraction process was used for further work. Since leaves have a high content of chlorophylls that may produce pro-oxidative effects, the leaf extracts were first separated into chlorophyll-enriched and chlorophyll-depleted fractions

on a column as follows. The 70 ml bed volume XK 16/26 (1.6×26 cm) column (GE Healthcare, Montreal, PQ) was packed with silicic acid and washed with 2 volumes of ethanol followed by 2 volumes of 20% ethanol. The leaf extracts were dissolved in double distilled water and 5 mL was applied to the top of the column at a concentration of 10 mg/mL. The column was then washed continuously with the 20% ethanol until the eluate (chlorophyll-enriched fraction) was clear and had no trace of green colour. After a clear colour was obtained, the non-chlorophyll fraction was collected by continuous washing with two column volumes of 20% ethanol. The 20% ethanol (chlorophyll-enriched) and (chlorophyll-depleted) fractions were concentrated in a rotary evaporator and the residues freeze-dried for assay of antioxidant properties.

8.2.4 Total phenolic content

The total phenolic content (TPC) of each extract was determined using the Folin–Ciocalteu method (Hoff & Singleton, 1977) with some modifications. A standard calibration curve was prepared using 25–350 mg/mL gallic acid concentration in 50% (v/v) methanol. The leaf extracts were also diluted with 50% methanol to a concentration range of 600–1,400 mg/mL. A 0.25 mL aliquot of Folin-Ciocalteu reagent was added to 0.25 mL of gallic acid solution or the sample and then mixed. After standing in the dark at room temperature for 5 min, 0.5 mL of 10% sodium carbonate solution was added followed by 4 mL of double distilled water. The contents were mixed and incubated in the dark for 1 hr. The intensity of the green color was then measured at 725 nm using an Ultraspec UV–visible spectrophotometer (GE Healthcare, Montreal, PQ, Canada). TPC was expressed as milligrams gallic acid equivalents (GAE) per gram of dry leaf powder (mg GAE/g).

8.2.5 Total flavonoid content

The total flavonoid content (TFC) was determined colorimetrically according to the method described by Nabavi et al. (2013b) with modifications (Olarewaju et al., 2018). An aliquot of 30 mL extracts in methanol was sequentially mixed with 90 mL of methanol, 6 mL of 10% aluminum chloride, 6 mL of 1 M potassium acetate, and 168 mL of double distilled water, followed by incubation in the dark at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm in a Synergy H4 multi-mode microplate reader (Biotek Instruments, Winooski, Vermont). TFC was calculated as rutin equivalent from a rutin calibration curve (0.05, 0.1, 0.125, 0.25, 0.5, and 1 mg/mL in methanol).

8.2.6 UHPLC MS/MS Analysis of Polyphenolic Compounds

An Agilent 1290 UHPLC system (Santa Clara, CA, USA) coupled with an HSS T3 2.1 _ 100 mm 1.7 µm column from Waters Corp (Milford, MA, USA) was used to perform UHPLC analysis of the vegetable extract and fractions. Samples were mixed with distilled water, vortexed, and passed through a 0.2 µm filter. Then, a 5 µL portion of the filtrate was injected onto the column followed by elution at a flow rate of 0.5 mL/min using mobile phases A and B (0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively) at 40 ^{o}C. The following gradients were used: initial holding time 0.5 min, mobile phase B ramped up to 50% after 5 min, 95% after 6 min, held for 1 min, and re-equilibrated for 1.5 min. Compounds were identified using a diode array detector at a wavelength range of 230–640 nm in 2 nm increments and a frequency of 5 Hz. The mass spec was carried out in an Agilent 6550 QTOF (Santa Clara, CA, USA) at 200 ^{o}C, using a drying gas pressure of 18 psi, 40 psi nebulizer and 350 ^{o}C sheath gas, a pressure of 12 psi, and 3500 V capillary with a 1000 V nozzle, and ran in positive ion electrospray at a frequency of 3Hz and acquisition from 30–1700 m/z. The MS/MS was

performed at a narrow quadruple setting (1.3 atomic mass units), using 10, 20, and 40 eV collision energy and 30–1700 m/z. The compounds were identified using MS/MS fragmentation patterns and quantified based on the MS peak area.

8.2.7 Estimation of chlorophyll content

Dried leaf powders (0.5 g) were extracted with 40 mL of 80% acetone according to a previous method (Rajalakshmi & Banu, 2015) and the extract centrifuged at 7500 g for 5 min. The supernatant was transferred into a glass container and the extraction procedure repeated until the residue became colorless; the extracts were then pooled together. Absorbance of the pooled extract was read at 645 nm (A645) and 663 nm (A663) against the solvent (80% acetone) blank. The concentrations of chlorophyll a, chlorophyll b and total chlorophyll were calculated using the following equation (Rajalakshmi and Banu, 2015):

Total chlorophyll: 20.2(A645) + 8.02(A663)
Chlorophyll a: 12.7(A663) – 2.69(A645)
Chlorophyll b: 22.9(A645) – 4.68(A663)

8.2.8 DPPH radical scavenging assay

The scavenging activity of vegetable leaf extracts against DPPH radical was determined using a previously described method (Aluko & Monu, 2006), which was modified for a 96-well clear flat-bottom plate. The leaf extracts were dissolved in 0.1 M sodium phosphate buffer, pH 7.0 containing 1% (w/v) Triton X-100 to give 0.0625, 0.125, 0.25, 0.5, and 1 mg phenolics/mL assay concentrations. DPPH was dissolved in methanol to a final concentration of 100 mM. A 100 mL aliquot of the plant extract solution was mixed with 100 mL of the DPPH solution in the 96-well plate and incubated at room temperature in the dark for 30 min. The absorbance values of the blank and samples were measured at 517 nm using the Synergy H4 multi-mode microplate reader. The blank consisted of buffer only instead of the plant extract while BHT was used as a

positive control. The percentage DPPH radical scavenging activity of the extracts was determined using the following equation.

$$\text{DPPH inhibition (\%)} = \frac{\text{Abs of blank} - \text{Abs of sample}}{\text{Abs of blank}} \times 100$$

8.2.9 Metal ion chelation

The metal ion chelating activity (MCA) was measured using a modified method as described by Xie et al. (2008). Briefly, a 1 mL solution of leaf extracts, BHT (final assay concentration of 0.0625, 0.125, 0.25, 0.5, and 1 mg phenolics/mL) or distilled water (blank) was added to 925 mL of water and 0.05 mL of 2 mM $FeCl_2$ in a reaction tube. After mixing, 25 mL of 5 mM Ferrozine solution was added and vortexed thoroughly. The mixture was then allowed to stand at room temperature for 10 min and an aliquot of 200 mL pipetted into a clear bottom 96-well plate. The absorbances of blank and samples were measured at 562 nm using the Synergy H4 multi-mode microplate reader. Percentage metal chelating effect was calculated using the following equation:

$$\text{Metal chelation activity (\%)} = \frac{\text{Abs of blank} - \text{Abs of sample}}{\text{Abs of blank}} \times 100$$

8.2.10 Ferric reducing antioxidant power (FRAP)

FRAP activity was measured using a previously described protocol (Benzie & Strain, 1999), which was slightly modified as follows. The FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), 10 mM TPTZ prepared in 40 mM HCl, and 20 mM $FeCl_3$ $6H_2O$ in the ratio of 10:1:1. The leaf extracts (40 mL) were mixed with 200 mL of working FRAP reagent in a 96-clear well microplate to give 0.0625–1.0 mg phenolics/mL final assay concentration followed by absorbance measurement at 593 nm in the Synergy H4 multimode microplate reader. A standard curve for $FeSO_4.7H_2O$ was prepared using a concentration of 25–

150 mM. The results were expressed in mM of Fe^{2+} reduced per gram of extract using the calibration curve of the $FeSO_4$ standard.

8.2.11 Total antioxidant capacity

Total antioxidant capacity (TAC) of the leaf extracts was evaluated using the phosphomolybdenum method as previously described (Prieto et al., 1999) with slight modifications. A 0.25 mL aqueous aliquot of the leaf extract was combined with 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) to give assay concentrations in the 7.5–20 mg phenolics/mL range. The tubes containing the reaction mixtures were incubated at 95 ^{o}C for 90 min, allowed to cool to room temperature, and absorbance measured at 695 nm using the Synergy H4 multimode microplate reader with distilled water as blank. A calibration curve was prepared using 2.0–4.5 mg/mL gallic acid and the absorbance of each sample was used to determine the gallic acid equivalent using the standard curve.

8.2.12 Hydroxyl radical scavenging assay

The hydroxyl radical scavenging assay (HRSA) was modified based on a method described by Fasakin et al. (2011). 1,10- phenanthroline (3 mM) and samples (1 mg/mL) were prepared using 0.1 M sodium phosphate buffer (pH 7.4). $FeSO_4$ (3 mM) and 0.01% hydrogen peroxide were separately prepared in distilled water. An aliquot (50 µL) of the sample was first added to a clear, flat bottom 96-well plate followed by addition of 50 µL of 1,10-phenanthroline and 50 µL of $FeSO_4$. To initiate reaction in the wells, 50 µL of hydrogen peroxide solution was added to the mixture, which was then covered and incubated at 37 °C for 1 h with shaking. The absorbance of the colored reaction mixtures was measured at 10 min intervals for 1 h in a microplate reader at a wavelength of 536 nm. The blank was prepared in a similar manner as the sample but was made

up of 50 µL of the phosphate buffer in place of the sample. The reaction rate (ΔA/min) was then used to evaluate the HRSA value as follows:

$$\text{HRSA (\%)} = \frac{\Delta A/\text{min of blank} - \Delta A/\text{min of sample}}{\Delta A/\text{min of blank}} \times 100$$

8.2.13 Superoxide scavenging assay

The superoxide radical scavenging activity was determined according to the method described by Famuwagun et al. (2020). The samples (80 µL of 1 mg/mL solution) were each mixed with 80 µL of 50 mM Tris–HCl buffer (pH 8.3) containing 1 mM EDTA in a clear bottom 96-well plate. Then, 40 µL of 1.5 mM pyrogallol dissolved in 10 mM HCl was added to each well. The reaction rate (ΔA/min)s was monitored as increase in absorbance at 420 nm for 4 min at room temperature. The blank mixture, (ΔA/min)b contained the Tris–HCl buffer but no leaf fraction. The superoxide radical scavenging activity of the leave samples was calculated using the following equation:

$$\text{Superoxide scavenging activity (\%)} = \frac{\Delta A/\text{min of blank} - \Delta A/\text{min of sample}}{\Delta A/\text{min of blank}} \times 100$$

8.2.14 *In-vitro* acetylcholinesterase inhibition assay

The assay for AChE activity was conducted using Ellman's colorimetric method with acetylthiocholine iodide (ATCI) as substrate (Khan & Ab Ghani, 2012). Briefly, 25 µL of 15 mM ATCI, 75 µL of 3 mM DTNB, and 50 µL of 50 mM Tris HCl, pH 8.0 containing 2% (w/v) bovine serum albumin (BSA) were mixed with 25 µL of each sample or 10–50 µg/mL galanthamine (standard drug) in a microplate. The blank reaction contained all these reagents except the sample or galanthamine. This was followed by addition of 25 µL AChE (0.26 U/mL) dissolved in buffer containing 1% BSA and the reaction samples were incubated at room temperature for 30 min. The absorbance was then measured in the Synergy H4 multimode

microplate reader at 405 nm for 20 min at 37 ^{o}C. Percentage inhibition of AChE activity was calculated as follows.

$$\text{AChE inhibition (\%)} = \frac{\text{Abs (B)} - \text{Abs (S)}}{\text{Abs (B)}} \times 100$$

where, Ab (B) is the absorbance of the blank (uninhibited reaction) and Ab (S) is the absorbance of the sample (inhibited reaction).

8.2.15 α-Amylase inhibition assay

The α-amylase inhibitory activity of leaf extracts was determined using the method described by Karakaya et al. (2018b) with slight modifications. The samples (dried leaf extracts) were dissolved in 0.02 M sodium phosphate buffer containing 0.006 M NaCl, pH 6.9. A 100 µL aliquot of each sample (assay concentrations of 1.1-2.0 mg/mL) and 100 µL of α-amylase enzyme solution (1 mg/mL) were added to test tubes and allowed to incubate at 37 ^{o}C for 10 min. After incubation, 100 µL of 1% (w/v) starch solution was added to test tubes and the reaction mixture incubated at 37 ^{o}C for 10 min. The reaction was terminated by adding 200 µL of dinitro-salicylic acid (DNSA) a color reagent (96 mM DNSA, 2 M sodium potassium tartrate tetrahydrate and 2 M NaOH) followed by incubation in a boiling water bath at 100 ^{o}C for 5 min. The reaction mixture was cooled down to room temperature, after which 3 mL of double distilled water was added. A 200 µL aliquot of the reaction mixture was then transferred to a 96-well microplate and the absorbance read at 540 nm using a Synergy™ H4 microplate reader (Biotek™, Vermont, USA) at 37 ^{o}C. Acarbose (drug) was used as the positive control. Percentage inhibitions of all samples were calculated using the equation:

Inhibition (%) = [((Ac − (As − Asb)) / Ac] * 100

Ac = Absorbance of the control As = Absorbance of the sample, Asb = Absorbance of the sample blank. (Sample blank = sample without enzyme).

8.2.16 α-Glucosidase inhibition assay

The inhibitory activity against α-glucosidase was assayed according to previously described methods (Ranilla et al., 2010; Shobana et al., 2009) with slight modifications. Briefly, 300 mg of rat intestinal acetone powder was homogenized in 9 mL of 0.9% (w/v) NaCl solution and centrifuged at 12,000 x g for 30 min; the clear supernatant was used as a source of α-glucosidase enzyme. Polyphenol extracts were dissolved in 0.1 M sodium phosphate buffer, pH 6.9. Fifty µL of extracts (assay concentrations of 1-5 mg/mL) were mixed with 50 µL of α-glucosidase enzyme (assay concentration = 8.33 mg/mL) in a 96-well microplate and incubated at 37 ^{o}C for 15 min. Following incubation, 100 µL of 5 mM 4-nitrophenyl α-D-glucopyranoside (PNP-glycoside) solution (in 0.1 M sodium phosphate buffer, pH 6.9) was added to each well and absorbance read continuously at 405 nm for 30 min (at every 30 s interval) using the Synergy™ H4 microplate reader set at 37 ^{o}C. A blank reading (no enzyme added) was subtracted from each well. α-glucosidase activity was quantified by measuring the p-nitrophenol released from the PNP-glycoside at 405 nm. Acarbose was assayed using the same protocol and served as a positive control. The α-glucosidase inhibitory activity (%) was calculated using the equation:

Inhibition (%) = [((Ac -Acb) − (As -Asb)) / [Ac − Acb] *100

Ac = Absorbance of the control, Acb = Absorbance of the control blank, As = Absorbance of the sample, Asb = Absorbance of the sample blank. (Sample blank = sample without enzyme).

8.2.17 Pancreatic lipase (PL) inhibition assay

PL inhibitory activity of the extracts was determined using the method described by (Tang et al., 2016) with slight modifications. PL activity was determined by measuring the release of 4-methylumbelliferone (4-MU) from the substrate 4-methylumbelliferyl oleate (4-MU oleate). A 25 µL aliquot of samples (assay concentrations of 0.5-2.5 mg/mL) dissolved in Tris

buffer (13 mM Tris-HCl, 150 mM NaCl and 1.3 mM $CaCl_2$, pH 8.0) and 225 µL of a 0.5 mM 4-MU oleate solution were mixed in a 96-well microplate and incubated for 15 min at 37 °C. After incubation, 25 µL of PL solution (assay concentration = 3.125 U/mL) was added to start the enzyme reaction and then incubated at 37 ^{o}C for 1 hr. After incubation, the amount of 4-methylumbelliferone released by the lipase was measured with a fluorimeter at an excitation wavelength of 340 nm and emission wavelength of 450 nm. Orlistat served as a positive control and was analyzed using same protocol. The PL inhibitory activity (%) was calculated using the equation:

Inhibition (%) = [Ac − As] /Ac] * 100

Ac = Absorbance of the control, As = Absorbance of the sample.

The concentration of extract that reduced enzyme activity by 50% (IC_{50}) was obtained by non-linear regression analysis of a plot of PL inhibition (%) versus the sample concentrations using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA).

8.2.18 Trypsin inhibitor assay

Inhibition of trypsin activity was determined using the method of Souza et al. (2016) with slight modifications. Inhibition of trypsin activity was determined by measuring the residual enzyme activity of the substrate BApNA, at pH 7.5 in the presence of the sample (leaf extract and column fractions). Trypsin (200 µL, at 60 µg/mL assay concentration), dissolved in Tris-HCl buffer (pH 7.5) containing 0.02 M $CaCl_2$, was pre-incubated for 5 min at 37 °C with 200 µL of the buffer (full enzyme activity) or with 200 µL of sample (inhibited reaction). Enzyme catalysis was initiated by adding 500 µL of 1 mM BApNA prepared with 1% (v/v) DMSO in Tris buffer. After 10 min at 37 °C, the reaction was stopped by adding 100 µL of 30% (v/v) aqueous acetic

acid. The extent of the enzymatic hydrolysis of the substrate was evaluated by recording the increase in absorbance at 410 nm.

8.2.19 Chymotrypsin Inhibition Assay

Chymotrypsin inhibitory activity was determined following the method described by El-latif (2014). Briefly, 200 µL of chymotrypsin (dissolved in 0.01 M Tris-HCl buffer containing 0.02 M $CaCl_2$, pH 8.0; assay concentration of 20 µg/mL) and 200 µL of samples (dissolved in the same buffer) were pre-mixed and incubated for 15 min at 37 ^oC. The reaction was started by the addition of 500 mL of 1 mM BTpNA (N-Benzoyl-L-tyrosine p-nitroanilide) prepared in 0.01 M Tris-HCl buffer containing 0.02 M $CaCl_2$ and 40% (v/v) ethanol, pH 8.0. Following incubation for 15 min at 37 ^oC, the reaction was terminated by the addition of 100 µL of 30% (v/v) acetic acid. A 200 µl aliquot of the reaction mixture was transferred to a 96-well microplate and absorbance measured using a microplate reader at 410 nm. Chymotrypsin inhibitory activity was determined by measuring the release of p-nitroaniline from the BTpNA substrate. 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) was used as a positive control. The chymotrypsin inhibitory activity (%) was calculated using the equation:

Inhibition (%) = [Ac − As] /Ac] x 100

Ac = Absorbance of the control

As = Absorbance of the sample

8.2.20 Mode of enzyme inhibition

The mode of inhibition of the enzymes α-glucosidase or trypsin by leaf extracts was determined by studying enzyme activities at various substrate and sample concentrations as described above. The inhibition pattern was then determined from the double reciprocal Lineweaver-Burk plots, in which the inverse of the initial rate was plotted against the inverse of

the substrate concentration in the presence or absence of the extracts. PNP-glycoside in the concentration range 0.5-3 mM and BApNA in the 0.25-2.5 mM range were used as substrates for α-glucosidase and trypsin reactions, respectively. The range of concentrations of leaf extracts used for the inhibitory kinetics was 0.05-1.75 mg/mL (α-glucosidase) and 1-10 µg/mL (trypsin). The Lineweaver-Burk plots and kinetic parameters were obtained using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA).

8.2.21 DPP-IV inhibitory activity assay

DPP-IV inhibitory activity was measured in triplicate using DPP-IV assay kit (Cayman Chemical, Ann Arbor, MI, USA). Briefly, the samples were diluted in the assay buffer (20 mM Tris-HCl pH 8.0 containing 100 mM NaCl and 1mM EDTA) to the desired concentrations. In a 96-well microplate, the activity well (assay control well) contained 30 µL of assay buffer, 10 µL of DPP-IV enzyme and 10 µL of the solvent used to dilute the sample. The background well (blank well) contained 40 µL of assay buffer and 10 µL of solvent while the inhibitor well (sample well) contained 30 µL of assay buffer, 10 µL of DPP-IV enzyme and 10 µL of sample/standard. The reaction rate was initiated by adding 50 µL of substrate (5 mM H-Gly-Pro conjugated to aminomethylcoumarin) to all the wells and incubated for 30 min at 37 ^{o}C. The plate was read after incubation using an excitation and emission wavelength of 360 and 465 nm respectively in a fluorimeter (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA). The fluorescence intensity (FI) of the background well was subtracted from the activity and inhibitor well. The percentage DPP-IV inhibition was calculated using the following equation:

$$\% \text{ DPP-IV inhibition} = \frac{\text{FI of control well} - \text{FI of sample well}}{\text{FI of control well}} \times 100$$

8.2.22 ACE Inhibition Assay

The method described by Oluwagunwa et al. (2019) was used to determine ACE-inhibitory activity of the leaf extract and column fractions. ACE, FAPGG (ACE substrate), and samples were individually dissolved in 50 mM Tris-HCl buffer, pH 7.5 containing 0.3 M NaCl. A 10 µL aliquot of ACE (assay activity of 25 mU) was added to each well containing 170 µL of 0.5 mM FAPPG and 20 µL of samples at 37 ^{o}C. The buffer was used as blank (uninhibited reaction), while captopril, an ACE-inhibitory drug, was used as standard and assayed using a similar protocol. Absorption (Abs) was read at 345 nm at 1 min intervals for 30 min to determine the reaction rate. The slope of the blank or sample reactions was used to calculate the percentage ACE inhibition as follows:

$$\text{ACE inhibition (\%)} = \frac{\Delta\text{Abs of blank} - \Delta\text{Abs of sample}}{\Delta\text{Abs of blank}} \times 100$$

8.2.23 Renin Inhibition Assay

In vitro inhibition of the activity of human recombinant renin assay was conducted according to the method described by Oluwagunwa et al. (2019) using the Renin Inhibitor Screening Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). Prior to the assay, renin buffer was diluted with 50 mM Tris–HCl, pH 8.0, containing 100 mM NaCl. The renin protein solution was diluted 20 times with assay buffer before use, and the assay buffer was pre-warmed to 37 ^{o}C before initiating the reaction in a fluorometric microplate reader (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA) maintained at 37 ^{o}C. The reaction mixture contained: (1) 20 µL substrate, 160 µL assay buffer, and 10 µL Milli-Q water for the background wells; (2) 20 µL substrate, 150 µL assay buffer, and 10 µL Milli-Q water for control (uninhibited) wells; and (3) 20 µL substrate, 150 µL assay buffer, and 10 µL polyphenol extract sample for the inhibited wells. The reaction was initiated by adding 10 µL renin to the inhibited and uninhibited wells

only. The microplate was shaken for 10 s to mix and incubated at 37 ^{o}C for 15 min followed by recording fluorescence intensity (FI) using an excitation wavelength of 340 nm and emission wavelength of 490 nm. FI of the background wells was subtracted from those of the control and sample wells and percentage inhibition calculated as follows:

$$\text{Renin inhibition (\%)} = \frac{\text{FI of control well} - \text{FI of sample well}}{\text{FI of control well}} \times 100$$

8.2.24 Intrinsic fluorescence emission

The method described by Li and Aluko (2006) was used to record intrinsic fluorescence spectra on the Jasco FP-6300 spectrofluorometer (JASCO, Tokyo, Japan) at 25 ^{o}C with a 1 cm path length cuvette. Sample (leaf extract/fractions) stock solutions (10 mg/mL) and enzymes (PL or trypsin or α-amylase) were prepared in 13 mM Tris-HCl buffer containing 150 mM NaCl and 1.3 mM $CaCl_2$, pH 8 for PL or 20 mM sodium phosphate, containing 6 mM NaCl, pH 6.9 for amylase. The enzyme and sample solutions were then mixed to obtain assay concentrations of 1 mg/mL and 6.25 or 12.5 µg/mL, respectively, which were then used for fluorescence emission measurement. The fluorescence spectra were recorded at excitation wavelengths of 275 nm with emission recorded from 280 to 450 nm. Buffer emission spectrum was subtracted form those of the respective samples to obtain reported spectrum of each enzyme/extract mixture.

8.2.25 Measurements of circular dichroism (CD) spectra

The CD spectra of enzyme/leaf extract/fractions mixtures (α-amylase, trypsin and PL) complexes were measured at 25 ^{o}C in a J-810 spectropolarimeter (JASCO, Tokyo, Japan) using the spectral range of 190-240 nm (far-UV) for secondary structure determinations and 250-320 nm (near-UV) for tertiary structure according to the method described by Omoni and Aluko (2006). Stock solutions of vegetable extract and that of the enzymes (PL and α- amylase) were prepared as described above for intrinsic fluorescence. The extract and enzyme solutions were

mixed to give assay concentrations of 1-3 mg/mL and 10 mg/mL, respectively. The secondary structure was determined using a cuvette with path length of 0.05 cm while the tertiary structure was measured using a cuvette with 0.1 cm path length. The enzyme spectra obtained after subtraction of the respective buffer spectrum.

8.2.26 Statistical analysis

Minimum of duplicate determinations were used to obtain mean values and standard deviations. For statistical analysis, analysis of variance was used while significant differences ($p < 0.05$) between mean values were determined by the Duncan's multiple range tests. The IBM SPSS statistical package (version 24) was used for all statistical analyses.

8.3 Results and Discussion

8.3.1 Total phenolic (TPC) and total flavonoid (TFC) contents

Polyphenols are the most widespread secondary metabolites in the plant kingdom that are gaining more acceptance due to their health benefits. Based on their chemical structure, they are natural antioxidants with the ability to scavenge free radicals and chelate metal ions. It has been reported that the antioxidant activity of phenol is mainly due to its redox properties, hydrogen donors and singlet oxygen quenchers (Cosme et al., 2020; Lin et al., 2016). Therefore, in the present study, TPC of TO, CH and NC were estimated using a modified Folin–Ciocalteau method and the results are presented in Table 8.1. The results show that TPC was higher in TO (736.11 ± 4.29 mg GAE/g) than in CH (724.21 ± 3.74 mg GAE/g) and NC (688.49 ± 1.72 mg GAE/g). The values obtained in this study are higher than those reported for kangkong (27.65 ± 1.45 mg GAE/g) (Sultana et al., 2020), root of Vitex negundo (72.11 ± 0.73 mg GAE/g) (Rana et al., 2019) and picralima seed (356.53 ± 4.67 mg GAE/g) (Akinwunmi & Amadi, 2019), which may be due to differences in the materials or solvents used for extraction. The values reported for

Ficus carica cultivars (233.14 ± 12.25 µg GAE/mL to 311.83 ± 6.93 µg GAE/mL) are lower than those reported in this study (Shahinuzzaman et al., 2020). Other factors that may contribute to these differences include response to water stress, specie of the plant and other environmental conditions (Rana et al., 2019; Sultana et al., 2020). Flavonoids are considered the most abundant polyphenolic compounds and they are an important factor in the estimation of polyphenol accumulation by plants (Olarewaju et al 2018). The TFC content (Table 8.1) is reported as rutin equivalent (RE). The results show that TO extract had the highest TFC content (2887.07 µg/g RE) followed by CH (1454.66 µg/g RE) and NC (543.76 µg/g RE). Overall, the results suggest that regular consumption of these vegetables could contribute to significant flavonoid intake. This is important because consumption of flavonoid-containing fruits and vegetables has been suggested to be associated with protection against cancer and heart diseases (Sultana et al., 2021)

Table 8.1. Total phenol (TPC), total flavonoid (TFC) and chlorophyll content of *Telfairia occidentalis* aqueous extract (TO) and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions.

Samples	**TPC (mg GAE/g)**	**TFC (µg RE/g)**	**Chlorophyll a (mg/g)**	**Chlorophyll b (mg/g)**	**Total Chlorophyll (mg/g)**
CH	724.21 ± 3.74	1454.66 ± 88.5	14.82 ± 0.06	31.42 ± 0.11	46.22 ± 0.16
NC	688.49 ± 1.72	543.76 ± 0.00	9.53 ± 0.09	15.77 ± 0.23	25.29 ± 0.21
TO	736.11 ± 4.29	2887.07 ± 49.17	16.81 ± 0.09	21.05 ± 0.28	37.84 ± 0.21

8.3.2 UHPLC MS/MS Analysis

Antioxidants from plants such as polyphenols are becoming more important as a form of dietary intake. The medicinal value of plants lies in the bioactive constituents, such as alkaloids, tannins, coumarins, terpenoids, phenolic compounds that have been known to bear valuable therapeutic activities (Engwa et al., 2019; Phaniendra et al., 2015; Repetto et al., 2009). Therefore, identification of phenolic compounds in plants is very important because this helps in their characterization and their efficient uses as important plant resources. The major polyphenolic compounds identified in the TO leaf extract as well as the CH and NC fractions are shown in Table 8.2. It was found that the extract and the fractions have the same phenolic compounds but at different concentrations. The identified compounds were mainly flavonoid glycosides of quercetin and kaempferol. The two main compounds found in the extract and fractions were quercetin O-rutinoside and kaempferol O-rutinoside. TO extract had the highest concentration of the quercetin O-rutinoside followed by NC and CH fractions. CH fraction had the highest concentration of kaemferol O-rutinoside followed by TO extract and NC fraction. Quercetin is an antioxidant that has been reported to scavenge radicals, inhibit lipid peroxidation, and chelate metals (Anand David et al., 2016; Bentz, 2017; Salehi et al., 2020; Treml & Šmejkal, 2016) and may be the main compound that contributed to the antioxidant activities of the extract and column fractions.

8.3.3 Chlorophyll content

Chlorophyll pigments are one of the most abundant natural pigments in nature and indispensable for life. They are consumed in our diet when ingested along with green vegetables and fruits (Chen & Roca, 2018; Wang et al., 2020). Chlorophyll is responsible for the green colour of plants and is an antioxidant compounds present and stored in the chloroplast of green

leafy plants (Kamble et al., 2015; Li et al., 2019). It has different health and medicinal benefits such as chelation of chemical carcinogens, scavenging of free radicals, reducing cancer risk (Chen & Roca, 2018) and also plays an important role in reducing blood glucose level, enhanced digestion and decreased allergenicity (Mulay, 2019; Sultana et al., 2020). Chlorophyll-a is the main photosynthetic pigment responsible for the production of energy in plants while chlorophyll-b consists of accessory pigments (Kamble et al., 2015; Sultana et al., 2020).

Table 8.1. Main polyphenolic compounds of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

Samples	Polyphenolic compounds	Retention time (min)	m/z (Da)	MS/MS (Da)	Concentration (mg/g)
CH	Quercetin O-rutinoside	3.4	611	303, 449	1.352
	Kaempferol O-rutinoside	3.4	595	287	8.481
NC	Quercetin O-rutinoside	3.4	611	303, 449	1.691
	Kaempferol O-rutinoside	3.4	595	287	3.589
TO	Quercetin O-rutinoside	3.4	611	303, 449	8.357
	Kaempferol O-rutinoside	3.4	595	287	7.051

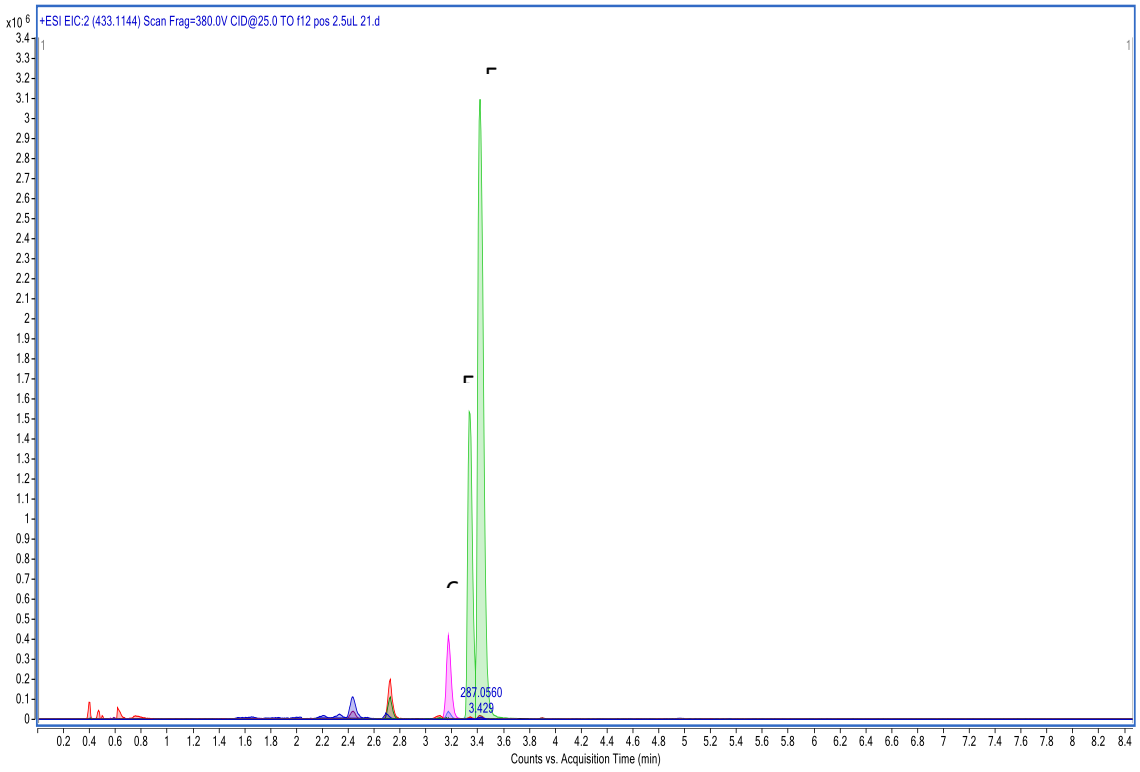

Figure 8. 1 MS chromatogram profile of aqueous extract of *Telfairia occidentalis* (TO) leaf extract

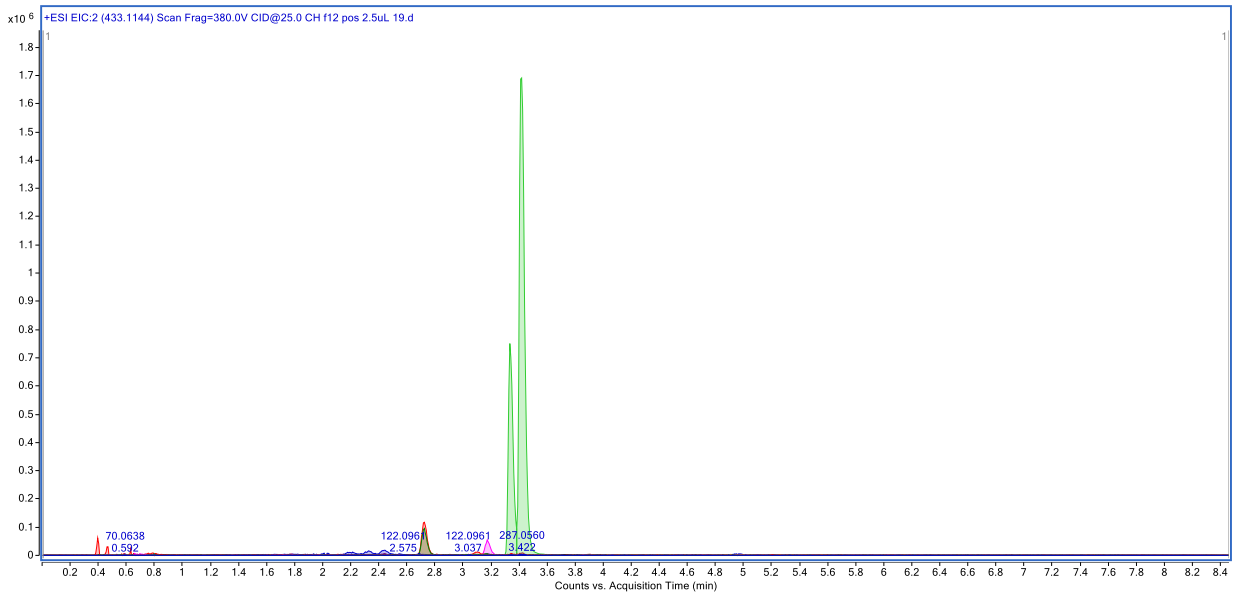

Figure 8. 2 MS chromatogram profile of aqueous extract of *Telfairia occidentalis* leaf chlorophyll-enriched (CH) fraction

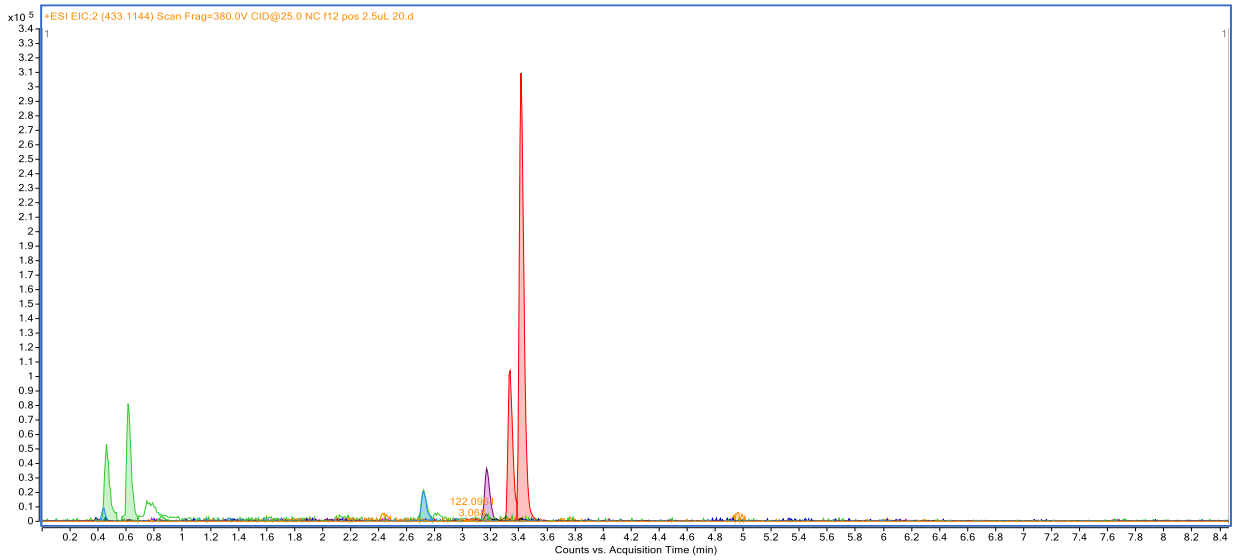

Figure 8. 3. MS chromatogram profile of aqueous extract of *Telfairia occidentalis* leaf chlorophyll-depleted (NC) fraction

The results obtained for chlorophyll content of the extract and fractions are shown in Table 8.1. The CH fraction had the highest total chlorophyll content followed by TO extract while NC fraction had the lowest value. Chlorophyll-a was found to be highest in TO extract and lowest in NC fraction. CH, NC and TO had higher chlorophyll-b than chlorophyll-a and this agrees with the work of Rajalakshmi and Banu, 2015. Based on previous report, it has been established that chlorophyll-a is three times more abundant than chlorophyll-b (Kamble et al., 2015), which is different from what was obtained in this study. This variation may be due to the reduced solubility of chlorophyll-a in water, which was used for the leaf extraction (Sultana et al., 2020).

8.3.4 DPPH radical scavenging activity

The DPPH scavenging assay is a rapid technique for screening the radical scavenging activity of synthetic compounds, isolated natural compounds, crude plant extracts and foods. DPPH is an assay that measures the ability of antioxidants to react with DPPH radical at 517 nm. It is a stable organic nitrogen-centered free radical with a dark purple color. Upon reaction with an antioxidant, the purple color becomes colorless (Famuwagun et al., 2020). The DPPH radical scavenging activities of the leaf extract fractions are presented in Fig. 8.2. The results showed that DPPH scavenging ability of NC fraction (61.19 ± 0.16%) increased with maximum scavenging activity at 0.5 mg/mL while the CH fraction (57.60 ± 0.42%) and TO extract (55.39 ± 0.16%) had maximum scavenging activity at 0.25 mg/mL. The DPPH radical scavenging activities of NC fraction was higher than that of CH fraction and TO extract. This support the findings of Fasakin et al. (2011) that chlorophyll-depleted fraction of *Vernonia amygdalina* and *Gongronema latifolium* leaves are better DPPH radical scavengers than the chlorophyll-enriched fraction. The DPPH radical scavenging activities reported in this work for all the fractionated

extracts were lower than those reported for *Telfairia occidentalis* (71.66%) in another study (Kambizi et al., 2017). The pathogenesis of several disease conditions such as diabetes, cancers, inflammation, and other cardiovascular diseases has been linked with the production of free radicals within the body. Due to fewer negative side effects, cheaper cost, and natural abundance when compared to drugs, antioxidants from plant sources are important as potential tools in preventing the development of chronic human disease conditions (Kambizi et al., 2017). The results from this study show that the column fractions are better scavengers of free radicals than the whole leaf extract. However, because of the demonstrated DPPH radical scavenging activity of the extract, consumption of the TO vegetable may help in preventing the onset of some of diseases caused my toxic free radicals.

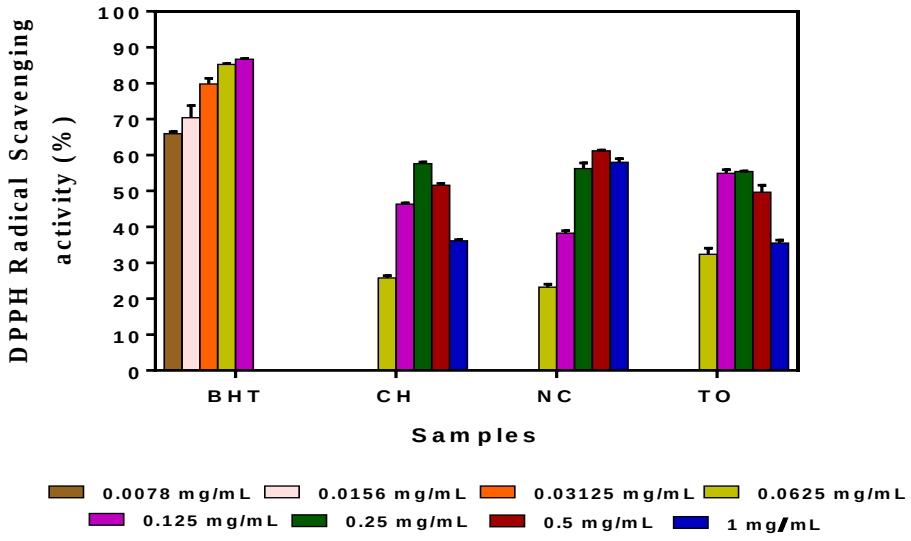

Figure 8. 4. DPPH radical scavenging activity of aqueous extract of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

8.3.5 Metal chelation activity

Metal chelating ability is one of the methods used to evaluate the ability of a compound to prevent metal-catalyzed generation of toxic free radicals (Fasakin et al., 2011). The ferrous ion

Fe^{2+} is a transition metal with the ability to initiate formation of free radicals whether by gaining or losing electrons (Sudan et al., 2014). The most important transition metals implicated in human diseases are iron and copper because they initiate lipid peroxidation and formation of hydroxyl radicals (Engwa et al., 2019; Phaniendra et al., 2015b; Repetto et al., 2009). Chelation of metal ions is, therefore, important in reducing the formation of free radicals. The chelating ability of TO, CH and NC were evaluated, and the results (Fig 8.3) show a dose-dependent activity. NC fraction (chlorophyll-depleted fraction) has the highest chelating power (75.55 ± 0.22%) at 1 mg/mL final concentration while the highest chelating ability for TO (67.21 ± 0.59%) and CH (62.13 ± 0.34%) was obtained at 0.5 and 1 mg/mL, respectively. The result obtained for TO extract in this study also correspond with that obtained for TO extract as reported by Olarewaju et al. (2018). The results are also higher than those obtained for Indian spinach (46.27± 1.31%), kangkong (31.61 ± 0.45%) and okra (26.33 ± 1.88%) (Sultana et al., 2020b). Excessive metal ion accumulation can lead to a variety of diseases in the body, including oxidative damage and neurodegenerative diseases like Alzheimer and Parkinson's (Aparadh et al., 2012; Sudan et al., 2014). The ability of plant extracts to chelate metal ion is of great benefit because they could help to reduce the toxic effect of metals in the body. Chelation therapy is a chemical procedure used to remove heavy metals or iron overload in the body (Ebrahimzadeh et al., 2008; Sudan et al., 2014). Therefore, the ability of these extract and fractions to chelate metal ions may be of therapeutic importance.

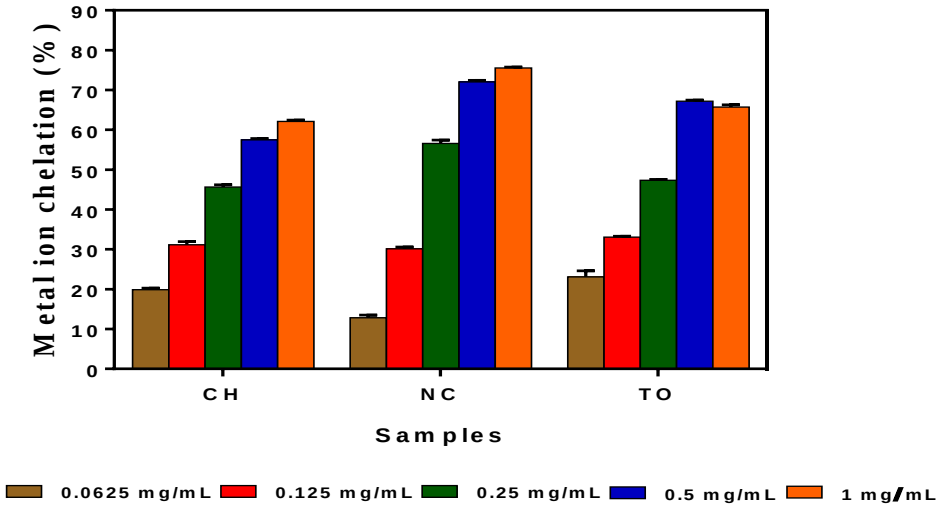

Figure 8. 5. Metal ion chelation activity of aqueous extract of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

8.3.6 Ferric reducing antioxidant power (FRAP)

The ability of the plant extracts to reduce ferric ions was determined using the FRAP assay. This is the measurement of the ability of a compound to donate electrons, which leads to conversion of Fe^{3+} to Fe^{2+} (Costa et al., 2015). Ability of the extract and fractions to act as a reducing agent is reported in Fig. 8.4. The results show a dose-dependent increase in FRAP for the extract and fractions as the values for TO, CH and NC increased at higher sample concentrations. The higher the absorbance, the better the reducing power of the tested sample. The TO extract exhibited the highest value (10.33 mmol Fe^{2+}) when compared to the fractions. This implied that TO have the highest reducing power followed by CH (9.79 mmol Fe^{2+}) and NC (4.69 mmol Fe^{2+}). Aryal et al. (2019) also reported a dose-dependent reducing power for *Solanum nigrum*. The results suggest that removal of chlorophyll from the extract diminished reducing power as evident in the lower FRAP for NC. The presence of multiple aromatic rings in chlorophylls may have provided more electrons to reduce ferric ion when compared to the NC.

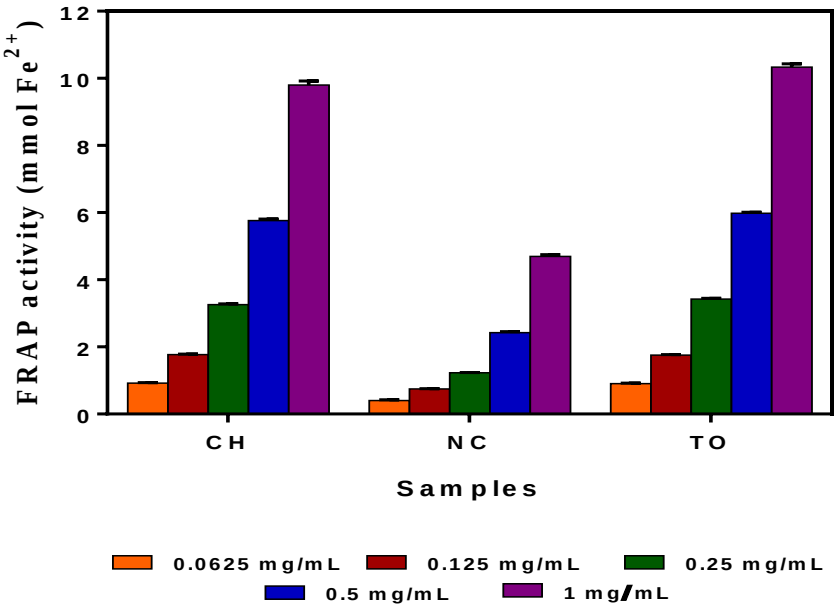

Figure 8. 6. Ferric reducing antioxidant power (FRAP) of aqueous extract of *Telfairia occidentalis* (TO) leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

8.3.7 Total antioxidant capacity (TAC)

Results of the TAC for CH, NC and TO are presented in Fig. 8.5. This method is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/Mo(V) complex with a maximal absorption at 695 nm. The results reveal that the TAC of the extract is in the increasing trend with increasing concentration and TO have a higher capacity than the fractions. Even after the removal of some of the chlorophylls, the NC fraction still had comparable TAC. The values obtained in this study compared well with the values obtained for TO extracts as reported by Olarewaju et al. (2018).

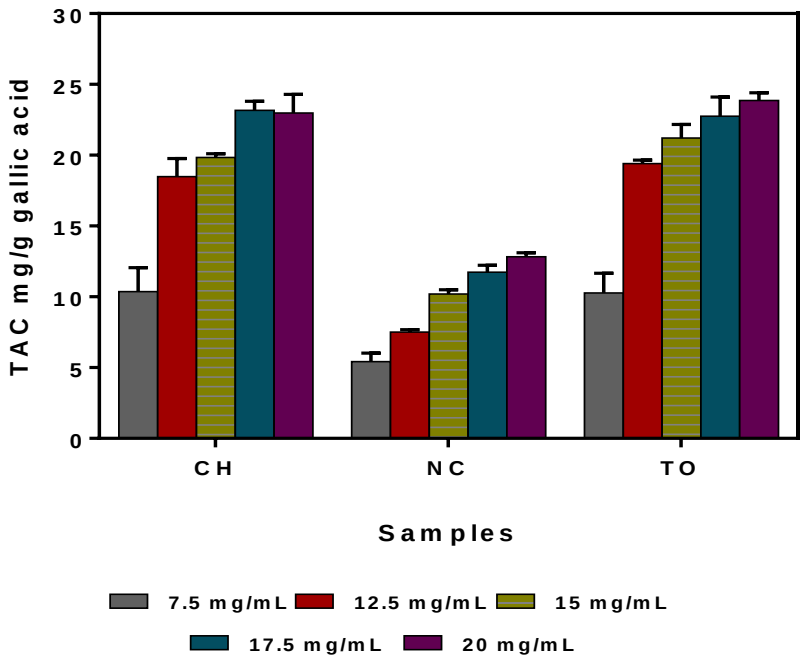

Figure 8. 7. Total antioxidant capacity of aqueous extract of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

8.3.8 Hydroxyl radical scavenging activity

The hydroxyl radical (•OH) is one of the highly toxic ROS that induce tissue damage by reacting with cellular macromolecules such as nucleic acid, proteins, and polyunsaturated fatty acids. Activity of the hydroxyl radical within the cell contributes to the development of various diseases such as cancer, diabetes, atherosclerosis, and aging (Phaniendra et al., 2015b). The breakdown of superoxide anions and hydrogen peroxide in the body can give rise to the production of hydroxyl radical thus causing oxidative stress. Therefore, scavenging hydroxyl radical will increase the antioxidant defense status of the body thus preventing the development of chronic diseases (Karthishwaran et al., 2018; Pizzino et al., 2017c). In this study, the hydroxyl radical scavenging ability of the fractions and extract were evaluated using an assay similar to the physiological system whereby hydroxyl radical was produced by the reaction of Fe^{2+} and hydrogen peroxide (Fenton reaction). The hydroxyl radical scavenging activity of the fractions

and extract are reported in Fig. 8.6. The results showed that the TO and NC had significantly higher ($p<0.05$) scavenging ability than the CH fraction. The levels of the •OH-scavenging activity displayed by all the fractions show that they can potentially be used as antioxidants *in vivo*. The •OH-scavenging activity of TO extract was not detected in the previous report of this study (Olarewaju et al., 2018) when TO extract at the individual fertilizer application was used. This suggests that combining the extracts together (T1 and T2) and fractionation enhanced its •OH-scavenging activity.

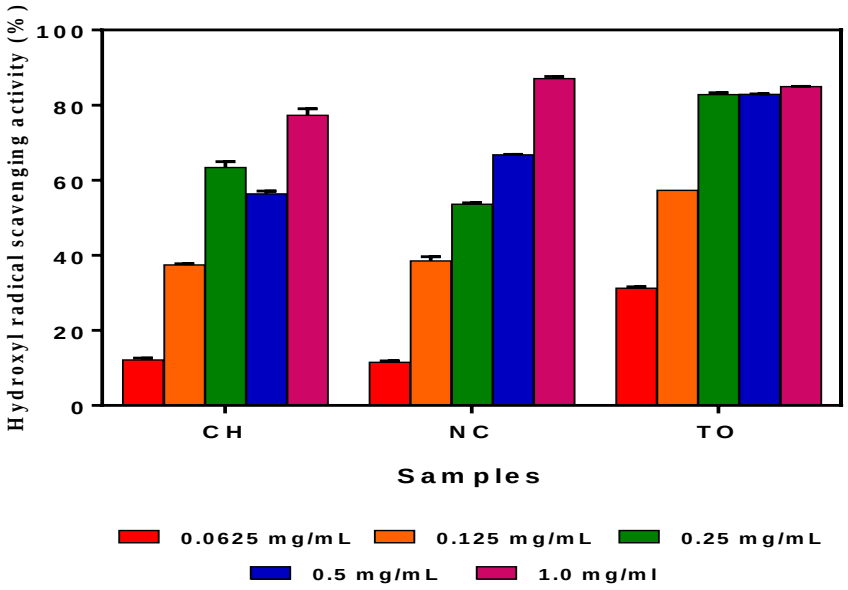

Figure 8.8. Hydroxyl radical scavenging activity of aqueous extract of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

8.3.9. Superoxide radical scavenging activity

One major test of antioxidant ability is its effectiveness in reacting or scavenging radicals generated in chemical model systems (Bajpai et al., 2017). The superoxide radical is produced by oxidation of molecular oxygen and this reaction is accelerated by the presence of transition metals such as copper or iron. Superoxide anions are precursors to active free radicals, which can react with biological macromolecules and causing tissue damage. The superoxide radical has

also been observed to directly initiate lipid peroxidation and plays a key role in the formation of other ROS such as hydroxyl radicals, which cause oxidative damage to lipids, proteins, and DNA (Bajpai et al., 2017; Fasakin et al., 2011; Venkatachalam & Muthukrishnan, 2012). In this study, the superoxide radical scavenging activities of TO, CH and NC are presented in Fig. 8.7. The values ranged from 14.17-19.38% for CH, 15.41-17.91% for NC and 7.08-13.54% for TO. The scavenging activity increases as the concentration increased for CH and NC and then decreased after reaching a maximum at concentration of 0.5 mg/mL. However, activity of the TO extract decreased as the concentration increases. CH fraction had the highest superoxide radical scavenging ability followed by NC and TO. The results obtained in this study are lower than those reported for ethanol extracts of *D. gangeticum* with 14.12-54.06% superoxide radical scavenging ability. Fractionation and mixing of TO60T1 and TO60T2 enhanced the superoxide radical scavenging ability of TO extracts because the values obtained in the current study were not achievable using the crude extract as previously reported (Olarewaju et al., 2018). This shows that the fractions are better scavengers of superoxide radicals than the extract, which indicates reduced antagonistic effects after separation of the extract molecules. A previous report has shown that plant flavonoids are good antioxidants that can scavenge free radicals (Gangwar et al., 2014) and their presence in TO, CH and NC could have contributed to the observed scavenging effects. However, the superoxide radical scavenging values obtained in this work are lower than those reported for *Rumex crispus* root extract and its fractions (Eom et al., 2020).

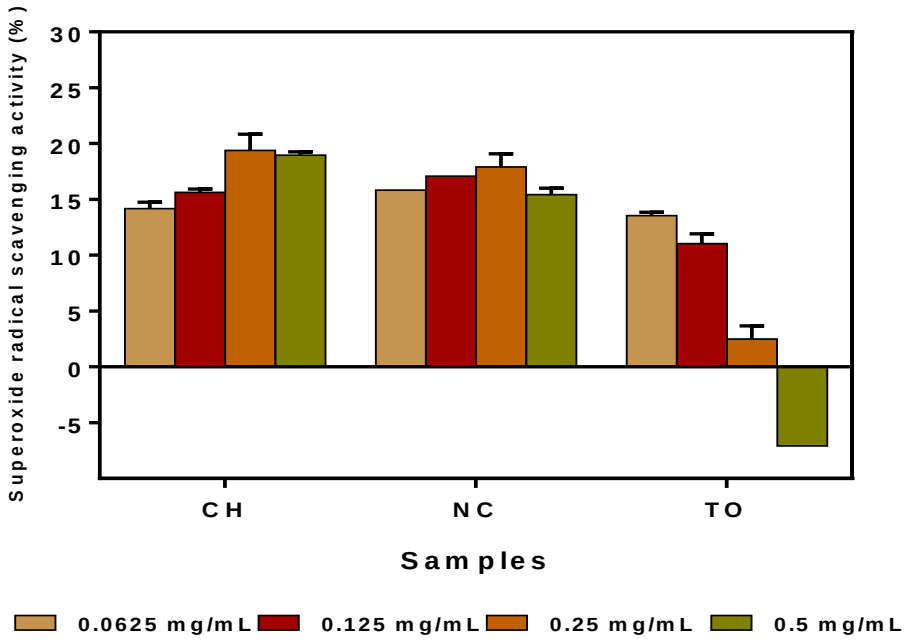

Figure 8.9. Superoxide radical scavenging activity of aqueous extract of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

The ability of NC fraction to have better antioxidant activity than CH fraction for most of the assay determined may be due to the higher content of chlorophyll a in CH than in NC. Report has shown that chemical instability of chlorophyll a affect their antioxidant activity and this report correspond with the findings of Lanfer-Marquez et al. (2005) which found that chlorophyll a had the weakest antioxidant properties when compared with other chlorophyll derivatives. It has also been found that the structure and configuration of chlorophylls affect their antioxidant properties. Different results have been reported for chlorophylls a and b. Some results have shown that chlorophyll b exhibit a better antioxidant property than chlorophyll a based on the functional group attached to the side chain of their structures. The mode of the mechanism at which this antioxidant activity is performed are contradictory based on different studies. Thus, the higher content of chlorophyll a in CH fraction more than NC fraction may have affected its

antioxidant properties. The structural characteristics of chlorophyll could also be used to justify the effect of the antioxidant properties. This can be based on the hypothesis which suggests that the presence of aldehyde functional group (-CHO) instead of a methyl functional group give a better antioxidant activity (Fernandes et al., 2017; Hsu et al., 2005; Lanfer-Marquez et al., 2005; Pérez-Gálvez et al., 2020). Identification of the structures of CH, NC and TO and their mechanism of action will better explain the varying result obtained in this study.

8.3.10 *In vitro* inhibition of acetylcholinesterase activity

Neurodegenerative diseases are now a major health concern due to the growing ageing population and have been recognized as a global healthcare problem (International et al., 2015; Langa, 2015, 2018; Global Health and Ageing, 2011). Due to limited treatment and some side effects associated with currently available drugs, there is need for new treatment alternatives that could alleviate symptoms and delay disease progression. Inhibition of AChE activity is considered as an important management strategy for Alzheimer's disease (AD) because inhibition of this enzyme prevents excessive hydrolysis of ACh in the brain, which is required to maintain cholinergic neurotransmission (Howes et al., 2020; Oboh et al., 2014). Screening of natural products from plants as a new strategy in AD treatment is cost effective as this could provide alternatives to the existing synthetic drugs. The ability of the TO extract, CH and NC fractions to inhibit *in vitro* activity of acetylcholinesterase (AChE) was investigated and the results are presented in Fig. 8.8. The results revealed that all the vegetable extracts inhibited AChE activity in a dose-dependent manner. However, the NC fraction (16.34 ± 0.19%) had the highest AChE inhibitory activity at 0.025 mg/mL with a mean average inhibition of 15.33% followed by CH fraction (16.14 ± 2.34% at 0.00625 mg/mL) with a mean average inhibition of 14.06% and TO extract (15.47 ± 0.85% at 0.0125 mg/mL) with a mean average inhibition of

12.77%. The determined acetylcholinesterase (AChE) inhibitory activity agreed with some earlier reports where plant extracts inhibited acetylcholinesterase (Ferreira et al., 2020; Sanchis et al., 2020). This study demonstrated that extracts and fractions of *Telfairia occidentalis i*nhibited AChE and the results are consistent with earlier data reported for the aqueous extracts of TO (Olarewaju et al., 2018). However, the results obtained in this study are lower than those reported for ethanol extract of TO and *Amaranthus cruentus* by Oboh et al. (2014). Differences in the results might be due to variations in the composition of extraction solvents and source of AChE used in the inhibition assay. Though the results obtained in this study are low, there is some promise as potential AChE inhibitor.

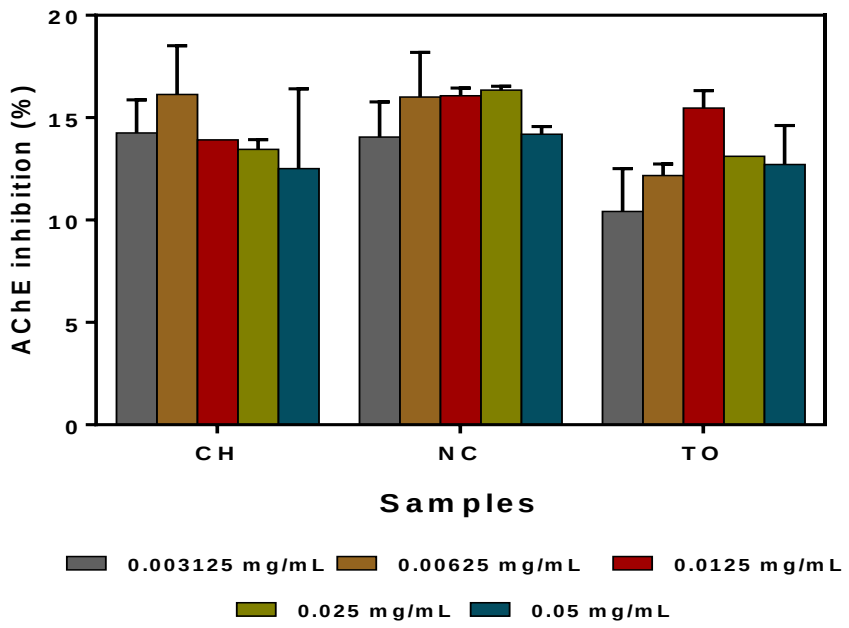

Figure 8.10. *In vitro* inhibition of acetylcholinesterase activity of aqueous extract of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

8.3.11 α-Amylase inhibition

Exposure to elevated blood glucose levels and overstimulation of pancreatic β-cells can cause a deficiency in insulin, in addition to dysregulation of glucose and lipid metabolism, thus

giving rise to diseases such as diabetes, obesity and cardiovascular disorder (D'Amico et al., 2019; Knudsen et al., 2019; Yuxue Zheng et al., 2020). α-Amylase is a key digestive enzyme responsible for the hydrolysis of starch into smaller units especially glucose, which when present at excessive levels in the blood stream can cause diseases such as diabetes and obesity (Karim et al., 2017; Tan et al., 2017). Therefore, inhibiting this enzyme activity is an effective way to reduce starch digestibility for preventing the development of these diseases. The inhibitory effects of CH, NC and TO against α-amylase was investigated and the values obtained at different sample concentrations as well as their IC_{50} values are presented in Fig 8.9 and Table 8.3. The results show that the catalytic activity of α-amylase decreased as the concentration of CH, NC and TO increased. The IC_{50} of acarbose, CH, NC and TO was determined to be 0.001 ± 0.02, 0.999 ± 0.02, 1.165 ± 0.01 and 0.919 ± 0.06 mg/mL respectively. Among the three samples, TO extract exhibited the highest inhibition against α-amylase and the lowest IC_{50} (Table 8.3), which indicate stronger synergistic interactions of the polyphenols when compared to the separated fractions. Results from this study showed that TO is the best inhibitor of α-amylase followed by CH and NC. Even with removal of chlorophyll, the NC still was able to inhibit α-amylase, which indicate that the chlorophylls were not the only determinants of the activity obtained for TO extract. Polyphenols are known to form complexes with proteins based on their ability to form hydrogen bond between their hydroxyl group and the amino acid residue in the active or non-active sites of α-amylase (Sun et al., 2016). Although all the three samples (extract, CH, and NC) contained the same non-chlorophyllic phenolic compounds (kaempferol O-rutinoside and quercertin O-rutinoside), the stronger inhibitory activity of TO against α-amylase may be attributed to the higher concentration of these compounds. Tan et al. (2017) reported that the fractions obtained from black turtle bean and black soybean are better inhibitors

of α-amylase than the crude extract. An opposite result of this report was obtained in this study where the extract (TO) inhibited α-amylase better than the fractions (CH and NC). The results obtained in this study showed that TO extract (IC_{50} value of 0.919 ± 0.06 mg/mL) is a better α-amylase inhibitor than the black turtle bean (IC_{50} value of 2.69 ± 0.12 mg/mL) and black soybean (IC_{50} value of 2.25 ± 0.01 mg/mL) extracts (Tan et al., 2017).

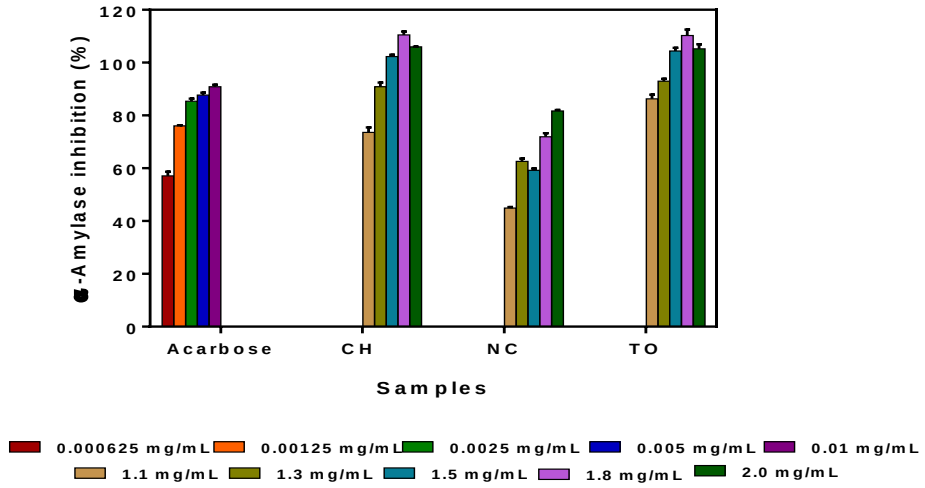

Figure 8.11. α-amylase inhibition of aqueous extract of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

Table 8.3. IC_{50} values of α-amylase, pancreatic lipase (PL), and dipeptidyl peptidase-IV (DPP-IV) inhibition of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

Samples/standards	α-Amylase (mg/mL)	PL (mg/mL)	DPP-IV (mg/mL)
CH	0.999 ± 0.02	1.080 ± 0.02	0.237 ± 0.03
NC	1.165 ± 0.01	1.272 ± 0.01	0.291 ± 0.02
TO	0.919 ± 0.06	1.121 ± 0.02	0.267 ± 0.02
Acarbose	0.001 ± 0.02		
Orlistat		1.811 ± 0.01	

8.3.12 α-Glucosidase inhibition

Another important membrane-bound enzyme involved in starch digestion is α-glucosidase. It breaks down oligo- and di-saccharides to produce glucose, which is then absorbed by the body. It is found in the epithelium of the small intestine and catalyzes the final step in the digestion of carbohydrates to release absorbable monosaccharides thus giving rise to increased blood glucose levels (Awosika & Aluko, 2019; Zhang et al., 2015). One therapeutic approach in the management and prevention of disease symptoms associated with diabetes is by delaying glucose absorption. Therefore, inhibition of carbohydrate digestive enzyme activity, especially α-glucosidase is very important in achieving reduced blood glucose levels (Awosika & Aluko, 2019; Rouzbehan et al., 2017). In this study, a dose-dependent inhibition activity was observed for the inhibition of α-glucosidase by TO extract and its fractions (Fig. 8.10). The inhibitory activity increased with increase in concentration with the highest inhibition at 5 mg/mL except for NC fraction, which had its highest inhibition at 4 mg/mL followed by decreases. The inhibitory activity of samples TO, CH and NC against α-glucosidase was lower than that of the standard acarbose (80.66% at 1 mg/mL). Based on the highest concentration tested (5 mg/mL), TO had the highest inhibitory activity followed by CH and NC. Using the mean inhibitory activity, CH (34.78%) had better inhibitory activity than TO (33.09%) and NC (27.67%). The values obtained in this study are lower than those obtained for brinjal (70.78% at 10 mg/mL), *Cornus capitata* leaves (98.37% at 50 µg/mL) and *Roylea cinerea* leaves (7.01% at 50 µg/mL) (Bhatia et al., 2019; Sultana et al., 2020a). The differences in these results may be due to variations in the source of enzyme (rat intestinal acetone powder or yeast α-glucosidase). With the rising interest in obtaining new and effective α-glucosidase inhibitors that have minimum side effects, the TO extract and its fraction have shown that they could serve as potential

antidiabetic or anti-obesity agents. The presence of bioactive polyphenolic compounds such as quercetin O-rutinoside and kaempferol O-rutinoside could have contributed to the observed α-glucosidase inhibitory properties of TO, CH and NC (Rouzbehan et al., 2017). For example, it has been reported that apigenin and kaempferol may be responsible for the inhibition of α-glucosidase by Labiatae (an herb) extracts (Rouzbehan et al., 2017). The ability of a sample to inhibit α-glucosidase has been shown to be dependent on the type of extraction solvent used. The methanol extracts of *Solanum melongena* and *Solanum macrocarpon* had higher activity than those obtained in this study and the ethyl acetate extract of bitter gourd had stronger activity than hexane, methanol, and chloroform extracts (Nwanna et al., 2013; Sulaiman et al., 2013). The lower inhibition rate of CH, NC and TO may be due to the aqueous extraction used, which may have solubilized less amounts or varieties of polyphenolic compounds when compared to organic solvents.

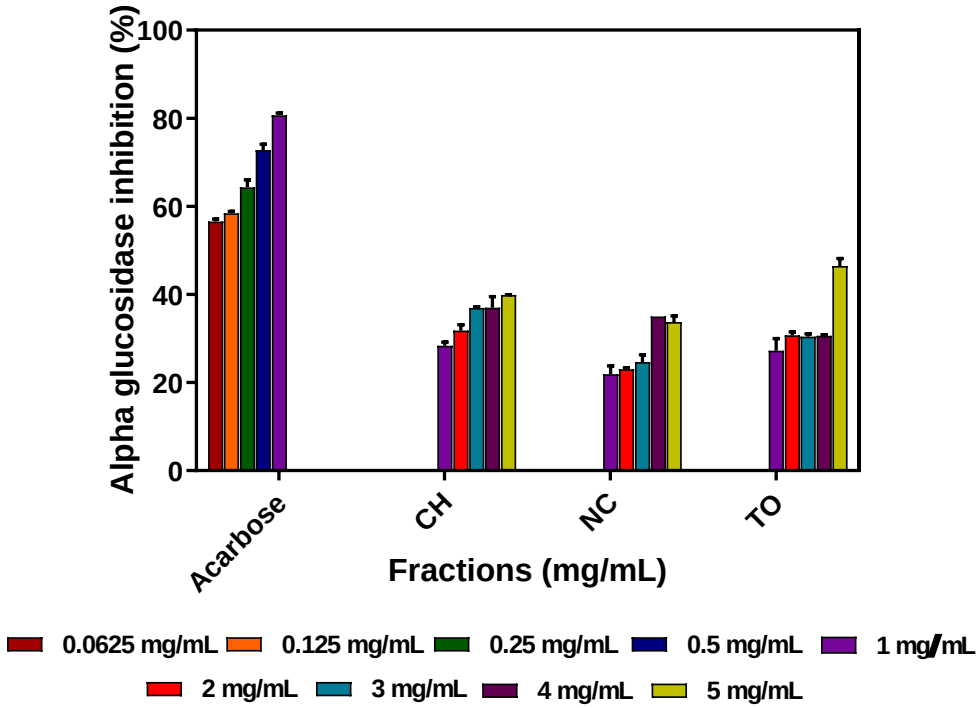

Figure 8.12. α-Glucosidase inhibition of aqueous extract of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

8.3.13. Pancreatic lipase (PL) inhibition

The incidence of diabetes and obesity worldwide has increased significantly due to lifestyle changes and the growing consumption of foods high in fat and carbohydrates (Sellami et al., 2017). The most effective approach in preventing the occurrence of obesity and type 2 diabetes (T2D) is by reducing the amount of dietary fat that is been digested and absorbed. PL is an important enzyme secreted in the pancreas that is responsible for the breakdown of about 50-70% of the triglycerides in the stomach and excessive activity could lead to high caloric intake (Awosika and Aluko, 2019; Sultana et al., 2020). Therefore, inhibition of PL activity will reduce the amount of fat that can be absorbed in the small intestine thus preventing weight gain (Awosika & Aluko, 2019). For example, a previous study reported that a TO-fortified diet induced a significantly lower serum triacylglycerols levels when fed to rat (Ugwu et al., 2010).

Due to its high potency in inhibiting PL, orlistat is the only health approved drug for the treatment of obesity. When combined with diet and physical activity, it can result in about 10% weight loss, but its use is associated with some undesirable adverse effects such as diarrhea, bloating, flatulence, incontinence, and abdominal cramping (Patil et al., 2015; Sellami et al., 2017; Sultana et al., 2020a). There is, therefore, the need for natural inhibitors of PL that have little or no side effects. The IC_{50} values for PL inhibition by TO extract, CH and NC fractions were determined, and the results (Fig. 8.11 and Table 3) show the dose-dependent activities. Results from this study show that more than 50% reduction in lipase activity was achieved; therefore, IC_{50} values were calculated to measure potency of enzyme inhibition. The lower the IC_{50}, the more active it is at inhibiting an enzyme. The findings showed that CH fraction and TO were the most effective lipase inhibitors with IC_{50} values of 1.08 ± 0.02 and 1.12 ± 0.02 mg/mL, respectively. NC fraction was the least potent inhibitor among the samples tested with IC_{50} value of 1.27 ± 0.01 mg/mL. It is also noticeable that NC fraction also had the weakest inhibitions of α-amylase and α-glucosidase activities. The results obtained in this study are weaker than those obtained for Indian spinach with an IC_{50} of 0.277 mg/mL (Sultana et al., 2020b). Therefore, this extract and its fractions might be a target for the development of anti-obesity drug.

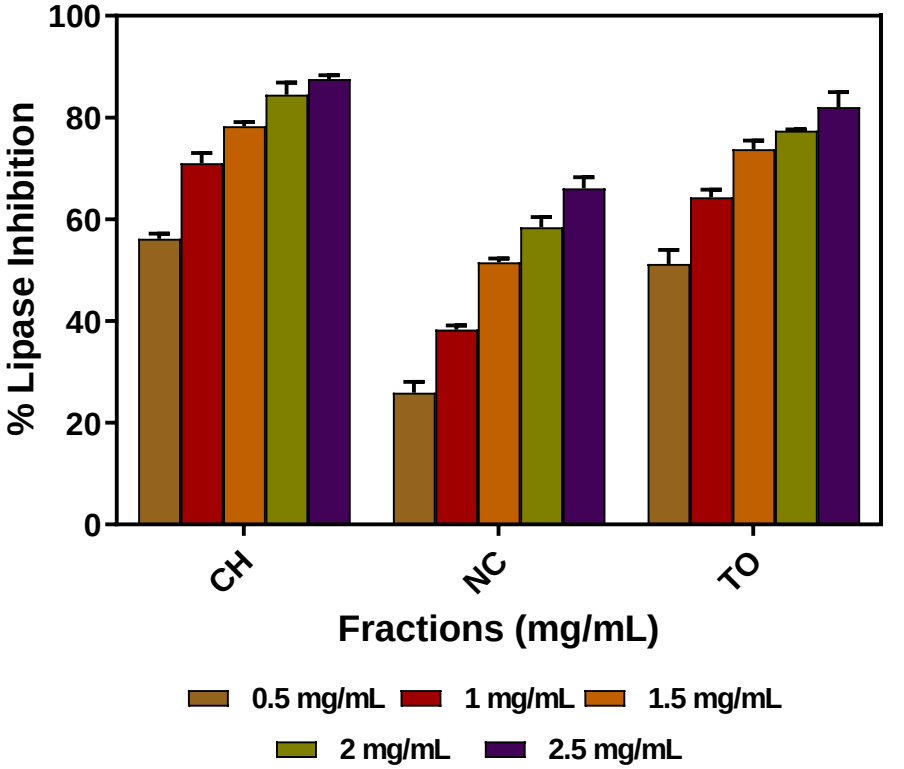

Figure 8.13. Pancreatic lipase inhibition of aqueous extract of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

8.3.14 Dipeptidyl-peptidase-iv (DPP-IV) inhibition

Dipeptidyl peptidase-IV (DPP-IV) inhibitors are a new class of oral hypoglycemic agents used in the treatment of diabetes mellitus. Studies have shown that DPP-IV inhibitors offer several beneficial health advantages, including reduced risk of cardiovascular diseases. The inhibitors regulate glucose level, reduce blood pressure, reduce oxidative stress, prevent weight gain, reduce inflammatory markers, improve endothelial function, improve lipid profile and decrease platelet aggregation in type 2 diabetes (T2D) patients (Mohanty et al., 2019; Scheen, 2013). Although it has high therapeutic effects in treating T2D, its use has some limitations that have raised concerns on their overall safety. These limitations include pancreatitis risk, high cost of treatment and different types of cancers such as pancreatic and thyroid cancer (Elashoff et al., 2011; Mohanty et al., 2019; Monami et al., 2013). Therefore, it is necessary to search for other

novel DPP-IV inhibitors of natural origin with little or no side effects in addition to being safe and cost effective. In this study, DPP-IV inhibitory activity of TO, CH and NC were evaluated, and their efficacy compared to the positive control using an *in vitro* assay. Results of the present study (Fig 8.12 and Table 3) demonstrated that CH (92.78 ± 0.09%) and TO (92.68 ± 1.18%) exhibited significant DPP-IV inhibitory effects when compared to NC (69.39 ± 0.78%). Higher DPP-IV inhibition of CH and TO can be attributed to the presence of chlorophyll in the extract and fraction. Removal of chlorophyll had a significant effect on DPP-IV inhibition as shown by the reduced potency of the NC fraction. Studies have shown that natural compounds isolated from medicinal plants could reduce hyperglycemia due to their biological activities in ameliorating diabetes (Choudhury et al., 2017; Li et al., 2019; Tran et al., 2020). The ability of TO, CH and NC to inhibit DPP-IV may be attributed to the presence of kaempferol O-rutinoside and quercetin O-rutinoside, which is supported by a previous study (Fan et al., 2013) showing that different polyphenolic compounds isolated from plants are strong inhibitors of DPP-IV. Other types of food bioactive such as protein hydrolysates, grape seed procyanidin and various plants have also been identified as DPP-IV inhibitors (González-Abuín et al., 2012). Anthocyanins from blueberry-blackberry wine blends have also been reported to inhibit enzymes involved in carbohydrate regulation, including DPP-IV (Johnson et al., 2013). Other polyphenols with reported DPP-IV inhibition activity include anthocyanins cyanidin 3,5-diglucoside isolated from the juice of Aronia berries and cyanidin 3-glucoside found in blackberry-blueberry wine blend. They were found to inhibit DPP-IV with an IC_{50} of 5.5 µM and 0.42 µM, respectively (Fan et al., 2013; Kozuka et al., 2015). Bower et al. (2014) also reported that phenolic compounds such as cirsimaritin, hispidulin and naringenin found in the extracts of Mexican oregano and rosemary are effective inhibitors of DPP-IV with IC_{50} values of 0.43, 0.49 and 2.5

μM, respectively. The IC_{50} values showed that CH (0.237 ± 0.03 mg/mL) had a stronger inhibitory activity against DPP-IV when compared with TO (0.267 ± 0.02 mg/mL) and NC (0.291 ± 0.02 mg/mL). Due to their high inhibitory activities, TO, CH and NC may provide significant anti-diabetic activity when consumed. The ability of TO extract and its fractions to inhibit DPP-IV activity supports a previous report about the anti-diabetic effect of this vegetable (Onuoha et al., 2017). The synergistic interactions of kaempferol O-rutinoside and quercetin O-rutinoside may have contributed to the strong DPP-IV inhibitory activity exhibited by CH, NC and TO in this study.

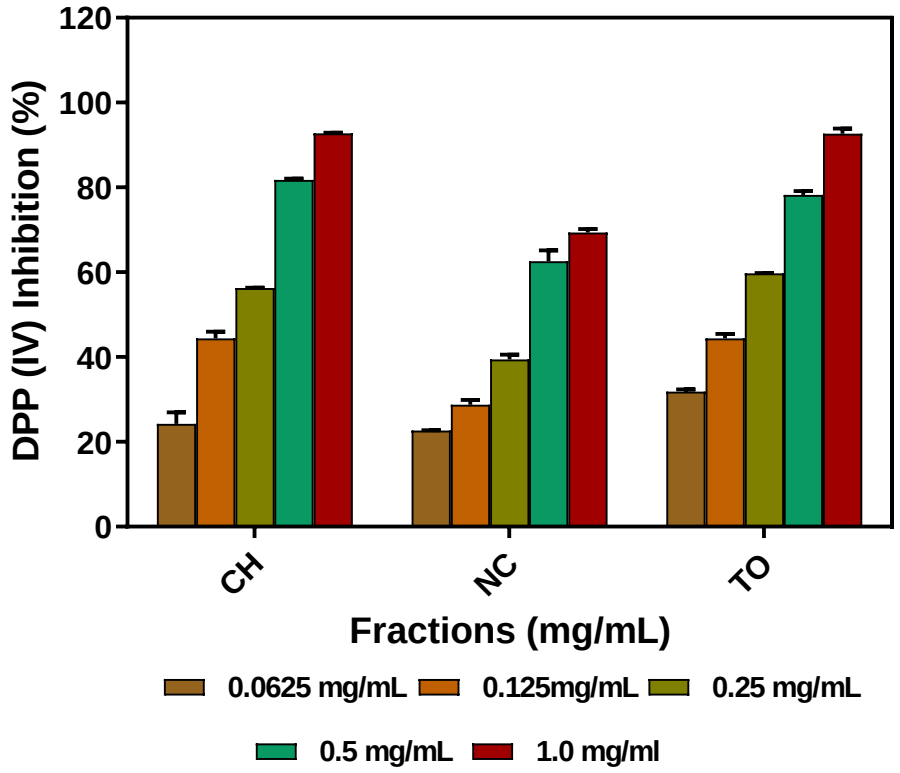

Figure 8.14. DPP-IV inhibition of aqueous extract of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

8.3.15 Trypsin and Chymotrypsin Inhibition

Results of the percentage trypsin- and chymotrypsin-inhibitory activities of TO, CH, and NC are presented in Figs 8.13 and 8.14. Generally, the results showed that NC (4.14 ± 1.09- 32.10 ± 0.81%) fraction had lower inhibitory activity when compared with CH (21.16 ± 1.63- 62.05 ± 1.90%) fraction and TO (18.15 ± 0.09- 56.93 ± 0.81%) extract. The trypsin inhibitory activity was dose-dependent with the inhibitory activity increasing as the dose of the fractions and extract increased with maximum inhibitory activity at 0.25 mg/mL after which the activity decreased. The NC fractions had a maximum inhibitory activity at 1 mg/mL. CH fraction had significantly higher values when compared with NC fraction and TO extract, indicating that the chlorophylls are the major contributors to the observed enzyme activity inhibitions. When

compared with the standard AEBSF (101.7% at 0.125 mg/mL), the fractions and TO extract have lower enzyme inhibition rates. The percentage chymotrypsin inhibition by the CH, NC fractions and TO extract was also concentration dependent, as shown in Fig. 14. Inhibitory activities of the samples were in the same range with that of the standard AEBSF (71.33% at 0.5 mg/mL). The inhibitory activity of CH fraction and TO increased as the sample concentration increased with maximum activity at 0.03125 mg/mL after which the activity reduced while inhibitory activity of NC decreased. Based on the results of the highest concentration tested (0.0625 mg/mL), the CH fraction had the highest inhibitory activity of 62.97% among all the samples. From the results, it can be established that NC fraction had weaker chymotrypsin and trypsin inhibitory activities than CH and TO, though all the three samples are better inhibitors of chymotrypsin than trypsin. The enzyme inhibition results indicate that fractionation improved the trypsin- and chymotrypsin-inhibitory capacity of the TO extract. The results suggest that there may have been polyphenol antagonism within the TO extract (unfractionated), which could have contributed to reduced activity against trypsin and chymotrypsin when compared to the CH fraction.

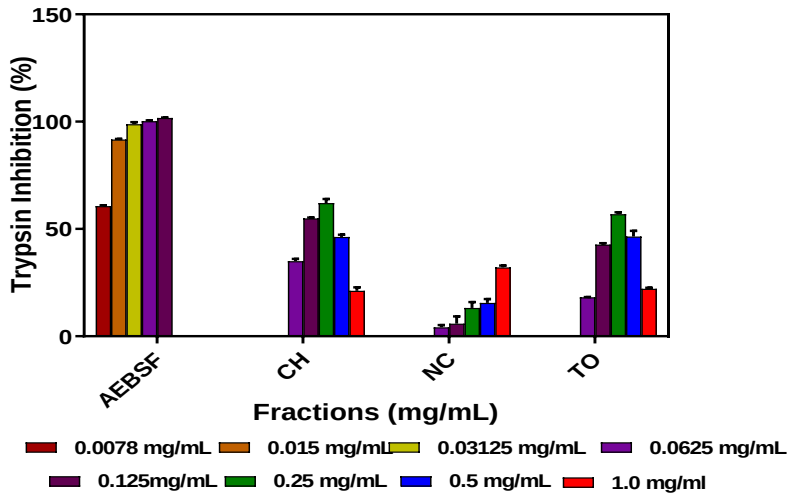

Figure 8.15. Trypsin inhibition of aqueous extract of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

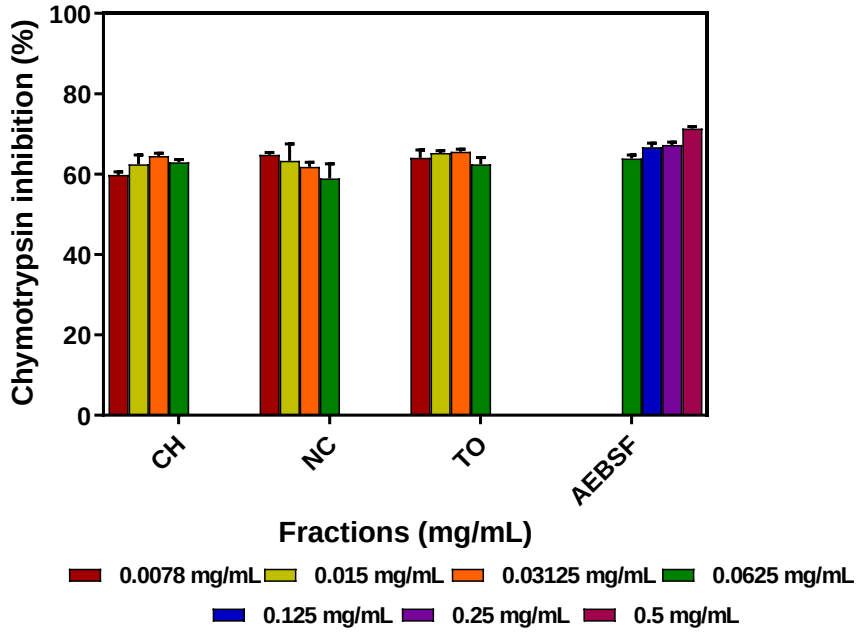

Figure 8.16. Chymotrypsin inhibition of aqueous extract of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

8.3.16 ACE and Renin Inhibition

Hypertension is one of the major risks of cardiovascular diseases affecting majority of people in the whole world (Ademiluyi et al., 2015). The results showed that both the extract and the fractions inhibited ACE in a dose-dependent manner (1-5 mg/mL), however, TO extract had the highest inhibitory effect while CH has the lowest inhibition (Fig 8.17). Based on the results, the chlorophyll-depleted fraction (NC) has better ACE inhibition (67.03%) than the chlorophyll-enriched (CH) fraction (63.8%) at 2 mg/mL assay concentration. There is a strong correlation between the ACE inhibitory activity of the extract and fractions and their total phenolic content. This could suggest that polyphenolic constituents of the samples are responsible for their activity. As previously reported (Oluwagunwa et al., 2019), the TO extract contain phenolic compounds such as rutin, myricetin and caffeic acid and this may be responsible for its inhibitory property against ACE. The results suggest that TO extract could interfere with the renin-angiotensin system (RAS) by inhibiting ACE activity. Identification of ACE inhibitors as a modern therapeutic tool has advanced the treatment and management of hypertension. The result reported for ACE and renin inhibition in this study are different from those reported by Ajibola et al. (2011) for chlorophyllic and non-chlorophyllic fractions of *Vernonia amygdalina* (VA) and *Gongronema latifolium* (GL). The result showed that the chlorophyllic fractions of VA and GL are better inhibitors of ACE and renin when compared with the non-chlorophyllic fractions. The ability of NC fractions to inhibit ACE more than CH may be due to other active compounds present in the fraction. ACE inhibitors are known to improve endothelial function and increase bradykinin concentration in the blood (Ancion et al., 2019). The results of this study show that the extract and the fractions are good inhibitors of ACE and could be used as therapeutic agents in the management of hypertension.

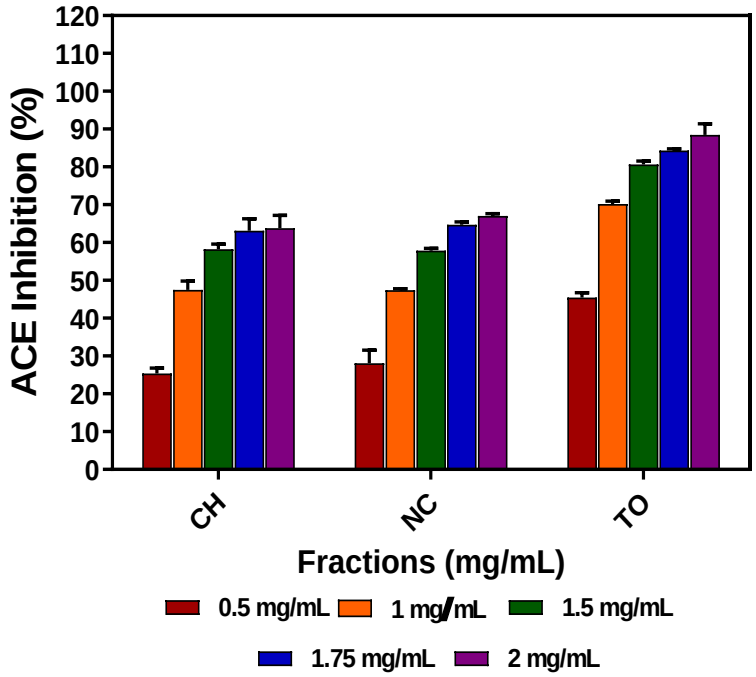

Figure 8.17. Inhibition of ACE by aqueous extract of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

Unlike ACE inhibition, ability of TO extract and its fractions to inhibit renin activity shows that there was an increase in the inhibition rate for TO while effect of the fractions (CH and NC) decreased as the concentration increased (Fig 8.18). The inhibitory rate is low when compared with aliskerin (74 .76 ± 4.56% at 0.1 mg), which is a standard renin inhibitor. Although NC had better inhibition rate than CH for ACE, reverse is the case for renin where it had the least inhibition. The result of the renin inhibition correlate with the report of Ajibola et al. (2011), where the chlorophyllic fractions of VA and GL have better inhibition of renin than the non-chlorophyllic fractions. The higher renin inhibitory activity of TO may be attributed to the

combined effects of the chlorophyll and non-chlorophyll phenolic compounds. Oluwagunwa et al. (2019) reported that the TO extract is mainly composed of caffeic acid and this contributed to its antihypertensive properties. ACE and renin are important enzymes responsible for maintaining vascular tension in the mammalian body and their inhibition is, therefore, an important therapeutic tool in the treatment of high blood pressure (Aluko, 2019). Results obtained in this study are lower than those obtained for brinjal (79.64 ± 7.63%) (Sultana et al., 2020a) but this may be due to differences in sample composition.

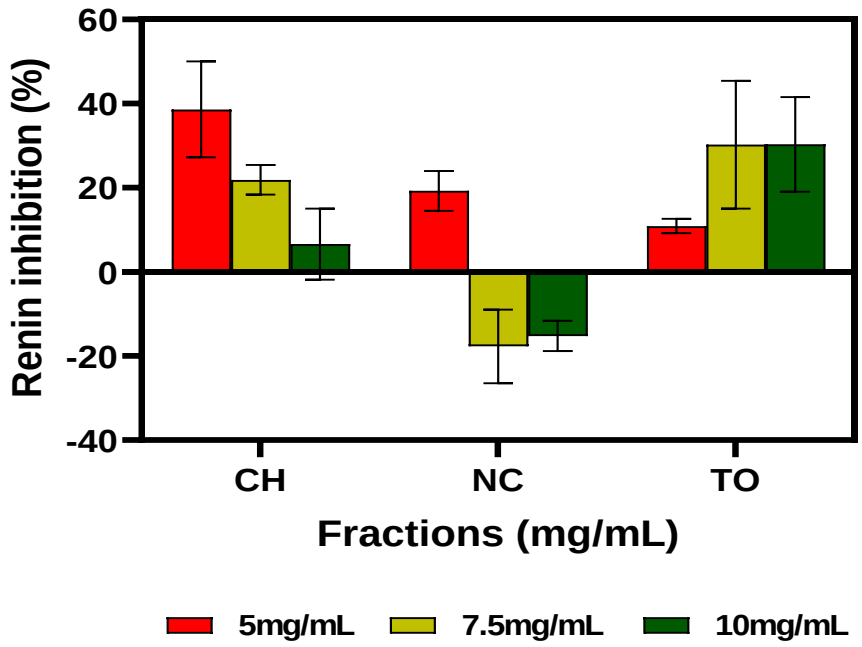

Figure 8.18. Inhibition of renin by aqueous extract of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

8.3.17. Intrinsic fluorescence intensity

Fluorescence measurements give information about the molecular environment in the vicinity of the chromophore molecule. It measures the conformational changes of proteins and estimates the modification of tryptophan through fluorescent quenching at an excitation wavelength of 290 nm

and emission wavelength between 320 and 390 nm (Schmid, 1989). The fluorescence emission spectra obtained for trypsin, α-amylase, and PL with and without the addition of TO, CH and NC are presented in Fig. 8.19-8.21. In all cases, a decrease in the fluorescence intensity of the enzymes was observed as the concentration of the samples increased. In Fig. 19, it was observed that there was a complete quenching of the amylase protein for TO extracts. In Fig. 8.20, the interaction of NC, CH and TO with trypsin shows that there was an absorption at 280 nm and an emission at 339 nm for trypsin alone and a slight red shift to 340 and 345 nm as the concentration of CH, NC and TO increased from 6.25 µg/mL to 12.5 µg/mL. As the concentration of CH, NC and TO increased, the fluorescence intensity of α-amylase and PL decreased gradually, and the position of the maximum emission wavelength was significantly red-shifted from 342 nm to 350 nm for α-amylase and from 349 nm to 351 nm for PL suggesting that there is a change in protein conformation. The decrease in the intensities and the red shift of peaks shows that the microenvironments of tryptophan residues were altered and became more hydrophilic due to the interactions of NC, CH and TO. The results also suggest that CH, NC and TO bind to the enzyme and this led to the modification of the natural tertiary structure of these enzymes.

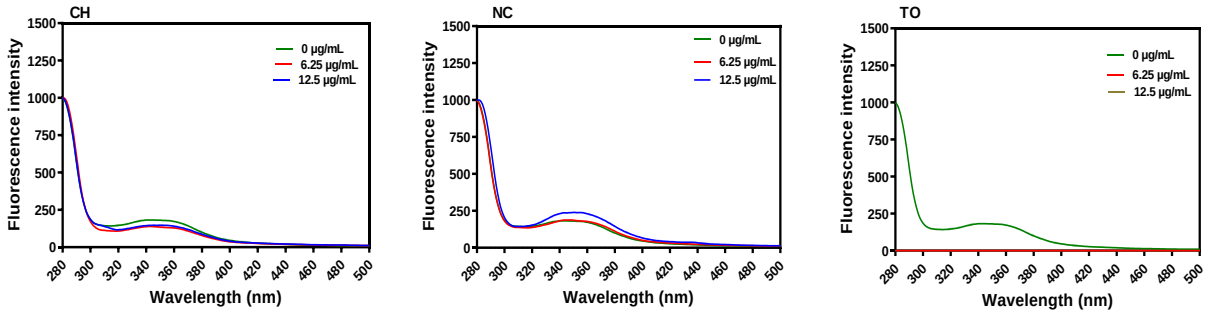

Figure 8.19. Intrinsic fluorescent of α- amylase with aqueous extract of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

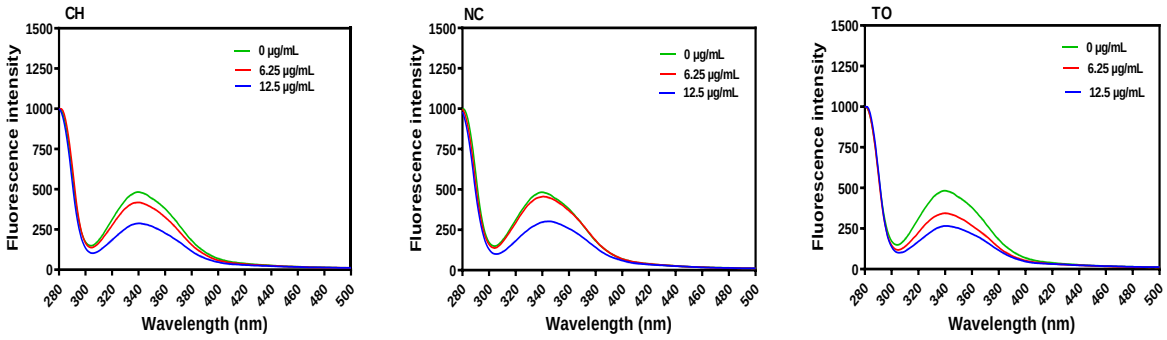

Figure 8.20. Intrinsic fluorescent of trypsin with aqueous extract of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

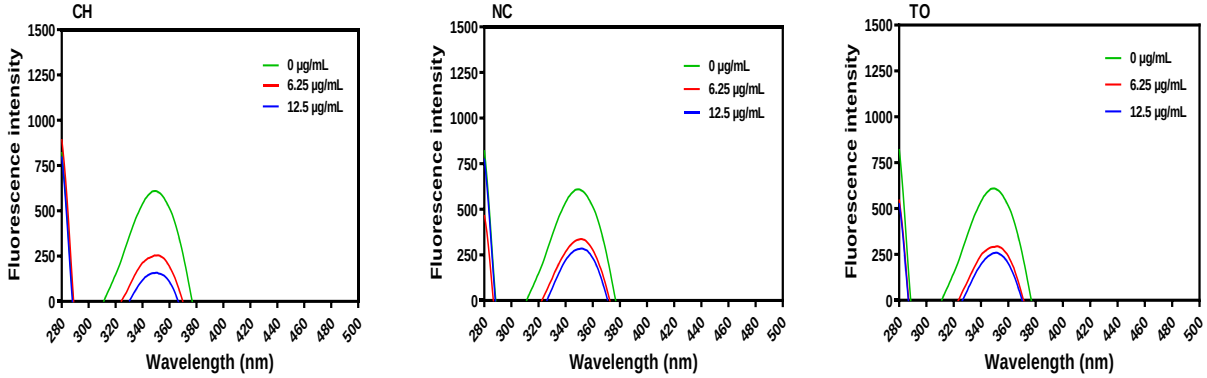

Figure 8.21. Intrinsic fluorescent of pancreatic lipase with aqueous extract of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

8.3.18 Circular dichroism (CD)

CD is an important technique in the structural characterization of proteins, especially for secondary structure determination (Schmid, 1989). It is a sensitive spectroscopic method to monitor conformational changes in chiral molecules that absorb light to different extents (Zeng et al., 2016). As shown in Figures 8.22-8.24, the far-UV CD spectra of α-amylase, lipase and trypsin were therefore recorded in the absence and presence of CH, NC and TO. In the CD

spectra of trypsin (Fig 8.23), CH and TO showed higher negative ellipticity as concentration was increased, which indicate an increase in secondary structure conformation (Schmid, 1989). However, when the concentration of NC increased, there was a decrease in the negative ellipticity of trypsin implying reductions in secondary structure conformations. The results show that NC fraction had a negative effect on trypsin secondary structure while CH and TO exhibited positive influence. The PL showed two positive bands at 195 nm and 213 nm, and a negative band at 231 nm while addition of CH and NC showed positive bands between 194-196 nm. The negative intensities of PL increased as the TO concentration increased, which suggest a positive influence on secondary structure conformation. For the α-amylase spectrum, all the samples had two negative spectra at 207 nm and 230 nm with a negative shoulder at 220, which is consistent with the presence of high levels of α-helix structure (Schmid, 1989). As the concentration of TO increased, there was a strong modification of the secondary structure, especially at 3 mg/mL, which indicate strong interactions of the polyphenolic compounds with the enzyme protein.

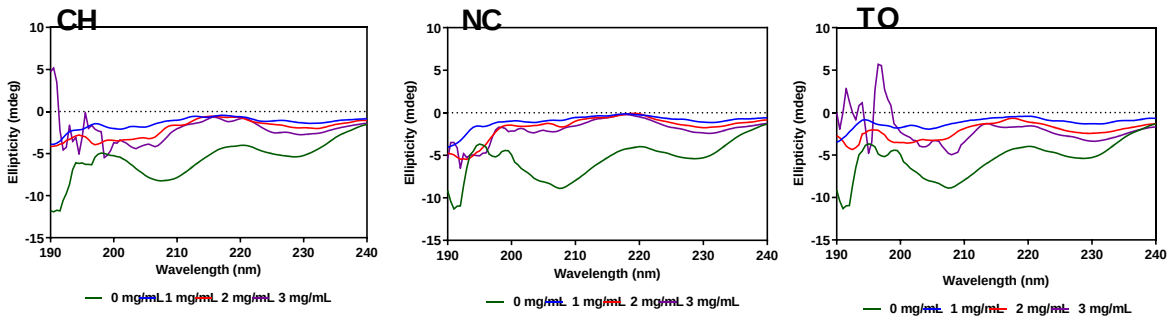

Figure 8.22. Far-UV CD of α-amylase of aqueous extract of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

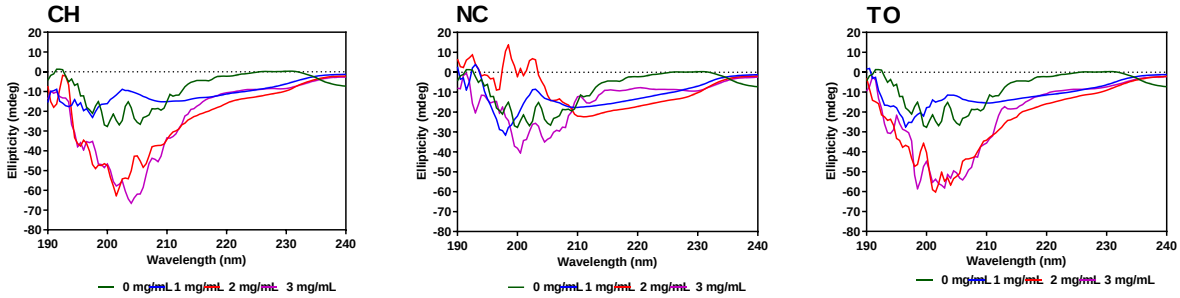

Figure 8.23. Far-UV CD of trypsin of aqueous extract of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

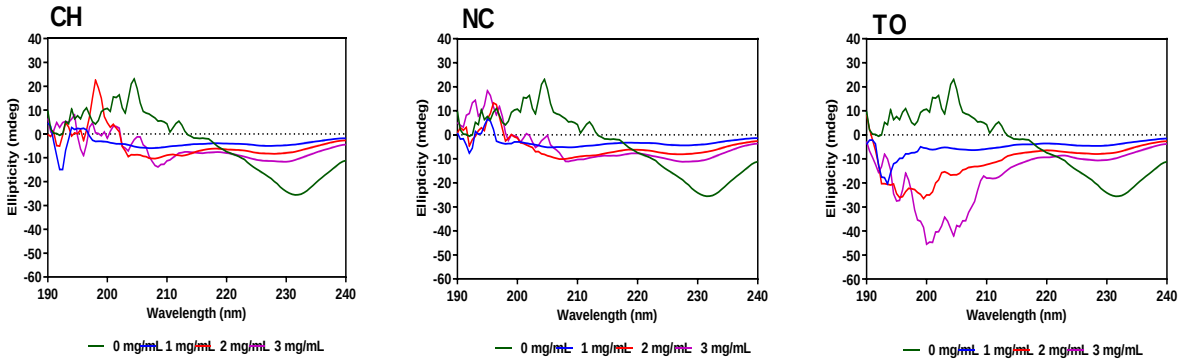

Figure 8.24. Far-UV CD of pancreatic lipase of aqueous extract of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

In the near-UV spectra (Figures 8.25-8.27), the α-amylase enzyme had a positive band at 270 nm while CH, NC and TO had negative bands at 268 nm with a weak negative peak ellipticity at 296 nm. Addition of CH extract led to an increase in the ellipticity of the negative band at 268 nm as the concentration increased, which reflect movement of the aromatic groups into a more hydrophobic environment (Schmid, 1989). In contrast, the ellipticity reduced with the addition of NC fraction and disappeared in the presence of TO extract, indicating reductions in tertiary conformation of α-amylase and shift of the aromatic groups to hydrophilic environments. The near-UV CD spectra for PL show a decrease in the ellipticity as the concentration of CH, NC and TO increased (Fig 8.27). The negative peak ellipticity at 298-300 nm and a slight positive peak at 269-272 nm indicate that tyrosine and tryptophan may be responsible for the observed structure (Schmid, 1989). The results suggest addition of TO, CH and NC led to denaturation of PL into a more open structure and shifting of aromatic groups into hydrophilic environments. Trypsin (Fig 8.26) near-UV CD spectra show three negative ellipticity peaks at 258 nm, 272 nm, and 298-300 nm, which represent the presence of phenylalanine, tyrosine and tryptophan, respectively (Schmid, 1989). The addition of CH, NC and TO led to decreased ellipticity and suggests unfolding of the trypsin protein structure into a more unordered structure that exposed the aromatic groups to a hydrophilic environment. The results indicate reduced tertiary conformation of trypsin into a less compact structure in the presence of TO, CH, and NC. Overall, addition of CH, NC and TO led to changes in the conformation of α-amylase, pancreatic lipase, and trypsin from a compact to an unfolded structure, which could explain the observed reductions in catalytic activities.

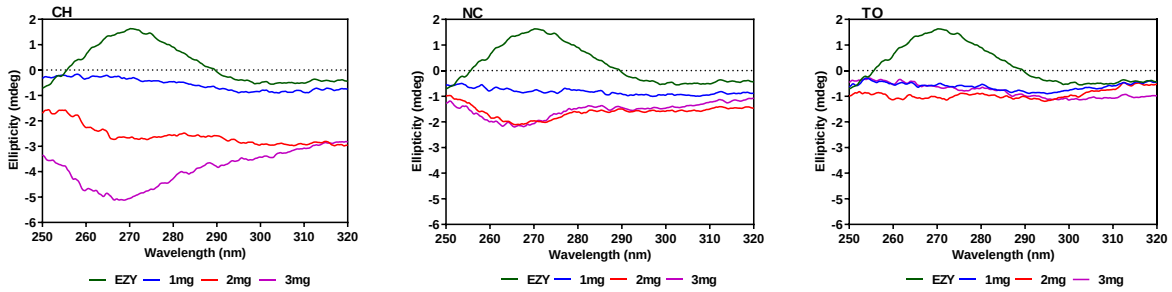

Figure 8.25. Near-UV CD of α-amylase of aqueous extract of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

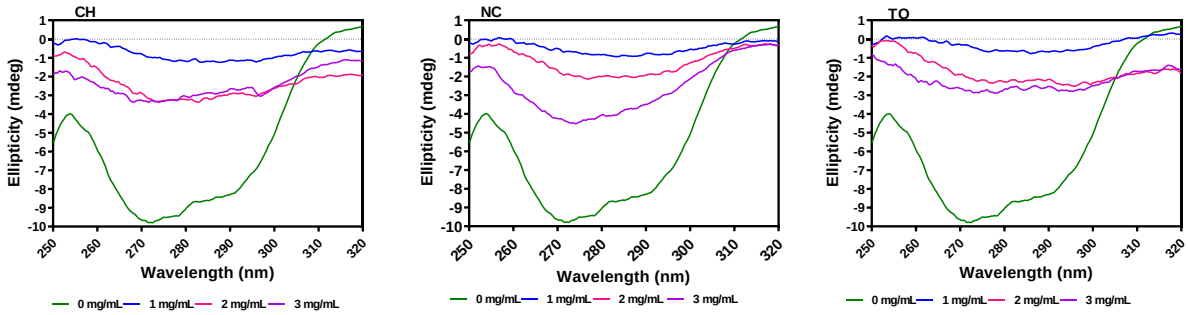

Figure 8.26. Near-UV CD of trypsin of aqueous extract of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

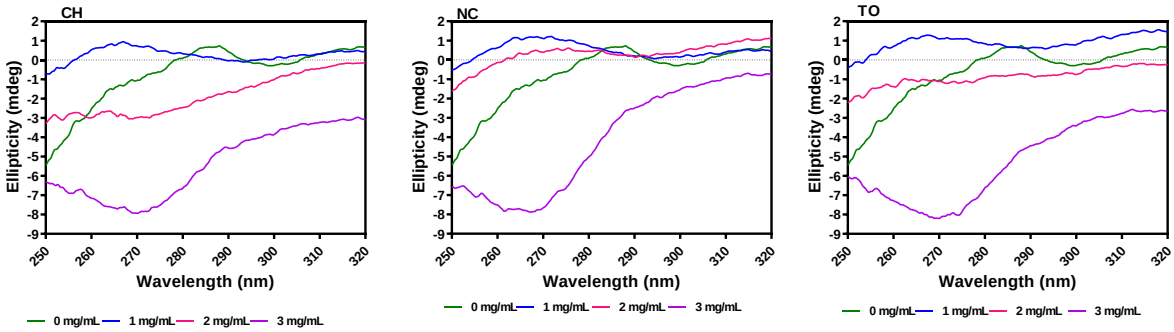

Figure 8.27. Near-UV CD of pancreatic lipase of aqueous extract of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

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CHAPTER NINE

9.1 GENERAL DISCUSSION AND CONCLUSION

The research presented in this thesis has reported on a series of investigations undertaken to determine the antioxidant, antihypertensive, anticholinesterase activity and the ability to inhibit carbohydrate-digestive enzymes and their potential effects on improving human health. This work has also identified the major phytochemicals (antioxidants) present in the three vegetables and has shown that they have positive *in vitro* activity against enzymes that have been implicated in human diseases such as diabetes, obesity, Alzheimer's, and hypertension. The novel findings and conclusions that can be drawn from this study are summarized below:

i. The first study shows that aqueous extracts of the three vegetables *Amaranthus viridis* (AV), *Solanum macrocarpon* (SM) and *Telfairia occidentalis* (TO) had *in vitro* antioxidant properties with the ability to scavenge DPPH, reduce ferric metal ions and chelate transition metals ions, an indication of their potential use to prevent, manage or reduce disorders arising from over production of ROS/free radicals in foods or in human tissues. This work has potentially established the claim that these vegetables contain antioxidative compounds such as gallic acid, rutin, myricetin and caffeic acid and can be utilized in the development of functional foods that will reduce excessive production of ROS/free radicals that may cause oxidative stress related disorders. Application of nitrogen fertilizer to the vegetables had a negative influence on the accumulation of polyphenolic compounds, which suggests that the plants channeled excess nutrients towards vegetative growth. The optimum dose at which the bioactivity of these vegetables was enhanced was at 60 kg N/ha of fertilizer. It was also established that the vegetables inhibited acetylcholinesterase, an enzyme implicated in the development of

Alzheimer's disease and thus could be used in the formulation of nutraceuticals in the management of neurodegenerative disorders. Based on the outcome of this study, it was found that TO vegetable extracts had the highest value for all the parameters tested when compared with AV and SM extracts. This is the first reported study in scientific literature to report the effect of fertilizer micro-dosing on bioactive properties of vegetable leaf polyphenolic extracts.

ii. In study two and three, the ability of AV, TO and SM to act as antihypertensive agents were investigated. Results obtained showed that TO extracts significantly ($p<0.05$) inhibited ACE activity while AV inhibited renin *in vitro*, the major causative agents of human systemic hypertension. Oral administration of the most active extracts of AV and TO extracts to spontaneously hypertensive rats (SHRs) at 100 mg/kg body weight showed that AV extract significantly lowered blood pressure (BP) and heart rate in SHRs. The *in vivo* BP-lowering effects of the extract did not correspond directly to their *in vitro* renin-ACE inhibition activities, which suggest other factors may have contributed to the *in vivo* antihypertensive effects. This is the first study in scientific literature to report modulation of heart rate by natural polyphenolic compounds, which could lead to the development of highly potent agents against cardiovascular diseases. Oral administration of SM polyphenols at 500 mg/kg BW gave rise to a highly viscous extract and thus preventing the availability of the polyphenols. Based on the 100 mg/kg BW of extract used in this study, the human equivalent dose is 16.22 mg/kg.

iii. Study four tested the ability of the three vegetables to inhibit trypsin and to determine the kinetics of inhibition. It was found that all the extracts obtained from the three vegetables AV, SM and TO inhibited trypsin with AV having the highest inhibition rate. The mode

of inhibition was competitive showing that the extracts were able to bind at the active site of the enzyme thus altering its activity. Because of the established role of trypsin-like proteases in viral infections and within the context of the current Covid-19 pandemic, this demonstrate that the ability of leaf polyphenols to inhibit trypsin activity constitutes a significant contribution to scientific literature.

iv. This work is also the first report of the ability of vegetable chlorophylls to inhibit *in vitro* activities of α-amylase, α-glucosidase, and pancreatic lipase. The result obtained showed that the extracts from the three vegetables are better inhibitors of α-amylase than α-glucosidase. They also inhibited pancreatic lipase with TO extracts having lowest IC_{50} which is indicative of strong enzyme inhibitory effects. The mode of enzyme inhibition is competitive meaning that they attach to the protein or to the active site of the enzyme thus inhibiting its activity. Inhibition of these enzymes suggests that these vegetable extracts could serve as potential use in the reduction of blood glucose and caloric intake by preventing intestinal digestion of polysaccharides thus maintaining body weight. Intrinsic fluorescence and CD data from this study revealed that there were changes to the enzyme structures, which was dependent on extract concentrations. It was found that the TO extract had the strongest inhibitory activity, which was reflected in its ability to change the secondary and tertiary structures of the enzymes. Overall, TO extracts gave the best outcome and could be proposed as a suitable agent for the management of obesity and diabetes. Due to the high antioxidant properties of the non-chlorophyll fraction (NC) obtained in this study, it could improve the utilization of these vegetables in the food industry thus solving the barrier of consumer acceptance of vegetable products due to their colour.

Due to the side effect and high cost of synthetic drugs in the management of human disease such as diabetes, hypertension and obesity, interest in functional foods obtained from plants has significantly increased recently due to factors such as consumer health awareness, market opportunity, and new emerging scientific findings linking foods/components to optimal health. It is therefore, concluded that these vegetables could serve as suitable functional ingredients in the food industry to produce drug alternatives in the management of human diseases. The vegetables could also find application in food formulations where their extracts can be mixed with beverages or taken as smoothies. The results obtained in this study will also be of great benefit to farmers through increase production of these vegetables and promote international trade thus boosting their consumption among consumers in search of health promoting food alternatives to drugs. In conclusion, this study confirms that agronomic practices such as fertilizer application have significant impact on the bioactive properties of AV, SM and TO. The use of fertilizer micro-dosing as opposed to the broadcasting method improve soil fertility and vegetable production at high level thus enhancing vegetable consumption and farming sustainability. This method could also aid in reducing the risk associated with the growing global populations demand for food.

CHAPTER TEN

10.1 Limitations of the study

Although this study involved a lot of tools and methodology both *in vitro* and *in vivo*, not all aspect of the experimental work was covered due to time constraints and finance. Some of the limitations in this research are listed below:

1. In addition to the aqueous extraction of vegetable flour, the use of organic solvent such as methanol and ethanol would have provided additional information and probably give different outcomes. However, the use of aqueous extraction, which was adopted in this study provides a processing method that is more compatible with industry practices and consumers demands.

2. The AChE inhibition study involved only the *in vitro* assays but tests on animal disease models of dementia or Alzheimer's are required to confirm the outcomes of the *in vitro* assays and the potential health benefits of the extracts to human health. Successful animal trials will need to be followed by appropriate human intervention trials to confirm anti-neurodegeneration properties of the vegetable leaf extracts.

3. Administration of the active compounds found in these vegetables to experimental animal could further establish their potential use as nutraceuticals in the management of human disease.

4. Evaluation of the levels of endogenous antioxidant enzymes such superoxide dismutase, glutathione peroxidase and catalase would have been an added advantage in validating the *in vitro* antioxidant properties.

10.2 Suggestions for future research

Finally, based on the findings of this study, there is need for *in vivo* study to validate the *in vitro* outcomes of the vegetable extracts against digestive enzymes. Because of the positive outcomes as demonstrated by the high *in vitro* inhibition values against α-glucosidase, α-amylase, trypsin, and pancreatic lipase, it is worthwhile conducting a long-term *in vivo* study using experimental animals to validate the observed results before conducting a clinical human trial. The cytotoxicity of the extracts should be tested using dose-dependent studies to determine if the extracts have any negative effects before it is administered to experimental animals. This will also provide information on the safe levels and right concentration of oral administration dosage that is needed to ensure the safety of the experimental animals. Some limitations still exist in the utilization of these vegetable extracts or their products as functional food ingredients in the food industry and human health applications. These limitations are due to the green color of the vegetables. Studies have shown that fortifying foods with vegetables will not only improve its bioactivity but also reduce caloric intake from high calorie diets. Therefore, the outcome of this work, which showed effective depletion of chlorophylls in the leaf extracts could be used to improve the color of vegetable-fortified products for increased consumer acceptability. With the high inhibition result for the *in vitro* assay of pancreatic lipase, the kinetics of enzyme inhibition would increase its industrial applications in food industry and pharmaceutical industry as an agent for drug development in the control of obesity.

APPENDICES
APPENDIX A: SUPPLEMENTAL INFORMATION

S6.1 Intrinsic fluorescence of trypsin enzyme

Trypsin is one of the important digestive enzymes responsible for some biological functions such as immune response, reproduction, cell death and differentiation (Gombos et al., 2008; Ding et al., 2015). Interaction of trypsin with foreign matters affects its structure thus altering its physiological function. To investigate the effect of PEs on trypsin, fluorescence spectroscopy was performed. In the absence and presence of PEs at different concentrations (6.25-50 µg/mL), Fig. S6a-d showed the fluorescence emission spectra of trypsin. It could be seen that the fluorescence intensity of trypsin gradually decreased with increasing concentration of the AV PEs and gradually increased for the SM40T1 PEs. The maximum emission peak occurred at 340 nm for native trypsin while the maximum emission peak for trypsin with the addition of PEs occurred at 342 nm (6.75 µg/mL). There was a significant red shift for SM40T1 and TO60T1 PE from 338 nm at 6.25 ug/mL to 346 nm at 50 µg/mL. This indicated that the specific biochemical reaction between these extracts and trypsin was formed after the addition of PE. SM40T1 PE was able to bind to the active site of trypsin, which brought about the quenching of the fluorescence of trypsin. This red shift of maximum emission wavelength suggests that there is change in the microenvironment of trypsin (Ding *et al.*, 2015). The result obtained for the interaction of trypsin with PE was also similar with those reported for the interaction of tetrabromobisphenol A, tetrabromobisphenol S with bovine trypsin (Ding *et al.*, 2015). Ren et al., (2019) also reported a gradual decrease in the fluorescent intensity of trypsin with a significant red shift upon the addition of resveratrol.

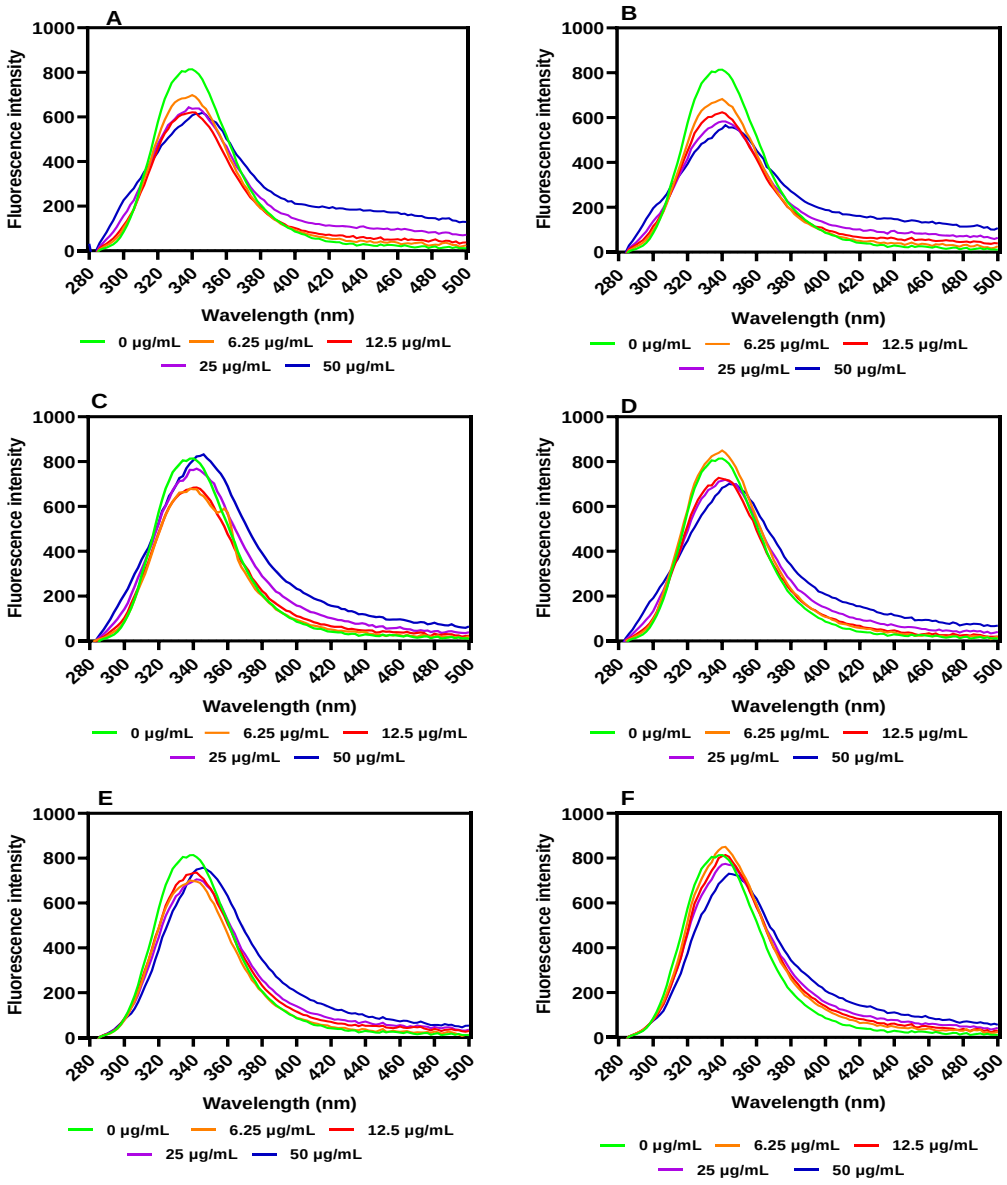

Figure S6. 1. Intrinsic fluorescent of trypsin with *Amaranthus viridis* leaves (AV80T1 (A) and AV80T2 (B)), *Solanum macrocarpon* (SM40T1 (C) and SM40T2 (D)) and *Telfairia occidentalis* (TO60T1 (E) and TO60T2 (F)) at different concentrations ranging from 0 µg/mL to 50 µg/mL. Vegetables were grown under different nitrogen fertilizer doses (40 kg/ha), that were applied before (T1) or after (T2) planting

S6.2 Circular Dichroism (CD)

S6.2.1 Far-UV CD

The CD spectrum of native trypsin exhibited (Fig. S6.2) a strong negative band between 207 nm and a weak positive band at 193 nm, indicating that trypsin consisted predominantly of α-helical sheets. After the addition of PE, at 1 mg, the shape of the spectrum shifted to the lower right and the negative band ranged from 207-210 nm while the positive band at 193 nm increased continuously and range from 195-206 nm, which indicates the presence of more β-sheet structure. As the concentration of PE increased the fold changes and the mean ellipticity of the samples decreased on the negative side and increased on the positive side indicating a loss of the α-helix shape to give rise to β-sheets. This supports the findings of Rawel et al., (2002) that interaction of myricetin with soy proteins give rise to a decrease in α-helix and an increase in β-strand and turn. It can be deduced that the phenolic compounds present in these extracts are responsible for the conformational changes observed in this study. At 3 mg, there is a wider stretch of the folds and a loss of the α-helix shape. The trypsin-PE complex show that the extracts was able to inhibit the activity of trypsin thus affecting its structure. This result also corresponds with the intrinsic fluorescence obtained for trypsin. Study has shown that interaction of polyphenols with proteins/enzymes can cause changes in their secondary structure (Roy et al., 2012).

S6.2.2 Near-UV CD

At 3 mg/mL, the reaction of PEs with trypsin resulted in unordered spectra suggesting loss of the fine structure. The result also corresponds with previously reported data of trypsin showing a competitive mode of inhibition thus indicating that PEs was able to inhibit trypsin at its active site thereby affecting its activity (Olarewaju et al., 2019). This suggests that the

aromatic side chains are disordered when the enzyme unfolds. Studies have shown that the shape and the

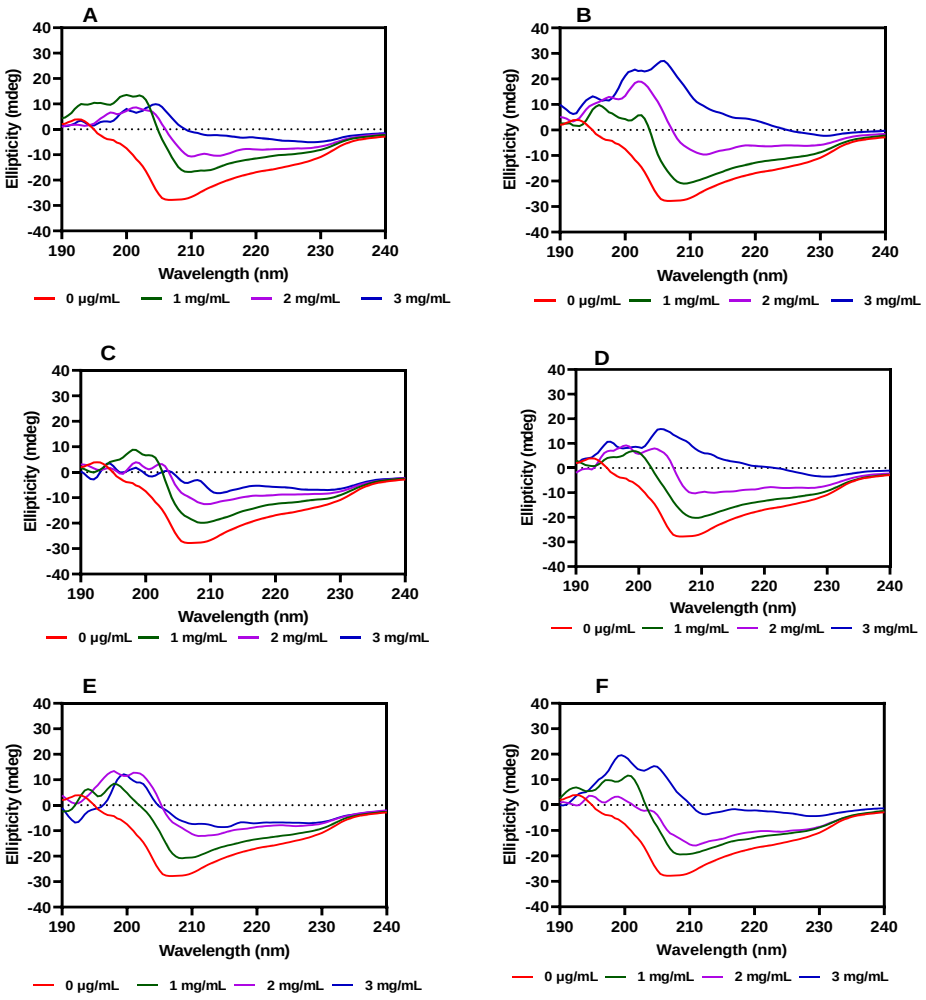

Figure S6. 1. Far-UV CD of trypsin with *Amaranthus viridis* leaves (AV80T1 (A) and AV80T2 (B)), *Solanum macrocarpon* (SM40T1 (C) and SM40T2 (D)) and *Telfairia occidentalis* (TO60T1 (E) and TO60T2 (F)) at different concentrations (0 - 3 mg/mL). Vegetables were grown under different nitrogen fertilizer doses (20, and 40 kg/ha), that were applied before (T1) or after (T2) planting

magnitude of the near-UV CD spectrum is altered by factors such as the type of aromatic amino acid, spatial arrangement, mobility, and the nature of the environment which is based on the type

of bond and polarity (Malomo and Aluko, 2015; Arise et al., 2017). Different studies have shown that interaction of polyphenols with enzymes can result in conformational changes in the structure of the enzymes (Rawel et al., 2002, 2005; Kroll et al., 2003; Gupta et al., 2010).

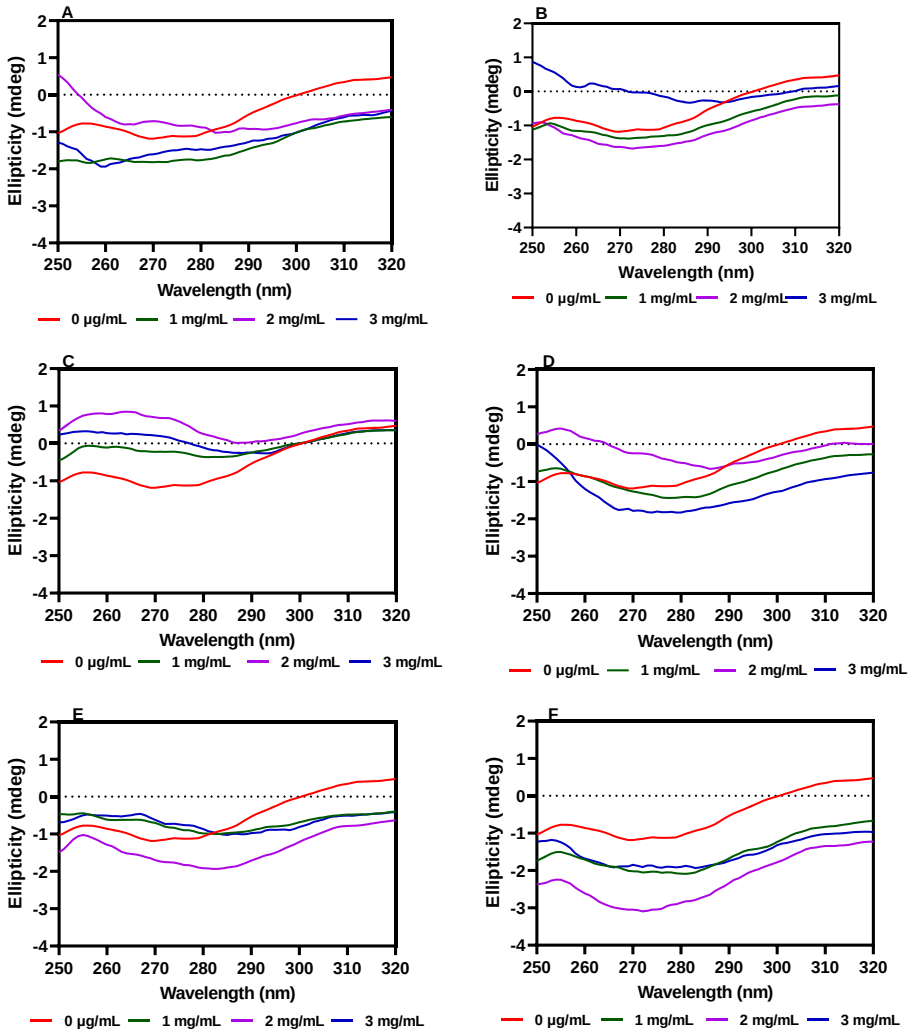

Figure S6. 2. Near-UV CD of trypsin with *Amaranthus viridis* leaves (AV80T1 (A) and AV80T2 (B)), *Solanum macrocarpon* (SM40T1 (C) and SM40T2 (D)) and *Telfairia occidentalis* (TO60T1 (E) and TO60T2 (F)) at different concentrations (0 - 3 mg/mL). Vegetables were grown under different nitrogen fertilizer doses (20, and 40 kg/ha), that were applied before (T1) or after (T2) planting

S6.3 References

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APPENDIX B: SUPPLEMENTAL INFORMATION

S7.1 α-Glucosidase inhibition

Regulation of blood glucose is vital in the management of diabetes and α-glucosidase is the key enzyme responsible for the glucose production from dietary carbohydrates during intestinal digestion (Uraipong and Zhao, 2016; Ercan and El, 2016). Therefore, its inhibition is more important than α-amylase and considered to be an effective strategy for the control of diabetes by reducing the amount of glucose available for absorption (Yu et al., 2011). The ability of the vegetable extracts to inhibit α-glucosidase activity *in vitro* was investigated and the results are presented in Figures S7.1a-c, which reveal a dose-dependent (1-5 mg/mL) pattern. However, as revealed by the graphs, TO80T1 (54.60%) had a higher inhibitory activity for α-glucosidase than SM and AV extracts. AV60T1 (24.46%) and SM0T1 (51.38%) had the highest inhibitory activity for AV and SM leaf extracts, respectively. The results obtained for the leaf extracts in this study are lower than the methanol extracts of *Solanum macrocarpon* (71.77 %) and *Solanum melongena* (63.24 %) as reported by Nwanna et al. (2013). The results are also lower than those reported for methanol extracts of *Cornus capitate* and *Roylea cinera* leaves, which were analyzed at 25 µg/mL (Bhatia et al., 2019). The lower inhibitory effects observed in this work may be due to the use of aqueous extraction, which may have isolated less active polyphenolic compounds when compared to the solvent extraction used in previous works. The three-way ANOVA data indicate that the extract from control leaves (0 kg urea N/ha), which suggest a negative effect for urea application just as observed also for α-amylase inhibition (Table S7.1). However, unlike α-amylase, the time at which the fertilizer was applied did not affect the inhibitory activity of α-glucosidase. Among the three vegetables, TO (45.97%) had the highest α-amylase inhibition while AV (20.16 %) had the lowest inhibition. The results also suggest that

the significantly higher content of caffeic acid in TO (Olarewaju et al., 2018; Oluwagunwa et al., 2019) may be responsible for the superior α-amylase inhibitory activity.

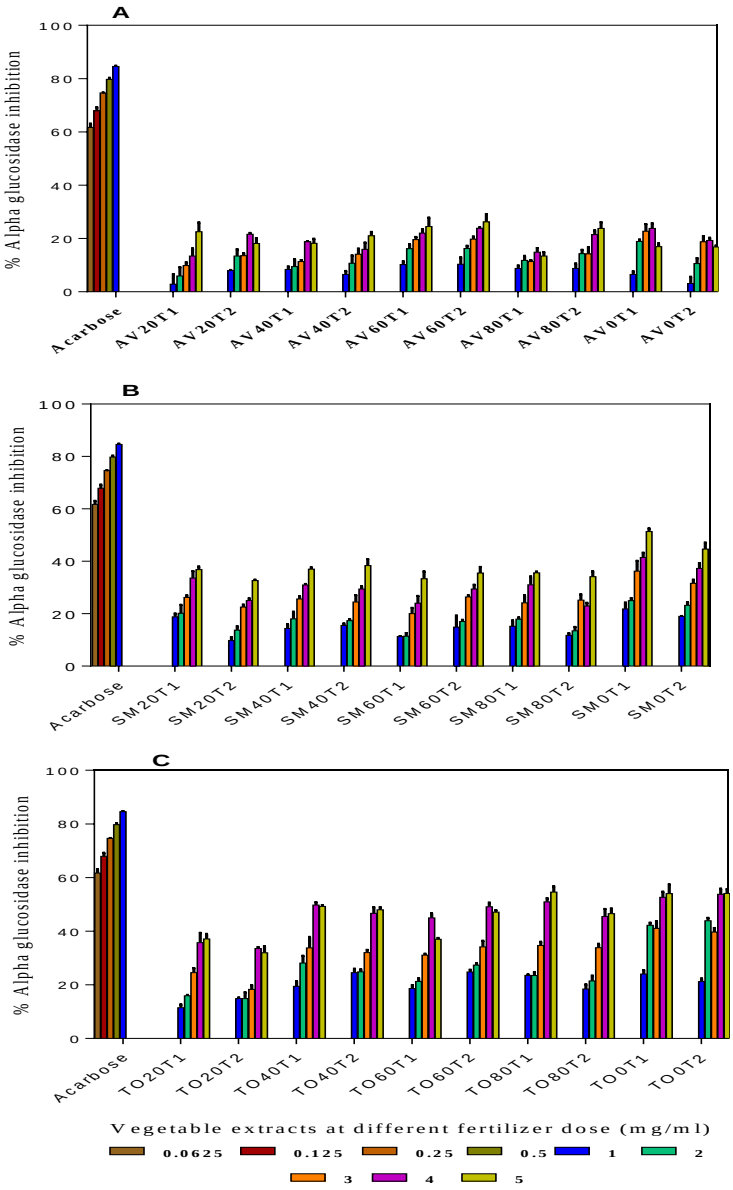

Figure S7. 1. α-glucosidase inhibition of aqueous extracts from the dried leaves of A - *Amaranthus viridis* leaves (AV), B - *Solanum macrocarpon* (SM) and C - *Telfairia occidentalis* (TO). Vegetables were grown under different nitrogen fertilizer doses (0, 20, 40, 60, and 80 kg/ha), that were applied before (T1) or after (T2) planting.

Table S7. 1. Results from 3-way ANOVA and Duncan's test of the effects of vegetable variety, (VV); fertilizer dose, (FD); fertilizer application time, (FAT); *Telfairia occidentalis*, (TO); *Solanum marcrocarpon*, (SM); *Amaranthus viridis*, (AV); (0T-80T as fertilizer dosing treatments) on α-glucosidase inhibitory activity of vegetable extracts.

Parameters	**Mean intensity for VV**			**Mean intensity for FD**					**Mean intensity for FAT**	
	SM	AV	TO	20T	40T	60T	80T	0T	T1	T2
α-glucosidase[p1]	37.94[b] (0.351)	20.16[a] (0.351)	45.97[c] (0.351)	29.87[a] (0.453)	35.31[c] (0.453)	33.94[b] (0.453)	34.68[bc] (0.453)	39.65[d] (0.453)	34.73[a] (0.286)	34.60[a] (0.286)

a,b,c,d Mean intensity values (followed in brackets by the standard error of the mean) within the same variable ''vegetable variety'', ''fertilizer dose'' and ''fertilizer application time'' with the same letter within the same row (parameter) are not significantly different ($p < 0.05$). Units: p: % Concentrations: 1: 5 mg/mL.

S7.2 Kinetics of enzyme inhibition

The enzyme kinetic study investigated the mode of enzyme inhibition of the aqueous extracts obtained from the vegetables leaves using selected extracts with high inhibition rates. Establishing the mode of inhibition via kinetics studies is very important because it reveals the mechanism by which the samples act as enzyme inhibitory agents (Girgih et al., 2015). Results of the kinetics study (Fig S7.2a-f) show that the extracts have a competitive mode of inhibition for α-glucosidase because the Lineweaver-Burk plots show similar y-axis intercept but different for the x-axis. The Lineweaver-Burk plots confirm that the inhibitor preferentially binds to the enzyme active site to reduce substrate entry and conversion into products. The kinetic parameters for glucosidase shown in Tables S7.3 reveals that V_{max} for SM0T1 and T2 (0.11), T080T1 and T2 (0.11) and AV60T1 and T2 (0.098) remained unchanged while the Km values increased. The inhibition constant (Ki), which indicates the binding ability to the enzymes shows that the extracts with fertilizer application at the time of planting (SM0T1 and T080T1) are stronger inhibitors than those with fertilizer application two weeks after the time of planting for α-glucosidase and α-amylase respectively except AV60T2. SM0T2 has the highest Ki value while AV60T2 has the lowest Ki value.

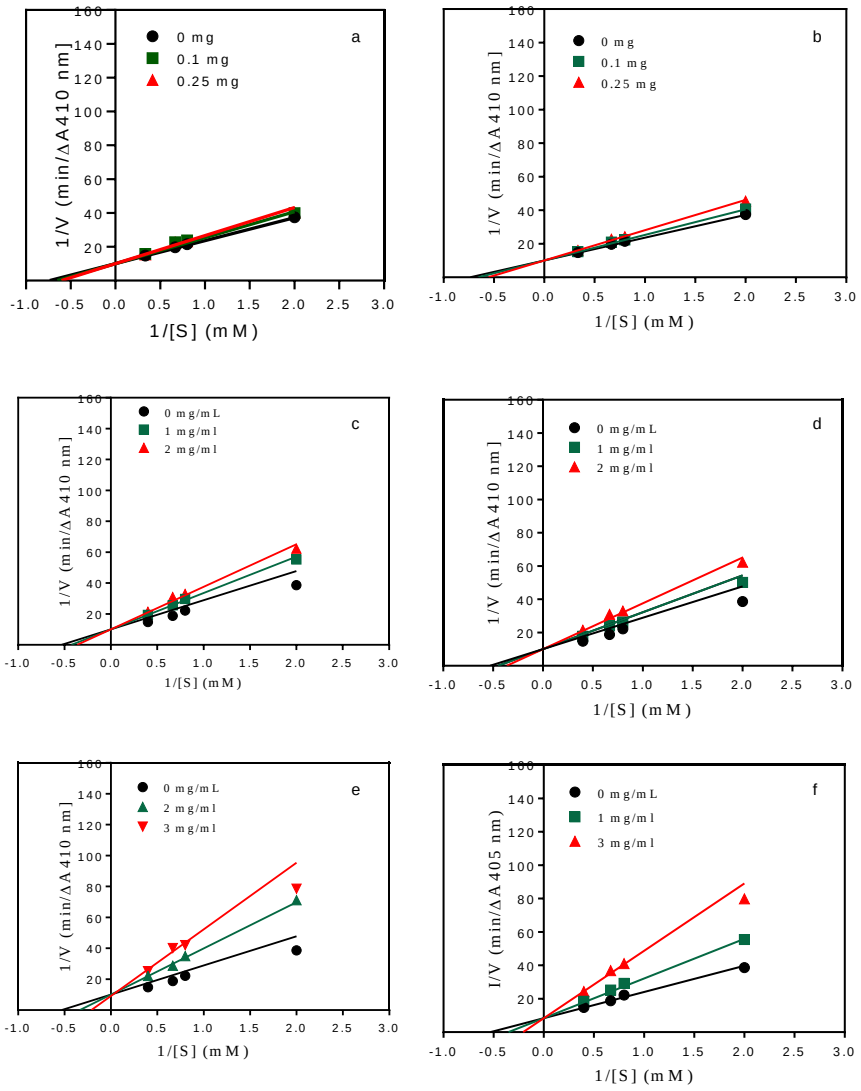

Figure S7. 2. Lineweaver-Burk plots of α- glucosidase inhibition of *Amaranthus viridis* leaves (a-AV60T1, b-AV60T2), *Solanum macrocarpon* (c-SM0T1, d-SM0T2) and *Telfairia occidentalis* (e-TO80T1, f- TO80T2). Vegetables were grown under different nitrogen fertilizer doses (0, 60 and 80 kg/ha), that were applied before (T1) or after (T2) planting

Table S7. 2a. Kinetic parameters for α-glucosidase inhibition by AV, SM and TO extracts

PARAMETERS	AV60T1			AV60T2			SM0T1		
Con (mg/mL)	0	0.1	0.25	0	0.1	0.25	0	1	2
Vmax	0.097±0.001	0.094±0.005	0.099±0.006	0.097±0.001	0.098±0.003	0.099±0.03	0.11±0002	0.0003±0.0002	0.0003±0.002
Km	1.36±0.04	1.54±0.19	1.66±0.23	1.36±0.04	1.53±0.10	1.81±0.10	1.89±0.19	2.35±0.16	2.76±0.51
Ki	1.44±0.47			0.91±0.08			1.79±0.15		

Results are presented as mean ± standard error. Km, Michaelis-Menten constant (mM); Vmax, maximum reaction velocity; Ki, enzyme-inhibitor dissociation constant (mg/ml)

Table S7. 3b. Kinetic parameters for α-glucosidase inhibition by AV, SM and TO extracts

PARAMETERS		SM0T2				TO80T1				TO80T2		
Con (mg/mL)		0	1	2	3	0	1	2	3	0	1	3
Vmax		0.11±0.006	0.11±0.004	0.11±0.010	0.11±0.018	0.11±0.006	0.099±0.008	0.10±0.008	0.11±0.03	0.12±0.006	0.12±0.006	0.12±0.017
Km		1.89±0.19	2022±0.15	2.76±0.51	3.96±0.98	1.89±0.19	2.26±0.31	2.99±0.38	4.75±1.65	1.89±0.19	2.85±0.22	4.85±0.92
Ki		2.01±0.14				1.75±0.105				1.92±0.096		

Results are presented as mean ± standard error. Km, Michaelis-Menten constant (mM); Vmax, maximum reaction velocity; Ki, enzyme-inhibitor dissociation constant (mg/ml)

This suggests that AV60T1 and AV60T2 are better inhibitors of α-glucosidase than SM and TO extracts. Among all the extracts, AV60T2 (Ki-0.91 ± 0.08 mg/mL) has the strongest ability to inhibit α-glucosidase followed by TO and SM extracts. SM0T2 (Ki-2.01 ± 0.14 mg/mL) is the weakest inhibitor among the extracts tested for α-glucosidase. This result does not correspond with the a-glucosidase inhibition result where TO have the highest inhibition rate. The ability of the extracts with fertilizer application at the time of planting to inhibit α-glucosidase more than those with fertilizer application two weeks after planting correlates with the results obtained for the percentage inhibitory activity. The concept of enzyme kinetics inhibition is important in the development of new drug therapy and has increased effective therapeutic agents for disease treatment. Enzyme inhibitors have also provided a better understanding of enzymatic transformations. Different mode of inhibition has been reported for various medicinal plants. Morinda lucida a traditional antidiabetic drug exhibited a competitive mode of inhibition (Kazeem et al., 2013) while Ajiboye et al., (2016) reported a non-competitive and uncompetitive mode of inhibition for α-glucosidase for Artocarpus heterophyllus stem bark. A mixed mode of inhibition was also reported for Labiatae extracts (Rouzbehan et al., 2017). Glucosidases are major enzymes responsible for the breakdown of carbohydrates into simple sugars, therefore these enzyme inhibitors have the potential to prevent the development of diabetes. The ability of these extracts to inhibit α-glucosidase indicate that they can be potential nutraceuticals in managing diabetes. Vmax, Km and Ki are the vital parameters that determine the mechanism by which the enzyme-inhibitor or enzyme-substrate reaction complex is formed. Vmax is the maximum velocity that reflects how fast the enzyme can catalyze the reaction. It is the maximum velocity reached when there are enough substrate molecules to saturate the active sites of the enzyme. Michaelis-Menten constant (Km) refers to the concentration of the substrate at which

the substrate saturates half of the active sites of the enzyme (Awosika and Aluko, 2019). The higher the Km, the more the amount of substrate needed to saturate the enzyme, indicating a lower affinity for the substrate. Ki is the dissociation constant, and it is reflective of the binding affinity of the inhibitor to the enzyme to form a complex. A low Ki value indicates higher binding affinity to the enzyme, which means a small concentration of the inhibitor is needed and vice versa.

S7.3 References

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APPENDIX C: SUPPLEMENTAL INFORMATION

S8.1 Kinetics of enzyme inhibition

The modes of inhibition of α-glucosidase, and trypsin activities were investigated via kinetic studies in the absence and presence of CH, NC and TO extract. Based on their ability to bind to proteins, phenolic compounds can inhibit the activities of carbohydrate-hydrolysing enzymes (Zhang et al., 2015). Results from Lineweaver-Burk plots (Figs S8.1-8.2) demonstrated that inhibition modes of all samples are competitive inhibitions. TO, CH and NC both have the same mode of inhibition for α-glucosidase and trypsin which may be due to the presence of the same phenolic compounds. The competitive mode of inhibition suggests that both the substrate and inhibitor compete for the same binding site in the enzyme. This indicate that the phenolic compounds present in TO, CH and NC bind to the active site of α-glucosidase, and trypsin thus making the enzyme unavailable for the substrate to bind. The y-intercept (1/Vmax) remains unaffected, and slope (Km/Vmax) increased with the concentration of the inhibitor. Since increases in substrate concentration could overcome the initial inhibition by the samples, the maximum velocity was the same for inhibited and un-inhibited reactions. However, binding of the samples to the active site of the enzyme necessitated an increase in substrate concentration required to reach half-maximal velocity, hence the Km values were higher for the inhibited reactions. The results are like our previous report, which also observed that the TO extract inhibited trypsin in a competitive manner (Olarewaju et al., 2019). The inhibition constant (Ki) provides an indication of the binding affinity of inhibitors to an enzyme target; lower values suggest stronger binding affinity than higher values. Therefore, TO extract has a stronger affinity to trypsin with a lower Ki (9.5 µg/mL) when compared to the fractionated samples (CH -10.5 µg/mL and NC – 14.0 µg/mL). The inhibition constant (Ki) revealed that NC fraction (0.158 ± 0.02 mg/mL) binds stronger to a-glucosidase than TO (0.232 ± 0.03) and CH (0.348 ±0.05

mg/mL). The result obtained here does not correspond with the α-glucosidase inhibition where CH had the highest inhibition rate followed by TO and NC.

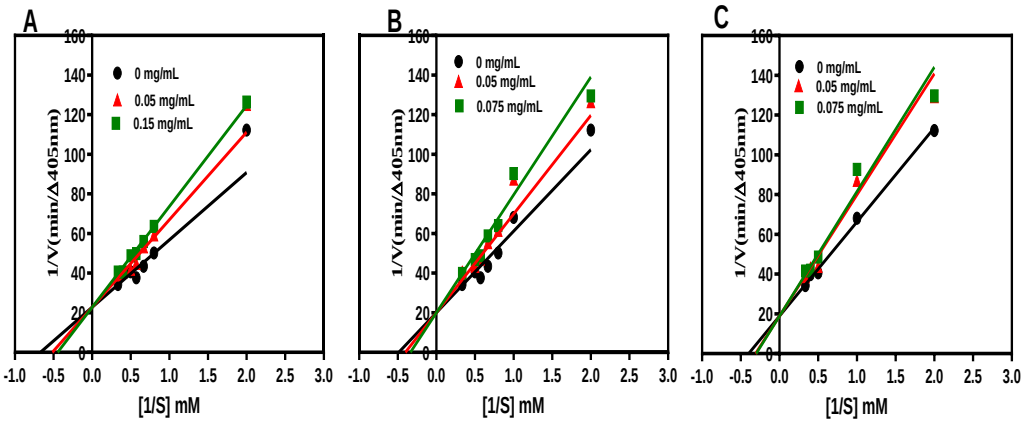

Figure S8. 1. Kinetic inhibition of α-glucosidase by aqueous extract of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

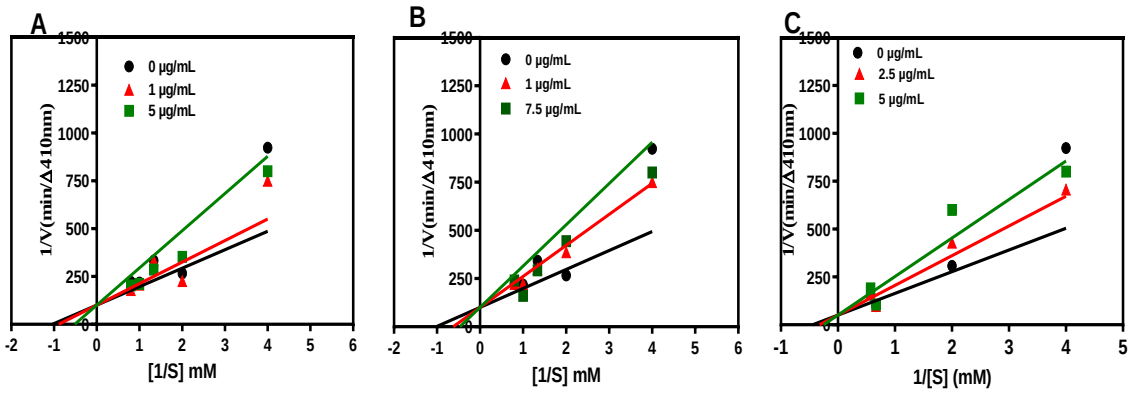

Figure S8. 2. Kinetic inhibition of trypsin by aqueous extract of *Telfairia occidentalis* leaf extract (TO), and the chlorophyll-enriched (CH) and chlorophyll-depleted (NC) fractions

S8.2 References

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